A Section 508–conformant HTML version of this article is available at https://doi.org/10.1289/EHP10239.

Phenol and Phthalate Effects on Thyroid Hormone Levels during Pregnancy: Relying on *In Vitro* Assays and Adverse Outcome Pathways to Inform an Epidemiological Analysis

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Background: Studies characterizing associations between phenols, phthalates and thyroid hormones during pregnancy produce inconsistent results. This divergence may be partly attributable to false positives due to multiple comparison testing of large numbers of chemicals, and measurement error as studies rely on small numbers of biospecimens despite high intra-individual variability in urinary chemical metabolite concentrations.

Objectives: This study employs *a priori* chemical filtering and expanded urinary biomonitoring to evaluate associations between phenol/phthalate exposures and serum thyroid hormones assessed during pregnancy.

Methods: A two-tiered approach was implemented: *a*) *In vitro* high-throughput screening results from the ToxCast/Tox21 database, as informed by a thyroid Adverse Outcome Pathway network, were evaluated to select phenols/phthalates with activity on known and putative molecular initiating events in the thyroid pathway; and *b*) Adjusted linear regressions were used to study associations between filtered compounds and serum thyroid hormones measured in 437 pregnant women recruited in Grenoble area (France) between 2014 and 2017. Phenol/phthalate metabolites were measured in repeated spot urine sample pools (median: 21 samples/women).

Results: The ToxCast/Tox21 screening reduced the chemical set from 16 to 13 and the associated number of statistical comparisons by 19%. Parabens were negatively associated with free triiodothyronine (T3) and the T3/T4 (total thyroxine) ratio. Monobenzyl phthalate was positively associated with total T4 and negatively with the T3/T4 ratio. Effect modification by iodine status was detected for several compounds (among them ΣDEHP and mono-*n*-butyl phthalate) that were associated with some hormones among women with normal iodine levels.

Conclusion: For these chemicals, screening for compounds with an increased likelihood for thyroid-related effects and relying on repeated urine samples to assess exposures improved the overall performance of multichemical analyses of thyroid disruption. This approach may improve future evaluations of human data for the thyroid pathway with implication for fetal health and may serve as a model for evaluating other toxicity outcomes. https://doi.org/10.1289/EHP10239

Introduction

During pregnancy, euthyroidism is crucial for normal fetal growth and development.[1] Even subtle alterations of thyroid hormone homeostasis can negatively impact the growing fetus and postnatal health.[2] Aside from iodine deficiency and preexisting thyroid diseases, exposure to environmental contaminants, specifically endocrine disruptors such as synthetic phenols and phthalates, are suspected to contribute to thyroid hormone dysregulation.[3,4] *In vivo* and *in vitro* data suggest that these compounds may disrupt thyroid hormone signaling by perturbing hormone biosynthesis, metabolic activation/inactivation, and associated negative feedbacks with the central hypothalamic-pituitary-thyroid (HPT) axis (as reviewed by Bruker-Davis,[5] Murk et al.,[6] Noyes et al.,[7] and Zoeller[8]).

Several epidemiological studies have explored associations between exposures to phenols and phthalates and thyroid hormone homeostasis during pregnancy. However, drawing conclusions from these studies is not straightforward, because results often differ. For example, urinary DEHP metabolites were shown to be associated with decreased thyroid stimulating hormone (TSH) and increased thyroxine (T4) concentrations in 2,521 pregnant women,[9] whereas opposing results (increased TSH and decreased T4) have been reported elsewhere ($n = 439$).[10] Result discrepancies across studies could be partly explained by differences in study designs, such as trimester of urine and blood collection; differences in participant's characteristics, such as iodine levels; and difference in exposures. In addition, due to their short half-lives and temporal variability in sources of exposure (e.g., diet, use of personal care products) high intra-individual variability in urinary concentrations has been reported for some of the studied compounds [e.g., intraclass correlation coefficients of about 0.2 for bisphenol A (BPA)[11–13]]. Studies often rely on a limited number of urine specimens to assess exposure, which is unlikely to be sufficient to reflect exposure over the full pregnancy term. That approach leads to classical measurement error and effect estimates biased toward the null that explain null findings.[14,15] Finally, due to the high number of hypotheses tested

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Supplemental Material is available online (https://doi.org/10.1289/EHP10239).

All authors declare they have no actual or potential competing financial interests.

Data used for this study is confidential. It can be provided on reasonable request toward the SEPAGES study comity.

The views expressed in this manuscript are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA.

Received 1 September 2021; Revised 1 August 2022; Accepted 7 October 2022; Published 9 November 2022.

(several thyroid hormones and exposures assessed, sometimes at repeated time points) family-wise error rate (FWER; probability of making one or more false discoveries) is likely to be elevated in these studies,[16] which may also explain result discrepancies across studies.

Herein, we relied on a two-tier approach. A thyroid adverse outcome pathway (AOP) network published by Noyes et al.[7] and results from *in vitro* higher-throughput screening (HTS) assays in the ToxCast/Tox21 database[17] were evaluated to select phenols and phthalates predicted to be bioactive modulators of molecular initiating events (MIEs) in thyroid toxicity pathway. We then studied associations between this restricted set of compounds and thyroid hormone concentrations in maternal blood. In comparison with a purely agnostic approach, our hypothesis-driven approach focused on compounds with a higher *a priori* likelihood for effects on thyroid hormone homeostasis and reduced the number of statistical tests performed to help mitigate the probability for false positive findings.

Methodology

Study Population

The prospective SEPAGES [Suivi de l'Exposition à la Pollution Atmosphérique durant la Grossesse et Effets sur la Santé (Assessment of air pollution exposure during pregnancy and effect on health)] cohort recruited 484 pregnant women from eight obstetrical ultrasonography practices located in Grenoble area of France, between July 2014 and July 2017.[18] Women were included based on the following criteria: ≥18 y of age, being pregnant for 19 gestational weeks or less, having a singleton pregnancy, residing in the study area, and planning to give birth in one of the four maternity clinics from the Grenoble area that were near the SEPAGES biobank.

Ethical agreements were obtained from the Comité de Protection des Personnes Sud – Est V (CPP) and the Comité Nationale de l'Informatique et des Libertés (CNIL), the French data privacy institution. All participating women gave written consent.

This analysis was restricted to the 437 pregnant women who did not report taking medication for any thyroid diseases (questionnaire completed during the first trimester that specifically asked about thyroid disorders) and had blood and urine samples collected during pregnancy (See flowchart in Supplemental Material, Figure S1).

Biospecimen Collection

Urine samples were collected over a week in the second trimester (median 17.7 gestational weeks (GW); 5th and 95th percentiles: 14.4, 20.0 wk, respectively), during which time women were requested to collect three spot urine samples per day (in the morning, midday, and evening). Samples were collected in 60 mL polypropylene tubes and stored in the participants' freezer (−20°C). At the end of the collection week, samples were transported on ice by a study fieldworker to the certified biobank of Grenoble University Hospital (bb-0033-00069). Samples were thawed overnight at 4°C, and for each subject a pool of the same volume of all the spots collected over the week were made following a previously validated protocol.[19] Although assessments of biomarker concentrations did not formally account for urine dilution of individual samples, biomarker concentrations assessed in such equal volume pools have been shown to correlate well with those assessed in a pool of all urine volume collected over 24 h or a week.[20] For each woman, this pool was aliquoted and stored at −80°C. Daily pools (equal volume pools of all samples collected over a day) were also made.

Assessments of Phenol and Phthalate Metabolite Concentrations

Pools of urine samples collected over a week were sent on dry ice with a temperature sensor to the Norwegian Institute of Public Health (NIPH), where measurements of phenol and phthalate metabolite concentrations were carried out (see Table 1 for a detailed list of biomarkers assessed). Phthalate and di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) biomarkers were analyzed and quantified using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS-MS).[21] Phenols were analyzed and quantified using ultra high-performance liquid chromatography coupled to mass spectrometry (UPLC-MS-MS).[22] The free and conjugated forms of phenol biomarkers were preliminarily measured in samples from 50 women. These preliminary measurements did not suggest external contamination,[23] so for the remaining participating women, we relied on analysis of the total form (free+conjugated). Bisphenols AF, B, F, and triclocarban were detected in <5% of the pooled samples and were not considered in our analysis.

Collection of Maternal Blood

Nonfasting maternal blood was collected by trained SEPAGES fieldworkers during a study visit at the participants' homes. For 85% of the women, blood was collected at the end of the urine collection week, and blood for the other 15% was collected several weeks after urine (median 9 wk, 5th and 95th percentiles: 6, 12, respectively). After collection, samples were transported on ice to the biobank of Grenoble University Hospital; there, blood was processed, and serum aliquots were stored at −80°C.

Measurements of Thyroid Hormone, Selenium, and Iodine Concentrations

TSH was quantified in maternal sera by LOCI Chemiluminescence on Dimension Vista analyzer (Siemens).[24] Serum concentrations of protein-bound and free T4 and 3, 5, 3′-triiodothyronine (T3) were quantified by RIA-Gnost (CisBio Bioassays). Maternal total T3 and T4 were obtained by summing the free and protein-bound concentrations. The ratio of total T3 to T4, an indicator of T4 deiodination into the bioactive T3 form, was then calculated. Selenium, an essential micronutrient required for biosynthesis of selenoproteins involved in the peripheral conversion of free T4 to free T3, as well as being vital antioxidants in the thyroid gland, was also measured in maternal sera using inductively coupled plasma mass spectrometry (ICP-MS).[25]

Iodine, an essential element in the synthesis of thyroid hormones, was measured in daily pooled samples of maternal urine using inductively coupled plasma mass spectrometry (ICP-MS).[26]

Tier 1: Relying on a Thyroid AOP Network and ToxCast/Tox21 In Vitro *HTS Database to Select Phenols and Phthalates*

The research effort herein relied on existing AOP networks that map the causal and putative MIEs in the thyroid pathway that have been demonstrated or hypothesized to be the chemical targets. To this end, there have been a number of efforts in the development and evolution of the thyroid AOP network, including an effort recently by Noyes et al.[7] Several ToxCast/Tox21 *in vitro* HTS assays were evaluated for chemical interactions with MIEs in the thyroid pathway.[17] MIEs targeted are highlighted in the Supplemental Material, Figure S2 (thick, green border in left-

Table 1. List of metabolites and parent compounds assessed in SEPAGES.

Parent compounds	Biomarkers assessed in SEPAGES urine samples	Biomarkers excluded from the statistical analysis due to low frequency of detection (<5%)	Biomarkers excluded from the statistical analysis because not identified as bioactive on relevant MIEs
Phenols			
Methylparaben	Methylparaben	—	X
Ethylparaben	Ethylparaben	—	X
Propylparaben	Propylparaben	—	—
Butylparaben	Butylparaben	—	—
Bisphenol A	Bisphenol A	—	—
Bisphenol S	Bisphenol S	—	—
Bisphenol F	Bisphenol F	X	—
Bisphenol B	Bisphenol B	X	—
Bisphenol AF	Bisphenol AF	X	—
Benzophenone-3	Benzophenone-3	—	—
Triclosan	Triclosan	—	—
Triclocarban	Triclocarban	X	—
Phthalates			
Diethyl phthalate (DEP)	Monoethyl phthalate (MEP)	—	X
Diisobutyl phthalate (DiBP)	Monoisobutyl phthalate (MiBP)	—	—
Dibutyl phthalate (DBP)	Mono-*n*-butyl phthalate (MnBP)	—	—
Butyl-benzyl phthalate (BBzP)	Monobenzyl phthalate monobutyl phthalate (minor) (MBzP)	—	—
Di(2-propylheptyl) phthalate (DPHP)	6-hydroxy-mono-propyl-heptyl phthalate (oh-MPHP)	—	—
Di(2-ethylhexyl) phthalate (DEHP)	Mono(2-ethylhexyl) phthalate (MEHP)	—	—
	Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	—	—
	Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)	—	—
	Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)	—	—
	Mono(2-methylcarboxyhexyl) phthalate (MMCHP)	—	—
Diisononyl phthalate (DiNP)	Mono(4-methyl-7-hydroxy-octyl) phthalate (OH-MiNP)	—	—
	Mono(4-methyl-7-oxo-octyl) phthalate (oxo-MiNP)	—	—
	Mono(4-methyl-7-carboxy-heptyl) phthalate (cx-MiNP)	—	—
Nonphthalate plasticizers			
Di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH)	2-(((hydroxy-4-methyloctyl)oxy)carbonyl)cyclohexanecarboxylic acid (oh-MINCH)	—	—
	2-(((4-methyl-7-oxyooctyl)oxy)carbonyl)cyclohexanecarboxylic acid (oxo-MINCH)	—	—

Note: —, included in our statistical analysis; MIE, molecular initiating event; X, excluded from our statistical analysis.

hand column) and include those involved in thyroid hormone biosynthesis in the thyroid gland (Na^+/I^- symporter [NIS,[27,28] thyroperoxidase (TPO[29])]; receptor-based interactions [thyroid hormone (TR) receptors (TRα, TRβ[30]) thyroid stimulating hormone (TSH) receptor,[31] and thyrotropin releasing hormone (TRH) receptor]; thyroid hormone peripheral tissue metabolism [iodothyronine deiodinases (DIO1, DIO2, DIO3[32,33])], and activation of hepatic T4 catabolism [e.g., constitutive androstane receptor (CAR), pregnane X receptor (PXR), uridine diphosphate glucuronosyl transferases (UDPGTs)]. The MIEs in the thyroid pathway have been described in detail elsewhere and readers are referred to reviews for additional background.[6,7,34]

ToxCast/Tox21 database outputs are typically presented as positive (hitcall = 1) or negative (hitcall = 0) with associated half-maximal activity concentration (AC_{50}) values and efficacy values (cutoff and maximum responses) for bioactive substances. To derive point estimates, raw chemical screening data in assay tests are processed through the ToxCast data analysis pipeline involving several steps in data normalization and dose–response modeling. ToxCast applies three dose–response models in its data evaluations: *a*) constant model with a constant value of zero response and only one parameter, the scale term; *b*) Hill model that is a constrained three parameter model; and *c*) Gain-Loss model that is a constrained five-parameter model. For assay results to be considered bioactive, the modeled concentration–response curves must meet three criteria: *a*) Hill or Gain-Loss curve fit models must be the winning models; *b*) the modeled curve fit top must exceed the efficacy cutoff for at least one dose; and *c*) the median response must exceed the efficacy cutoff. Automated flags identify potentially anomalous outputs. To further limit potential false positives, we also considered only ToxCast positive hitcalls that matched the following criteria: *a*) concentration–response curves had no more than three flags; *b*) response curve fits displayed a sigmoidal shape; *c*) more than one data point was above the efficacy cutoff; and *d*) data for low concentrations were present (i.e., AC_{50} should not have been extrapolated[35,36]). Concentration–response curves and associated data were extracted from the ToxCast chemistry dashboard for each chemical.[37] Those not meeting these criteria and excluded from the analysis are displayed in Supplemental Material, Figure S3.

Tier-2: Statistical Analyses

Exposure biomarker concentrations below the limit of detection (LOD) and between the limit of detection and the limit of quantification (LOQ) were singly imputed by values randomly selected between 0 and LOD and between LOD and LOQ, respectively, based on the estimated underlying distribution.[38,39] To limit the impact of between-subject variations in conditions related to urine processing (i.e., sample transport time from participant's home to the biobank, time during which the individual samples were thawed at 4°C during the pooling procedure) and assay (analytical batches), we standardized the measured phenol and phthalate metabolite concentrations when needed. We first estimated the associations between each biomarker concentration assessed in pools (natural log-transformed) and the factors

Table 2. Characteristics of pregnant women included in this study ($n = 437$, SEPAGES cohort, 2014–2017).

Characteristics	n (%)	Median (5th–95th percentiles)
Maternal age (years)	437	32.2 (26.5–39.0)
Gestational age at serum collection (weeks)	437	19.1 (15.9–28.0)
Gestational age at delivery (weeks)	436	40.0 (37.1–41.4)
Number of urine samples in pools	437	21 (17–21)
Education level		
≤2 y after high school	75 (17)	—
3–4 y after high school	116 (27)	—
≥5 y after high school	245 (56)	—
Missing	1	—
Parity		
Nulliparous	198 (45)	—
Parous	239 (55)	—
Maternal prepregnancy BMI (kg/m^2)		
<18.5	27 (6)	—
18.5 to <25	324 (75)	—
≥25	82 (19)	—
Missing	4	—
Child sex		
Male	235 (54)	—
Female	200 (46)	—
Missing	2	—
Vitamin use during pregnancy		
Yes	387 (91)	—
No	37 (9)	—
Missing	13	—
Smoking (first trimester)		
No	377 (94)	—
Yes	23 (6)	—
Missing	37	—

Note: —, no data; BMI, body mass index.

above-mentioned using adjusted linear regression. If processing/assay conditions were identified as associated with the biomarker urine concentrations ($p < 0.2$), we then used the measured biomarker concentrations and the estimated effects of the processing/assay conditions to predict standardized concentrations (i.e., concentrations that would have been observed if all samples had been processed under the same conditions and assayed in the same analytical batch).[40,41] We used these standardized concentrations in our statistical analyses.

To limit the impact of extreme values, thyroid hormone concentrations, as well as the T3/T4 ratio, were ln-transformed. Phenol and phthalate metabolite concentrations were considered as continuous (ln-transformed) variables, as well as categorized into tertiles, except for compounds detected in <70% of the samples that were dichotomized (detected/undetected). We computed the molar sum of metabolites from the same parent [e.g., di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DiNP) and DINCH parents, Table 1]. We used adjusted linear regression to assess the associations between each selected phenol and phthalate biomarker and thyroid hormone (TSH, T4, T3) or the T3/T4 ratio.

Adjustment factors for our statistical analyses were selected *a priori* and included variables likely to be common causes of both the exposures and the thyroid hormones without being likely consequences thereof and factors that were possible predictors of the thyroid hormones only,[42,43] such as maternal age (quadratic terms), body mass index (BMI, kilograms per square meter) before pregnancy (continuous), education level (three categories: ≤2 y after high school, 3–4 y after high school, and postgraduate or ≥5 y after high school), maternal smoking during the first trimester of pregnancy (Yes/No), parity (nulliparous and parous), gestational age at serum collection (continuous, weeks), hour of serum collection (categorized), maternal urinary iodine concentrations (ln-transformed, micrograms per liter), and selenium concentrations in sera during pregnancy (tertiles, micromoles per liter). Models were also adjusted for analytical batch for all hormones except TSH, for which no batch effect was detected. A directed acyclic graph representing these relationships is displayed in Supplemental Material, Figure S4. Missing values for covariates were handled using multiple imputation (20 imputed data sets). Effect estimates were reported as percentage change in the hormone concentrations for a doubling of urinary biomarker concentrations. For bisphenol S (BPS) and butylparaben, which were dichotomized, effect estimates represent percentage change in hormone concentrations between those with detected and undetected concentrations.

Betas were expressed as percentage change in outcomes for each doubling of urinary biomarker concentrations except for butylparaben and BPS, for which effect estimates represent percentage change in outcomes between those with undetected and detected urinary concentrations. These percentage changes were obtained from the original betas using the following formulae: $(2^{\beta} - 1) \times 100$ for continuous exposures and $(e^{\beta} - 1) \times 100$ for categorial exposures (butylparaben and BPS). We report in the "Results" section all associations with $p < 0.05$. Associations with p–values between 0.05 and 0.10 were also reported as a trend when the biomarker was associated ($p < 0.05$) with another hormone.

Additional Analyses

Iodine status might modify the associations between chemical exposure and thyroid hormone levels.[44] For this reason, in sensitivity analyses, we explored modification by adding an interaction term between iodine levels and urinary phenol and phthalate metabolite concentrations. When an effect modification was suggested ($p_{interaction} < 0.10$), stratified analyses on iodine status were performed. For this analysis, we dichotomized iodine levels according to the World Health Organization (WHO) threshold for iodine deficiency in pregnant women (150 $\mu g/L$[45]).

Although serum samples were collected at the end of the urine collection week for most women, serum samples were collected for 15% of participants several weeks after the urine samples (median 9 wk, 5th and 95th percentiles: 6, 12, respectively). For this reason, we ran an additional analysis restricted to the 373 women for whom serum samples were collected at the end of the urine collection week.

For the main models, we plotted residuals to visually identify influential individuals. If any, we run a sensitivity analysis without these individuals to assess result's robustness.

Finally, we estimated the joint effects of the selected phenols and phthalates on each hormone using adjusted Bayesian Kernel Machine Regressions (BKMR; R package: bkmr). Such modeling allows estimation of mixture effect and identification of the important components of the mixture. It also accommodates for nonlinear relationships and correlated exposures.[46,47] Categorical exposures (BPS and butylparaben) were not considered in this analysis. Each exposure biomarker concentration was standardized [i.e., divided by their standard deviation (SD)]. For each model, we ran 50,000 iterations, dropped the first 25,000, and kept every fifth iteration among the last 25,000 for inference. The overall mixture effect was given by a figure showing the expected change in hormone concentration with concomitant increase quantiles of all exposure biomarkers, relative to when they are fixed at their 25th percentile. When this graph was suggestive of an effect of the mixture, we provided the posterior inclusion probability (PIP) for each biomarker and plotted the estimated effect of an increase from the 25th to 75th percentile in a single biomarker concentration when all other biomarker concentrations were fixed at either their 25th, 50th, or 75th percentiles.

Table 3. Distribution of phenol and phthalate metabolite concentrations in a pool of repeated urine samples collected over a week [median (percentiles 5 and 95) number of samples in each pool: 21 (17–21); $n = 437$ pregnant women from the SEPAGES cohort, 2014–2017].

				Standardized[a,b] concentrations			Measured concentrations[a]			
				Percentiles			Percentiles			
Metabolites	LOD/LOQ	Percentage >LOD	Percentage >LOQ	5th	50th	95th	5th	50th	95th	Rho[c]
Phenols										
Bisphenol A	0.04/0.1	99.5	99.3	0.63	1.86	8.54	0.67	2.08	8.93	0.95
Bisphenol S	0.1/0.4	25.2	20.6	—	—	—	<LOD	<LOD	2.9	—
Benzophenone-3	0.04/0.1	100	98.6	0.18	0.86	25.2	0.25	1.20	35.6	0.97
Triclosan	0.04/0.1	98.2	98.2	0.21	0.91	189	0.21	0.91	189	1.00
Methylparaben	0.04/0.1	100	100	2.15	10.6	234	2.53	12.0	278	0.94
Ethylparaben	0.04/0.1	99.7	99.8	0.27	0.89	38.2	0.24	0.71	32.3	0.93
Propylparaben	0.04/0.1	84	67.3	0.00	0.34	55.7	0.01	0.45	71.5	0.95
Butylparaben	0.07/0.2	24.7	11	—	—	—	<LOD	<LOD	0.72	—
Phthalate metabolites										
MEP	0.2/0.5	100	100	6.42	24.1	130	6.52	24.5	141	0.97
MiBP	0.2/0.5	100	10	6.34	15.1	47.1	6.61	18.3	57.5	0.87
MnBP	0.2/0.5	100	100	4.68	10.6	32.8	5.41	12.7	41.3	0.97
MBzP	0.07/0.2	100	100	1.46	4.44	16.3	1.54	4.77	17.3	1.00
oh-MPHP	0.07/0.2	100	99.5	0.50	0.86	2.77	0.41	0.87	2.92	0.82
MEHP	0.2/0.5	100	99.1	0.74	2.36	8.47	0.74	2.33	8.54	0.97
MEHHP	0.2/0.5	100	100	3.21	7.04	24.2	3.17	7.10	24.0	0.97
MEOHP	0.2/0.5	100	100	2.26	4.96	17.4	2.25	5.25	17.31	0.97
MECPP	0.7/2	100	99.8	5.11	9.96	27.5	5.23	10.6	31.2	0.95
MMCHP	0.7/2	99.3	99.1	4.17	7.56	19.8	4.98	9.35	25.0	0.91
ΣDEHP	—	—	—	0.05	0.11	0.32	0.06	0.12	0.35	0.97
oh-MiNP	0.1/0.25	100	100	1.70	4.87	28.9	1.70	4.87	28.9	1.00
oxo-MiNP	0.1/0.25	100	99.5	0.83	2.17	14.8	0.89	2.26	17.9	0.96
cx-MiNP	0.4/1	100	100	2.58	4.68	26.8	2.53	4.65	28.6	0.90
ΣDiNP	—	—	—	0.02	0.04	0.21	0.02	0.04	0.22	0.98
Nonphthalate plasticizer										
oh-MINCH	0.07/0.2	100	100	0.75	1.77	18.6	0.45	1.50	16.3	0.87
oxo-MINCH	0.07/0.2	99.8	99.8	0.63	1.51	13.5	0.37	1.13	13.3	0.89
ΣDINCH	—	—	—	0.00	0.01	0.11	0.00	0.01	0.10	0.88

Note: Bisphenols AF, B, F, and triclocarban were detected in <5% of the pooled samples and were not displayed in this table. —, no data; cx-MiNP, mono-4-methyl-7-carboxyoctyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DINCH, di(isononyl)cyclohexane-1,2-dicarboxylate; DiNP, diisononyl phthalate; LOD, limit of detection; LOQ, limit of quantification; MBzP, monobenzyl 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; MMCHP, mono-2-carboxymethyl hexyl phthalate; MEP, monoethyl phthalate; MiBP, monoisobutyl phthalate; MiNP, monoisononyl phthalate; MnBP, mono-*n*-butyl phthalate; oh-MINCH, 2-(((Hydroxy-4-methyloctyl) oxy) carbonyl) cyclohexanecarboxylic acid; oh-MiNP, mono-4-methyl-7-hydroxyoctyl phthalate; oxo-MiNP, mono-4-methyl-7-oxooctyl phthalate; oxo-MINCH, 2-(((4-Methyl-7-oxooctyl) oxy) carbonyl) cyclohexanecarboxylic acid; oh-MPHP, mono-6-hydroxy-propylheptyl phthalate; ΣDEHP, molar sum of the five DEHP metabolites; ΣDiNP, molar sum of the three DiNP metabolites; ΣDINCH, molar sum of the two DINCH metabolites.
[a]Concentrations in micrograms per liter, except for ΣDEHP, ΣDiNP and ΣDINCH, which are provided in micromoles per liter.
[b]Concentrations were standardized for sample transport time from participant's home to the biobank, during which time the individual samples were thawed at 4°C during the pooling procedure or analytical batches when these variables were associated with the measured biomarker concentrations ($p < 0.2$).
[c]Spearman correlation coefficient between measured and standardized concentrations.

Analyses were carried out with Stata/SE (version 15.1; StataCorp LLC) and R (version 4.0.4; R Development Core Team).

Results

Characteristics of Study Participants

Median of maternal age at recruitment was 32.5 y old (Table 2). The majority (55%) already had a child and were highly educated (56% had pursued education for ≥5 y after high school). Most (74%) had a BMI in normal range (18.5–25 kg/m^2), and 6% reported smoking during their first trimester of the pregnancy (after they knew they were pregnant). Median of gestational age at birth was 40 wk, and 54% of the infants were males. Median (percentiles: 5th and 95th) number of samples in each pool was 21 (17 and 21).

Table 4. Distributions of serum thyroid hormone concentrations, selenium and iodine assessed in serum or urine (iodine) of pregnant women of the SEPAGES cohort.

		Percentiles				
	n[a]	5th	25th	50th	75th	95th
Total T4 (ng/mL)	437	75.5	85.4	95.6	105.3	122.9
Free T4 (pg/mL)[a]	435	5.4	6.4	7.2	8.4	10.4
Total T3 (ng/mL)[a]	405	0.9	1.0	1.2	1.3	1.7
Free T3 (pg/mL)	437	1.7	1.9	2.1	2.3	2.6
TSH (mUI/L)	437	0.6	0.9	1.3	1.8	2.6
Iodine (µg/L)	437	31.6	56.7	89.3	134.5	271.6
Selenium (µmol/L)	364	0.8	0.9	1.0	1.1	1.2

Note: T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.
[a]Lower n for free T4, total T3, and selenium are due to insufficient serum quantity to perform all the assessments for a few women.

Distribution of Maternal Urinary Phenol and Phthalate Concentrations

Phenol and phthalate metabolite concentrations have been described elsewhere.[23,48] Briefly, bisphenols AF, B, F, and triclocarban were detected in <5% of the pooled samples and were not considered in our analysis. Except for butylparaben and BPS detected in 25% of the samples, frequencies of detection for the other compounds were above 83% (Table 3).

Distribution of Serum Thyroid Hormones

For TSH, 95% of the women ($n = 414$) were within the reference range defined for pregnant women by the French Health Authority (0.358 to 2.500 mUI/L for samples collected during the first trimester and 0.358 to 3.000 mUI/L for samples collected later in pregnancy). Median iodine urinary concentration assessed in urine daily pools was relatively low (89 µg/L; Table 4), and

Table 5. Summary of information extracted from the U.S. EPA ToxCast screening library (https://comptox.epa.gov/dashboard/chemical_lists/TOXCAST, version 3.3; accessed November 2020).

	Peripheral TH metabolism[a]	TH synthesis			Induction of xenobiotic receptor (liver)					Hypothalamic-pituitary feedback		TR transactivation	
Compound	DIO inhibition[a]	TSHR binding	TPO inhibition	NIS inhibition	CAR	AhR	PPAR	PXR	UDPGT1A1	TR_β	TRHR	TR_α	TR_β
Triclosan	X	—	X	X	X	X	X	X	—	X[b]	X[c]	—	X[b]
Methylparaben	NA	—	NA	NA	—	—	—	—	—	—	—	—	—
Ethylparaben	NA	—	NA	NA	—	—	—	—	—	—	—	—	—
Butylparaben	NA	—	NA	—	X	—	X	X	—	—	—	—	—
Propylparaben	NA	—	NA	NA	X	—	X	X	—	—	—	—	—
Benzophenone-3	NA	—	—	NA	X	X	X	X	NA	—	—	—	—
Bisphenol S	NA	—	—	NA	—	—	X	—	NA	—	—	—	—
Bisphenol A	NA	X[d]	X	X	X	—	—	X	—	X[b]	—	—	X[b]
Diethyl phthalate	NA	—	NA	NA	—	—	—	—	—	—	—	—	—
Benzylbutyl phthalate	NA	—	—	X	X	—	X	X	—	—	—	—	—
Di(2-ethylhexyl)phthalate	NA	—	NA	NA	—	—	—	X	X	—	—	X[b]	—
Dibutyl phthalate	NA	—	NA	X	—	—	—	X	—	—	—	—	—
Diisobutyl phthalate	NA	—	NA	X	—	—	X	—	—	—	—	—	—
Diisononylcyclohe1ane-1,2-dicarbo1ylate	NA	—	NA	NA	—	—	—	X	—	—	—	—	—
Diisononyl phthalate	NA	—	NA	NA	—	—	—	X	NA	—	—	—	—
Bis(2-propylheptyl) phthalate	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: ToxCast positive hitcalls were only considered if they matched the following criteria: Curves should have less than three flags; curves should display a sigmoidal shape; there should be more than one isolated data point above efficacy cut-off; and data for low concentrations should be present (i.e., AC_{50} should not have been extrapolated[35,36]). —, compound identified as non-bioactive *in vitro*; AC_{50}, activity concentration, 50%; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; DIO, iodothyronine deiodinase; MIE, molecular initiating event; NA, compound not assessed in ToxCast for this MIE; NIS, sodium–iodide symporter; PPAR, peroxisome proliferator–activated receptor; PXR, pregnane X receptor, TH, thyroid hormone; TPO, thyroperoxidase; TR, thyroid hormone receptor; TRHR, thyrotropin releasing hormone receptor; TSHR, thyroid stimulating hormone receptor; UDPGT, uridine diphosphate glucuronosyltransferase; U.S. EPA, U.S. Environmental Protection Agency; X, compound identified as bioactive *in vitro* (either antagonist or agonist activity).

[a]Includes inhibition of the three DIO isoforms (1, 2, and 3).

[b]Antagonists.

[c]Both agonist and antagonist.

[d]Agonist.

80% of the women had a iodine urinary concentration lower than the WHO guideline for pregnant women.[45]

Tier 1: Relying on a Thyroid AOP Network and an Existing In Vitro *HTS Database to Select Phenols and Phthalates*

Three compounds (methylparaben, ethylparaben, and diethyl phthalate) were not bioactive at any of the thyroid-related MIEs evaluated and so were not included in the statistical analysis (Table 5; Supplemental Material, Excel Table S1). Among the phenols and phthalates assessed in SEPAGES, one phthalate, [bis (2-propylheptyl) phthalate (DPHP)] was not screened for thyroid activity in ToxCast. We nevertheless retained its metabolite in the tier-2 statistical analysis, based on thyroid toxicity (i.e., thyroid gland hypertrophy/hyperplasia) reported in adult male rats in a two-generation reproductive toxicity study.[49]

This selection step allowed us to restrict our set of 16 (13 individual compounds and 3 molar sums) initially considered chemicals to 13, leading to a reduction in the number of tests performed in our main analysis (Table 6) by approximatively 19%.

Tier 2: Associations of Selected Phenol and Phthalate Metabolites with Thyroid Hormone Concentrations

Parabens. Propylparaben was negatively associated with the T3/T4 ratio [$\beta = -0.5\%$ (95% confidence interval (CI): −0.9, −0.1) for each doubling in propylparaben concentration]. This compound also tended to be negatively associated with TSH ($\beta = -1.4\%$; 95% CI: −2.8, 0.1) and free T3 ($\beta = -0.3\%$; 95% CI: −0.6, 0.0). Based on model residuals, we identified three individuals with low TSH concentrations (≤ 0.2 mUI/L) that may drive the association with this hormone. Their exclusion indeed led to an attenuated effect estimate for the association between propylparaben and TSH: $\beta = -0.9\%$; 95% CI: −2.1, 0.4, Supplemental Material, Table S1.

Other phenols. The analysis relying on exposure biomarkers categorized in tertiles showed a negative association between BPA and TSH that decreased by 6.8% (95% CI: −19.5, 7.8) and 16.3% (95% CI: −27.8, −3.0) in the second and third concentration tertiles in comparison with the first (Table 7). For triclosan, estimates were suggestive of a U-shaped association with TSH, that decreased by 21.3% (95% CI: −31.7, −9.4) and 9.1% (95% CI: −21.4, 5.1) in the second and third triclosan concentration tertiles, respectively in comparison with the first.

We did not observe associations between benzophenone-3, butylparaben, BPS, and thyroid hormone concentrations in our main analysis.

Phthalates. Monobenzyl phthalate monobutyl phthalate (minor) (MBzP), a metabolite of butylbenzyl phthalate (BBzP), was positively associated with total T4 ($\beta = 1.3\%$, 95% CI: 0.0, 2.6). This metabolite also tended to be negatively associated with the T3/T4 ratio that on average decreased by 1.4% (95% CI: −2.9, 0.2) for each doubling in MBzP urinary concentration (Table 6). Mono-6-hydroxy-propylheptyl phthalate (oh-MPHP), a metabolite of DPHP, was negatively associated with TSH ($\beta = -7.4\%$, 95% CI: −13.8, −0.4). However as for propylparaben, this association was driven by the three individuals with the lowest TSH values [β of -2.6% (95% CI: −8.3, 3.4) after exclusion]. No other phthalate metabolite was associated with thyroid hormones in our main analysis (Table 6).

Additional analysis restricted to women for which serum sample was collected at the end of urine collection. Restricting our analysis to the 373 women who had their blood withdrawn at the end of the urine collection week did not strongly impact the results. As expected by the sample size decrease, *p*-values increased slightly, but effect sizes generally were similar except for butylparaben, which was negatively associated with the T3/

Table 6. Adjusted associations between phenol and phthalate metabolite concentrations and thyroid hormones (ln-transformed) in the SEPAGES cohort.

	TSH (mUI/L, $n=437$)			Total T4 (ng/mL, $n=437$)			Free T4 (pg/mL, $n=435$)			Total T3 (ng/mL, $n=405$)			Free T3 (pg/mL, $n=437$)			Ratio T3/T4 ($n=405$)		
	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value
Bisphenol A	−4.3	[−8.9, 0.6]	0.08	0.2	[−0.9, 1.4]	0.73	0.0	[−1.2, 1.3]	0.95	0.7	[−0.7, 2.1]	0.35	−0.1	[−1.2, 0.9]	0.82	0.1	[−1.2, 1.5]	0.84
Bisphenol S[b]	1.7	[−11.4, 16.7]	0.81	0.2	[−2.9, 3.4]	0.92	1.6	[−1.9, 5.1]	0.37	2.4	[−1.6, 6.5]	0.24	1.4	[−1.4, 4.4]	0.32	1.4	[−2.4, 5.3]	0.47
Triclosan	0.0	[−2.1, 2.1]	0.99	−0.1	[−0.6, 0.4]	0.73	−0.2	[−0.7, 0.4]	0.56	−0.3	[−0.9, 0.3]	0.28	0.0	[−0.4, 0.5]	0.89	−0.2	[−0.8, 0.4]	0.49
Propyl paraben	−1.4	[−2.8, 0.1]	0.07	0.3	[−0.1, 0.6]	0.15	0.2	[−0.1, 0.6]	0.20	−0.2	[−0.6, 0.3]	0.43	−0.3	[−0.6, 0.0]	0.07	−0.5	[−0.9, −0.1]	0.03
Butylparaben[b]	−2.7	[−15.4, 11.9]	0.70	1.1	[−2.1, 4.4]	0.52	−0.2	[−3.7, 3.5]	0.93	−1.6	[−5.5, 2.5]	0.45	−2.0	[−4.8, 1.0]	0.19	−3.2	[−6.9, 0.7]	0.10
Benzophenone 3	−0.2	[−2.9, 2.5]	0.87	0.1	[−0.5, 0.8]	0.66	−0.2	[−0.9, 0.5]	0.55	−0.1	[−0.9, 0.6]	0.71	−0.5	[−1.1, 0.1]	0.08	−0.2	[−1.0, 0.5]	0.57
MBzP	−1.0	[−6.5, 4.7]	0.72	1.3	[0.0, 2.6]	0.04	0.7	[−0.7, 2.1]	0.34	0.2	[−1.4, 1.9]	0.82	−0.3	[−1.4, 0.9]	0.65	−1.4	[−2.9, 0.2]	0.08
MiBP	−1.5	[−8.1, 5.6]	0.67	0.9	[−0.7, 2.5]	0.28	0.3	[−1.4, 2.0]	0.74	0.2	[−1.8, 2.2]	0.86	0.4	[−1.0, 1.8]	0.59	−0.6	[−2.5, 1.3]	0.50
MnBP	−3.7	[−10.5, 3.6]	0.31	1.1	[−0.5, 2.8]	0.18	0.0	[−1.8, 1.8]	1.00	0.7	[−1.4, 2.8]	0.52	0.9	[−0.6, 2.5]	0.25	−0.6	[−2.6, 1.5]	0.58
oh-MPHP	−7.4	[−13.8, −0.4]	0.04	−0.2	[−1.9, 1.4]	0.78	0.4	[−1.4, 2.3]	0.65	−0.1	[−2.1, 1.9]	0.89	−0.1	[−1.6, 1.4]	0.87	−0.3	[−2.2, 1.6]	0.75
ΣDiNP	−2.6	[−7.6, 2.6]	0.32	0.0	[−1.2, 1.2]	0.99	0.7	[−0.6, 2.1]	0.28	0.3	[−1.1, 1.9]	0.65	0.5	[−0.5, 1.6]	0.33	0.2	[−1.3, 1.6]	0.83
ΣDINCH	−1.3	[−5.6, 3.1]	0.55	−0.4	[−1.4, 0.6]	0.43	0.2	[−0.9, 1.3]	0.73	0.1	[−1.1, 1.4]	0.86	−0.2	[−1.1, 0.7]	0.64	0.3	[−0.9, 1.5]	0.64
ΣDEHP	−5.3	[−11.7, 1.5]	0.12	0.5	[−1.1, 2.1]	0.57	0.8	[−1.0, 2.6]	0.39	0.2	[−1.7, 2.3]	0.82	0.6	[−0.8, 2.1]	0.41	−0.6	[−2.5, 1.3]	0.53

Note: Analyses were adjusted for maternal age, BMI before pregnancy, education level, maternal smoking during the first trimester of pregnancy, parity, gestational age at serum collection, time of serum collection, and maternal urinary iodine concentrations and selenium concentrations in sera during pregnancy. Models were also adjusted for analytical batch for all hormones but TSH, for which no batch effect was detected. Effect estimates represent percent change in outcomes for each doubling of urinary biomarker concentrations except for butylparaben and BPS, for which effect estimates represent percent change in outcomes between those with undetected and detected urinary concentrations. Percentage changes were obtained from the original betas using the following formulae: $(2^{\beta}-1)\times 100$ for continuous exposures and $(e^{\beta}-1)\times 100$ for categorial exposures (butylparaben and BPS). BMI, body mass index; BPS, bisphenol S; CI, confidence interval; DEHP, di(2-ethylhexyl) phthalate; DINCH, di(isononyl)cyclohexane-1,2-dicarboxylate; DiNP, diisononyl phthalate; MBzP, Monobenzyl phthalate; MiBP, monoisobutyl phthalate; MnBP, mono-*n*-butyl phthalate; oh-MPHP, mono-6-hydroxy-propylheptyl phthalate; ΣDEHP, molar sum of DEHP metabolites; ΣDiNP, molar sum of DiNP metabolites; ΣDINCH, molar sum of DiNCH metabolites; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

[a]Expressed as percentage change in the studied outcome.

[b]Categorized as follows: undetected/detected.

T4 ratio (β = −4.1%; 95% CI: −8.0, 0.0) and free T3 (β = −2.9%; 95% CI: −6.0, 0.3). A negative association between benzophenone-3 and free T3 also appeared (β = −0.6%; 95% CI: −1.2, 0.0) (Supplemental Material, Table S2).

Interaction with iodine levels. Interaction with iodine levels ($p_{interaction} < 0.10$) was observed for several exposure–thyroid hormone pairs (Supplemental Material, Table S3). After stratification for iodine status, a negative association between ΣDEHP and TSH emerged among women with normal iodine concentration (Table 8). In the normal iodine group, we also observed a positive association between MnBP and total T3 (β = 5.6%; 95% CI: −0.1, 11.7) and free T3 (β = 3.9% ; 95% CI: −0.5, 8.4; Table 8).

Joint effect. Analyses relying on BKMR suggested a negative association between the mixture of the 13 selected chemicals and the T3/T4 ratio (Figure 1; Supplemental Material, Table S4). These negative associations seemed to be driven by MBzP and propylparaben, the two compounds with the highest PIP (Figure 2; Supplemental Material, Table S5). They were both negatively associated with this ratio in the unipollutant model. Most of the other compounds were considered has noninfluential by BKMR (PIP and effect estimates of 0; Figure 2; Supplemental Material, Table S5). No association with the mixture was highlighted for the other hormones (Figure 1).

Discussion

Compound selection based on *in vitro* bioactivity allowed us to reduce the number of tests performed in our main analysis by approximatively 19% (from 16 to 13 compounds) and provides a biologically based screen to help limit the propagation of chance findings and false positives. Relying on within-subject pools of repeated urine samples collected during pregnancy, we highlight associations between individual prenatal exposures to several phenols and phthalates, as well as the mixture, and maternal thyroid hormone concentrations. Associations were mainly seen with serum TSH, free T3, and the T3/T4 ratio. Most of the observed associations were negative (either monotonic decrease or U-shaped associations). A few associations with ΣDEHP and MnBP were modulated by urinary iodine concentrations and observed only among participants with iodine concentrations above 150 μg/L. However, careful interpretation is required due to the relatively small numbers of women in this group ($n = 87$).

Parabens

Propylparaben was negatively associated with free T3 and the T3/T4 ratio. A similar pattern was observed for butylparaben in our analysis restricted to the 373 women for whom serum samples were collected at the end of the urine collection week. Propylparaben was also identified as a major contributor of the negative association observed between the mixture and T3/T4 ratio. Among the few studies that have explored associations between parabens and thyroid hormone concentrations during pregnancy,[50,51] only one assessed the T3/T4 ratio and did not report association for propylparaben and butylparaben.[50] To our knowledge, no study assessed free T3, limiting comparison with our results. Although studies in animal are also scarce, exposure to butylparaben has been shown to increase TPO activity and reduce DIO activity.[52] Regarding other potential mechanisms by which parabens may affect thyroid hormone homeostasis, the *in vitro* HTS data indicated activation of xenobiotic nuclear receptors (e.g., CAR, PXR) regulating expression of genes encoding metabolizing enzymes, which could in turn enhance thyroid hormone (Table 5).

Table 7. Adjusted associations between phenol and phthalate metabolite concentrations coded in tertiles and thyroid hormones (ln-transformed) in the SEPAGES cohort.

	TSH (mUI/L, $n=437$)			Total T4 (ng/mL, $n=437$)			Free T4 (ng/mL, $n=435$)			Total T3 (ng/mL, $n=405$)			Free T3 (ng/mL, $n=437$)			Ratio T3/T4 ($n=405$)		
	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value
Bisphenol A																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	−6.8	[−19.5, 7.8]	0.34	0.7	[−2.6, 4.1]	0.70	3.7	[0.0, 7.6]	0.05	2.7	[−1.5, 7.1]	0.21	0.2	[−2.8, 3.3]	0.90	1.3	[−2.7, 5.5]	0.52
Tertile 3	−16.3	[−27.8, −3.0]	0.02	1.9	[−1.5, 5.4]	0.28	2.7	[−1.0, 6.7]	0.16	2.6	[−1.7, 7.1]	0.24	1.2	[−1.9, 4.4]	0.44	−0.3	[−4.3, 3.9]	0.88
Triclosan																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	−21.3	[−31.7, −9.4]	0.00	−0.1	[−3.3, 3.2]	0.96	2.0	[−1.6, 5.7]	0.28	1.1	[−2.9, 5.4]	0.59	2.0	[−1.0, 5.1]	0.18	0.9	[−3.0, 5.0]	0.64
Tertile 3	−9.1	[−21.4, 5.1]	0.20	0.1	[−3.2, 3.5]	0.95	0.3	[−3.3, 4.1]	0.87	−1.6	[−5.6, 2.6]	0.45	0.8	[−2.2, 3.9]	0.60	−1.4	[−5.3, 2.7]	0.49
Propylparaben																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	3.4	[−10.6, 19.6]	0.65	2.6	[−0.7, 6.1]	0.12	3.3	[−0.4, 7.2]	0.08	1.7	[−2.4, 6.0]	0.42	−1.2	[−4.1, 1.9]	0.45	−1.4	[−5.2, 2.6]	0.50
Tertile 3	−13.7	[−25.6, 0.2]	0.05	2.5	[−1.0, 6.2]	0.16	2.4	[−1.4, 6.4]	0.22	−1.8	[−6.0, 2.6]	0.42	−2.8	[−5.8, 0.3]	0.08	−4.3	[−8.3, −0.2]	0.04
Benzophenone 3																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	−11.2	[−23.3, 2.8]	0.11	1.5	[−1.8, 4.9]	0.39	2.9	[−0.8, 6.7]	0.12	1.6	[−2.6, 5.9]	0.46	2.4	[−0.7, 5.5]	0.13	1.5	[−2.5, 5.6]	0.48
Tertile 3	−5.4	[−18.3, 9.5]	0.46	0.6	[−2.7, 4.0]	0.73	−0.5	[−4.1, 3.2]	0.78	0.3	[−3.9, 4.6]	0.90	−0.8	[−3.7, 2.3]	0.61	−0.1	[−4.1, 4.1]	0.97
MBzP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	4.3	[−9.9, 20.6]	0.57	1.2	[−2.1, 4.6]	0.46	1.6	[−2.1, 5.3]	0.40	−1.2	[−5.2, 3.0]	0.56	0.8	[−2.2, 3.9]	0.59	−2.5	[−6.3, 1.4]	0.21
Tertile 3	−1.3	[−14.6, 14.1]	0.86	2.8	[−0.5, 6.3]	0.10	3.7	[0.0, 7.6]	0.05	−1.7	[−5.7, 2.5]	0.43	0.0	[−3.0, 3.1]	0.99	−5.0	[−8.7, −1.1]	0.01
MiBP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	6.8	[−7.8, 23.8]	0.38	1.3	[−2.0, 4.8]	0.43	1.6	[−2.1, 5.5]	0.39	−1.0	[−5.1, 3.3]	0.64	−0.6	[−3.6, 2.5]	0.72	−1.8	[−5.7, 2.3]	0.38
Tertile 3	0.1	[−13.8, 16.1]	0.99	1.4	[−2.0, 4.9]	0.42	1.2	[−2.5, 5.0]	0.54	−2.1	[−6.2, 2.1]	0.32	0.1	[−3.0, 3.2]	0.95	−2.9	[−6.8, 1.1]	0.15
MnBP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	5.4	[−9.0, 22.1]	0.48	3.2	[−0.2, 6.7]	0.06	−1.0	[−4.6, 2.7]	0.58	0.3	[−3.8, 4.6]	0.88	−1.0	[−4.0, 2.0]	0.51	−1.9	[−5.8, 2.1]	0.35
Tertile 3	0.7	[−13.2, 16.9]	0.93	1.3	[−2.1, 4.7]	0.47	−0.5	[−4.1, 3.3]	0.81	0.9	[−3.4, 5.3]	0.70	0.8	[−2.3, 4.0]	0.62	−0.5	[−4.5, 3.7]	0.81
oh-MPHP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	−7.7	[−20.4, 6.9]	0.28	−1.3	[−4.6, 2.1]	0.43	1.1	[−2.6, 4.9]	0.58	3.0	[−1.2, 7.5]	0.16	2.2	[−0.9, 5.4]	0.17	3.4	[−0.7, 7.7]	0.11
Tertile 3	−8.8	[−21.3, 5.8]	0.22	−1.3	[−4.6, 2.1]	0.44	0.7	[−3.0, 4.5]	0.72	0.9	[−3.3, 5.2]	0.70	1.6	[−1.4, 4.8]	0.30	0.8	[−3.2, 5.0]	0.70
ΣDiNP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	−0.9	[−14.4, 14.7]	0.90	0.5	[−2.8, 3.9]	0.79	0.8	[−2.8, 4.6]	0.67	−0.5	[−4.6, 3.7]	0.80	2.0	[−1.0, 5.2]	0.19	−1.1	[−5.0, 3.0]	0.60
Tertile 3	−1.5	[−15.0, 14.3]	0.85	−0.3	[−3.6, 3.2]	0.88	1.6	[−2.1, 5.4]	0.41	0.0	[−4.2, 4.4]	0.99	2.1	[−1.0, 5.3]	0.19	0.1	[−4.0, 4.3]	0.98
ΣDINCH																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	3.2	[−10.9, 19.5]	0.67	−0.4	[−3.7, 3.1]	0.84	−1.5	[−5.1, 2.2]	0.41	−0.7	[−4.9, 3.6]	0.74	−0.5	[−3.5, 2.6]	0.74	−0.2	[−4.3, 4.0]	0.92
Tertile 3	−6.9	[−19.8, 7.9]	0.34	−0.5	[−3.8, 2.9]	0.77	1.0	[−2.7, 4.8]	0.59	0.2	[−4.0, 4.6]	0.92	0.5	[−2.5, 3.7]	0.74	0.0	[−4.0, 4.2]	1.00
ΣDEHP																		
Tertile 1	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—
Tertile 2	2.5	[−11.7, 18.9]	0.75	0.0	[−3.3, 3.5]	1.00	2.0	[−1.8, 5.9]	0.31	2.1	[−2.1, 6.5]	0.34	0.0	[−3.1, 3.1]	0.99	0.3	[−3.7, 4.5]	0.87
Tertile 3	−5.0	[−17.9, 9.9]	0.49	−0.1	[−3.4, 3.4]	0.97	2.0	[−1.7, 5.9]	0.29	0.5	[−3.7, 4.8]	0.82	1.2	[−1.9, 4.3]	0.45	−0.8	[−4.8, 3.3]	0.70

Note: Effect estimates represent percent change in outcomes. Percentage changes were obtained from the original betas using the following formula: $(e^{\beta}-1)\times 100$. Analyses were adjusted for maternal age, BMI before pregnancy, education level, maternal smoking during the first trimester of pregnancy, parity, gestational age at serum collection, time of serum collection, maternal urinary iodine concentrations and selenium concentrations in sera during pregnancy. Models were also adjusted for analytical batch for all hormones but TSH for which no batch effect was detected. —, reference; BMI, body mass index; CI, confidence interval; DEHP, di(2-ethylhexyl) phthalate; DINCH, di(isononyl)cyclohexane-1,2-dicarboxylate; DiNP, diisononyl phthalate; MBzP, monobenzyl phthalate; MiBP, monoisobutyl phthalate; MnBP, mono-*n*-butyl phthalate; oh-MPHP, mono-6-hydroxy-propylheptyl phthalate; ΣDEHP, molar sum of DEHP metabolites; ΣDiNP, molar sum of DiNP metabolites; ΣDINCH, molar sum of DINCH metabolites; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

[a]Expressed as percent change in the studied outcome.

Table 8. Adjusted associations between phenols, phthalate metabolites, and thyroid hormones (ln-transformed) stratified for iodine urinary levels.

	TSH (mUI/L)			Total T4 (ng/mL)			Total T3 (ng/mL)			Free T3 (ng/mL)			Ratio T3/T4		
	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value	β[a]	95% CI	*p*-Value
Triclosan															
Iodine <150 μg/L	—	—	—	−0.3	[−0.89, 0.19]	0.21	—	—	—	—	—	—	—	—	—
Iodine ≥150 μg/L	—	—	—	1.0	[−0.09, 2.15]	0.07	—	—	—	—	—	—	—	—	—
MBzP															
Iodine <150 μg/L	—	—	—	—	—	—	−0.8	[−2.55, 1.02]	0.39	−1.0	[−2.25, 0.27]	0.12	—	—	—
Iodine ≥150 μg/L	—	—	—	—	—	—	1.7	[−2.77, 6.39]	0.45	1.6	[−1.79, 5.12]	0.35	—	—	—
MnBP															
Iodine <150 μg/L	—	—	—	—	—	—	−0.6	[−2.85, 1.79]	0.64	0.2	[−1.48, 1.83]	0.85	−1.6	[−3.83, 0.62]	0.15
Iodine ≥150 μg/L	—	—	—	—	—	—	5.6	[−0.08, 11.70]	0.05	3.9	[−0.48, 8.38]	0.08	2.0	[−3.23, 7.62]	0.45
ΣDEHP															
Iodine <150 μg/L	−2.6	[−9.98, 5.44]	0.52	—	—	—	—	—	—	—	—	—	—	—	—
Iodine ≥150 μg/L	−20.5	[−32.80, −6.02]	0.01	—	—	—	—	—	—	—	—	—	—	—	—

Note: Stratified analyses were only conducted for the biomarker outcome pairs for which an interaction with iodine status was detected (*p*-values for the interaction term biomarker – iodine <0.01; all *p*-values for interaction are displayed in Supplemental Material, Table S3). —, not computed; CI, confidence interval; ΣDEHP, molar sum of DEHP metabolites; MBzP, monobenzyl phthalate; MnBP, mono-*n*-butyl phthalate; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

[a]Expressed as percentage change in the studied outcome.

Other Phenols

When categorized in tertiles, BPA was negatively associated with TSH. This result was in line with those of two previous studies,[53,54] whereas for one the association was seen only among women with a prepregnancy BMI >23 kg/m^2.[54] Five other human studies reported null associations with TSH.[50,55–58] We did not observe any association between BPA and the other hormones assessed nor with the T3/T4 ratio, whereas a few previous studies did.[50,55,57] Changes in thyroid hormone concentrations have also been reported in pregnant females (and/or their offspring) following low-dose exposures in experimental animal models.[59–61] These results, along with ToxCast data indicating BPA bioactivity on several relevant MIEs in the thyroid pathway, strengthen the overall body of evidence of its potential to perturb thyroid hormone signaling during sensitive developmental periods.

We did not observe any association for BPS. One study, with a bigger sample size and a higher frequency of detection than ours, reported a positive association between BPS and total T4.[56]

We observed a non-monotonic decrease in TSH concentrations with increased triclosan urinary concentrations. Wang et al. also reported a U-shape association with TSH,[62] whereas other studies report no association with TSH[50,51,57]; however, only one has explored nonmonotonic associations.[57] Mechanisms by which triclosan may affect thyroid hormone homeostasis include inhibiting DIO, TPO, and NIS, binding TR_β, and activating xenobiotic nuclear receptors (Table 5), which generally aligns with other mechanistic evidence.[63–68] Additionally, an *in vitro* screening assay indicated some capacity for triclosan to inhibit iodotyrosine deiodinase involved in iodide recycling in the thyroid gland.[69]

Phthalates

MBzP, a metabolite of BBzP, was positively associated with total T4 and negatively with the T3/T4 ratio. MBzP was also one of the major contributors of the negative association observed between the mixture and T3/T4 ratio. To the best of our knowledge, associations with the T3/T4 ratio has been evaluated in only two studies.[10,70] Consistent with our results, one reported a negative association,[70] whereas the other did not.[10] Our results for total T4 are not aligned with previous studies that reported a negative[9] or no association with T4.[10,43,70] BBzP can up-regulate the transcriptional activity of NIS.[71] Consistent with results herein, it is possible up-regulation of NIS may prompt increased iodine intake into the thyroid and increased thyroid hormone production. The fact that we observed a positive association only with total T4 and not total T3 might partly be related to difference in half-lives across hormones with T3 (1 d) having shorter half-life than T4 (5 to 7 d).

Effect modification by iodine concentrations were observed for two phthalates, ΣDEHP and MnBP. Only Villanger et al. examined interactions with iodine concentrations.[44] That study relied on a factor analysis and reported interactions between iodine status and the factor containing MnBP along with MiBP and MBzP. However, associations were observed for TSH and total and free T4, whereas we observed associations with total and free T3. Villanger et al. did not report interactions with the factor containing ΣDEHP.

Use of AOP Network and In Vitro *HTS to Select Phenols and Phthalates*

In comparison with an agnostic approach, the hypothesis-driven selection of phenols and phthalates using the thyroid AOP network and *in vitro* HTS assays allowed for the reduction of the number of statistical tests performed and limited FWER. However, the complexity of the thyroid system that includes tightly controlled compensatory signaling, and differences in pharmacokinetic

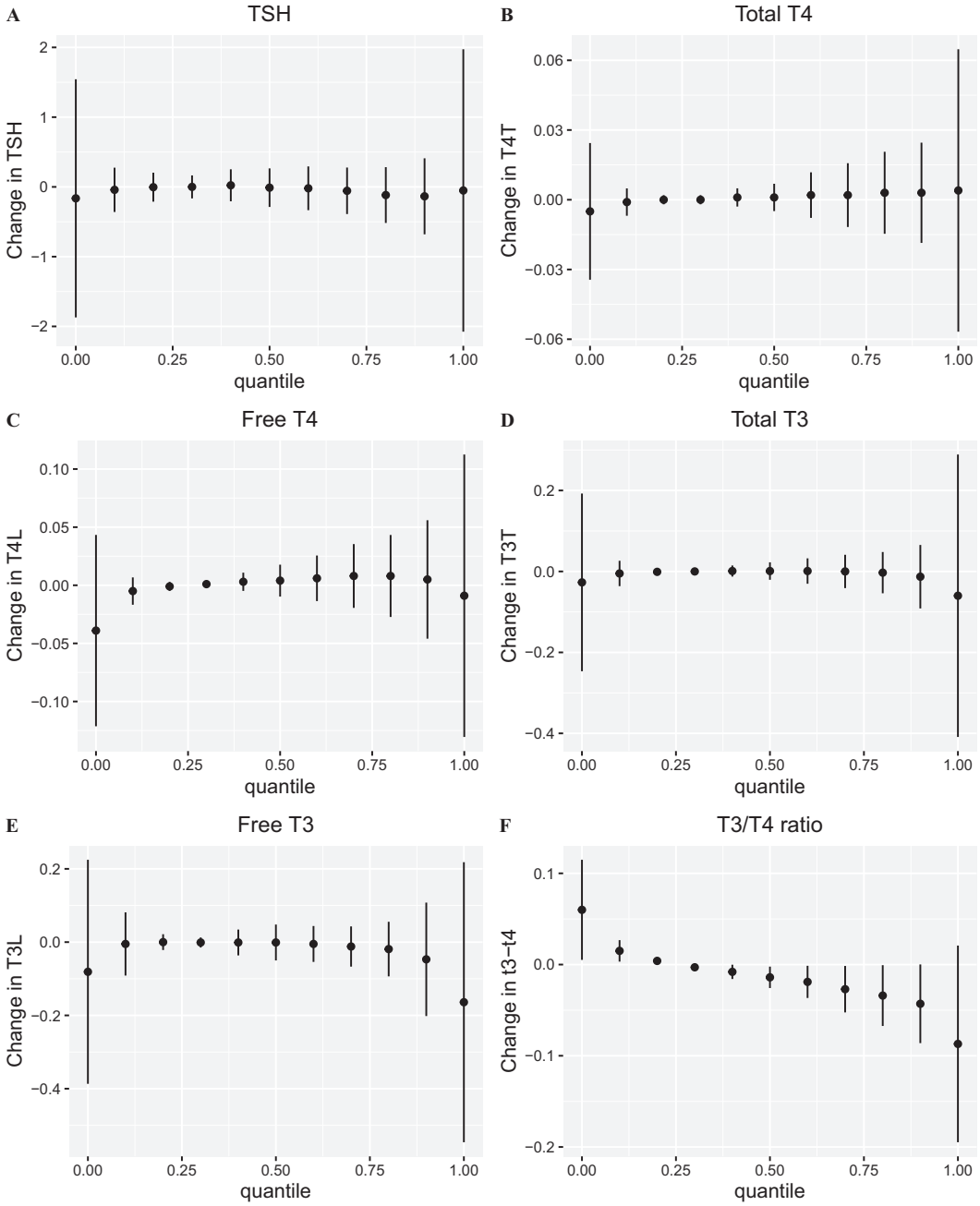

Figure 1. Expected changes and 95% CIs in (A) TSH, (B) total T4, (C) free T4, (D) Total T3, (E) free T3, and (F) T3/T4 ratio associated with concurrently increasing quantiles of all exposure biomarkers, relative to when all concentrations are fixed at their 25th percentile. Note: Numerical value of effect estimates and 95% CIs are reported in Supplemental Material, Table S4. Analyses were adjusted for maternal age, BMI before pregnancy, education level, maternal smoking during the first trimester of pregnancy, parity, gestational age at serum collection, time of serum collection, maternal urinary iodine concentrations and selenium concentrations in sera during pregnancy. Models were also adjusted for analytical batch for all hormones but TSH for which no batch effect was detected. BMI, body mass index; CI, confidence interval; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

parameters *in vivo* and *in vitro*, make predictions with *in vitro* screening data a challenge. Adaptive responses at higher levels of biological organization also may impart some protection to chemicals, with *in vitro* assays typically predicting effects at lower dose levels (more sensitive). This complexity may explain why the direction of associations observed in our study are not always aligned with those described in AOPs for the targeted MIEs. In addition, although several of the phthalates and phenols evaluated in this study activated xenobiotic nuclear receptors (and the mechanistic literature suggests a role for up-regulated T4 catabolism pathways), the performance of these and other assays in predicting effects at putative targets in the thyroid hormone conjugation and excretion pathway is an area of ongoing study. In addition, the ToxCast *in vitro* HTS assays rely on testing individual chemicals, so the potential role of chemical mixtures in affecting thyroid regulation was not considered in chemical selection. Finally, it is possible that a direct chemical effect on another biological target (e.g., immune system/inflammation[72]) may prompt secondary effects on thyroid regulation that in turn elicit effects elsewhere in the body, depending on the life stage, timing, severity, and duration of the hormonal disruption. By filtering for compounds with bioactivity at thyroid MIEs, we may have excluded chemicals that elicit thyroid disruption by secondary pathways. Nonetheless, although beyond the scope of this study, integration of biological pathway

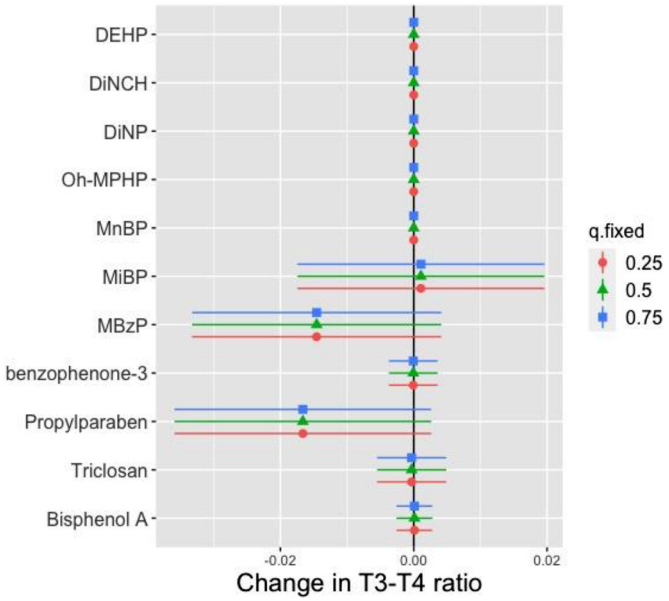

Figure 2. Estimated effect and 95% CI of an increase from the 25th to 75th percentile in a single biomarker concentration on T3/T4 ratio when all other exposure biomarkers are fixed at either the 25th, 50th, or 75th percentiles. Note: Numerical value of effect estimates and 95% CI are reported in Supplemental Material, Table S5. Analyses were adjusted for maternal age, BMI before pregnancy, education level, maternal smoking during the first trimester of pregnancy, parity, gestational age at serum collection, time of serum collection, maternal urinary iodine concentrations and selenium concentrations in sera during pregnancy. Models were also adjusted for analytical batch for all hormones but TSH for which no batch effect was detected. BMI, body mass index; CI, confidence interval; DEHP, di(2-ethylhexyl) phthalate, DINCH, di(isononyl)cyclohexane-1,2-dicarboxylate; DiNP, Diisononyl phthalate; ΣDEHP, molar sum of the five DEHP metabolites; ΣDINCH, molar sum of the two DINCH metabolites; ΣDiNP, molar sum of the three DiNP metabolites; MBzP, monobenzyl phthalate; MiBP, monoisobutyl phthalate; MnBP, mono-*n*-butyl phthalate; oh-MPHP, mono-6-hydroxy-propylheptyl phthalate; T3, triiodothyronine; T4, thyroxine.

models such as the AOP network approaches used herein provide a framework from which to begin capturing some of these more complex modes of action and effects of thyroid hormone reductions in pregnant women exposed to environmental chemicals. The approach herein allowed for the selection of chemicals with increased biological plausibility for interacting with MIEs in the thyroid pathway and thus provided a useful application in mixture and exposome studies that test larger sets of chemicals.

Other Strengths and Limitations

We relied on within-subject pools of repeated urine samples collected over a week to assess exposure. This approach is of importance for compounds with high intra-individual variability, such as BPA and DEHP metabolites (intraclass correlation coefficients of about 0.2–0.3[11–13]). For such compounds, reliance on multiple urine samples during relevant time windows should lead to decreased measurement error and, for a given sample size, increased power.[15] SEPAGES correlation coefficients between two non-consecutive weeks of pregnancy was still relatively low for some compounds,[23,48] highlighting the need to collect repeated biospecimens in sensitive time windows. For most women (85%), urine samples were collected the week preceding blood draws, allowing for evaluation of short-term effects of phenols and phthalates. In addition to thyroid hormone concentrations, we also explored associations with the T3/T4 ratio. Although clinically relevant, this indicator, which was negatively associated with MBzP, propylparaben, and butylparaben, as well as the mixture, has not been extensively studied in association with prenatal exposure to phenols and phthalates. In our study population, 80% of the women were below the WHO threshold for iodine deficiency. Such low iodine concentrations have been previously described among pregnant women from France[73,74] and other European countries.[75] Further studies should consider this essential element, given results here and among other researchers[44] that effects of some phthalates and phenols on the thyroid may be modulated by iodine status.

Although we accounted for many potential confounders, residual confounding cannot be ruled out. There are indeed other synthetic chemicals known to disrupt thyroid functioning that were not assessed in our study, and that may have confounded the observed associations (e.g., halogenated BPA compounds such as tetrabromobisphenol A). In addition, we did not assess thyroid antibodies, which might be important predictors of thyroid hormone concentrations. We also did not recruit women early enough to assess thyroid hormones during the first trimester of pregnancy, a key period during which the fetal thyroid gland is immature and the fetus is dependent on maternal sources of thyroid hormone. Despite our chemicals' *a priori* selection, the number of associations tested was still high. We did not apply any formal correction for multiple comparisons. This and the fact that *in vitro* data may not always be predictive of *in vivo* biological events suggest that part of the associations we observed may still have resulted from chance findings and thus should be interpreted cautiously.

Conclusion

Relying on pools of multiple urine samples and on a novel hypothesis-driven method to reduce false positives and chance findings, we observed negative associations between several phenols and phthalates and TSH, free T3, and the T3/T4 ratio. Given widespread exposure to these compounds in the general population and the crucial role of thyroid hormones in development, the impact on fetal and child health might be substantial.

Acknowledgments

The SEPAGES study group includes: E. Eyriey, A. Licinia, A. Vellement (Groupe Hospitalier Mutualiste, Grenoble), I. Pin, P. Hoffmann, E. Hullo, C. Llerena (Grenoble University Hospital, La Tronche), X. Morin (Clinique des Cèdres, Echirolles), A. Morlot (Clinique Belledonne, Saint-Martin d'Hères), J. Lepeule, S.L.-C., C.P., I.P., J. Quentin, V. Siroux, and R.S. (Inserm, CNRS, University Grenoble Alpes IAB research center).

The authors acknowledge M. Rolland and K. Supernant for data management; P. Jedynak, O. Coiffier, and M. Ouidir for their help with the implementation of the BKMR mixture model; L. Bajard for her help accessing and interpreting ToxCast data; and M. Barbagallo for her help downloading data from the ToxCast chemistry dashboard. The authors are also thankful to I. Druwe and H. Ru of the U.S. Environmental Protection Agency (U.S. EPA) for their helpful reviews of the manuscript.

This work was funded by ANSES (CNAP, EST-2016-121). D.N. was supported by a doctoral grant from Université Grenoble Alpes. The SEPAGES cohort was supported by the European Research Council (N°311765-E-DOHaD), the European Community's Seventh Framework Programme (FP7/2007-206 – N 308333-892 HELIX), the French Research Agency – ANR (ANR-19-CE36-0003-01; ANR-12-PDOC-0029-01, ANR-14-CE21-0007, ANR-18-CE36-005, ANR-15-IDEX-02, ANR-15-IDEX5 and GUMME project), the French Agency for Food, Environmental and Occupational Health & Safety – ANSES (EST-2019/1/039, EST-2016-121), the Plan Cancer (Canc'Air project), the French Cancer Research Foundation Association de Recherche sur le Cancer – ARC, the French Endowment Fund

AGIR for chronic diseases – APMC, PRENAPAR), the French Endowment Fund for Respiratory Health and the French Fund – Fondation de France (CLIMATHES – 00081169).

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