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Longitudinal Associations between Prenatal Exposure to Phthalates and Steroid Hormones in Maternal Hair Samples from the SEPAGES Cohort

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1 **Longitudinal associations between prenatal exposure to phthalates and steroid**
2 **hormones in maternal hair samples from the SEPAGES cohort**

3
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43 **Abstract**

44 We aimed to assess phthalate-hormone associations in the new-generation SEPAGES
45 cohort (2014-2017, France) using improved exposure and outcome assessments.
46 Metabolites from 7 phthalate compounds and the replacement di(isononyl)cyclohexane-
47 1,2-dicarboxylate (DINCH) were measured in within-subject pools of repeated urine
48 samples collected at the second and third pregnancy trimesters (≈ 21 samples/trimester).
49 Metabolites from 5 steroid hormones were measured in maternal hair samples collected
50 at delivery reflecting on cumulative levels over the previous weeks-months. Adjusted
51 linear regression and bayesian weighted quantile sum (BWQS) mixture models were
52 performed. Each doubling in third trimester urinary MBzP concentrations was
53 associated with average increases of 13.3% (95%CI: 2.65, 24.9) for \sum cortisol, 10.0%
54 (95%CI: 0.26, 20.7) for \sum cortisone, 17.3% (95%CI: 1.67, 35.4) for 11-
55 dehydrocorticosterone and 16.2% (95%CI: 2.20, 32.1) for testosterone, together with a
56 suggestive 10.5% (95%CI: -1.57, 24.1) increase in progesterone levels. Each doubling
57 in second trimester urinary di-isononyl-phthalate (\sum DiNP) concentrations was inversely
58 associated with testosterone levels (-11.6%; 95%CI: -21.6, -0.31). For most hormones, a
59 non-significant trend towards a positive phthalate mixture effect was observed at the
60 third but not the second trimester. Our study showed that exposure to some phthalate
61 metabolites, especially MBzP, may affect adrenal and reproductive hormone levels
62 during pregnancy.

63

64 **Keywords:** endocrine disruption, phthalate, DINCH, DiNP, MBzP, cortisol,
65 testosterone, pregnancy

66

67 **Synopsis (30 words max.):** This study implementing improved exposure and outcome
68 assessment methods showed that some phthalate metabolites can influence adrenal and
69 reproductive hormone levels during pregnancy.

70

71 **1. Introduction**

72 Maternal steroid hormone regulation during pregnancy is critical to maintain a
73 healthy pregnancy and achieve an optimal fetal development.^{1,2} For example, the
74 equilibrium between glucocorticoids (e.g., cortisol) and its precursor progesterone is
75 critical to maintain pregnancy and switch the maternal immune response towards fetal
76 tolerance.^{3,4} Contrastingly, a disequilibrium in progesterone and/or glucocorticoids
77 predisposes to inflammation, which may lead to placental insufficiency and poor fetal
78 growth.³ Regarding the hypothalamus-pituitary-adrenal (HPA) axis, corticotropin-
79 releasing hormone (CRH) and cortisol play a major role in the timing of labor, and have
80 been associated with prematurity and low birth weight in epidemiologic studies.^{2,5} In
81 relation to the hypothalamus-pituitary-gonadal (HPG) axis, women presenting higher
82 testosterone levels, such as cases of polycystic ovarian syndrome (PCOS) also show a
83 higher risk of preterm birth, fetal growth restriction and pregnancy complications.⁶⁻⁹
84 Overall, disruptions in steroid hormone levels during pregnancy may result in adverse
85 pregnancy and birth outcomes.³

86 Phthalates are a family of high-volume chemicals used in a wide range of
87 commercial products. However, some of them are considered endocrine disruptors and
88 can interfere with hormonal regulation.¹⁰ High molecular weight phthalates (> 250 Da;
89 ester side-chain lengths of five or more carbons) are employed as plasticizers in the
90 production of polyvinyl chloride (PVC) plastics, and are found in a variety of products
91 such as food contact materials,¹¹ building and construction materials (e.g., vinyl
92 flooring, floor tiles, wall coverings and furniture upholstery), medical devices (tubing,
93 catheters, blood/dialysis bags), and toys.^{12,13} Low molecular weight phthalates (< 250
94 Da; ester side-chain lengths of one to four carbons) are used as solubilizing agents in the
95 formulation of cosmetics and personal care products (e.g., fragrances), and as coatings

96 of some pharmaceuticals.¹³ Despite some regulations in Europe, recent birth cohorts and
97 biomonitoring studies show that phthalate exposure is still widespread with more than
98 90% of the European population showing detectable concentrations in urine.^{14,15} In
99 addition, newer phthalates such as bis(2-propylheptyl) phthalate (DPHP) and the non-
100 phthalate substitute 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH) have
101 recently entered the market, showing increasing concentrations in humans over time.^{14,16}
102 Since these new chemicals have a similar structure than those they replace and could
103 potentially exert deleterious effects,^{17,18} there is a need for continuous biomonitoring
104 and surveillance.^{19,20}

105

106 Phthalates and their metabolites have been shown to interfere with steroid
107 hormone regulation in laboratory animals through a variety of mechanisms that depend
108 on age and sex.^{10,21} Through the construction of an adverse outcome pathway (AOP)
109 network, Baken et al. has shown that exposure to specific phthalates (diethyl-hexyl
110 phthalate (DEHP), monoethyl-hexyl phthalate (MEHP) and dibutyl-phthalate (DBP)
111 among others) could be related to hormonal alterations and reproductive problems in
112 experimental animals.²¹ These effects depended on sex and exposure timing and can be
113 mediated through peroxisome-proliferator activated receptors and other pathways
114 implicating the estrogenic (ER) and glucocorticoid (GR) receptors.²¹ In addition to the
115 ability of some phthalates to interact with the (HPG) axis, the adrenal gland has recently
116 emerged as another target of phthalate toxicity, with DEHP and DBP being able to
117 influence adrenal gland histology and steroidogenesis in both male and female rats.^{22,23}
118 However, mechanistic data on the impact of phthalates on the HPA axis is still
119 limited.²⁴

120

121 The human evidence is very limited, with some studies in pregnant women
122 finding associations between specific phthalate metabolites and either increased or
123 decreased testosterone levels in maternal serum^{25,26} or urine,²⁷ and reduced serum
124 progesterone levels.^{25,28} One study showed increased cord blood cortisol levels related
125 to several phthalate metabolites among women carrying female fetuses, while reduced
126 cortisol levels were reported among those who carried male fetuses in association with
127 DEHP metabolites.²⁹ Another study found that prenatal exposure to DEHP metabolites
128 was associated with reduced cord blood cortisol and cortisone levels and increased
129 adrenal androgens.³⁰ These previous studies showed associations that varied by
130 exposure at different pregnancy trimesters and in some cases were sex-specific.

131

132 Limitations of previous observational studies include the assessment of phthalate
133 metabolites in spot urine samples, which leads to exposure misclassification and
134 attenuation bias due to the short-half life and large within-day and within-week
135 variability of phthalate metabolites.^{31,32} Another shortcoming is that most studies used a
136 unique or few blood^{25,26,28-30} or urine²⁷ sample(s) to evaluate hormone levels, since
137 many of them are influenced by circadian rhythms and time-specific confounders.^{33,34}
138 Distinct collection protocols or biospecimens are needed to provide complementary
139 information about these hormone levels over an extended period of time (i.e., weeks or
140 months) while being less influenced by circadian rhythms and transient stressors (e.g.,
141 allergies, colds, transient moods).

142 Our objective was to study the longitudinal associations between prenatal
143 exposure to phthalates and maternal steroid hormones in a new-generation cohort. We
144 relied on the prospective collection of multiple urine samples during pregnancy to
145 improve exposure characterization.^{31,32} In addition, we collected hair samples at

146 delivery that allowed to reliably measure cumulative levels of steroid hormones over the
147 previous weeks to months.³⁵⁻³⁷

148

149 **2. Methods**

150 2.1 Study population

151 We used data from the French mother-child cohort SEPAGES that recruited 484
152 pregnant women at eight obstetrical ultrasonography practices of the Grenoble
153 metropolitan area from July 2014 to July 2017. Inclusion criteria were: singleton
154 pregnancies up to gestational week 19, being older than 18 years, living in the
155 metropolitan area of Grenoble and planning to deliver in one of the four Grenoble
156 maternity clinics. The research protocol was presented to 3360 women, with an
157 eligibility and participation rate of 69% and 21%, respectively.³⁸

158 The current study included mothers with adrenal and reproductive hormones
159 assessed in hair samples collected at delivery and urinary phthalate metabolites
160 measured at the second trimester (T2) (N = 382) and third (T3) trimester (N = 378) of
161 pregnancy (Supporting Information Figure S1). The SEPAGES study was approved by
162 the Ethics Committee (CPP) Sud-Est V and the National Commission on Informatics
163 and Liberty (CNIL). After study staff explained all details to participants, pregnant
164 women signed the written informed consent.

165 2.2 Repeated urine collection and exposure assessment

166 Pregnant women were asked to collect 3 urine samples per day (morning,
167 midday and evening), for 7 consecutive days, twice during pregnancy: at the second
168 trimester [median (P25, P75) gestational age: 17 weeks (16-18)], and during the third
169 trimester [33 weeks (31-34)]. Women showed excellent compliance to the urine
170 collection protocol: the median (P25, P75) number of biospecimens collected per

171 pregnant woman was 21 (20, 21) samples/trimester at both T2 and T3 collection weeks.
172 Women stored urine samples in their freezer until the SEPAGES field workers picked
173 them up at the end of each collection week for transport to a certified biobank (ISO
174 9001 standard, Grenoble University Hospital, bb-0033-00069). For each of the two
175 prenatal urine collection weeks, within-subject pools were conducted by combining
176 equal volumes of all spot urine samples obtained over a collection week (Supporting
177 Information Figure S2). This pooling strategy, that does not consider urinary dilution of
178 each spot sample, was previously validated as a good proxy of the urine concentrations
179 that would have been obtained in the pool of the whole volume of all individual spot
180 samples collected.³⁹ We have indeed showed that, when an equal volume of each
181 individual urine sample is pooled, as was the case of the SEPAGES cohort,
182 standardization by specific gravity or creatinine is not needed, and can be even
183 counterproductive for some compounds such as bisphenol A.³⁹

184

185 Aliquots of weekly pools were stored at -80°C before being sent on dry ice
186 with a temperature sensor to the Norwegian Institute of Public Health (Oslo, Norway), a
187 certified European laboratory that has participated in recent inter-laboratory
188 comparisons.⁴⁰ The concentration of 13 phthalate and 2 non-phthalate plasticizer
189 metabolites were measured (Supporting Information Table S1) using high performance
190 liquid chromatography coupled to mass spectrometry.⁴¹ The limits of detection (LOD)
191 and quantification (LOQ) ranged from 0.07 to 0.4 ng/ml and 0.20-0.50 ng/ml,
192 respectively. Urine specific gravity was assessed in each weekly pool using a handheld
193 Atago PAL 10-S refractometer.

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196 2.3 Hair steroid hormones

197 Around delivery (1-3 days post-partum), at the maternity ward, mothers
198 collected their own maternal hair samples using material and protocol provided by
199 SEPAGES fieldworkers. Once collected, hair samples were stored at ambient
200 temperature by the maternity staff until it was transported to the biobank where they
201 were stored at -80°C. Hair samples from 408 SEPAGES mothers were sent to the
202 Applied Metabolomics Research Laboratory of the IMIM-Hospital del Mar Medical
203 Research Institute (Barcelona, Spain), where the first 3 cm (scalp side, corresponding to
204 the hair growth of the third trimester^{42,43}) were used to assess ten steroid hormones (5
205 hormones and 5 metabolites, Table 2) using a previously validated liquid
206 chromatography-tandem mass spectrometry (LC-MS/MS) methodology.⁴⁴ The
207 hormones and metabolites assessed were: cortisol and its metabolites 20 α -
208 dihydrocortisol and 20 β -dihydrocortisol; cortisone and its metabolites 20 α -
209 dihydrocortisone, 20 β -dihydrocortisone and β -cortolone; 11-dehydrocorticosterone;
210 testosterone; and progesterone. The selection of these hormones was based on their
211 reliable measurement in hair samples,⁴⁴ their relevance to pregnancy, and their
212 representativeness of both HPG and HPA hormonal axes. During the analytical
213 procedure, 2 samples broke during centrifugation and 10 had a sample weight below the
214 minimum required. Additionally, 12 samples did not pass analytical quality controls for
215 most hormones and were excluded from our analysis. Thus, data on steroid hormones
216 was available for 384 women. Figure S3 graphically depicts the relationships among
217 these hormones (Supporting Information).

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222 **2.4 Statistical Analysis**

223 2.4.1 Phthalate biomarkers

224 Concentrations below the LOD and between the LOD-LOQ were imputed by
225 values randomly selected between 0 and LOD and between LOD and LOQ,
226 respectively, considering the underlying distribution of concentrations.^{45,46}

227 If needed (i.e., sample processing and analytical conditions affecting the
228 measured concentrations), phthalate metabolite concentrations were standardized using
229 a two-step approach to correct for between-sample variations in the processing and
230 chemical analysis of urine samples.^{47,48} The following sampling conditions were
231 considered: time of urine sample transportation between the home of participants to the
232 biobank, the time that samples were maintained at 4 °C in the course of the pooling
233 process, and analytical batches. First, the associations between each natural log-
234 transformed phthalate biomarker and the three above-mentioned conditions were
235 estimated running separate linear regressions adjusted for maternal age, education, pre-
236 pregnancy body mass index (BMI), parity, date, season, pregnancy trimester of sample
237 collection and urinary specific gravity. Second, we used the measured phthalate
238 concentrations and the estimated effects of processing/assay conditions associated with
239 phthalate concentrations (p-value < 0.20) to predict standardized concentrations, that is,
240 concentrations that would have been obtained if all samples had been processed under
241 the same conditions and assayed in the same batch. Details have been described in
242 Guilbert et al., (2021) and Mortamais et al., (2012).^{47,48} Since high correlations
243 (Spearman rho >0.80) between these standardized and non-standardized phthalate
244 concentrations have been previously reported,⁴⁹ the current work provides descriptive
245 statistics only for standardized concentrations, which were used in all phthalate-

246 hormone analyses. In the case of phthalates metabolites belonging to a same parent-
247 compound, molar sums ($\mu\text{mol/L}$) were computed (i.e., $\sum\text{DEHP}$, sum of diisononyl
248 phthalate metabolites ($\sum\text{DiNP}$) and $\sum\text{DINCH}$). Urinary phthalate metabolite
249 concentrations were natural log-transformed to reduce the skewness of distributions and
250 the influence of extreme values.

251 2.4.2 Hormone biomarkers

252 Some steroids presented a moderate percentage of non-detected values (range
253 between 0 and 26%). As for exposure biomarkers, non-detects were imputed by values
254 randomly selected between 0 and the LOD, considering the estimated distribution of the
255 quantified hormone concentrations.^{45,46} Such method results in a balanced distribution
256 of imputed values, providing unbiased or minimally biased parameter estimates when
257 30% or less of data are below detection limits.⁴⁶

258 To reduce the number of statistical comparisons, we computed the molar sum of
259 cortisol and cortisone and their metabolites by summing their concentrations divided by
260 their molecular weight. Five outcomes were thus included in the main analysis:
261 $\sum\text{cortisol}$, $\sum\text{cortisone}$, 11-dehydrocorticosterone, testosterone and progesterone. In
262 addition, two hormones ratios were computed: the $\sum\text{cortisol}$ to $\sum\text{cortisone}$ ratio as an
263 indicator of corticoid activity that may be predictive of hypertensive disorders;⁵⁰ and the
264 testosterone to $\sum\text{cortisol}$ ratio as an indicator of the general (im)balance between the
265 mutually inhibiting HPG and HPA axes that has been related to antisocial and risk-
266 taking behaviors.⁵¹ The two ratios were considered as secondary outcomes to be
267 interpreted alongside with the primary outcomes. All hormones and ratios were natural-
268 log transformed to reduce the skewness of distributions.⁵¹

269

270 2.4.3 Covariates and confounding factors

271 Data on potential covariates were self-reported using questionnaires
272 administered by a fieldworker during study visits and clinical examinations.
273 Confounding factors and outcome predictors were selected *a priori* based on causal
274 knowledge using a Directed Acyclic Graph (DAG)⁵² (Supporting Information Figure
275 S4). All models were adjusted for: maternal age at conception (continuous), pre-
276 pregnancy body mass index (BMI, underweight/normal weight *vs.* overweight/obese
277 mothers), education level (below *vs.* Master's degree or above), active smoking during
278 pregnancy (smoking at any trimester *vs.* non-smoker), passive tobacco exposure
279 (exposed to indoor tobacco smoke ≥ 1 hour/week at any trimester *vs.* non-exposed),
280 parity (nulliparous *vs.* uniparous/multiparous), maternal anxiety/depression score at
281 third trimester using the French version of the Hospital Anxiety and Depression scale
282 (continuous),⁵³ infant sex and season of hair collection (winter *vs.* remaining seasons
283 since hair samples collected in winter showed significantly lower corticosteroid levels
284 compared to other seasons).

285 We additionally controlled for technical variables such as hair analysis batch
286 (dichotomous) and time elapsed (continuous) between hair sampling and hormone
287 measurement. The slight number of missing values for some covariates (see Table 1
288 footnote) was singly imputed with the variable median or mode value.

289 2.4.4 Associations between phthalates and hormones

290 The linearity of the relationship between phthalate metabolites and hormones
291 was tested using generalized additive models (GAMs). Most associations showed
292 effective degrees of freedom below 2,^{54,55} supporting linear relationships and so, the
293 modelling of phthalates as continuous variables (Supporting Information Figure S5).
294 Additionally, the few possible non-linear associations identified were not statistically
295 significant (GAM p-value > 0.05).

296 Adjusted linear regression models were then performed for each phthalate-
297 hormone pair at both the second and third trimester of pregnancy, since previous studies
298 showed associations may differ by trimester.²⁷⁻²⁹ Regression estimates were expressed
299 as percent change (PC) for each 2-fold increase in standardized urinary phthalate
300 biomarkers using the following formula: $([e^{(\ln 2 \times \beta)} - 1] \times 100)$.

301 A phthalate-sex interaction term was added to the model to identify potential
302 sex-specific associations, since previous studies also showed effect modification by this
303 variable.^{27,29} A p-value below 0.10 for the interaction term was considered as evidence
304 of effect modification,⁵⁶ in which case a sex-stratification of the specific association was
305 conducted.

306 2.4.5 Mixture model

307 Given the possibility of cumulative phthalate effects,⁵⁷ the overall mixture effect
308 of prenatal exposure to phthalates and DINCH on maternal hair hormone levels was
309 estimated using adjusted Bayesian Weighted Quantile Sum (BWQS) regression. BWQS
310 is a quantile-based approach that combines multiple independent variables additively
311 into a weighted index, with weights estimating the contribution of each component to
312 the mixture.⁵⁸ This model represents a novel Bayesian extension of the classic WQS
313 regression, counteracting its main limitations including higher stability of estimates,
314 higher statistical power (no data splitting into training and validation sets), and being
315 able to estimate mixture associations considering multiple chemicals with individual
316 positive, negative, and/or null effects simultaneously.⁵⁹ Phthalate and DINCH
317 metabolite concentrations were modeled in quartiles. Similar to linear regressions,
318 BWQS effect estimates (Beta1 and 95% Credible Intervals) were expressed as the PC in
319 hormone levels for each quartile increase in the mixture: $[(\exp(\text{Beta1}) - 1) * 100]$.

320

321 2.4.6 Sensitivity Analyses

322 The robustness of the main models was tested after: 1) additional adjustment for
323 urine specific gravity (continuous), to rule out any potential influence of urine
324 dilution;⁶⁰ 2) exclusion of extreme exposure and hormone values corresponding to
325 percentiles 1 and 99 of their respective distributions.

326

327 2.4.7 Interpretation and software

328 Statistical significance was set as p-value <0.05. A p-value between 0.05 and
329 0.10 was considered as possibly suggestive of an association. However, results were
330 interpreted not solely depending on statistical significance, but considering patterns of
331 associations and the previous evidence available to contextualize the findings.⁶¹ Thus,
332 associations with p-value <0.10 will only be considered meaningful if there is enough
333 previous toxicological or epidemiological support or if it follows a pattern of
334 associations (i.e., a non-isolated association). Regression analyses were carried out with
335 Stata version 14.2 (Stata Corp), while GAM (“mgcv” package) and BWQS models
336 using RStudio version 4.0.3 (RStudio Team, 2020).

337

338 3. Results

339 3.1 Characteristics of the study population

340 Mothers showed a high education level (58% reached Master’s degree or
341 higher), 16% were overweight/obese ($\geq 25 \text{ Kg/m}^2$), and conceived at a mean (SD) age
342 of 32.6 (3.9) years (Table 1). About half of the mothers (44%) were nulliparous at the
343 time of conception, and there was a slightly higher number of boys (54%) born in the

344 cohort. The prevalence of maternal active smoking was low (6%) compared to data in
 345 France,³⁸ while 17% of the women were exposed to environmental tobacco smoke.

346 Phthalates and DINCH metabolites were quantified in more than 95% of the
 347 urine samples at both pregnancy trimesters. The highest median urinary concentrations
 348 were observed for the sum of DEHP metabolites and the low molecular weight
 349 metabolite MEP (Supporting Information Table S2). Phthalate metabolites showed low-
 350 to-moderate estimates of Spearman correlation (rho range: 0.27-0.59) between the two
 351 pregnancy trimesters (Supporting Information Figure S6). In general, phthalate
 352 exposure levels were lower or similar to other contemporary European cohorts.^{14,62}
 353 Extended description of phthalate exposure levels in the SEPAGES cohort has been
 354 previously reported.¹⁴

355 Regarding hormones measured in hair samples, progesterone showed the highest
 356 levels (median: 76.5 ng/g), followed by Σ cortisone (median: 32.9 ng/g) and Σ cortisol
 357 (median: 6.1 ng/g) (Table 2). Σ Cortisol and Σ cortisone were highly correlated with
 358 their respective metabolites (rho > 0.84), with the exception of Σ cortisone and β -
 359 cortolone that were moderately correlated (rho: 0.43). Σ Cortisol and Σ cortisone levels
 360 were also highly correlated between them (rho: 0.86). While Σ cortisol and Σ cortisone
 361 moderately correlated with 11-Dehydrocorticosterone (rho: 0.56 and 0.67, respectively),
 362 their correlations with testosterone and progesterone levels tended to be lower
 363 (Supporting Information Figure S7).

364

365 **Table 1. Characteristics of the study population (N=382).**

Characteristics	Included (N=382)	Excluded (N=102)	P-value ^a
	N (%) or Mean (SD)	N (%) or Mean (SD)	
Mother's age at conception of index pregnancy (years)	32.6 (3.9)	32.2 (3.8)	0.41
Mother's anxiety/depression symptoms at 3 rd	10.4 (4.8)	11.0 (4.6)	0.29

trimester ^b			
Mother's pre-pregnancy BMI (kg/m ²)	22.3 (3.6)	22.8 (4.4)	0.09
< 25	320 (83.8%)	78 (76.5%)	
≥ 25	62 (16.2%)	24 (23.5%)	
Mother's education			0.40
Less than Master's degree	162 (42.4%)	48 (47.1%)	
Master's degree or higher	220 (57.6%)	54 (52.9%)	
Mother's tobacco consumption during pregnancy (=consumers ≥1 cigarette/day)	23 (6.0%)	6 (5.9%)	0.96
Mother's passive tobacco exposure (=exposed)	64 (16.8%)	24 (23.5%)	0.12
Parity			0.07
Nulliparous	167 (43.7%)	55 (53.9%)	
Uniparous/Multiparous	215 (56.3%)	47 (46.1%)	
Season of hair collection			0.09
Winter	75 (19.6%)	28 (27.5%)	
Remaining seasons	307 (80.4%)	74 (72.5%)	
Child sex (= boys)	206 (53.9%)	52 (53.1%)	0.88
Time elapsed from sample collection to assessment (years)	4.3 (0.78)	-	-
Analytical batch of hair analysis			
Batch 1	288 (75.4%)	-	-
Batch 2	94 (24.6%)	-	-

Abbreviations: BMI (Body Mass Index); N (Number); SD (Standard deviation).

Note: Percentage of missing covariates for included participants: maternal anxiety/depression score (5.0%), education (0.5%), pre-pregnancy BMI (0.5%), active (8.1%) and passive (5.5%) smoking. The remaining covariates had no missing values.

^a Categorical variables compared using the two-sample proportion test and continuous variables compared using the two-sample t test.

^b Hospital Anxiety and Depression score rated on a scale of 21. Anxiety and depression symptoms were summed, so the scores should be interpreted on a scale of 42.

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376 **Table 2. Hormone levels in the study population (N=382).**

Hormone levels in maternal hair at delivery	LOD	% >LOD	P25	Median	P75
∑Cortisol (nmoles/g) ^a	-	-	0.009	0.017	0.035
Cortisol (ng/g)	0.06	98	0.73	1.71	4.06
20α-dihydrocortisol (ng/g)	0.07	85	0.23	0.63	1.48
20β-dihydrocortisol (ng/g)	0.01	98	1.85	3.69	6.71
∑Cortisone (nmoles/g) ^a	-	-	0.050	0.091	0.156
Cortisone (ng/g)	0.10	95	4.77	12.0	22.5
20α-dihydrocortisone (ng/g)	0.02	99	3.80	8.27	15.7
20β-dihydrocortisone (ng/g)	0.02	97	2.60	5.74	9.82
β-cortolone (ng/g)	0.50	74	<LOD	4.54	7.66
11-Dehydrocorticosterone (ng/g)	0.10	88	0.91	1.82	2.98
Testosterone (ng/g)	0.10	89	0.23	0.42	0.65
Progesterone (ng/g)	0.10	100	30.8	76.5	166.6

377

378 ^a Molar sum of cortisol and cortisone metabolites. Median Σ Cortisol and Σ Cortisone levels expressed as
379 ng/g were approximately 6.1 and 32.9 ng/g, respectively.

380

381 3.2 Associations in the whole population

382 MBzP showed the most consistent pattern of associations with hair hormones
383 (Figure 1 and Supporting Information Table S3). Second trimester exposure to MBzP
384 tended to be associated with higher levels of all hormones, but only reaching borderline-
385 statistical significance for 11-dehydrocorticosterone levels (PC= 16.1%; 95%CI: -1.60,
386 35.6). Third trimester MBzP exposure was robustly and positively associated with most
387 of the hormones investigated. Each doubling in third trimester urinary MBzP
388 concentrations was associated with average increases of 13.3% (95%CI: 2.65, 24.9) for
389 Σ cortisol, 10.0% (95%CI: 0.26, 20.7) for Σ cortisone, 17.3% (95%CI: 1.67, 35.4) for
390 11-dehydrocorticosterone and 16.2% (95%CI: 2.20, 32.1) for testosterone, together with
391 a suggestive 10.5% (95%CI: -1.57, 24.1) increase in progesterone levels (Figure 1 and
392 Table S3). No associations with hormone ratios were found for MBzP at either the
393 second or third trimester.

394 Although without reaching statistical significance, third trimester urinary MiBP
395 concentrations tended to be positively associated with most of the hormones assessed in
396 hair samples (Figure 1 and Table S3), with a suggestive association (i.e., p-value < 0.10)
397 being observed for progesterone (PC= 12.7%; 95%CI: -1.51, 28.9).

398 Second trimester urinary Σ DiNP concentrations were inversely associated with
399 testosterone levels (PC= -11.6%; 95%CI: -21.6, -0.31) and the TC ratio (PC= -14.0%;
400 95%CI: -24.4, -2.15). Third trimester Σ DiNP exposure did not follow the same pattern
401 nor was associated with any hormone outcome.

402 Although maternal MEP exposure was associated with a lower CC ratio at both
403 second (PC= -4.98%; 95%CI: -9.05, -0.72) and third (PC= -4.18%; 95%CI: -8.12, -

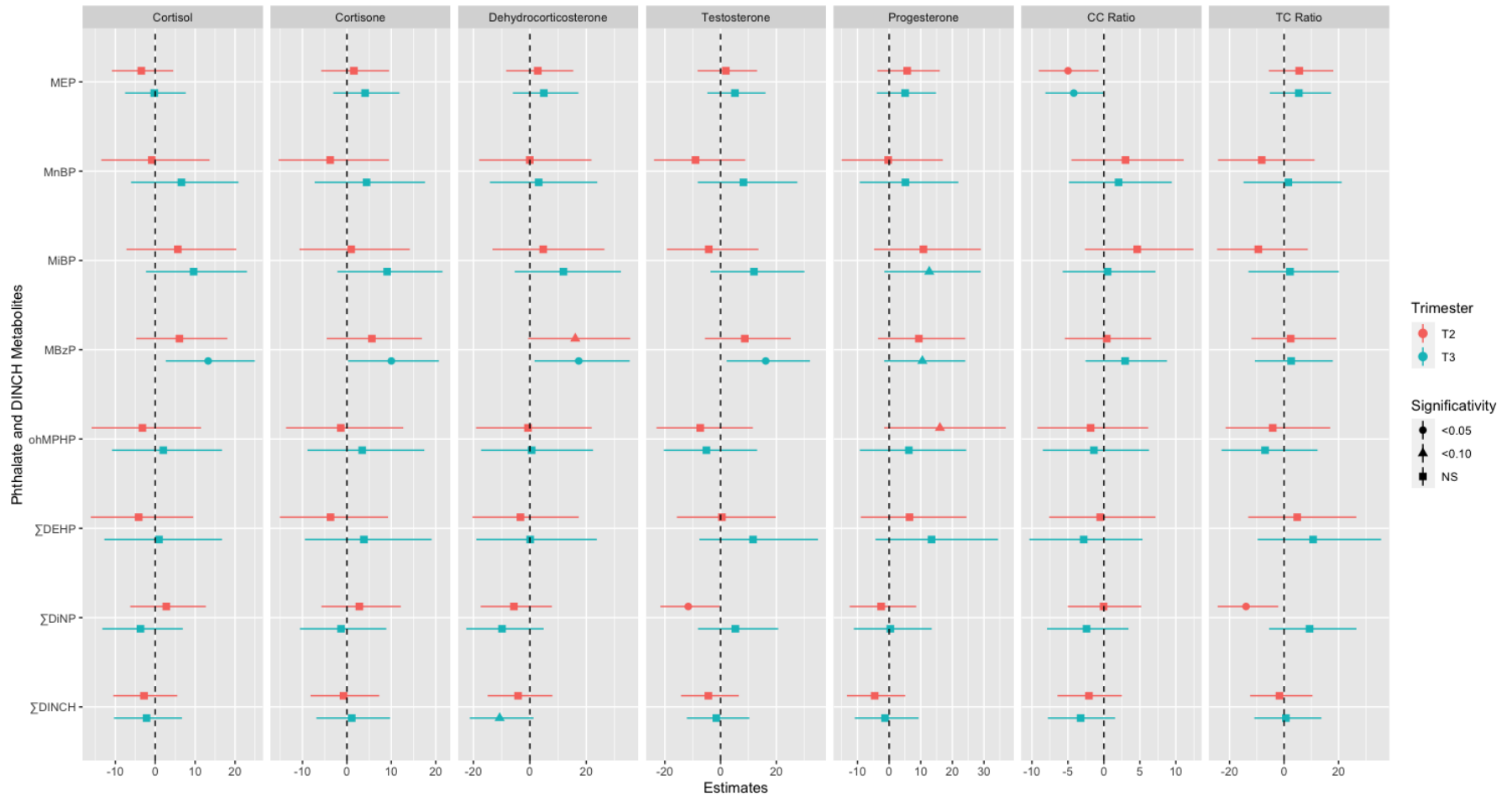
404 0.07) trimesters, this association appeared isolated, since MEP was not associated with
405 any of the primary hormone outcomes examined. Additionally, no meaningful
406 associations were observed for MnBP and \sum DEHP at any pregnancy trimester (Figure 1
407 and Supporting Information Table S3).

408

409

410

Figure 1. Adjusted associations between maternal prenatal urinary phthalate metabolite concentrations and hair hormones at delivery.



411

412 Note: Geometric figures (circles, triangles and squares) and error bars represent linear regression effect estimates with their corresponding 95% confidence intervals
 413 expressed as percent change (PC) in hormone levels for each doubling in exposure biomarkers. Models adjusted for: maternal age at conception (continuous), pre-pregnancy
 414 body mass index (BMI, normal weight vs. overweight/obese), education level (below vs. Master's degree or above), active smoking during pregnancy (smoker vs. non-
 415 smoker), passive tobacco exposure (exposed vs. non-exposed), parity (nulliparous vs. uniparous/multiparous), maternal anxiety/depression score at third trimester
 416 (continuous), infant sex and season of hair collection (winter vs. remaining seasons), hair analysis batch (dichotomous) and time elapsed (continuous) between hair sampling
 417 and hormone measurement. . Abbreviations: 11-DHC (11-dehydrocorticosterone); CC (Cortisol to Cortisone ratio); TC (Testosterone to Cortisol ratio).

418 3.3 Sex-specific associations

419 Few sex-specific associations were observed (Supporting Information Table S4).
420 Sex-stratified models showed that the previously observed second trimester inverse
421 \sum DiNP-testosterone association was driven by boys (PC= -21.9%; 95%CI: -34.3, -
422 7.15), while a null association was observed in girls (PC= 0.28%; 95%CI: -15.7, 19.3).

423 A sex-specific pattern was identified for MnBP, a compound not associated with
424 any hormone when boys and girls were studied together. In women carrying male
425 fetuses, second trimester MnBP exposure was positively associated with 11-
426 dehydrocorticosterone (PC= 40.4%; 95%CI: 3.53, 91.9), together with non-significant
427 increases in \sum cortisol (PC= 14.9%; 95%CI: -7.30, 41.4) and \sum cortisone (PC= 8.67%;
428 95%CI: -9.88, 32.0) levels, while women carrying female fetuses showed opposite
429 trends including significantly lower 11-dehydrocorticosterone levels (PC= -22.6%;
430 95%CI: -40.5, 0.00) (Table S4).

431

432 3.4 Mixture associations

433 BWQS models showed that the phthalate mixtures measured at the second and
434 third trimesters of pregnancy were not significantly associated with any of the hormones
435 investigated (Table 3). Notwithstanding, several suggestive positive associations were
436 observed for the third trimester mixture and testosterone (PC= 17.0%; 95%CI: -3.63,
437 41.6), \sum cortisone (PC= 11.0%; 95%CI: -4.28, 27.3) and progesterone (PC= 10.3%;
438 95%CI: -7.36, 30.2) (Table 3). Although in general phthalates showed similar mixture
439 weights, MBzP and MiBP tended to show slightly higher weights, in line with single-
440 pollutant models (Supporting Information Figure S8). The phthalate mixture at the third
441 trimester also tended to be associated with a lower CC ratio, which was mostly driven

442 by MEP in accordance with single-pollutant models (Supporting Information Figure
443 S8).

444
445 **Table 3. Mixture associations using Bayesian Weighted Quantile Sum (BWQS)**
446 **regression models.**

Hormones	Second trimester	Third trimester
	PC (95% CrI)	PC (95% CrI)
Σ Cortisol	-2.33 (-18.3, 16.2)	5.74 (-9.89, 23.5)
Σ Cortisone	0.06 (-14.0, 16.7)	11.0 (-4.28, 27.3)
11-Dehydrocorticosterone	-0.39 (-22.2, 27.5)	5.55 (-17.2, 32.9)
Testosterone	-4.50 (-22.3, 17.5)	17.0 (-3.63, 41.6)
Progesterone	4.64 (-12.9, 26.9)	10.3 (-7.36, 30.2)
CC Ratio	-3.22 (-13.4, 7.68)	-5.50 (-13.6, 3.44)
TC Ratio	-3.15 (-23.5, 23.3)	10.3 (-10.3, 35.9)

447
448 **Note:** Percent change (PC) in hormone levels per quartile increase of the phthalate mixture, with its
449 related 95% Credible Interval (95% CrI). The weight contributed by each chemical compound to the
450 most relevant mixture associations can be consulted in Figure S8. Mixture models were adjusted for the
451 same set of covariates included in single-pollutant models.
452

453 3.5 Sensitivity Analyses

454 The additional inclusion of urine specific gravity as a covariate in the models did
455 not materially change associations (Supporting Information Table S5).

456 The exclusion of extreme values attenuated the isolated associations observed
457 between MEP and the CC ratio that were no longer significant (Supporting Information
458 Figure S9). Associations observed at the third trimester for MBzP did not materially
459 change, while associations for third trimester MiBP were notably strengthened, showing
460 significant associations with Σ cortisol (PC= 13.6%; 95%CI: 0.92, 27.95), Σ cortisone
461 (PC= 15%; 95%CI: 2.71, 28.65), 11-dehydrocorticosterone (PC= 26.1%; 95%CI: 6.34,
462 49.42), testosterone (PC= 16.9% ; 95%CI: 0.45, 36.12) and progesterone (PC= 15.9%;
463 95%CI: 0.81, 33.24) levels (Supporting Information Table S6 and Figure S9).

464

465

466 **4. Discussion**

467 This cohort is the first to rely on within-subject pools of multiple urine samples
468 to assess phthalate exposure during gestation and to examine its association with
469 maternal hair samples collected at delivery to assess cumulative hormone levels. At the
470 second trimester, Σ DiNP concentrations were associated with lower testosterone levels
471 in all the sample, and especially among women carrying male fetuses. At the third
472 trimester, urinary MBzP concentrations were robustly and consistently associated with
473 higher levels of all the adrenal and reproductive hormones investigated. Third trimester
474 MiBP exposure followed a similar pattern of positive associations with all hormones,
475 that reached statistical significance after the exclusion of extreme values.

476

477 In line with single-pollutant results, a non-significant trend towards a positive
478 association with most hormones was observed for the phthalate mixture at the third - but
479 not second – trimester. Nevertheless, the lack of a significant mixture association seems
480 to imply that, in this study population, specific phthalate metabolites (Σ DiNP, MBzP
481 and to some extent MiBP) rather than a “cocktail effect” are the drivers of changes in
482 specific hormones.

483

484 **4.1 Biological plausibility and literature comparison**

485 MBzP and other phthalate monoesters exert a dose-dependent increase in the
486 gene expression of peroxisome proliferator-activated receptor gamma (PPAR γ) in
487 mouse Granulosa cells at environmentally relevant concentrations,⁶³ which may reduce
488 aromatase expression, leading to increased testosterone levels by reducing its

489 conversion to estradiol.²¹ Although this hypothesis could explain the increased
490 testosterone levels reported in our study, it does not explain the MBzP-related
491 systematic increase observed in all adrenal and reproductive hormones. Our MBzP
492 findings could result from one of the following modes of action: 1) A central
493 simultaneous increase in both hypothalamic gonadotropin and corticotropin releasing
494 hormones or their pituitary downstream hormones (for which we did not find supporting
495 evidence); 2) A peripheral increase in the rate-limiting step of steroidogenesis, that is,
496 an increased cholesterol delivery to the inner mitochondrial membrane by Steroidogenic
497 Acute Regulatory Proteins (StAR), or upregulation of CYP450scc (cholesterol side-
498 chain cleavage enzyme) that converts cholesterol into pregnenolone, the common
499 precursor of all steroid hormones (Supporting Information Figure S3).⁶⁴ We found *in*
500 *vitro* and epidemiological evidence supporting that MBzP can upregulate P450scc in
501 placental cells and tissue at human-relevant concentrations,^{65,66} while no relevant data
502 was found in relation to StAR. Although to our best knowledge this MBzP-upregulation
503 of P450scc has not been studied in other steroid organs with high P450scc expression
504 such as the adrenal glands and ovary,⁶⁷ a molecular docking study supported the ability
505 of MBzP to interact with P450scc.⁶⁸ Therefore, a MBzP-driven increase in
506 steroidogenic hormones through a multi-organ (placenta, adrenal glands and/or ovary)
507 upregulation of P450scc appears as a biologically plausible explanation that could be
508 further investigated in future experimental studies.

509 Although the current work is difficult to compare with previous studies due to
510 differences in matrices for hormone assessment (use of blood^{25,26,29} or urine²⁷ instead of
511 hair), our MBzP results are coherent with Pacyga et al., who reported a trend towards
512 positive associations between maternal prenatal urinary MBzP concentrations and the
513 sum of urinary testosterone metabolites measured three times during pregnancy among

514 434 U.S. women.²⁷ Other studies reported non-significant associations with serum
515 testosterone and progesterone, although the trend in estimates was again toward higher
516 hormone levels.^{25,26} Regarding corticosteroids, a positive association was reported
517 between first trimester urinary MBzP concentrations and cord blood cortisol to
518 cortisone ratio measured at birth among 553 Chinese mother-newborn pairs.²⁹ Despite
519 the small number of studies, the literature appears supportive of possible associations
520 between prenatal MBzP exposure and increased adrenal and reproductive hormone
521 levels during pregnancy.

522 MiBP showed a pattern of increased steroidogenesis similar to MBzP, that was
523 notably strengthened after exclusion of extreme exposure and hormone values.
524 Compared to MBzP, both the epidemiological^{25-27,29} and toxicological evidence for
525 hormonal effects during pregnancy is more limited and equivocal.⁶⁹ Although MiBP has
526 also been shown to interact with the P450scc enzyme, the specific study tested very
527 high doses in adult male mice,⁷⁰ being not comparable to our work. Additional data will
528 help to confirm or rule out our MiBP results.

529 DiNP is a phthalate replacement for DEHP. Although it is a less potent anti-
530 androgenic chemical than DEHP,⁷¹⁻⁷⁴ DiNP still shows endocrine disrupting abilities. In
531 adult female mice, DiNP exposure altered steroid hormone levels and ovarian
532 folliculogenesis at moderate doses, with DiNP decreasing serum testosterone levels at a
533 low to moderate dose of 100 µg/kg/day.^{75,76} In the current work we also observed
534 decreased testosterone concentrations in response to higher DiNP exposure, especially
535 among women carrying male fetuses. Since the human male fetus is able to synthesize
536 its own testicular testosterone between the first and second trimester,⁷⁷ we hypothesize
537 this association could reflect an anti-androgenic effect that may not only affect the
538 mother but also the developing male fetus. Although biologically plausible, this

539 association needs to be confirmed in future studies given the limited toxicologic and
540 epidemiologic data available for this phthalate replacement.

541 MnBP is known to exert anti-androgenic actions in male^{21,78,79} and female
542 reproductive organs.^{78,80-82} However, interspecies differences in glucocorticoids⁸³
543 precluded us from comparing our MnBP results to the rodent studies available.^{23,84}
544 Since comparison with other epidemiological studies is neither possible, the MnBP-
545 related increase in 11-dehydrocorticosterone levels among women carrying male fetuses
546 is difficult to interpret and needs to be confirmed in future studies.

547 Although MEP was negatively associated with the CC ratio at both second and
548 third trimesters, which could indicate a higher conversion of cortisol to its less active
549 metabolite cortisone, no associations were observed between MEP and cortisol or
550 cortisone individually. The lack of toxicological support, together with the fact that
551 when outliers were excluded the MEP-CC ratio associations disappeared, diminished
552 our confidence in this potential association.

553

554 4.2 Hair vs. other matrices for hormone assessment

555 Compared to saliva, urine and blood, cortisol in hair is thought to reflect mid to
556 long-term cumulative levels which are less affected by circadian rhythms and time-
557 varying stressors.⁴³ Although there are still some doubts about whether hair cortisol
558 represents long or mid-term cumulative concentrations,^{85,86} hair seems to provide
559 information at least on the previous weeks, constituting an advantage over saliva, urine
560 and blood spot samples which only represents the past few hours.⁸⁷ In a recent study
561 with a longitudinal design comparing hair and multiple saliva samples, Singh et al.,
562 2023 confirmed that hair cortisol is a reliable retrospective biomarker of at least the
563 previous six weeks.⁸⁷ Recent evidence suggests that not only corticosteroids but other

564 steroids such as reproductive hormones can be assessed in hair samples, possibly
565 leading to an improved hormone assessment in clinical and population studies.⁴²

566

567 In the current study, hair corticosteroids and reproductive hormones were
568 assessed in the last 3 centimeters of maternal hair obtained around delivery. Assuming
569 an average hair growth of 1 cm per month, the samples would reflect cumulative
570 hormone levels over the previous 3 months (the third trimester), or at least the previous
571 six weeks.^{43,87} Indeed, we observed more associations when phthalate metabolites were
572 assessed at the third compared to the second trimester. Since phthalates have short half-
573 lives, a possible explanation is that phthalate exposure at the third trimester may be
574 more biologically relevant to the cumulative period reflected by hair hormone analysis.

575

576 4.3 Clinical relevance

577 Regarding the extrapolation of population-based results to the clinic, it should be
578 taken into account that even moderate disturbances in the hormonal milieu can have
579 relevant effects particularly during fetal development.^{2,3,5,88} Additionally, exposure to
580 phthalates and their replacements is pervasive with virtually all human populations
581 showing quantifiable levels.¹⁴⁻¹⁶ In this setting, small shifts in the mean of a distribution
582 can produce large effects in their tails.⁸⁹⁻⁹²

583

584 The ovarian synthesis of maternal testosterone increases about 70% throughout
585 pregnancy.⁹³ Notwithstanding, testosterone levels are especially elevated in cases of
586 pre-eclampsia and PCOS (Kallak et al., 2017). Since these hyperandrogenic pregnancy
587 states have been related to preterm birth, fetal growth restriction and pregnancy
588 complications including diabetes,⁶⁻⁹ as well as a higher risk of metabolic and behavioral

589 problems in the offspring,^{94,95} the positive associations observed with MBzP (and
590 suggested for MiBP) could be of clinical relevance, especially at high-end exposure
591 levels.

592 At the end of gestation, glucocorticoid levels increase up to 20-fold compared to
593 those of mid-pregnancy, and pregnancy is thus conceptualized as a period of transient
594 hypercortisolism.³ Indeed, the placenta acts as a stress-sensitive organ that increasingly
595 secretes CRH levels that in turn increase adrenocorticotrophic hormone (ACTH)
596 secretion at the maternal pituitary, with consequently increased cortisol levels at the
597 adrenal glands, which become gradually hypertrophic throughout pregnancy. The
598 activation of the HPA axis, especially the release of CRH by the placenta, has been
599 proposed to function as a biological clock that can determine a preterm, term, or post-
600 term delivery.² Thus, our findings of increased hair cortisol and cortisone in response to
601 MBzP exposure could have an impact on the timing of labor, possibly leading to an
602 increased risk of preterm birth as shown by a recent meta-analysis [OR (95%CI) = 1.18
603 (1.01, 1.37)].⁹⁶

604

605 4.4 Strengths and limitations

606 The main strengths of this work are the improved methodologies that limited bias
607 arising due to daily variations occurring in both the phthalate metabolites and hormones
608 examined. This new-generation cohort is the first to perform a within-subject pooling of
609 multiple repeated urine samples, designed to reduce phthalate exposure
610 misclassification and attenuation bias.^{31,32} At the same time, our study is the first in the
611 field of endocrine disruption to use maternal hair samples obtained at delivery to
612 measure cumulative adrenal and reproductive hormone levels over the third trimester of
613 pregnancy.^{42,43} Among limitations are the medium-size of the cohort, especially when

614 examining sex-stratified associations, although we anticipate a substantially increased
615 statistical power compared to similar-sized cohorts collecting few urine samples.³¹
616 Another limitation was the lack of data for related steroid hormones such as estradiol or
617 the adrenal androgen dehydroepiandrosterone (DHEA). Finally, although we controlled
618 for the most relevant covariates reported in the literature, we cannot fully exclude the
619 possibility of residual or unmeasured confounding (e.g., diet quality or medications
620 containing phthalates). Additionally, the low number of women reporting hair dyes
621 during pregnancy (n = 7) prevented us from adjusting models with this variable.
622 However, we relied on a 7-day recall questionnaire completed during the second and
623 third pregnancy trimesters to estimate hair dye treatments, which may have probably
624 underestimated its use over the whole pregnancy.

625

626 Overall, this prospective study with improved exposure and outcome assessment
627 showed that maternal third trimester urinary MBzP concentrations were robustly and
628 consistently associated with increased levels of all the adrenal and reproductive
629 hormones evaluated in maternal hair samples at delivery, deserving further confirmation
630 and investigation of its possible clinical implications.

631

632 **Supporting Information**

633 Phthalate biomarkers assessed in SEPAGES; Urinary phthalate metabolite
634 concentrations; Adjusted main associations for single-pollutant models; Sex-stratified
635 phthalate-hormone associations; Sensitivity analysis of main model with additional
636 adjustment for specific gravity; Sensitivity analysis of main model with exclusion of
637 outliers; Study population flow-chart; Cohort design figure; Directed acyclic graph
638 (DAG) for covariate selection; Generalized additive models (GAMs); Spearman

639 correlation heatmap of phthalate metabolites; Spearman correlation heatmap of hair
640 hormones; Relative weights of BWQS mixture models; Figure of sensitivity analysis
641 excluding outliers.

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675

676 **Conflict of interest**

677 The authors declare no actual or potential competing financial conflicts of interest.

678

679 **References**

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