## Supplementary Material

# A Possible Impact of Antenatal Exposure to Environmentally Ubiquitous Phthalates Upon Male Reproductive Function at 20 Years of Age.

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## Content:

**Supplemental Materials and Methods**

*Reagents and standards*

All native and isotope labeled standards were synthesized by Institut Für Dünnschichttechnologie und Microsensorik (Teltow, Germany) or were purchased from Toronto Research Chemicals (Ontario, Canada) and Cambridge Isotope Laboratory (Andover, MA; distributed by VWR international), **Supplemental Table 2**. Acetone, isopropanol and ammonium acetate were obtained from Merck (distributed by VWR international). Acetonitrile and methanol were obtained from Fisher Scientific (Slangerup, Danmark). 4-methylumbelliferone, 4-methylumbelliferyl-β-D-glucuronide and formic acid were obtained from Sigma-Aldrich (Brøndby, Denmark). β-glucuronidase (Escherichia coli K12) was obtained from Roche Diagnostics (Mannheim, Germany). Milli-Q water was cleaned in a Millipore system (Synthesis A10). All chemicals were of analytical, HPLC or MS grade and all chemicals, solutions and lab wares were controlled for contamination with the actual phthalate metabolites before use. For method validation a serum pool collected randomly among colleagues was used.

*Preparation of calibration, quality control and validation materials*

A native stock solution containing 100 μg/mL of each native standard in 50% methanol was prepared. Eleven solutions (0.1-500 ng/mL) of the native stock solution solved in 50% methanol were prepared for calibration curves. Furthermore, for determination of limits of detections (LOD) and intra-day variation eight solutions (0.01-50 ng/mL) of the native stock solution were spiked in a randomly collected serum pool (beforehand tested for low or no content of the phthalate metabolites). The serum pool was also used unspiked and spiked at two different levels (Q low and Q high) with the native stock solution for determination of inter-day variation. All calibration and quality control materials were stored at -20°C until use. As solvent blank samples, Milli-Q water was used and treated as all other samples.

*Analytical method*

The total content of phthalate metabolites (sum of free and conjugated) in serum samples was measured by a newly developed method for simultaneous quantitative determination of 32 different phthalate metabolites (**Supplemental Table 1**) in human serum by isotope dilution TurboFlow-LC-MS/MS with preceding enzymatic deconjugation. After thawing, all serum samples, calibration and validation samples were mixed and aliquots of 100 µL were added 115 µL 1 M ammonium acetate buffer, pH 5.5. Twenty-five µL of internal standard solution (containing 5-40 ng/mL of the different labelled standards dissolved in 50% methanol) and for control of enzyme reaction 50 µL deconjugation mixture (100 ng/mL of 4-methylumbelliferyl β-D-glucuronide and $^{13}C_4$-methylumbelliferone dissolved in 1 M ammonium acetate buffer, pH 5.5) were added to all samples as previously described (Frederiksen et al. 2010). Immediately prior to the incubation for deconjugation all samples were added 10 µL of a freshly prepared enzyme solution (20 v/v % β-glucuronidase dissolved in 1 M ammonium acetate buffer, pH 5.5). The samples were mixed and incubated for 1.5 h at 37°C in a shaking water bath. The enzyme reaction was stopped by adding 50 µL 40% formic acid. To ensure same methanol concentrations in all preparations, the calibration samples (prepared in Milli-Q water added native standards dissolved in methanol) were added 100 µL Milli-Q water, while all control materials (spiked serum pool) and ordinary serum samples were added 50% methanol followed by mixing. Finally, samples were kept in the refrigerator at 4°C followed by 10 min centrifugation at 25,200 g and 4°C. Supernatants were transferred to HPLC vials.

For all extract clean-up, detection and quantification of phthalate metabolites in serum samples, an on-line TurboFlow-LC-MS/MS system (Thermo scientific Aria TLX-1 LC system coupled to TSQ Ultra triple quadrupole mass spectrometer from Thermo Fisher Scientific, San Jose, CA, USA) was used in combination with Aria operating software 1.6.3 and Xcalibur 2.1.0.1139 system software (ThermoFinnigan, Bellefonte, PA, USA). For sample extraction and chromatographic separation of analytes the TurboFlow-LC system was equipped with TurboFlow Cyclone P columns

(0.5 x 50mm) and Hypersil Gold aQ columns (4 x 50 mm, 3 µm particle size) (both from Thermo Scientific, Franklin, MA, USA). The MS/MS-system was equipped with a heated electrospray ionization source (HESI) and all samples were analyzed in negative mode. All sample batches were kept on the auto-sampler at 10°C. The injection volume was 100 µL with flow rate and solvent programming as shown in **Supplemental Table 3**. The optimized MS/MS interphase settings used are shown in **Supplemental Table 4** and the MS transitions, retention times, collision energies and S-lens settings optimized for each single analyte are shown in **Supplemental Table 5**.

*Operation procedure and method validation*

Matrix effect and ion suppression were investigated in duplicate calibration curves in Milli-Q water and serum pool at 8-10 concentration levels for each compound (0.1-50 ng/mL). The responses from standards prepared in the serum pool were plotted as a function of the responses from samples prepared in Milli-Q water. Subsequently, 95% confidence intervals were calculated for slopes and intercepts of the linear regression using the regression function in Analysis Toolpak for Microsoft Excel 2007. If the 95% confidence intervals included 1 for the slopes and 0 for the intercepts, no matrix effect was present. In cases where e.g. slopes for calibration curves made in serum and water differed (matrix effects) or where e.g. internal standard was different from the specific native analyte and thereby changed the slope of the calibration curves prepared in water from calibration curves prepared in serum, results were corrected for this matrix effect by dividing with the slope coefficient observed in calibration curves made in the serum pool (**Supplemental Table 6**).

For calibration curves, the ratio between the area of native standard and internal standard was plotted as a function of concentration of native standards. By linear regression based on area ratios (sample area/internal standard area) the concentration of unknown samples and the control material were determined.

For method validation and all other analysis two calibration curves in Milli-Q water were included at the beginning and the end of all sample batches. Furthermore, five calibration curves made in serum pool were used for estimation of the intra-day variability: Accuracy (% recovery) and precision (relative standard deviation (RSD)) were calculated for a low and a high concentration level (Q Low and Q High) of the five repeated calibration curves made in the serum pool (**Supplemental Table 7**). The five repeated calibration curves made in serum pool were used for determination of linearity and limit of detection (LOD). For this the approach described by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (ICH, 2005) on validation of analytical procedures was used: 3.3 times the standard error of the intercept of the calibration curve with the y-axis divided by the slope of the calibration curve using the five lowest calibration levels for each analyte (Frederiksen et al., 2013; Soeborg et al., 2013). The standard error of the intercept and the slope of the calibration curve were calculated using the regression function in Analysis Toolpak for Microsoft Excel 2007 (Microsoft Corp., Redmond, WA).

The inter-day variation (precision) was estimated from analysis of control material; quality control material was analyzed in triplicates in 14 batches over a period of three month (**Supplemental Table 8**).

**Supplemental references**

Frederiksen H., Jørgensen N. and Andersson A.-M. (2010) Correlations between phthalate metabolites in urine, serum and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. *J Anal Toxicol.* 49, 400-410

Frederiksen H., Aksglaede L., Sorensen K., Nielsen O., Main K.M., Skakkebæk N.E., Juul A., Andersson A.-M. (2013) Bisphenol A and other phenols in urine from Danish children and adolescents. *Int J Hygiene and Env Health*. 216, 710-720

Søeborg T., Frederiksen H., Johansen T.H., Fruekilde P., Juul A. and Andersson A.M. (2013) Serum concentrations of DHEA, DHEAS, 17$\alpha$-hydroxyprogesterone, $\Delta$4-androstenedione and testosterone in children determined by TurboFlow-LC-MS/MS. *Clin Chem Acta*. 419, 95-101

**Supplemental Table 1.** Phthalate diesters and their respective metabolites analyzed by LC-MS/MS

| Phthalate diester | Abbreviation | Human serum metabolite | Abbreviation | LOD* (ng/mL) |
|---|---|---|---|---|
| Di-methyl phthalate | DMP | Mono-methyl phthalate | MMP | 0.44 |
| Di-ethyl phthalate | DEP | Mono-ethyl phthalate | MEP | 0.65 |
| Di-iso-propyl phthalate | DiPrP | Mono-(4-oxopentyl) phthalate | MiPrP | 0.4 |
| | DPrP | Mono-propyl phthalate | MPrP | 0.23 |
| Di-iso-butyl phthalate | DiBP | Mono-iso-butyl phthalate | MiBP | 0.75 |
| Di-n-butyl phthalate | DnBP | Mono-n-butyl phthalate | MnBP | 0.61 |
| | | Mono-(3-hydroxybutyl) phthalate | MHBP | 0.22 |
| Butylbenzyl phthalate | BBzP | Mono-benzyl phthalate | MBzP | 0.26 |
| Di-n-pentyl phthalate | DPP | Mono-n-pentyl phthalate | MPP | 0.27 |
| | | Mono-(4-hydroxypentyl) phthalate | MHPP | 0.38 |
| Di-(2-ethyl-hexyl) phthalate | DEHP | Mono-(2-ethyl-hexyl) phthalate | MEHP | 0.74 |
| | | Mono-(2-ethyl-5-hydroxyhexyl) phthalate | MEHHP | 0.59 |
| | | Mono- (2-ethyl-5-oxohexyl) phthalate | MEOHP | 0.45 |
| | | Mono-(2-ethyl-5-carboxypentyl) phthalate | MECPP | 0.25 |
| | | Mono-(2-carboxymethyl-hexyl) phthalate | MCMHP | 0.39 |
| Di-n-hexyl phthalate | DHxP | Mono-n-hexyl phthalate | MHxP | 0.38 |
| | | Mono-(5-hydroxyhexyl) phthalate | MHHxP | 0.26 |
| | | Mono-(5-carboxypentyl) phthalate | MCPeP | 0.2 |
| Di-cyclohexyl phthalate | DCHP | Mono-cyclohexyl phthalate | MCHP | 0.27 |
| Di-n-heptyl phthalate | DHpP | Mono-n-heptyl phthalate | MHepP | 0.38 |
| | | Mono-(6-hydroxyheptyl) phthalate | MHHpP | 0.15 |
| | | Mono-(6-carboxyhexyl) phthalate | MCHxP | 0.23 |
| Di-octyl phthalate | DOP | Mono-octyl phthalate | MOP | 0.7 |
| | | Mono-3-carboxypropyl phthalate | MCPP | 0.19 |
| Di-iso-nonyl phthalate | DiNP | Mono-iso-nonyl phthalate | MiNP | 0.53 |
| | | Mono-hydroxy-iso-nonyl phthalate | MHiNP | 0.4 |
| | | Mono-oxo-iso-nonyl phthalate | MOiNP | 0.31 |
| | | Mono-carboxy-iso-octyl phthalate | MCiOP | 0.13 |
| Di-iso-decylphthalate | DiDP | Mono-iso-decyl phthalate | MiDP | 0.72 |
| | | Mono-(9-hydroxydecyl) phthalate | MHiDP | 0.31 |
| | | Mono-(9-oxodecyl) phthalate | MOiDP | 0.31 |
| | | Mono-(9-carboxynonyl) phthalate | MCiDP | 0.32 |

*LOD, Limit of detection (according to ICH algorithm) based on phthalate standards spiked in serum pool

**Supplemental Table 2.** Native and internal (isotope labeled) phthalate metabolite standards, CAS-numbers and suppliers

| Metabolite | Cas-numbers | Supplier | Internal standard | Supplier |
|---|---|---|---|---|
| MMP | 4376-18-5 | CIL | $^{13}C_2$-MMP | CIL |
| MEP | 2306-33-4 | CIL | $^{13}C_2$-MEP | CIL |
| MiPrP | 35118-50-4 | CIL | $^{13}C_2$-MEP | CIL |
| MPrP | 4376-19-6 | IDM | $^{13}C_2$-MEP | CIL |
| MiBP | 30833-53-5 | CIL | $D_4$-MiBP | IDM |
| MnBP | 131-70-4 | CIL | $^{13}C_2$-MnBP | CIL |
| MHBP | 57074-43-8 | TRC | $D_4$-MHBP | TRC |
| MBzP | 2528-16-7 | CIL | $^{13}C_2$-MBzP | CIL |
| MPP | 24539-56-8 | CIL | $D_4$-MPP | IDM |
| MHPP | | IDM | $^{13}C_2$-MCPP | CIL |
| MEHP | 4376-20-9 | CIL | $^{13}C_2$-MEHP | CIL |
| MEHHP | 40321-99-1 | CIL | $^{13}C_4$-MEHHP | CIL |
| MEOHP | 40321-98-0 | CIL | $^{13}C_4$-MEOHP | CIL |
| MECPP | 40809-41-4 | CIL | $D_4$-MECPP | IDM |
| MCMHP | 82975-93-7 | CIL | $^{13}C_4$-MCMHP | CIL |
| MHxP | 24539-57-9 | IDM | $D_4$-MHxP | IDM |
| MHHxP | | IDM | $D_4$-MHHxP | IDM |
| MCPeP | | IDM | $D_4$-MCPeP | IDM |
| MCHP | 7517-76-4 | TRC | $^{13}C_2$-MCHP | CIL |
| MHepP | | IDM | $D_4$-MHepP | IDM |
| MHHpP | | IDM | $D_4$-MHHpP | IDM |
| MCHxP | | IDM | $D_4$-MCHxP | IDM |
| MOP | 5393-19-1 | CIL | $^{13}C_2$-MOP | CIL |
| MCPP | 66851-46-5 | CIL | $^{13}C_2$-MCPP | CIL |
| MiNP | 519056-28-1 | CIL | $^{13}C_2$-MiNP | CIL |
| MHiNP | | IDM | $D_4$-MHiNP | IDM |
| MOiNP | 936022-00-3 | IDM | $D_4$-MOiNP | IDM |
| MCiOP | 898544-09-07 | IDM | $D_4$-MCiOP | IDM |
| MiDP | 297182-84-4 | CIL | $D_4$-MiDP | IDM |
| MHiDP | | IDM | $D_4$-MHiDP | IDM |
| MOiDP | | IDM | $D_4$-MOiDP | IDM |
| MCiNP | | IDM | $D_4$-MCiNP | IDM |
| 4-MB | 90-33-5 | Sigma | $^{13}C_4$-4MB | CIL |

TRC: Toronto Research Chemicals; CIL: Cambridge Isotope Laboratory; IDM: Institut Für Dünnschichttechnologie und Microsensorik (specific synthesis)

**Supplemental Table 3.** TurboFlow-LC system parameters: mobile phases, solvent gradients and flow-rate.

| | TurboFlow-column/loading pump[a] | | | | | | | Analytic column/eluting pump[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Step** | **Time (min)** | **Flow (µl/min)** | **% A** | **% B** | **% C** | **Tee** | **Loop** | **Flow (µl/min)** | **Gradient** | **% A** | **% B** |
| 1 | 0.00 | 0.3 | 100 | | | - | out | 0.7 | step | 95 | 5 |
| 2 | 1.00 | 0.1 | 100 | | | T | in | 0.6 | step | 95 | 5 |
| 3 | 2.00 | 1.5 | | 100 | | - | in | 0.7 | step | 95 | 5 |
| 4 | 2.48 | 1.5 | | 100 | | - | in | 0.7 | ramp | 75 | 25 |
| 5 | 2.50 | 1.5 | | | 100 | - | in | 0.7 | ramp | 74 | 26 |
| 6 | 3.50 | 0.2 | 20 | 80 | | - | in | 0.7 | ramp | 73 | 27 |
| 7 | 4.50 | 0.2 | 100 | | | - | out | 0.7 | ramp | 62 | 38 |
| 8 | 15.00 | 0.2 | 100 | | | - | out | 0.7 | ramp | 55 | 45 |
| 9 | 16.25 | 0.2 | 100 | | | - | out | 0.7 | ramp | 50 | 95 |
| 10 | 16.85 | 0.2 | 100 | | | - | out | 0.7 | ramp | 20 | 80 |
| 11 | 17.85 | 0.2 | 100 | | | - | out | 0.7 | ramp | | 100 |
| 12 | 19.85 | 0.2 | 100 | | | - | out | 0.7 | step | | 100 |
| 13 | 21.35 | 1.5 | 100 | | | - | out | 0.7 | step | 95 | 5 |
| 14 | 21.37 | 1.5 | 100 | | | - | out | 0.7 | step | 95 | 5 |
| 15 | 23.37 | 1.5 | 100 | | | - | out | 0.7 | step | 95 | 5 |

[a] Mobile phases for loading pump: A, 10 mM ammonium acetate; B, 0.1% formic acid in methanol; C, acetone/isopropanol/acetonitril (10:45:45)

[b] Mobile phases for eluting pump: A, water; B, methanol

**Supplementary table 4.** Optimized MS/MS interphase settings

| | **Negative mode** |
|---|---|
| Spray Voltage (V) | 3500 |
| Discharge current (V) | 4 |
| Vaporizer temperature (°C) | 400 |
| Capillary temperature (°C) | 220 |
| Sheath gas ($N_2$) pressure (units) | 40 |
| Auxiliary gas ($N_2$) pressure (units) | 15 |
| Ion sweep gas ($N_2$) pressure (units) | 0 |
| Collision gas (Ar) pressure (mTorr) | 1.5 |
| Declustering voltage (V) | 5 |
| Scan type | SRM |
| Chrome filter peak width (s) | 3 |
| Scan time (s) | 0.8 |
| Skimmer Offset (V) | 5 |
| Peak with for Q1 (Da) | 0.7 |
| Peak with for Q3 (Da) | 0.7 |
| MS run time (min) | 19.0 |
| Divert valve settings (min) | |
| 1. injection to waste | 0-0.15 |
| 2. injection to TSQ | 0.15-18.7 |
| 3. injection to waste | 18.7-19.0 |

SRM, selective reaction monitoring

**Supplemental Table 5.** Phthalate metabolites: Native and internal (labeled) precursor/product ions in negative mode $[M-H]^-$ and retention time (RT), collision energy and tube lens settings for native and internal ions, respectively.

| Native standard | Precursor/product ions | Internal standard | Labeled precursor/product ions | Retention time | Collision Energy | T-Lens |
|---|---|---|---|---|---|---|
| | m/z | | m/z | min | eV | V |
| MMP | 179 → 77 | $^{13}C_4$-MMP | 183 → 79 | 1.99 | 24, 23 | 108, 114 |
| MEP | 193 → 77 | $^{13}C_4$-MEP | 197 → 79 | 2.73 | 19, 18 | 130, 105 |
| MiPrP* | 207 → 77 | | | 3.81 | 19 | 119 |
| MPrP* | 207 → 77 | | | 4.16 | 19 | 119 |
| MiBP | 221 → 77 | $D_4$-MiBP | 225 → 81 | 6.29 | 20, 20 | 111, 106 |
| | 221 → 134 | | | 6.29 | 17 | 104 |
| MnBP | 221 → 77 | $^{13}C_4$-MnBP | 225 → 79 | 6.68 | 20, 20 | 111, 103 |
| MHBP | 237 → 121 | $D_4$-MHBP | 241 → 125 | 2.07 | 20, 20 | 109, 124 |
| MBzP | 255 → 183 | $^{13}C_4$-MBzP | 259 → 186 | 7.87 | 15, 15 | 112, 119 |
| MPP | 235 → 77 | $D_4$-MPP | 239 → 81 | 10.11 | 21, 22 | 129, 129 |
| MHPP** | 251 → 121 | | | 2.49 | 23 | 111 |
| MEHP | 277 → 134 | $^{13}C_4$-MEHP | 281 → 137 | 16.14 | 19, 18 | 134, 120 |
| MEHHP | 293 → 121 | $^{13}C_4$-MEHHP | 297 → 124 | 6.5 | 23, 23 | 118, 146 |
| MEOHP | 291 → 121 | $^{13}C_4$-MEOHP | 295 → 124 | 6.75 | 21, 20 | 141, 133 |
| MECPP | 307 → 159 | $^{13}C_4$-MECPP | 311 → 159 | 6.36 | 17, 15 | 112, 120 |
| | 307 → 121 | | | 6.36 | 17 | 112 |
| | 307 → 113 | | | 6.32 | 17 | 112 |
| MCMHP | 307 → 159 | $^{13}C_4$-MCMHP | 311 → 159 | 8.05 | 17, 15 | 105, 95 |
| MHxP | 249 → 77 | $D_4$-MHxP | 253 → 81 | 14.01 | 20, 28 | 118, 124 |
| MHHxP | 265 → 121 | $D_4$-MHHxP | 269 → 125 | 3.26 | 19, 20 | 154, 122 |
| MCPeP | 279 → 131 | $D_4$-MCPeP | 283 → 131 | 3.3 | 17, 15 | 109, 114 |
| MCHP | 247 → 97 | $^{13}C_2$-MCHP | 251 → 97 | 9.09 | 19, 19 | 126, 126 |
| MHepP | 263 → 77 | $D_4$-MHepP | 267 → 81 | 15.87 | 23, 27 | 123, 127 |
| MHHpP | 279 → 121 | $D_4$-MHHpP | 283 → 125 | 4.48 | 21, 21 | 128, 124 |
| MCHxP | 293 → 145 | $D_4$-MCHxP | 297 → 145 | 4.59 | 17, 17 | 106, 106 |
| MOP | 277 → 127 | $^{13}C_4$-MOP | 281 → 127 | 16.31 | 20, 19 | 120, 127 |
| MCPP*** | 251 → 103 | $^{13}C_4$-MCPP | 255 → 103 | 2.14 | 58, 58 | 96, 106 |
| MiNP | 291 → 77 | $^{13}C_4$-MiNP | 299 → 79 | 16.3 | 29, 29 | 140, 154 |
| MHiNP | 307 → 121 | $D_4$-MHiNP | 311 → 125 | 8.49 | 17, 23 | 112, 139 |
| MOiNP | 305 → 121 | $D_4$-MOiNP | 309 → 125 | 8.92 | 20, 23 | 128, 128 |
| MCiOP | 321 → 173 | $D_4$-MCiOP | 325 → 173 | 8.47 | 20, 18 | 122, 117 |
| MiDP | 305 → 77 | $D_4$-MiDP | 309 → 81 | 16.79 | 29, 29 | 137, 137 |
| MHiDP | 321 → 121 | $D_4$-MHiDP | 325 → 125 | 11.72 | 23, 22 | 121, 121 |
| MOiDP | 319 → 121 | $D_4$-MOiDP | 323 → 125 | 12.56 | 22, 23 | 125, 130 |
| MCiNP | 335 → 187 | $D_4$-MCiNP | 339 → 187 | 11.71 | 20, 18 | 113, 115 |
| 4-MB | 175 → 133 | $^{13}C_4$-4MB | 179 → 135 | 2.47 | 24, 23 | 110, 110 |

*13C4-MEP was used as internal standard

**13C4-MCPP was used as internal standard

***Could also be a metabolite of DnBP and other phthalate diesters

**Supplemental Table 6.** Matrix effects evaluated by slope and intercept with corresponding 95% confidence intervals (CI) when the responses at 10 concentration levels (range <LOD-100 ng/mL) for phthalate metabolite standards spiked in serum pool were plotted as a function of the responses at same concentration levels for standards prepared in Milli-Q water.

| | Slope | 95% CI | | Intercept | 95% CI | |
|---|---|---|---|---|---|---|
| **Metabolite** | **mean** | **lower** | **upper** | **mean** | **lower** | **upper** |
| MMP | 0.813 | 0.804 | 0.823 | **0.005** | **-0.001** | **0.011** |
| MEP | **1.016** | **0.979** | **1.052** | **0.002** | **-0.005** | **0.009** |
| MiPrP | 0.934 | 0.895 | 0.972 | **0.004** | **-0.002** | **0.010** |
| MPrP | 0.917 | 0.872 | 0.963 | **0.003** | **-0.006** | **0.011** |
| MiBP | **1.010** | **0.982** | **1.038** | **0.000** | **-0.004** | **0.005** |
| MnBP | **0.977** | **0.945** | **1.010** | **0.006** | **0.000** | **0.013** |
| MHBP | -0.078 | -0.713 | 0.558 | **0.442** | **-0.055** | **0.938** |
| MBzP | 0.194 | -0.456 | 0.843 | **0.384** | **-0.123** | **0.891** |
| MPP | 0.966 | 0.950 | 0.981 | **0.003** | **-0.008** | **0.014** |
| MHPP | **0.948** | **0.894** | **1.002** | **0.009** | **-0.039** | **0.058** |
| MEHP | 1.046 | 1.007 | 1.086 | 0.140 | 0.118 | 0.162 |
| MEHHP | 1.034 | 1.020 | 1.048 | **-0.002** | **-0.008** | **0.005** |
| MEOHP | **0.992** | **0.976** | **1.008** | **-0.002** | **-0.007** | **0.003** |
| MECPP | **0.994** | **0.986** | **1.003** | 0.005 | 0.001 | 0.009 |
| MCMHP | 1.028 | 1.008 | 1.049 | 0.021 | 0.016 | 0.026 |
| MHxP | 1.037 | 1.012 | 1.062 | **-0.009** | **-0.034** | **0.016** |
| MHHxP | 0.930 | 0.905 | 0.955 | **0.015** | **0.000** | **0.031** |
| MCPeP | 0.971 | 0.953 | 0.988 | **0.004** | **-0.007** | **0.015** |
| MCHP | **1.012** | **0.991** | **1.032** | **0.012** | **-0.004** | **0.028** |
| MHepP | 0.922 | 0.880 | 0.965 | **0.024** | **-0.010** | **0.059** |
| MHHpP | **0.984** | **0.964** | **1.003** | **0.006** | **-0.007** | **0.019** |
| MCHxP | 0.960 | 0.935 | 0.984 | **0.018** | **0.000** | **0.037** |
| MOP | 1.051 | 1.019 | 1.082 | **0.009** | **-0.021** | **0.039** |
| MCPP | 1.153 | 1.055 | 1.251 | **-0.008** | **-0.033** | **0.017** |
| MiNP | **1.039** | **0.990** | **1.088** | 0.060 | 0.025 | 0.096 |
| MHiNP | 1.060 | 1.039 | 1.081 | **-0.011** | **-0.030** | **0.009** |
| MOiNP | 1.043 | 1.018 | 1.068 | **-0.016** | **-0.042** | **0.009** |
| MCiOP | **1.003** | **0.990** | **1.015** | **0.000** | **-0.009** | **0.009** |
| MiDP | 0.908 | 0.851 | 0.965 | **0.003** | **-0.040** | **0.046** |
| MHIDP | 1.026 | 1.004 | 1.048 | **-0.005** | **-0.021** | **0.012** |
| MOIDP | 1.049 | 1.012 | 1.085 | **-0.014** | **-0.043** | **0.015** |
| MCINP | 1.033 | 1.009 | 1.057 | **-0.002** | **-0.021** | **0.017** |

Confidence intervals (according to ICH algorithm) for slopes crossing 1 and intercepts crossing 0 are marked in bold.

**Supplemental Table 7.** Method validation: Intra-day[a] accuracy and precision of quality control materials in Q low and Q high concentrations, limit of detection (LOD)[b] and linear range[c]

| | | | | Q Low | | | Q High | | |
|---|---|---|---|---|---|---|---|---|---|
| | Linear range | | LOD | Mean | RSD | Recovery | Mean | RSD | Recovery |
| Metabolite | ng/mL | R2 | ng/mL | ng/mL | % | % | ng/mL | % | % |
| MMP | 0.1-100 | 0.9601 | 0.44 | 1.84 | 9.3 | 91.8 | 9.93 | 7.3 | 99.3 |
| MEP | 0.5-500 | 0.9998 | 0.65 | 1.87 | 18.2 | 93.3 | 50.29 | 1.5 | 100.6 |
| MiPrP | 0.1-100 | 0.9989 | 0.4 | 1.24 | 21.9 | 124.3 | 11.12 | 6.7 | 111.2 |
| MPrP | 0.1-100 | 0.9992 | 0.23 | 0.89 | 22.7 | 89.5 | 11.19 | 1.7 | 111.9 |
| MiBP | 0.5-500 | 0.9997 | 0.75 | 5.26 | 10.5 | 105.2 | 47.64 | 3.4 | 95.3 |
| MnBP | 0.5-500 | 0.9998 | 0.61 | 5.42 | 9.8 | 108.3 | 47.05 | 1.7 | 94.1 |
| MHBP | 0.1-100 | 0.9988 | 0.22 | 4.77 | 14.3 | 95.4 | 47.75 | 3.5 | 95.5 |
| MBzP | 0.1-100 | 0.9998 | 0.26 | 4.91 | 10.0 | 98.2 | 99.86 | 2.1 | 99.9 |
| MPP | 0.1-100 | 0.9987 | 0.27 | 0.75 | 19.3 | 75.0 | 10.99 | 5.2 | 109.9 |
| MHPP | 0.1-100 | 0.9980 | 0.38 | 1.12 | 5.6 | 111.8 | 11.53 | 6.7 | 115.3 |
| MEHP | 0.1-100 | 0.9998 | 0.74 | 0.72 | 13.7 | 71.8 | 11.19 | 9.2 | 111.9 |
| MEHHP | 0.5-500 | 0.9998 | 0.59 | 2.15 | 9.5 | 107.4 | 49.26 | 2.2 | 98.5 |
| MEOHP | 0.5-500 | 0.9996 | 0.45 | 1.91 | 6.5 | 95.5 | 48.91 | 1.3 | 97.8 |
| MECPP | 0.5-500 | 0.9999 | 0.25 | 1.98 | 3.5 | 99.0 | 47.80 | 1.0 | 95.6 |
| MCMHP | 0.1-100 | 0.9988 | 0.39 | 1.15 | 11.2 | 114.7 | 12.04 | 8.7 | 120.4 |
| MHxP | 0.1-100 | 0.9996 | 0.38 | 0.76 | 24.8 | 76.2 | 10.82 | 8.5 | 108.2 |
| MHHxP | 0.1-100 | 0.9982 | 0.26 | 1.10 | 8.5 | 109.6 | 11.33 | 4.9 | 113.3 |
| MCPeP | 0.1-100 | 0.9994 | 0.2 | 1.02 | 6.8 | 101.7 | 10.90 | 3.0 | 109.0 |
| MCHP | 0.1-100 | 0.9990 | 0.27 | 1.98 | 8.7 | 99.0 | 11.27 | 3.9 | 112.7 |
| MHepP | 0.1-100 | 0.9970 | 0.38 | 2.08 | 6.1 | 103.9 | 12.12 | 2.7 | 121.2 |
| MHHpP | 0.1-100 | 0.9995 | 0.15 | 1.00 | 10.4 | 100.2 | 11.58 | 0.9 | 115.8 |
| MCHxP | 0.1-100 | 0.9997 | 0.23 | 0.97 | 15.5 | 97.0 | 11.36 | 2.1 | 113.6 |
| MOP | 0.1-100 | 0.9997 | 0.7 | 2.74 | 25.9 | 136.8 | 11.56 | 10.4 | 115.6 |
| MCPP | 0.1-500 | 0.9994 | 0.19 | 11.83 | 12.8 | 118.3 | 53.29 | 9.1 | 106.6 |
| MiNP | 0.1-100 | 0.9993 | 0.53 | 5.07 | 24.4 | 101.4 | 9.85 | 13.1 | 98.5 |
| MHiNP | 0.1-500 | 0.9994 | 0.4 | 0.98 | 9.0 | 97.5 | 50.14 | 1.8 | 100.3 |
| MOiNP | 0.1-500 | 0.9997 | 0.31 | 0.92 | 8.0 | 91.7 | 48.78 | 1.2 | 97.6 |
| MCiOP | 0.1-500 | 0.9999 | 0.13 | 0.98 | 4.9 | 97.5 | 49.28 | 2.3 | 98.6 |
| MiDP | 0.1-100 | 0.9957 | 0.72 | 1.96 | 31.2 | 98.2 | 8.92 | 19.0 | 89.2 |
| MHiDP | 0.1-500 | 0.9999 | 0.31 | 0.98 | 8.5 | 98.5 | 49.69 | 1.5 | 99.4 |
| MOiDP | 0.1-500 | 0.9995 | 0.31 | 0.95 | 8.9 | 95.3 | 49.73 | 1.4 | 99.5 |
| MCiNP | 0.1-500 | 0.9999 | 0.32 | 1.02 | 9.9 | 102.1 | 50.74 | 1.0 | 101.5 |

RSD: relative standard deviation; R2: mean of five R2-values

[a] Intra-day accuracy and precision based on five repeats of quality control materials prepared as serum pool spiked at a low and a high level.

[b] LOD, limit of detection (according to ICH algorithm) based on five repeated calibration curves prepared by spiking standards in serum pool in the range 0.1-50 ng/mL.

[c] linear range (according to ICH algorithm) based on two repeated calibration curves prepared in water.

**Supplemental Table 8.** Method validation: Inter-day precision[a] of quality control materials in Q low and Q high concentrations

| | Q Low | | Q High | |
|---|---|---|---|---|
| | Mean | RSD | Mean | RSD |
| Metabolite | ng/mL | % | ng/mL | % |
| MMP | 1.09 | 28.4 | 5.70 | 12.0 |
| MEP | 2.01 | 15.0 | 10.28 | 7.7 |
| MiPrP | 1.86 | 11.2 | 9.84 | 7.8 |
| MPrP | 1.97 | 7.5 | 9.85 | 6.9 |
| MiBP | 2.38 | 13.6 | 10.22 | 10.2 |
| MnBP | 2.42 | 15.9 | 10.62 | 6.9 |
| MHBP | 1.76 | 19.2 | 9.72 | 10.2 |
| MBzP | 1.98 | 14.4 | 10.23 | 6.0 |
| MPP | 1.76 | 11.8 | 9.45 | 10.7 |
| MHPP | 1.82 | 19.1 | 9.43 | 12.5 |
| MEHP | 2.06 | 19.6 | 10.00 | 10.4 |
| MEHHP | 1.89 | 10.7 | 9.94 | 6.0 |
| MEOHP | 1.90 | 9.8 | 9.79 | 5.7 |
| MECPP | 1.93 | 13.8 | 9.59 | 13.8 |
| MCMHP | 2.32 | 8.1 | 11.54 | 6.1 |
| MHxP | 1.93 | 5.5 | 9.80 | 5.4 |
| MHHxP | 2.06 | 5.3 | 10.15 | 4.8 |
| MCPeP | 2.03 | 6.5 | 10.15 | 5.7 |
| MCHP | 2.05 | 6.8 | 10.03 | 6.5 |
| MHepP | 1.85 | 17.5 | 9.63 | 16.1 |
| MHHpP | 2.02 | 4.6 | 10.04 | 4.8 |
| MCHxP | 1.91 | 7.4 | 9.55 | 5.7 |
| MOP | 2.11 | 13.4 | 9.99 | 11.2 |
| MCPP | 1.78 | 27.7 | 10.27 | 14.2 |
| MiNP | 1.73 | 26.7 | 9.89 | 14.6 |
| MHiNP | 2.01 | 7.9 | 9.96 | 6.1 |
| MOiNP | 1.94 | 9.8 | 9.69 | 9.0 |
| MCiOP | 1.97 | 11.0 | 10.28 | 10.0 |
| MiDP | 1.59 | 25.5 | 7.54 | 19.6 |
| MHiDP | 2.03 | 5.4 | 10.23 | 6.4 |
| MOiDP | 2.00 | 7.4 | 9.90 | 8.0 |
| MCiNP | 2.05 | 8.3 | 10.19 | 6.9 |

RSD: relative standard deviation

[a]Inter-day precision based on serum pool spiked with low and high amounts of standards, n=42 analyzed in 14 batches over a period of three month.

**Supplemental Table 9.** Pearson correlations between the ranked concentrations of maternal serum phthalate metabolites and their sums for all Raine Study participants (n=982).

| | ∑MBP$_{(i+n)}$ | ∑DEHPm | ∑DiNPm | ∑LMW phth.m | ∑HMW phth.m | ∑all phth.m | ∑DEHPm+DiNPm |
|---|---|---|---|---|---|---|---|
| MEP | **0.25**** | **0.16**** | **0.11**** | **0.75**** | **0.15**** | **0.66**** | **0.19**** |
| MiBP | **0.79**** | 0.05 | **0.10**** | **0.56**** | **0.07**** | **0.46**** | 0.12 |
| MnBP | **0.92**** | **0.27**** | **0.16**** | **0.69**** | **0.26**** | **0.65**** | **0.29**** |
| MEHP | **0.11**** | **0.80**** | 0.05 | **0.16**** | **0.62**** | **0.39**** | **0.59**** |
| MECPP | **0.20**** | **0.44**** | **0.26**** | **0.21**** | **0.39**** | **0.31**** | **0.47**** |
| MCMHP | **0.16**** | **0.55**** | 0.06 | **0.16**** | **0.39**** | **0.29**** | **0.43**** |
| MiNP | **0.15**** | **0.07**** | **0.98**** | **0.14**** | **0.53**** | **0.28**** | **0.70**** |
| MCiOP | **0.08**** | **0.26**** | **0.33**** | **0.13**** | **0.31**** | **0.21**** | **0.37**** |

***P*<0.05; **P*<0.10.

| | MiBP | MnBP | MEHP | MECPP | MCMHP | MiNP | MCiOP |
|---|---|---|---|---|---|---|---|
| MEP | **0.19**** | **0.26**** | **0.09**** | **0.16**** | **0.10**** | **0.10**** | **0.13**** |
| MiBP | | **0.61**** | -0.04 | **0.16**** | **0.11**** | **0.11**** | 0.05* |
| MnBP | | | **0.18**** | **0.21**** | **0.18**** | **0.15**** | **0.10**** |
| MEHP | | | | 0.03 | **0.15**** | 0.03 | 0.05 |
| MECPP | | | | | **0.67**** | **0.22**** | **0.54**** |
| MCMHP | | | | | | 0.02 | **0.33**** |
| MiNP | | | | | | | **0.25**** |

****P*<0.05**; **P*<0.10.

**Supplemental Table 10.** Pearson correlations between the ranked concentrations of maternal serum phthalate metabolites and their sums for all male participants who took part in the Raine study 20 year follow-up study (n=216).

| | ∑MBP$_{(i+n)}$ | ∑DEHPm | ∑DiNPm | ∑LMW phth.m | ∑HMW phth.m | ∑all phth.m | ∑DEHPm+DiNPm |
|---|---|---|---|---|---|---|---|
| MEP | **0.22**** | 0.00 | -0.08 | **0.78**** | -0.06 | **0.63**** | -0.06 |
| MiBP | **0.79**** | -0.03 | **-0.16**** | **0.49**** | -0.11 | **0.39**** | -0.07 |
| MnBP | **0.91**** | **0.19**** | -0.10 | **0.60**** | 0.06 | **0.54**** | 0.09 |
| MEHP | 0.05 | **0.80**** | 0.11* | 0.06 | **0.61**** | **0.33**** | **0.61**** |
| MECPP | 0.03 | **0.48**** | 0.12* | 0.01 | **0.39**** | **0.23**** | **0.38**** |
| MCMHP | 0.13* | **0.52**** | 0.08 | 0.13* | **0.37**** | **0.30**** | **0.38**** |
| MiNP | -0.11 | 0.10 | **0.97**** | -0.10 | **0.63**** | **0.14**** | **0.66**** |
| MCiOP | -0.05 | **0.33**** | **0.27**** | -0.05 | **0.38**** | **0.18**** | **0.38**** |

***P*<0.05; **P*<0.10.

| | MiBP | MnBP | MEHP | MECPP | MCMHP | MiNP | MCiOP |
|---|---|---|---|---|---|---|---|
| MEP | **0.14**** | **0.20**** | -0.06 | 0.02 | 0.09 | -0.09 | 0.06 |
| MiBP | | **0.61**** | -0.08 | -0.04 | 0.10 | **-0.17**** | **-0.14**** |
| MnBP | | | **0.14**** | 0.04 | 0.11 | -0.11 | -0.04 |
| MEHP | | | | 0.06 | 0.10 | 0.10 | **0.14**** |
| MECPP | | | | | **0.75**** | 0.08 | **0.56**** |
| MCMHP | | | | | | 0.05 | **0.37**** |
| MiNP | | | | | | | **0.19**** |

****P*<0.05;** **P*<0.10.