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Ongoing exposure to endocrine disrupting phthalates and alternative plasticizers in neonatal intensive care unit patients

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ABSTRACT

Due to endocrine disrupting effects, di-(2-ethylhexyl) phthalate (DEHP), a plasticizer used to soften plastic medical devices, was restricted in the EU Medical Devices Regulation (EU MDR 2017/745) and gradually replaced by alternative plasticizers. Neonates hospitalized in the neonatal intensive care unit (NICU) are vulnerable to toxic effects of plasticizers. From June 2020 to August 2022, urine samples (n = 1070) were repeatedly collected from premature neonates (n = 132, 4–10 samples per patient) born at <31 weeks gestational age and/or <1500 g birth weight in the Antwerp University Hospital, Belgium. Term control neonates (n = 21, 1 sample per patient) were included from the maternity ward. Phthalate and alternative plasticizers' metabolites were analyzed using liquid-chromatography coupled to tandem mass spectrometry. Phthalate metabolites were detected in almost all urine samples. Metabolites of alternative plasticizers, di-(2-ethylhexyl)-adipate (DEHA), di-(2-ethylhexyl)-terephthalate (DEHT) and cyclohexane-1,2-dicarboxylic-di-isononyl-ester (DINCH), had detection frequencies ranging 30–95 %. Urinary phthalate metabolite concentrations were significantly higher in premature compared to control neonates ($p = 0.023$). NICU exposure to respiratory support devices and blood products showed increased phthalate metabolite concentrations ($p < 0.001$). Phthalate exposure increased from birth until four weeks postnatally. The estimated phthalate intake exceeded animal-derived no-effect-levels (DNEL) in 10 % of samples, with maximum values reaching 24 times the DNEL. 29 % of premature neonates had at least once an estimated phthalate intake above the DNEL. Preterm neonates are still exposed to phthalates during NICU stay, despite the EU Medical Devices Regulation. NICU exposure to alternative plasticizers is increasing, though currently not regulated, with insufficient knowledge on their hazard profile.

1. Introduction

Plasticizers are chemical compounds added to plastics, such as polyvinyl chloride (PVC), to make them flexible, soft and to extend their lifetime. Phthalates are the best-known and most widespread group of plasticizers. Human exposure to phthalates is ubiquitous and occurs from various environmental sources, including food contact materials, dust, pharmaceuticals, and household products (Panneel et al., 2021). Another exposure route is through plastic medical devices (PMDs), which for a long time have been mainly plasticized with di-(2-

Abbreviations: AP, Alternative plasticizer; BBzP, Benzylbutyl phthalate; BPD, Bronchopulmonary dysplasia; DEHA, Di-(2-ethylhexyl) adipate; DEHP, Di-(2-ethylhexyl) phthalate; DEHT, Di-(2-ethylhexyl) terephthalate; DEP, diethyl phthalate; DINCH, Cyclohexane-1,2-dicarboxylic di-isononyl ester; DF, Detection frequency; DiBP, Diisobutyl phthalate; DIDP, Di-isodecyl phthalate; DINP, Di-isononyl phthalate; DnBP, Di-n-butyl phthalate; DNEL, derived no effect level; EDC, Endocrine discrupting chemical; EDI, Estimated daily intake; GA, Gestational age; HI, Hazard index; HQ, Hazard quotient; LOQ, Limit of quantification; MDR, Medical Devices Regulation; MW, Molecular weight; NICU, Neonatal intensive care unit; PHT, phthalate; PMD, Plastic Medical Device; PVC, Polyvinyl chloride; SG, Specific gravity, Tris-(2-ethylhexyl) trimellitate (TOTM).

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ethylhexyl) phthalate (DEHP) (Malarvannan et al., 2019). Due to its low cost, enhanced flexibility, and physical stability, DEHP is incorporated in many invasive PMDs, such as intravenous (IV) infusion sets, respiratory support equipment and blood bags (Panneel et al., 2021). Most phthalates, such as DEHP, have been classified as carcinogenic, mutagenic or toxic for reproduction (CMR1b) and as endocrine disrupting chemicals (EDC) (ECHA, 2006). In May 2021, the EU applied the Medical Devices Regulation (MDR 2017/745), declaring that CMR1a/b and/or EDC substances in medical devices above a concentration of 0.1 wt% are subject to justification and labelling (Regulation (EU), 2017). The last decades, alternative plasticizers (APs), including di-(2-ethylhexyl) terephthalate (DEHT), diisononyl cyclohexane-1,2-dicarboxylate (DINCH), tris-(2-ethylhexyl) trimellitate (TOTM) and di-(2-ethylhexyl) adipate (DEHA) have been used as a substitute for DEHP (Panneel et al., 2021). However, since the EU MDR application, their use in PMD getting more justified.

The use of invasive PMDs for respiratory support, catheterization and nutrition increases the risk of phthalate and AP exposure in the neonatal intensive care unit (NICU). In addition, premature neonates are susceptible to potential toxic chemicals, since exposure happens at a critical developmental period with a still immature excretory function (Raybaud et al., 2013; Gubhaju et al., 2014). Due to their noncovalent binding to PVC, plasticizers can leach from plastic products into the human body, as shown by detection of their metabolites in the urine of critically ill adults and children (Huygh et al., 2015; Verstraet et al., 2016), and of neonates hospitalized in a NICU (Panneel et al., 2021; Stroustrup et al., 2018). Moreover, despite the EU MDR, recent studies hint at the ongoing use of DEHP in PMDs in the NICU (Panneel et al., 2023; Bernard et al., 2023). However, few clinical studies exist concerning neonatal exposure to APs (Panneel et al., 2021). In addition, most neonatal biomonitoring studies are based on spot urine sampling at admission or discharge, not accounting for temporal variability of exposure, and are lacking individual risk assessment. Therefore, the goal of this study was to assess current exposure of premature neonates to both phthalates and alternative plasticizers during the entire NICU stay. In addition, we aimed to identify sources of such exposure and to estimate the associated health risks. We hypothesized to find higher urinary plasticizer metabolite concentrations in samples of patients with more PMD exposure, with exposure being the highest in the first days after birth.

2. Materials and methods

2.1. Study population

From June 2020 to August 2022, the Plastic-NICU prospective cohort (Clinicaltrials.gov identifier NCT05404815 (Cleys et al., 2023) included 132 premature neonates born at <31 weeks gestational age (GA) and/or <1500 g birth weight, in a tertiary NICU at the Antwerp University Hospital (Belgium). We focused on this group of extreme premature neonates, because they typically spend significant time in the NICU during a critical developmental period. Exclusion criteria were poor prognosis with impending early neonatal death, and/or a language barrier preventing informed consent from being obtained. Term neonates ($n = 21$; GA $\geq$ 37 weeks) were recruited from the maternity ward as a healthy control group without NICU exposure. Written informed consent was obtained from all parents before inclusion. The Antwerp University Hospital Ethical Committee (Ref. 2003022, EDGE 000701) approved this study.

2.2. Exposure assessment

After inclusion within 48 h after birth, urine samples from NICU neonates were collected on day 1–2–3 of postnatal age and weekly thereafter until 10 weeks postnatal age or NICU discharge, by placing cotton gauzes in the infant's diaper. For the control neonates, a single urine sample was collected within 48 h after birth, also by placing cotton gauzes in the infant's diaper. In addition, to assess *in utero* exposure, maternal urine samples of both NICU and control neonates were collected once within 48 h after giving birth, in pre-cleaned urine polypropylene containers. For NICU neonates, PMD use was recorded daily in electronic case report forms, from birth until NICU discharge. For each urine sample, exposure to a specific PMD type was scored (yes or no) if it was used in a 48-hour window before urine collection, as described by Stroustrup et al. (Stroustrup et al., 2018). In addition, for each urine sample, PMD use was further categorized based on phthalate-content derived from *ex vivo* studies (Panneel et al., 2021), into "low" (gastric tube and/or urinary catheter), "moderate" (parenteral nutrition and/or non-invasive respiratory support) and "high" (invasive respiratory support and/or blood products (transfusion of either red blood cells, platelets or plasma)) exposure, as categorized by Stroustrup et al. (Stroustrup et al., 2018).

2.3. Analysis of urinary metabolites of phthalates and alternative plasticizers

Urinary metabolites of the most widely used phthalates and alternative plasticizers (DEHA, DEHT, DINCH) were measured at the Toxicological Centre, University of Antwerp, using validated methods based on solid-phase extraction and liquid-chromatography coupled to tandem mass spectrometry (Bastiaensen et al., 2020). An appropriate method for the quantification of TOTM metabolites in urine samples is currently being developed. Therefore, the validation of this method in addition to the application on NICU samples will be disseminated in a separate manuscript. Details of the analytical procedures, quality assurance, and targeted compounds are given in the Appendix (Table A1–A3). Urinary specific gravity (SG) was measured at room temperature using a hand-held refractometer (Euromex RF.6612, Euromex Arnhem, Holland).

2.4. Statistical analysis

Database set-up and statistical analysis were performed in JMP Pro 17 (JMP®, Version *16*. SAS Institute Inc., Cary, NC, 1989–2023). Detection frequencies (DF) represent the proportion of samples in which the targeted metabolite was detected above its limit of quantification (LOQ) (Bastiaensen et al., 2020). For further statistical analysis, compounds with a DF > 50 % were considered, with concentrations < LOQ substituted by DF*LOQ. Concentrations were corrected for urinary dilution by SG using formula 1, with C_{SG} and C_{raw} being the SG-normalized and raw concentrations, and SG_{ref} and SG_{spec} reflecting the population median and specific sample SG.

$$C_{SG} = C_{raw} \times \left(\frac{SG_{ref} - 1}{SG_{spec} - 1} \right) \quad (1)$$

Sums of metabolites within the same parent compound were calculated by adding the molar concentrations of metabolites, and then converted to concentrations in ng/mL by multiplying with the molecular weight (MW). $\sum$PHT and $\sum$AP reflect the sum of all phthalate and AP metabolites (ng/mL), respectively. Based on inspection of bar charts and normal quantile plots, all metabolites did not follow a normal distribution, consequently nonparametric statistical tests were used. Spearman's rank correlation coefficients were used to assess correlation between two continuous variables. Wilcoxon-rank-sums-tests were conducted to compare concentrations in subgroups within our study population (Kruskal-Wallis-Test if $\geq$three levels), and the Wilcoxon signed rank test when comparing with other populations. A two-sided p-value < 0.05 was considered statistically significant.

2.5. Hazard estimation

For risk assessment analysis, we calculated estimated daily intakes

(EDI) using formula 2 - based on urinary metabolite concentrations (C_m), body weight (BW), 24 h urine excretion at sampling (V_{24}, calculated by diaper weight every three hours), fractional urinary excretion factors (F_{UE}), and MW of parent compounds (MW_p) and their metabolites (MW_m) (Frederiksen et al., 2014). EDI for the sum of different metabolites of the same parent compound was calculated by dividing each metabolite concentration by its MW, then summing the molar concentrations to be used in the formula. Formula 3 was used to calculate hazard quotients (HQ), by dividing the EDI for a specific plasticizer by its derived no effect levels (DNEL) (Frederiksen et al., 2014). DNEL values indicate the daily human exposure that is assumed without adverse health effects, extrapolated from experimental animal studies (Appendix, Table A4) (ECHA, 2023). HQ ≤ 1 indicates that exposure is not expected to lead to adverse effects. Due to a common toxicological mechanism, the EDI of DEHP, diethyl phthalate (DEP), benzylbutyl phthalate (BBzP), di-n-butyl phthalate (DnBP), and diisobutyl phthalate (DIBP) were merged to EDI-$\sum_{PHT}$ and evaluated against a group-DNEL, as previously reported (EFSA Panel on Food Contact Materials et al., 2019). DEHP was identified as index compound since it has the most robust underlying toxicological dataset. Consequently, the group-DNEL was established to be 36 μg/kg/d, expressed as DEHP equivalents (EFSA Panel on Food Contact Materials et al., 2019). Considering cumulative risk assessment of different plasticizers, hazard index (HI) values were calculated by summing all HQ estimates within each subject (Formula 4) (Frederiksen et al., 2014). A HI > 1 indicates a high risk of cumulative exposure.

$$EDI\left(\frac{\mu g}{kg \times d}\right) = \frac{C_m\left(\frac{ng}{mL}\right) \times V_{24}\left(\frac{mL}{24h}\right) \times MW_p\left(\frac{g}{mol}\right)}{1000 \times MW_m\left(\frac{g}{mol}\right) \times BW(kg) \times F_{UE}} \quad (2)$$

$$HQ = \frac{EDI\left(\frac{\mu g}{kg \times d}\right)}{DNEL\left(\frac{\mu g}{kg \times d}\right)} \quad (3)$$

$$HI = \sum HQ_{PHT+DINP+DIDP+DEHA+DEHT+DINCH} \quad (4)$$

3. Results

3.1. Study demographics

Detailed study demographics can be found in the Appendix (Table A5)). Preterm infants (n = 132; 62 boys, 70 girls), with a median GA of 28^3 weeks^{days} (IQR 26^5-29^6) and a median birth weight of 1170 g (IQR 850–1370) were enrolled. Premature prelabour rupture of membranes and pre-eclampsia were the most common causes of premature birth. The majority (93 %) of mothers had received antenatal corticosteroids. Late-onset sepsis (antibiotic duration of ≥48 h) and bronchopulmonary dysplasia (BPD, defined as need for supplemental oxygen or respiratory support at 36 weeks postmenstrual age) were the most common perinatal complications. During a median NICU hospitalization of 42 days (IQR 24–71), a total of 941 urine samples was collected with a median of 6 samples per patient (IQR 4–8). In addition, we collected a single urine sample from 104 NICU mothers, 21 control neonates and 25 control mothers. Neonatal and maternal numbers differ due to exclusion of samples collected more than 48 h postpartum, and mothers giving birth to twins.

3.2. Urinary metabolite profile

Table 1 shows detection frequencies (DF) and distributions of all targeted compounds, full names of all metabolites are given in the Appendix (Table A3)). Results refer to NICU neonatal samples, unless stated otherwise. Primary (MEHP; mono-ester metabolites derived after hydrolysis) and secondary (MEHHP, MEOHP, MECPP; derived after respectively hydroxylation, oxidation, and carboxylation) metabolites of DEHP were detected in 87–99 % of urine samples, and are hereafter grouped as ∑DEHP. Metabolites of other endocrine disrupting phthalates (DEP, DnBP, DiBP, and BBzP) were also present in 95–100 % of samples. Metabolites of di-isononyl phthalate (DINP) and di-isodecyl phthalate (DIDP) had a DF of respectively 22 and 66 %. AP metabolites were less frequently detected. The primary metabolite of DEHA, MEHA, had a DF of 62 %, its secondary metabolites 5-HO-MEHA 29 % and 5-oxo-MEHA 4 %. MEHTP and 5-OH-MEHTP, metabolites of DEHT, were detected in 66–83 % of NICU samples. From the last studied AP, DINCH, secondary metabolites OH-MINCH and Cx-MINCH were quantified in 95 % and 92 % of samples, while the primary metabolite MINCH had a DF of 44 %.

3.3. NICU neonates compared to term control neonates

NICU neonates showed significantly higher levels of DEHP metabolites (∑DEHP) compared to control neonates (Table 1, Fig. 1). Urinary metabolite concentrations of two (DEHA, DEHT) out of the three considered APs were significantly higher in NICU than control neonates (Table 1). In addition, NICU neonatal levels of all studied phthalates and APs were significantly higher than in NICU maternal samples (Table 1). Interestingly, NICU mothers showed also higher levels of DEHP metabolites compared to control mothers, while no differences were observed for AP metabolites (Table 1). It is important to note that, even though DEHP levels are statistically higher in NICU compared to control neonates, there is a wide range within all NICU samples (Fig. 1).

3.4. Determinants of exposure

3.4.1. Perinatal factors

Univariate predictor analysis (Appendix, Table A6) revealed intrauterine growth restriction (IUGR, birth weight <10th percentile), GA at birth and "clinical risk index for babies" (CRIB) score II (Parry et al., 2003) as birth characteristics associated with increased exposure to different plasticizers ($p < 0.05$). No differences were found based on gender. Neonates with IUGR had significantly higher urinary levels of all phthalate metabolites, as well of DEHA and DEHT metabolites, when compared to neonates without IUGR. Likewise, GA < 28 weeks and a CRIB-score of 11–15 were associated with significantly higher levels of all phthalate and AP metabolites. In addition, neonates with neonatal morbidities as BPD, intraventricular hemorrhage and late-onset sepsis showed increased levels of all phthalates. Likewise, children with BPD had significantly higher levels of DINCH and DEHT metabolites.

3.4.2. PMD exposure

Table 2 displays metabolite concentrations in urine sampled within 48 h after exposure to predefined PMD categories. Exposure to peripheral or central lines and parenteral nutrition showed no significant differences in urinary DEHP metabolite concentrations compared to unexposed neonates. Respiratory support ($p < 0.001$) and blood product administration ($p < 0.001$) were associated with significantly higher DEHP-metabolites. Post-hoc analysis showed higher levels with invasive ($p < 0.001$) and non-invasive ventilation ($p = 0.010$) than without respiratory support. Within non-invasive ventilation, support with high-flow nasal cannula showed higher DEHP metabolite levels than with continuous positive airway pressure ($p = 0.011$). Observations are similar for ∑PHT, except being higher with presence of a central line. Urinary metabolites of DEHA and DEHT were lower ($p < 0.05$) in every PMD exposure group. DINCH-metabolites, on the other hand, were higher after blood product administration or invasive ventilation, like phthalate metabolites.

Since neonates are often exposed to multiple PMDs simultaneously and urine excretion reflects whole body exposure, univariate urine analysis can be disturbed by concurrent exposure. As such, forward

Table 1
Summary statistics urinary concentrations of all phthalate and AP metabolites.

Parent	Metabolite	LOQ	NICU-B (132)				Control-B (n = 21)				NICU-M (n = 104)				Control-M (n = 25)			
			DF (%)	P25	Median	P75	DF (%)	P25	Median	P75	DF (%)	P25	Median	P75	DF (%)	P25	Median	P75
DEHP	MEHP	0.2	96.5	1.1	3.2	10.3	100.0	1.2	2.0	4.9	81.7	0.3	0.8	2.5	48.0	0.2	0.2	0.8
	MEHHP	0.2	91.9	0.5	1.2	3.0	81.0	0.5	0.8	2.0	93.3	0.6	1.6	4.1	84.0	0.5	1.3	2.4
	MEOHP	0.2	87.1	0.3	0.8	2.0	81.0	0.2	0.6	2.0	95.2	0.5	1.1	2.5	84.0	0.4	1.0	1.8
	MECPP	0.2	99.7	4.3	9.2	22.6	95.2	2.5	6.7	16.2	96.2	1.3	2.7	5.7	96.0	0.8	1.8	3.7
	∑DEHP	NA	NA	12.8	26.1	59.6	NA	8.3	13.4*	59.6	NA	4.8	9.5***	23.3	NA	2.4	6.0	11.4
DEP	MEP	0.4	97.8	2.9	5.0	10.7	100.0	1.9	8.9	15.0	94.2	2.2	4.2	18.4	92.0	1.3	5.7	18.3
DnBP	MnBP	0.4	100.0	8.1	15.2	31.7	100.0	11.1	27.6	51.7	100.0	2.8	5.0	9.5	96.0	2.2	4.9	7.4
DiBP	MiBP	0.4	99.4	3.2	6.1	11.5	100.0	8.4	24.1	52.8	95.2	1.3	2.9	6.7	88.0	1.2	4.1	10.5
BBzP	MBzP	0.2	95.5	0.9	1.9	5.0	90.5	1.8	3.0	8.8	90.4	0.3	1.1	2.6	88.0	0.3	0.8	1.4
DINP	7-Cx-MINP	0.4	21.5	0.1	0.1	0.1	47.6	0.1	0.1	1.4	43.3	0.1	0.1	0.9	32.0	0.1	0.1	1.0
DIDP	6-Cx-MIDP	0.2	32.1	0.1	0.1	0.3	66.7	0.1	0.5	0.9	50.0	0.1	0.1	0.3	32.0	0.1	0.1	0.2
	6-OH-MIDP	0.2	60.4	0.1	0.3	1.0	61.9	0.1	0.3	1.3	68.3	0.1	0.3	0.9	40.0	0.1	0.1	0.6
	6-oxo-MIDP	0.2	6.7	0.0	0.0	0.0	52.4	0.0	0.2	0.5	44.2	0.0	0.0	0.3	20.0	0.0	0.0	0.0
	∑PHT	NA	NA	38.8	68.9	124.3	NA	44.3	83.0	152.1	NA	17.7	30.6***	76.9	NA	15.5	27.8	75.6
DEHA	MEHA	0.4	61.6	0.2	0.6	2.2	4.8	0.2	0.2	0.2	5.8	0.2	0.2	0.2	0.0	0.2	0.2	0.2
	5-OH-MEHA	0.2	0.2	0.1	0.1	0.2	28.6	0.1	0.1	0.2	18.3	0.1	0.1	0.1	4.0	0.1	0.1	0.1
	5-oxo-MEHA	0.2	0.2	0.0	0.0	0.0	14.3	0.0	0.0	0.0	55.8	0.0	0.2	1.4	68.0	0.0	0.5	1.9
	∑DEHA	NA	NA	0.4	1.3	3.6	NA	0.4	0.4***	0.6	NA	0.4	0.8**	2.8	NA	0.4	1.0	2.9
DEHT	MEHTP	0.4	82.5	0.9	6.2	44.0	57.1	0.3	0.5	3.9	15.4	0.3	0.3	0.3	0.0	0.3	0.3	0.3
	5-OH-MEHTP	0.2	66.0	0.1	0.3	0.9	61.9	0.1	0.6	1.5	54.8	0.1	0.3	0.7	44.0	0.1	0.1	0.3
	∑DEHT	NA	NA	2.1	10.3	62.4	NA	1.0	2.6**	8.5	NA	0.6	0.8***	1.5	NA	0.6	0.6	0.9
DINCH	OH-MINCH	0.2	94.7	0.5	0.9	1.8	95.2	0.5	2.8	4.9	71.2	0.2	0.4	0.6	72.0	0.2	0.3	0.5
	Cx-MINCH	0.2	92.2	0.5	1.1	2.2	95.2	0.4	1.4	3.1	66.3	0.2	0.4	0.9	52.0	0.2	0.2	0.6
	∑DINCH	NA	NA	2.4	4.7	10.1	NA	1.6	7.0	12.3	NA	0.8	1.3***	2.3	NA	0.7	1.1	1.6
	∑AP	NA	NA	7.8	19.7	64.1	NA	5.9	11.8*	24.2	NA	2.8	4.8***	12.9	NA	2.6	4.4	6.3

Concentrations are specific-gravity-adjusted and given in ng/mL.
NICU neonates (NICU-B) are compared to control neonates (control-B) and NICU-mothers (NICU-M), respectively.
P-values are derived from the Wilcoxon Rank Sums Test (p-value ≥ 0.05 not displayed, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).
DF, detection frequency; NA, not applicable ND; NICU-B, NICU neonates; NICU-M, NICU-mothers; Control-B, control neonates; Control-M, control mother.

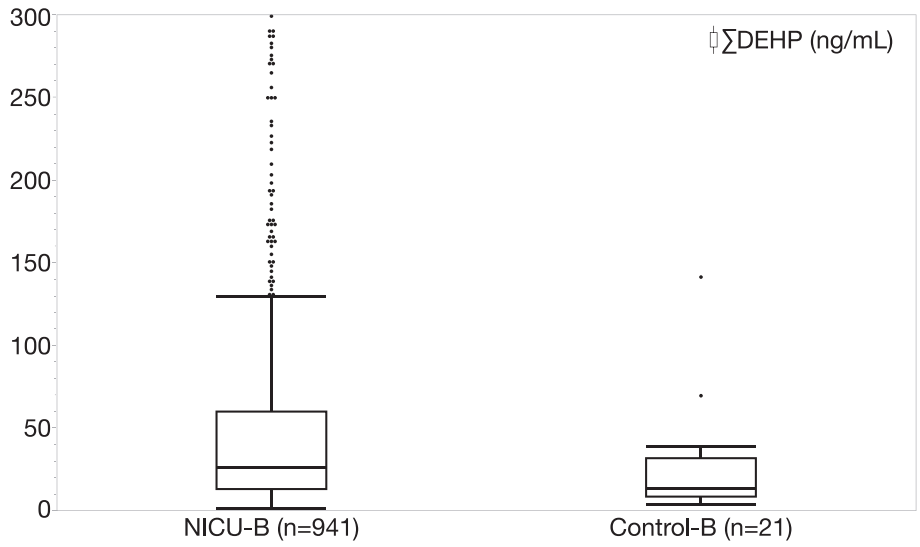

Fig. 1. Comparison of the sum of DEHP metabolites between NICU and control neonates. DEHP metabolites are specific-gravity-corrected and expressed in ng/mL; NICU-B represents NICU neonates and control-B represents control neonates. 941 NICU samples were collected from 132 NICU neonates. 21 control samples were collected from 21 control neonates. Boxplots present median, P25, and P75 values. P-values are derived from the Wilcoxon Rank Sums Test.

Table 2
Association of medical equipment exposure categories with urinary concentrations of summed phthalate and AP metabolites.

PMD Category	Exposure level (nr. of urine samples)	∑DEHP	∑PHT	∑DEHA	∑DEHT	∑DINCH	∑AP
Peripheral line	No (n = 720)	26.2	80.2	1.4*	12.2**	4.6	20.9*
	Yes (n = 221)	25.1	90.7	1.2	4.0	5.2	17.5
Central line	No (n = 419)	28.4	74.6	1.6***	17.0***	4.9	23.1***
	Yes (n = 522)	23.1	85.9**	1.1	5.8	4.6	17.3
Parenteral nutrition	No (n = 420)	30.2	77.9	1.6***	17.6***	5.0	23.6***
	Yes (n = 521)	22.0	84.6	1.1	5.6	4.6	17.0
Blood product administration	No (n = 886)	24.4	77.4	1.3*	10.1	4.6	18.6
	Yes (n = 55)	191.4***	259.5***	1.0	11.7	6.7**	29.3*
Mode of respiratory support	None (n = 193)	21.4	74.6	1.5*	13.1*	3.5	18.6
	Non-invasive (n = 629)	26.4*	72.7	1.4	9.2	4.7	18.6
	Mechanical ventilation (n = 119)	53.8***	160.2***	0.9	11.0	6.1*	23.6
Respiratory support	No (n = 193)	21.4	74.6	1.5	13.1*	3.5	18.6
	Yes (n = 748)	28.2**	83.4*	1.3	9.4	5.0*	19.8
Non-invasive ventilation	HFNC (n = 306)	30.6***	78.2*	1.7**	18.5***	4.6	27.1***
	CPAP (n = 323)	19.7	69.6	1.1	4.3	4.7	14.0
PMD exposure category	Low (n = 127)	19.6	71.4	1.4*	14.8*	4.7	18.0
	Moderate (n = 678)	24.6*	72.4*	1.4	9.4	4.5	18.6
	High (n = 136)	67.1***	163.5***	0.9	9.2	6.1	23.0

Concentrations are specific-gravity-adjusted and median values are given in ng/mL.
P-values were derived from the Wilcoxon Rank Sums Test when 2 groups, Kruskal-Wallis when more than 2 groups, and the Steel-Dwass test to correct for post-hoc multiple comparison (p-value ≥ 0.05 not displayed, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).
CPAP, continuous positive airway pressure; HFNC, high-flow nasal cannula; NA, not applicable; PMD exposure categories - "low" (gastric tube and/or urinary catheter), "moderate" (parenteral nutrition and/or non-invasive respiratory support) and "high" (invasive respiratory support and/or blood products).

stepwise linear regression, imputing all PMD exposure categories and the above-mentioned perinatal factors, revealed blood transfusion ($p < 0.001$) and mechanical ventilation ($p = 0.001$) to be the main predictors of higher phthalate metabolite concentrations. Likewise, "high" and "moderate" PMD exposure were associated with higher concentrations of phthalate metabolites ($p < 0.001$) (Table 2).

3.4.3. Time trend analysis

Fig. 2 shows a non-linear evolution of DEHP exposure, with metabolite concentrations gradually increasing after birth until week four postnatal age, and then decreasing until discharge (Spearman's rho 0.23, $p < 0.001$). When grouping all phthalate metabolites, the concentration peak is reached at week three postnatally. Given univariate predictor analysis revealed GA at birth to be associated with increased exposure to different plasticizers, we divided our population in extreme (<28 weeks GA) and very (Stroustrup et al., 2023; Sampson and de Korte, 2011; Serrano et al., 2016; Lagerberg et al., 2015) weeks GA, as defined by the WHO (Ohuma et al., 2023). Neonates born at <28 weeks GA show higher DEHP exposure levels, at every measured time point, than those born at 28–31 weeks GA (Fig. 2). The metabolites of DEHA, DEHT and DINCH increased slightly over time (Appendix, Fig. A1), reflected by weak though statistically significant ($p < 0.001$) correlation coefficients of respectively 0.16, 0.15, and 0.23.

3.5. Hazard estimation

Fig. 3 shows the combined EDI of all phthalates (EDI-$\sum_{PHT}$) during NICU stay. The majority of median EDIs were below their respective DNEL with HQs < 1. Nevertheless, the EDI-$\sum_{PHT}$ exceeded the group-DNEL in 10 % of samples. The maximum EDI-$\sum_{PHT}$ exceeded the group-DNEL by 24 times. Next, the EDI of DEHA was above the DNEL in 8 % of samples (Appendix, Table A4), with the maximum EDI 9 times

Table 3
Comparison of concentrations of urinary phthalate and AP metabolites in different NICU- and control neonatal exposure studies.

Country, Year	Population	MEHP	MEHHP	MEOHP	MECPP	∑DEHP	MEHA	MEHTP	5-OH-MEHTP	MINCH	OH-MINCH	Cx-MINCH
USA, 2009 (Calafat et al., 2009)	NICU(n = 54)	22	267	256	n/a	1203	n/a	n/a	n/a	n/a	n/a	n/a
Finland, 2014 (Frederiksen et al., 2014)	NICU, GA 24-37w (n = 67)	1	8.6	6.1	18	45	n/a	n/a	n/a	n/a	n/a	n/a
Norway, 2016 (Strommen et al., 2016)	NICU, GA 24-33w, BW < 1500 g (n = 46)	57	122	68	1215	1441	n/a	n/a	n/a	n/a	n/a	n/a
USA, 2018 (Stroustrup et al., 2018)	NICU, BW < 1500 g (n = 64)	7.1	11.8	11.9	49.7	95	n/a	n/a	n/a	n/a	n/a	n/a
France, 2019 (Pinguet et al., 2019)	NICU (n = 104)	38.2	49.6	36.2	n/a	190	0.1 (76 %)	<LOQ (30 %)	<LOQ (97 %)	n/a	0.1 (70 %)	0.1 (93 %)
Belgium, 2021 (Bastiaensen et al., 2021)	Adolescents (n = 416)	n/a	n/a	n/a	n/a	n/a	n/a (4 %)	n/a (1 %)	0.5 (87 %)	n/a (6 %)	1.1 (95 %)	1.0 (98 %)
France, 2021 (Philippat et al., 1987)	Term neonates – 2 m (n = 152)	2.4	0.9	0.8	5.2	9.3	n/a	n/a	n/a	n/a	0.3 (75 %)	n/a
Czech Republic, 2022 (Urbancova et al., 2022)	Term neonates (n = 315)	0.1	0.2	0.2	2.9	3.4	n/a	n/a	n/a	<LOQ (0 %)	0.1 (5 %)	0.1 (5 %)
Belgium, 2023Plastic-NICU	NICU, GA < 31w/BW < 1500 g (n = 941/132)	3.2	1.2	0.8	9.2	20.1	0.6 (62 %)	6.2 (83 %)	0.3 (66 %)	0.2 (44 %)	0.9 (95 %)	1.1 (92 %)

Concentrations are given in ng/mL. For AP metabolites, detection frequencies (%) are added.
AP, alternative plasticizers; BW, birth weight; GA, gestational age; NICU, neonatal intensive care unit; LOQ, limit of quantification; USA, United States of America.

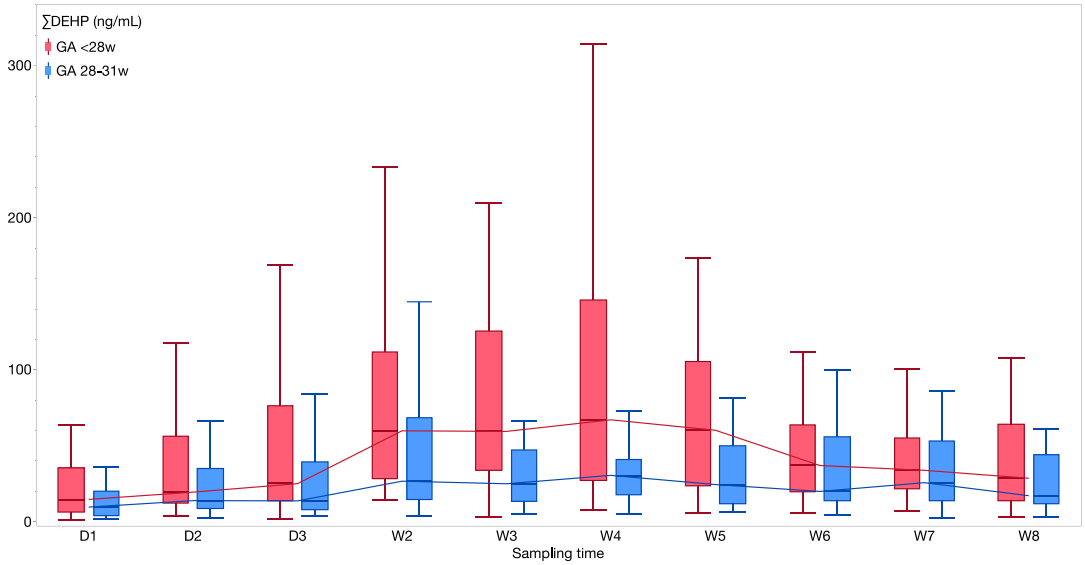

Fig. 2. Time trend of DEHP exposure during NICU stay. DEHP exposure is shown by the sum of DEHP metabolites (∑DEHP). Time is defined as postnatal age, D1-3 postnatal day 1 to 3, W2-8 postnatal week 2 to 8. Concentrations are SG-corrected and expressed in ng/mL. Boxplots present median, P25, and P75 values. Time trend lines connect median values at each time point. Neonates born at <28 weeks gestational age (GA) are represented by red boxplots, neonates born at 28–31 weeks GA are presented by blue boxplots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

above the DNEL. EDI-values for DEHT, DINCH and DIDP were mostly far below their DNEL, corresponding to negligible HQs (Appendix, Table A4). The median HI over all samples was 0·467, indicating a safe exposure level. However, in 24 % of urine samples the HI exceeded 1, with combined contribution of $\sum_{PHT}$ and DEHA up to 99 %. Over their entire NICU stay, 29 % of neonates had at least one day a HQ-PHT ≥ 1, while 57 % of the neonates in this study had at least one day a cumulative HI ≥ 1. Contingency analysis confirmed that neonates exposed to blood products (82 %) and invasive respiratory support (67 %) had a significantly higher proportion of HI values ≥1 (Chi-Squared test, $p < 0.001$).

4. Discussion

4.1. Evolution of NICU plasticizer exposure

We assessed current exposure of premature neonates in the NICU to both legacy phthalates and alternative plasticizers using repeated urine sampling. Most phthalate metabolites were detected in 90–100 % of samples, reflecting omnipresent phthalate exposure in the NICU. However, compared to other NICU biomonitoring studies conducted in the past 20 years (Stroustrup et al., 2018; Bernard et al., 2023; Frederiksen et al., 2014; Calafat et al., 2009; Strommen et al., 2016; Pinguet et al.,

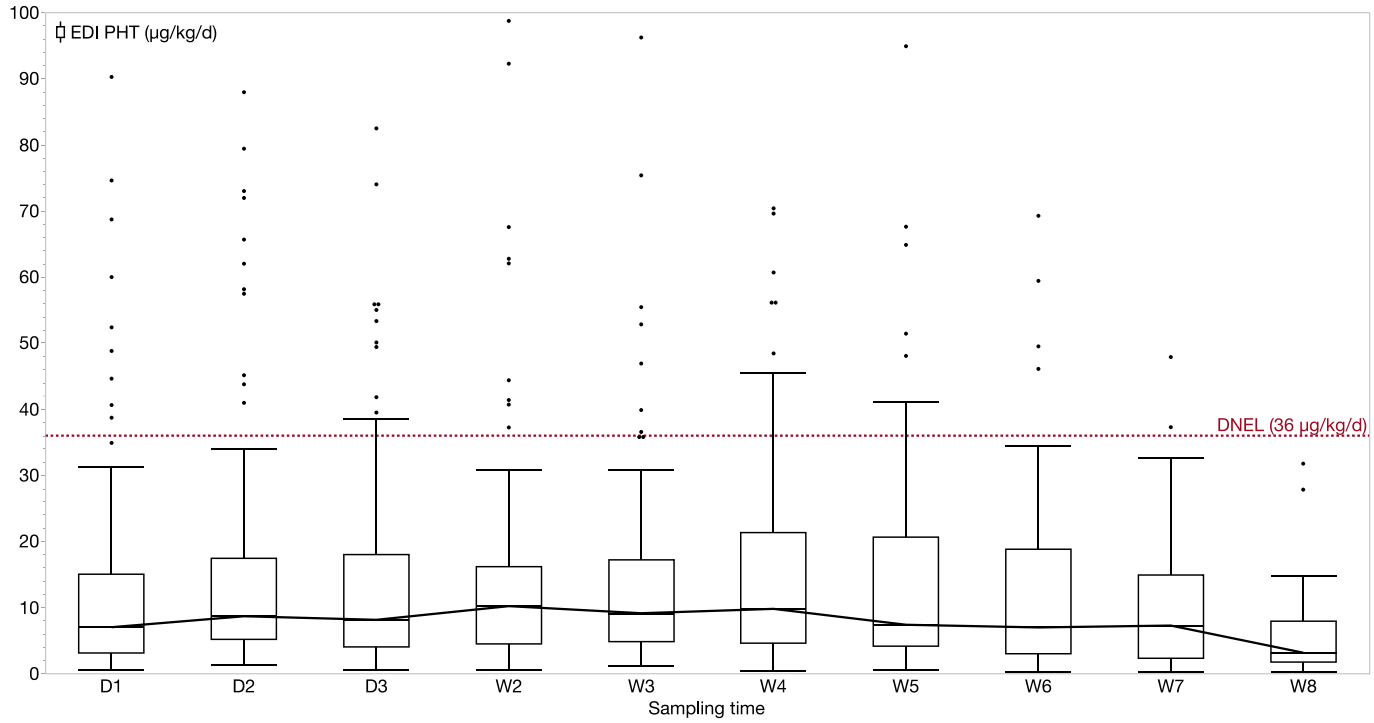

Fig. 3. Time trend of estimated intake of all phthalates during NICU stay. Estimated daily intake of phthalates (EDI-∑PHT) is expressed in µg/kg/d. Time is defined as postnatal day 1 to 3 (D1-3), and postnatal week 2 to 8 (W2-8). Boxplots present median, P25, and P75 values. The full line connects median EDI-values at each time point. The red dotted line represents the derived no effects level (DNEL, 36 µg/kg/d), associated with health hazard if exceeded.

2019), median DEHP metabolite concentrations were 10–50 times lower in this Flemish cohort (Table 3). This could be explained by regional differences in the manufacturing and distribution of PMDs, and by the EU MDR which influences the European PMD and plasticizer use. Nevertheless, median concentrations were still significantly higher compared to healthy control neonates not exposed to PMDs (Table 3) (Philippat et al., 1987; Urbancova et al., 2022). In addition, rather than pooling all samples of each subject, our study showed a wide range of metabolite concentrations changing over time, explained by corresponding PMD exposure (Appendix, Fig. A2).

In contrast to phthalates, few studies have published data on hospitalized patient exposure to APs, especially in neonates. One French pilot study detected metabolites of DEHA, DEHT and DINCH in single spot urine samples from 104 NICU neonates (Pinguet et al., 2019). Compared with their results, our study shows increasing levels of those metabolites (Table 3), indicating an inverse trend compared to phthalate exposure. Likewise, AP metabolites in NICU samples in our study had significantly higher urinary levels compared to other studies assessing urinary concentrations of term neonates (Philippat et al., 1987; Urbancova et al., 2022) or adolescents (Bastiaensen et al., 2019) (Table 3). In addition, our results provide insight in the exposure profile of different APs. DEHA for example was *ex vivo* estimated to have the highest migration potential of all tested APs (Van Vliet et al., 2011; Den Braver-Sewradj et al., 2020). Nevertheless, only its primary metabolite MEHA was frequently detected. DEHT, a DEHP isomer with the substituents in the para- compared to the *ortho*-position, showed *ex vivo* a significantly lower migration rate from PVC than DEHP (Panneel et al., 2021), but its metabolites were detected in 66–83 % of NICU urine samples. DINCH is a non-aromatic analogue of DINP with similar mechanical properties (Engel et al., 2018). Substitution of DINP with DINCH in PMDs might explain the high DF of DINCH- (>90 %) compared to DINP-metabolites (22 %).

4.2. Plastic medical devices as sources of exposure

Although showing decreasing concentrations of phthalate metabolites over the previous decades and despite changing regulations (Regulation (EU), 2017), our results indicate ongoing phthalate exposure to neonates in the NICU. In line with previous research, PMD use in the NICU was still associated to significantly higher DEHP levels in urine sampled within 48 h after exposure, in particular in neonates receiving respiratory support or blood transfusion (Stroustrup et al., 2018; Stroustrup et al., 2023). Indeed, DEHP is known be used in blood bag systems since it increases membrane stability of red blood cells, reducing hemolysis (Sampson and de Korte, 2011). Recently developed blood bags plasticized with APs as DINCH (Serrano et al., 2016; Lagerberg et al., 2015) or DEHT (Larsson et al., 2021) have shown equal to better blood product storage capacity. Regrettably, a recent survey revealed that blood centers in Europe are concerned with manufacturers practically implementing DEHP-free blood bags (Razatos et al., 2022). Likewise, *ex vivo* models simulating respiratory support show DEHP to leach from ventilation circuits (Bouattour et al., 2020). However, the exact sources of exposure from (non-) invasive respiratory support equipment (*e.g.,* endotracheal tube, ventilation circuit) remains challenging to quantify (Panneel et al., 2021). Therefore, *ex vivo* models need to match experimental conditions as much as possible to the clinical situation. Unfortunately, the MDR does not prevent manufacturers from using substances of very high concern, such as phthalates, in medical devices “where justified”. In addition, until the end of 2025, DEHP can still be used in PMDs, albeit with appropriate labelling on the packaging (Regulation (EU), 2017). AP metabolites, on the other hand, had lower concentrations than phthalate metabolites. DINCH metabolites were less clearly associated with PMD use, correlating with *ex vivo* leaching studies showing considerably lower intrinsic migration potential than DEHP (Engel et al., 2018). DEHA and DEHT metabolite concentrations being lower after PMD exposure might be explained by exposure via gastric tubes. Although mostly present from birth until discharge, enteral nutrition via these tubes, and corresponding possible plasticizer

exposure, increases with postnatal age. This warrants further investigation in *ex vivo* leaching studies.

4.3. Time trend of exposure

Although new methods have been developed to detect phthalates and APs in other matrices, like hair (Cleys et al., 2023), it is clear that urine, due to its availability and the possibility to calculate daily intakes, remains a cornerstone in assessing neonatal exposure to plasticizers. Previous NICU studies relied mostly on spot urine samples, except for the New York based NICU-HEALTH (Stroustrup et al., 2018), and French ARMED (Bernard et al., 2023) cohorts. Our study on the other hand combined repeated urine analysis with a daily inventory of PMD exposure, allowing for time trend analysis. Given premature neonates often reside for an extended period in the NICU, our results provide an overview of plasticizer exposure from birth to discharge. As such, phthalate exposure follows a non-linear trend increasing towards week four postnatally. This could be explained by changing PMD-exposure over time, as associations of ∑DEHP to PMD exposures remain present at each time point (Appendix, Fig. A2). In contrast, urinary metabolites of all studied APs increased slightly but continuously during NICU stay. This might be explained by leaching from PMDs during enteral nutrition, which increases proportionally with increasing postmenstrual age.

In addition, maturation of neonatal clearance capacity might also influence exposure profiles over time. Oral exposure studies in adult populations found the half-life of most DEHP metabolites to be 6–12 h (Koch et al., 2006), but the half-life in premature infants is presumably longer due to immature liver and kidney function (Gubhaju et al., 2014; Allegaert et al., 2017). First, activity of cytochrome P450 enzymes, responsible for phase I biotransformation of phthalates (*e.g.,* oxidation, reduction and hydrolysis), is suggested to increase with postnatal age (Allegaert et al., 2017). Secondly, phase II biotransformation of phthalate metabolites includes glucuronidation in order to facilitate renal elimination. However, glucuronyl transferase activities are immature right after birth (Demirel et al., 2016), and conjugation can be even lower in premature neonates, leading to delayed excretion. Lastly, once solubilized by glucuronidation, phthalates are excreted proportionally to the glomerular filtration rate (Gubhaju et al., 2014). Maturation of renal elimination capacity is a continuous process, already started *in utero,* with a 2–4 fold increase in glomerular filtration rate in the first 4 weeks of postnatal age (Allegaert et al., 2017).

Our results indicate that cohort studies correlating outcomes with single time point exposure are prone to miss relevant exposure in many children and might under- or overestimate health effects. Therefore, the detection of developmental windows associated with increased exposure and/or decreased excretion (both in terms of gestational age and postnatal age), and the incorporation of exposure during these windows are essential for future studies assessing health effects of neonatal plasticizer exposure.

4.4. Risk of exposure

Despite decreasing overall phthalate exposure compared to previous studies, EDIs of phthalates still exceeded the DNEL in 10 % of the samples, with maximum values reaching 24 times this threshold. Moreover, over their entire NICU stay, 29 % of premature neonates had at least once a phthalate EDI above the DNEL, while 57 % of the neonates had at least once a HI above 1, indicating cumulative plasticizer exposure that may have detrimental effects on long-term development. Our EDI calculations indicate a decreased phthalate exposure compared to previous studies. Frederiksen et al., who calculated phthalate EDI values for 67 premature neonates born between 2006 and 2008 in a Finnish cohort, showed that at day seven of life, more than 80 % of those infants had a phthalate EDI above the DNEL (Frederiksen et al., 2014). Similarly, Strommen et al showed that phthalate EDI exceeded the group-DNEL in 90 % during the first week of life in 40 premature infants included in 2010 (Strommen et al., 2022).

In addition, neonatal phthalate exposure is increasingly being associated with non-endocrine health effects. Although phthalates were initially not considered neurotoxic pollutants, associations between prenatal or early childhood exposure to phthalates and alterations in multiple domains of neurodevelopment have been reported in non-NICU populations (Engel et al., 2021; Lucaccioni et al., 2021). In addition, NICU-based exposure to phthalate mixtures has been associated with infant neurobehavioural performance (Stroustrup et al., 2018). However, the long-term neurodevelopmental impact of iatrogenic exposure to phthalates and alternative plasticizers during NICU stay remains largely unknown (Lucaccioni et al., 2021). Similarly, epidemiological studies show strong positive associations between prenatal or early childhood phthalate exposure and respiratory morbidity in childhood, as asthma development and increased respiratory tract infections (Li et al., 2017; Wu et al., 2020). Premature neonates are especially vulnerable to exogenous toxicants since the perinatal period is a critical window for lung maturation, specifically alveolarization (Woods et al., 2016). Indeed, inhaled phthalates, leaching from respiratory support systems, could act directly at these vulnerable peripheral airways (Strommen et al., 2016). Moreover, exposure to phthalate mixtures in the NICU, with DEHP predominance, has been associated with development of BPD (Stroustrup et al., 2023). However, to our knowledge, no studies have assessed the association between phthalate exposure in the NICU and respiratory function in later life (Panneel et al., 2021).

4.5. Strengths and limitations

One of this study's strengths is that it was designed to address shortcomings of past biomonitoring studies assessing plasticizer exposure in the NICU. As such, metabolite concentrations were adjusted for urine dilution using specific gravity (Stroustrup et al., 2023) instead of creatinine, since urinary creatinine in neonates is highly dependent on maturity and body weight (Rios et al., 2021). In addition, repeated urine sampling allowing time trend analysis provides an important insight in the entire scope of NICU exposure. Moreover, the measurement of more than 1000 urine samples ensured robustness of exposure estimation of premature neonates to phthalates and APs. Next, this study is unique in its patient-oriented approach, focusing on the exposome of plasticizers within each neonate, instead of being either phthalate- or AP-oriented. Individual risk assessment based on urinary excretion is another strength compared to previous studies. Lastly, this large prospective observational study is the starting point of a prospective follow up cohort assessing respiratory and neurodevelopment in a vulnerable population.

Nonetheless, this study has some limitations. First, extreme premature neonates in a Belgian single center cohort do not represent the entire NICU population, both in terms of maturity and ethnic background. However, providing insight in their PMD-exposure profiles and a comparison to control neonates, these data might serve as a proxy for other neonatal populations. We are aware of the fact that the control subjects are not comparable in terms of physical or clinical characteristics, as PMD as a source for plasticizer exposure is virtually absent in these subjects. On the other hand, there is no better control population available for subjects in their early development. The low number of control (n = 21) compared to NICU (n = 132) subjects was due to practical difficulties to enroll healthy neonates within the scope of this study. Next, calculating EDI values in infants can be challenging. The F_{UE} elimination factors, one of the most important parameters in EDI models, are based on oral exposure in adult populations (Koch et al., 2003), while premature infants have reduced glomerular filtration rate and tubular secretion compared to adults (Strommen et al., 2022). In addition, a large proportion of neonatal plasticizer exposure occurs intravenously (Panneel et al., 2021). Therefore, calculating EDI from neonatal urinary concentrations might underestimate *in vivo* exposure

(Strommen et al., 2022). Since remaining data gaps regarding clinically relevant endpoints make risk assessment insufficient (Bui et al., 2016) and NICU neonates are exposed to a mixture of plasticizers, the possible risk of cumulative exposure is important to be considered. Therefore, follow-up of the patients described in this Plastic-NICU cohort can give insight in correlation between neonatal plasticizer exposure and prematurity-associated life-long morbidity.

5. Conclusion

This study maps NICU neonatal exposure to both phthalate and alternative plasticizers using repeated urine sampling. It shows ongoing exposure of premature neonates to endocrine disrupting phthalates, despite changing legislation. More immature children, especially receiving respiratory support and/or blood products, are at increased risk of exposure above safe levels. This work provides insight in the trend of such exposure during NICU stay, with phthalate exposure increasing the first few days of life until four weeks postnatal. 57 % of neonates had at least once an estimated cumulative plasticizer exposure that may have detrimental effects on long term development.

CRediT authorship contribution statement

Lucas Panneel: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Paulien Cleys:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Giulia Poma:** Writing – review & editing, Supervision, Data curation. **Yu Ait Bamai:** Writing – review & editing, Supervision, Formal analysis. **Philippe G. Jorens:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Adrian Covaci:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition. **Antonius Mulder:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lucas Panneel reports financial support was provided by Research Foundation Flanders. Paulien Cleys reports financial support was provided by Research Foundation Flanders. Co-author Adrian Covaci is co-editor of Environment International If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw anonymized data that support the findings of this study are available from the corresponding author upon reasonable request. Individual participant data will not be available.

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Ethical approval

Prior to inclusion, written informed consent was obtained from the neonates' parents. This study was approved by the Antwerp University Hospital Ethical Committee (Ref. 2003022, EDGE 000701).

Appendix A. Supplementary material

Supporting information including detailed instrumental and quantification parameters and supplementary tables and figures can be found in the supplementary appendix, as referenced in the text. Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108605.

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