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Abstract

Perfluoroalkyl substances (PFASs) are environmental contaminants that have been shown to exert toxic effects, which are dependent upon concentration, in animals and humans. No specific data on the exposure of preterm infants to PFASs are available. We aimed to quantify the potential exposure of preterm infants to PFASs through human milk (HM), to be compared to the exposure data recently reported for infants by EFSA. The amount of PFASs in ten preterm (PHM) and ten donor HM (DHM) samples was evaluated and the expected daily intake (EDI) at full enteral feeding was calculated. This EDI was compared to the mean and the 95th centile dietary exposure ranges at the lower bound for infants issued by EFSA. The calculated median EDI for total PFASs was 20.72 ng/kg/day (range 10.72-107.84) for PHM and 17.92 ng/kg/day (range 6.4-28.96) for DHM, which were both higher than mean exposure ranges reported for infants (2.4-12.2 ng/kg/day). The calculated EDI for DHM was far more similar to the 95th centile (4.5-27.9 ng/kg/day) dietary exposure ranges. For PHM samples higher EDI values were obtained, with 4 out of 10 samples exceeding the upper limit of the 95th centile range.

Conclusion: The exposure of preterm infants to PFASs through HM feeding might exceed reference values reported for older and healthier infants. Given the immunological and developmental vulnerability of preterm infants, the risks related to their exposure to PFASs should be further investigated, also focusing on how maternal exposure and subsequent transfer through HM feeding can be reduced.

Keywords

Perfluoroalkyl substances; human milk; preterm infant

Introduction

Perfluoroalkyl substances (PFASs) is the collective name for a vast group of fluorinated compounds with high thermal, chemical and biological inertness [1]. PFASs are ubiquitous in the environment and have been shown to exert toxic effects, which are dependent upon concentration, in animals and humans [2]. Potential clinical effects of these substances on human health are under investigation, and seem to include impaired immune and thyroid function, liver and kidney disease, metabolic dysregulation, reproductive toxicity, altered development, and cancer [3].

Recently, the European Food Safety Authority (EFSA) performed a risk assessment for the sum of four PFASs sharing toxicokinetic properties and showing similar accumulation and long half-lives [4]. The established group tolerable weekly intake of 4.4 ng/kg/week was significantly lower than the values previously reported by EFSA for sodium perfluoro-1-octanesulfonate (PFOS) and perfluoro-n-octanoic acid (PFOA) individually [1]. Mean exposure ranges in infants, toddlers and older children were also detailed, showing the highest exposures in infants compared to adults and other children.

PFASs maternal transfer to the offspring can occur both prenatally, as these substances can cross the placenta, and postnatally, through breastfeeding [5–7]. To date, studies evaluating the excretion of PFASs through human milk (HM) have been focused on mothers of term infants, showing that PFASs concentration in HM is highly variable and dependent upon a range of maternal and environmental factors [8].

No data on the specific exposure of preterm infants to PFASs are currently available. Preterm infants constitute a highly vulnerable population, facing a huge risk of infection and infection-related morbidity and mortality. For these infants, HM, either mother's own milk or donor milk (DHM), represents the optimal source of nutrition [9]. In this perspective, we aimed to quantify

65 the potential exposure of preterm infants to PFASs through HM, to be compared to the
66 exposure data recently reported for infants by EFSA.
67
68 **Materials and methods**
69 *Ethics*
70 The study was approved by the Ethical Committee of Sant'Orsola-Malpighi Hospital, Bologna,
71 Italy (study ID 60/2016/U/Tess). Women who provided HM samples signed a written informed
72 consent.
73
74 *HM samples*
75 HM samples (10 mL each) were collected using a breast pump into sterile polypropylene
76 containers, immediately frozen at -20 °C and then delivered to the analytical laboratory.
77 Three different types of HM were collected:
78 • Term HM (THM): HM samples from mothers who had delivered at term (37-40 weeks
79 gestational age [GA]), recruited at the Nursery of Sant'Orsola-Malpighi Hospital within 48
80 hours from delivery.
81 • Preterm HM (PHM): HM samples from mothers of very preterm infants (GA<32 weeks),
82 recruited at the Neonatal Intensive Care Unit of Sant'Orsola-Malpighi Hospital within one
83 week from delivery.
84 • DHM: HM samples collected from donors of the HM Bank of Bologna, who are usually
85 recruited as donors within three months after delivering their own baby.
86 To evaluate preterm infants' potential exposure through both preterm and donor HM, total
87 PFASs concentration in each sample was multiplied for the recommended milk intake at full
88 enteral feeding (160 mL/kg/day), obtaining an estimated PFASs daily intake (EDI –
89 ng/kg/day). This EDI was compared to the mean and the 95th centile dietary exposure ranges

at the lower bound for the infants age (< 12 months old) group reported in the scientific opinion issued by EFSA [4].

Sample analysis

Samples were prepared according to a previously validated procedure [10]. Briefly, milk underwent protein precipitation with acetone, followed by a two-step SPE purification, first on an Oasis® HLB cartridge (Waters, Milford, MA, USA) and then on a Supelclean™ ENVI-Carb™ cartridge (Supelco, Sigma Aldrich, St. Louis, MO, USA) before LC-MS/MS analysis. Chromatographic separation was achieved with a Waters Acquity UPLC® binary pump, equipped with the PFC isolator kit to prevent from background contamination, and using a Waters Acquity UPLC® BEH C18 column (50 × 2.1 mm, 1.7 µm) kept at 45 °C (Waters, Milford, MA, USA). The mobile phase was a mixture of ammonium acetate 20 mM aqueous solution and methanol under programmed conditions at a constant flow rate of 0.5 mL/min over 6 min, and 10 µL of each sample was injected. The LC was interfaced with a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA), operating in negative electrospray ionization (ESI−) mode with capillary voltage set at 2.00 kV. Samples were analysed in multiple reaction monitoring (MRM) mode, selecting two transitions for each analyte and one for the relative internal standard. The monitored transitions, and the relative cone voltage and collision energy, are reported in Supplementary Table 1. Data were acquired and processed using MassLynx 4.1 software (Waters, Milford, MA, USA).

A matrix-matched calibration curve and quality control (QC) samples were freshly prepared during each day of analysis to assess the performance of the method in terms of linearity, specificity, accuracy and precision.

Statistical analysis

In each HM sample, the individual concentration of four PFASs (PFOS, PFOA, sodium perfluoro-1-hexanesulfonate [PFHxS], and perfluoro-n-nonanoic acid [PFNA]) was evaluated, and the sum of the obtained values was calculated. Following the advice by EFSA, lower bound PFASs levels, using zero for non-detects, were used, as these were deemed more realistic than upper bound levels [4].

Statistical analysis was performed using IBM SPSS Statistic v.20. Data distribution was checked for normality using the Shapiro-Wilk test and non-parametric tests were then used. Differences in PFASs concentration among the three groups were evaluated using the Kruskal-Wallis test with Dunn's post-hoc comparisons.

A p value ≤ 0.05 was considered as statistically significant.

Results

Ten PHM samples and ten DHM samples were analysed. Fifteen THM samples were used for comparison.

PFASs concentrations in the three groups of HM samples are reported in Table 1. There were no significant differences in PFOS, PFOA, PFHxS, and total PFASs concentrations among the three groups. PFNA concentration differed among groups, with THM concentration being the lowest.

Mean contribution (%) of individual PFASs was similar to that reported by EFSA for infants, with PFOA representing the main contributor to total PFASs (mean 58.7%, interquartile range [IQR] 37.2-73.7%), followed by PFOS (mean 37.9%, IQR 21.4-58.6%). The contribution of PFNA was lower (mean 3.4%, IQR 0-5.1%), while PFHxS was virtually absent.

The calculated median EDI for total PFASs was 20.7 ng/kg/day (range 10.7-107.8) for PHM and 17.9 ng/kg/day (range 6.4-29.0) for DHM. Both these values were considerably higher than mean lower bound exposure ranges reported for infants (2.4-12.2 ng/kg/day) [4].

140 The calculated EDI for DHM was far more similar to the 95th centile (4.5-27.9 ng/kg/day)
141 dietary exposure ranges reported by EFSA. For PHM samples higher EDI values were
142 obtained, with 4 out of 10 samples exceeding the upper limit of the 95th centile range (Figure
143 1). The EDI for THM was also calculated using 160 ml/kg/day as reference for the purpose of
144 comparison: median THM EDI was 21.6 ng/kg/day, with a range of 9.3-97.2 ng/kg/day. This
145 might represent an under- or over-estimation of actual PFASs daily intake, as healthy term
146 infants are usually fed "on demand" and precise milk intake is not easily quantifiable.
147

148 **Discussion**

149 The exposure of preterm infants to PFASs through HM feeding appears to be considerably
150 higher than mean exposure values recently reported by EFSA. A high variability in PFASs
151 concentration was documented in both donor and preterm HM, with a discrete proportion of
152 samples collected from mothers of preterm infants exceeding the upper exposure limit reported
153 for the infant population.
154 There is growing concern over PFASs as environmental contaminants, and for this reason they
155 have been subject to voluntary industry substitutions and regulatory actions [11]. It is well
156 known that the main routes of exposure to PFASs in infants are placental transfer and
157 breastfeeding [7]. As for preterm infants, it is unknown whether a reduced length of gestation
158 affects the amount of PFASs that is transferred from the mother to the foetus. However, it is
159 likely that, similarly to what happens in term infants, HM constitutes the main source of PFASs
160 also for preterm infants.
161 The recent scientific opinion issued by EFSA has indicated the immune system as a "prime
162 target" for PFASs, highlighting a positive association between maternal PFASs concentrations
163 and later risk of infection in the offspring, as well as between higher PFOS and PFNA
164 concentration and reduced antibody response to vaccination [4]. No specific link between

165 PFASs exposure and preterm infants' immune-related risk has been described so far. However,
166 it seems reasonable to speculate that preterm infants, especially those born at the lowest GAs,
167 might constitute a high-risk population in terms of PFASs exposure. Indeed, these infants face
168 a high risk of infection, due to the reduced transfer of protective maternal antibodies during
169 gestation and the relatively immature immune system. Neonatal infections might have a strong
170 impact on later life in this population, with impaired neurodevelopment following neonatal
171 sepsis documented in former extremely preterm infants up to 10 years of age [12]. Furthermore,
172 as for vaccine response, it has been recently shown that, despite the retrieval of protective
173 antibody levels for most antigens (except for *Haemophilus influenzae* type B) following a
174 routine vaccination schedule, postimmunization antibody levels in preterm infants were
175 significantly lower than those retrieved in a historical cohort of term infants [13].
176 The present study provides preliminary evidence that the exposure of preterm infants to PFASs
177 through HM, either own mother's or donor milk, might exceed reference values reported for
178 older and healthier infants. Human milk represents the optimal source of nutrition for preterm
179 infants, thanks to its unique bioactive properties, which have been associated to numerous
180 beneficial clinical effects in this population [9]. Given the immunological and developmental
181 vulnerability of preterm infants, the risks related to their exposure to PFASs should be further
182 investigated, also focusing on how maternal exposure and subsequent transfer through HM
183 feeding can be reduced.
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229

230 **Table 1.** Concentrations (ng/L) of four of the most often detected perfluoroalkyl substances

231 (PFASs) in human milk (HM). Values are reported as median (interquartile range).

232

	Term HM (n = 15)	Preterm HM (n = 10)	Donor HM (n = 10)	p
PFOS	47 (41-85)	34 (22-178)	26 (18-33)	0.115
PFOA	87 (77-115)	90 (68-108)	72 (62-92)	0.183
PFHxS	0 (0-0)	0 (0-00)	0 (0-00)	0.513
PFNA	0 (0-0)	6 (0-12)	20 (0-24)	0.013
Total PFASs	135 (118-208)	130 (96-296)	112 (93-135)	0.309

233
234

235 **Figure legends**

236

237 **Figure 1.** Calculated expected daily intake (EDI) of perfluoroalkyl substances (PFASs) in
238 preterm infants at full enteral feeding (160 ml/kg/day) through donor human milk (DHM) and
239 preterm human milk (PHM). Solid lines represent the upper (in red) and lower (in blue) limit
240 for mean total daily exposure (TDI) at lower bound (2.4-12.2 ng/kg/day), and dashed lines the
241 upper (in red) and lower (in blue) limit for the 95th centile TDI at lower bound (4.5-27.9
242 ng/kg/day).

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