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Concentrations and determinants of lead, mercury, cadmium, and arsenic in pooled donor breast milk in Spain

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A R T I C L E I N F O

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A B S T R A C T

Aim: To measure concentrations of lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) in longitudinally collected donor breast milk samples and to determine associated factors.

Methods: Pb, Hg, Cd, and As concentrations were measured in 242 pooled breast milk samples from 83 donors to a Human Milk Bank in Spain, in 2015–2018, determining their association with the donors' sociodemographic profile, dietary and lifestyle habits, and post-partum time, among other factors, and with the nutritional characteristics of samples. Mixed-effect linear regression was used to identify predictors of Hg and As concentrations in breast milk and mixed-effect logistic regression to identify predictors of the presence of Pb and Cd.

Results: As was the element most frequently detected in milk samples (97.1%), followed by Hg (81.2%), Pb (50.6%), and Cd (38.0%). Their median breast milk concentrations were 1.49 μg/L, 0.26 μg/L, 0.14 μg/L, and <0.04 μg/L, respectively. Concentrations of As were higher in breast milk from primiparous donors, while Hg was higher in donors with a greater intake of fatty fish and meat and lower in samples collected after a longer post-partum time and with higher lactose content. Detection of Pb was higher among multiparous donors, those gaining weight since before pregnancy, and ex-smokers and was lower in samples collected more recently and from donors with greater intake of red meat and eggs. Cd detection was higher for donors with university education and those with greater intake of fried and canned food and more frequent use of hand cream and was lower for donors with greater bread intake.

Conclusions: These findings reveal relatively high As concentrations, moderate Hg concentrations, and low Pb and Cd concentrations in pooled donor breast milk. Several factors including post-partum time, parity, smoking habit, and the intake of certain food items were associated with the metal content of milk samples.

1. Introduction

The benefits for infants and mothers of breastfeeding are well documented (Labbok, 2001; Lawrence, 2000). Breast milk is the best source of nutrition for both full-term and preterm infants. It contains fats, carbohydrates, proteins, and other important dietary components, contributing to the growth, immunity, and development of the infant (Ballard and Morrow, 2013). Nevertheless, breast milk is also known to be a pathway for the maternal excretion of environmental chemicals, and there have been numerous reports worldwide on the presence of

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different types and concentrations of persistent organic pollutants (POPs) and toxic metals/metalloids due to past or current maternal exposure (Gil and Hernández, 2015; LaKind et al., 2018; Rebelo and Caldas, 2016).

Lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) are widespread environmental metals that top the list of priority hazardous substances published by the Agency for Toxic Substances and Disease Registry (Agency for Toxic Substances and Disease Registry, 2019). Pb, Hg, As, and to a lesser degree Cd can readily pass through the placental barrier from the maternal bloodstream into the fetal circulation (Esteban-Vasallo et al., 2012) and can be excreted *via* breast milk after birth, with the amount transferred to the milk depending on their chemical form and distribution in maternal blood fractions (Gundacker and Zödl, 2005). Pb, Hg, Cd, and As are considered as persistent contaminants; however, unlike POPs, they do not bind to fat and therefore do not usually accumulate at higher concentrations in breast milk than in blood; hence, infants are likely exposed to higher levels of these metals before birth than during breastfeeding (Solomon and Weiss, 2002). Nonetheless, the metal content of breast milk is an important additional pathway of postnatal exposure and is likely to reflect intrauterine exposure (Solomon and Weiss, 2002) or even the lifetime exposure of the mother (LaKind et al., 2018). For instance, lactation can be accompanied by enhanced bone resorption due to the demand for calcium of nursing infants, which can mobilize Pb stored in maternal bone and contribute to the Pb content of breast milk (Ettinger et al., 2006). Pb and Hg are toxic for the reproductive system and the developing nervous system, Cd and As are known to be human carcinogens (Diamanti-Kandarakis et al., 2009; IARC, 2016), and all four metals are suspected endocrine disruptors (Diamanti-Kandarakis et al., 2009; Mendiola et al., 2011).

Pb and Hg have been the most investigated toxic metals in breast milk worldwide, with less research on the presence of As (Cherkani-Hassani et al., 2019; Rebelo and Caldas, 2016; Vollset et al., 2019). Breast milk concentrations of Cd are among the lowest reported (Cherkani-Hassani et al., 2017; Rebelo and Caldas, 2016) most likely because Cd-binding milk proteins have a greater affinity for calcium, which is abundant in breast milk (Vahter et al., 2002). Mothers who smoke or have a higher intake of certain food items (*e.g.*, fish) may be exposed to higher levels of Cd, Pb, and Hg (Bassil et al., 2018; Rebelo and Caldas, 2016; Vollset et al., 2019). With regard to As, higher breast milk concentrations were found in mothers from areas with elevated levels of As in drinking water (Samanta et al., 2007). Some studies have described a trend towards lower breast milk concentrations of toxic metals at later stages of lactation, but this has yet to be definitively established (Chao et al., 2014; García-Esquinas et al., 2011; Leotsinidis et al., 2005).

It is accepted that the benefits of breastfeeding generally outweigh the risks posed by the presence of environmental chemicals in the milk (Mead, 2008). However, it is important to improve knowledge on the exposure of infants to environmental chemicals and on changes in the exposure of mothers during lactation, particularly in the setting of the neonatal intensive care unit (NICU). However, only one recent study has been published on concentrations of toxic metals in donated breast milk given to hospitalized preterm newborns (Oliveira et al., 2020). Recent reports on toxic metals in breast milk from Spanish women are based on the analysis of Pb, Hg, and Cd in 100 samples collected at week 3 postpartum in Madrid (García-Esquinas et al., 2011) and of Pb, Hg, Cd, and As, among other trace elements, in 170 samples gathered in the city of Santiago de Compostela in Northern Spain (Mandiá et al., 2021). Our group recently reported the concentration profiles of various perfluoroalkyl substances (PFAS) and environmental phenols in donor breast milk from a Human Milk Bank in Granada, Southern Spain (Iribarne-Duran et al., unpublished results; Serrano et al., 2021). In the present study, we examined concentrations of Pb, Hg, Cd, and As and associated factors in longitudinally gathered breast milk samples from the same donors.

2. Materials and methods

2.1. Study population

During 2015–2018, 275 mature breast milk samples were obtained from 83 donors to the Regional Milk Bank of the Virgen de las Nieves University Hospital, Granada (Southern Spain) at different times postpartum (never before 2–3 weeks post-partum). All potential participants were invited to participate in this study. Exclusion criteria for the donors were previously described in detail (Serrano et al., 2021) and included: positive serology for HIV, syphilis, or hepatitis B or C; risk factor for sexual transmitted disease; transplantation in previous 6 months; current smoking or drug habit; and high consumption of alcohol (>20 g/day) or caffeine-containing drinks (>30 g/day). After providing their written informed consent, participating donors completed a questionnaire on socio-demographic characteristics and reproductive and lifestyle factors and donated milk samples for the analysis of environmental chemicals. The research protocol was approved by the Biomedical Research Ethics Committee of Granada. This study included 242 out of 275 milk samples from 83 donors with sufficient volume for the analysis of trace elements. Information on dietary habits and the use of personal care products (PCPs) was gathered for a sub-sample of 78 participants who provided a total of 228 samples.

2.2. Breast milk sample collection

Participating donors were asked by the milk bank to collect milk over a period of 1–4 consecutive weeks by manual expression and/or breast pump and to keep the samples frozen at −20 °C in their refrigerator until delivery to the bank. On arrival at the milk bank, samples were stored at −30 °C without ever breaking the cold chain. Immediately before pasteurization by the Holder method (within 2 weeks of arrival at the bank), samples collected from each donor were thawed and then pooled, obtaining an aliquot of 5–30 mL that was stored at −20 °C until analysis. The day of pasteurization was recorded as the donation date, and the interval between the first sample expressed by the mother and the donation date never exceeded 6 weeks. The median number of donations per woman was two, ranging from one to thirteen (four from 25% of participants and seven from 10%).

2.3. Sample preparation

First, 0.5 mL of milk sample was microwave digested in quartz vessels with 0.5 mL of HNO_3 (Suprapur, Merck, Darmstadt, Germany) using the Ethos UP system (Milestone, Shelton, CT, USA) programmed with 1800 W as maximum power and 210 °C as temperature limit (ramp time 20 min; hold time 15 min; cooling time 60 min). The digested solution was then transferred to a decontaminated tube for later analysis. Before their utilization, the quartz vessels were vigorously cleaned, soaked for 24 h in 10% HNO_3, thoroughly rinsed with Milli-Q® water, and dried at 80 °C for about 2 h. A certified reference material was used as quality control (ERM-BD151 Skimmed milk powder). Approximately 0.5 g of certified reference material was reconstituted with 4.5 mL of Milli-Q® water. The resulting liquid milk was then digested, as were all study samples. All samples were diluted 1:5 with 1% HCl (Suprapur, Merck).

2.4. Metal analysis

Quantification of Pb, Hg, Cd, and As concentrations in breast milk was performed by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA) at the laboratory of the Department of Legal Medicine, Toxicology, and Physical Anthropology of the University of Granada (Spain). A calibration curve was prepared for each element in ultrapure water (Milli-Q) with 2% HNO_3 (Suprapur, Merck) and 1% HCl (Suprapur, Merck) using reference metal standard solutions (Agilent

Technologies) and analyzing blanks to correct the results. The instrument was tuned and performance parameters were checked before each analysis session. The quality of results was ensured by adding online multielement 400 μg/L internal standard solution with Sc, Ge, Ir and Rh to the samples. In addition, corresponding certified reference materials [National Institute of Standards and Technology NIST (USA) Trace Elements in Natural Water Standard Reference Material SRM 1640a and ERM-BD151 Skimmed milk powder] were reanalyzed together with a blank and an intermediate calibration standard every 12 samples. One in every twelve samples was also reanalyzed at the end of each session. Milk concentrations were expressed as μg/L. Limits of detection (LODs) were 0.10 μg/L for Pb, 0.05 μg/L for Hg, 0.04 μg/L for Cd, and 0.40 μg/L for As.

2.5. Explanatory variables

Information on potential explanatory variables was obtained from the questionnaires completed by donors on registration at the bank (donor selection questionnaire) and after inclusion in this study (research questionnaire). The research questionnaire was interviewer administered at the time of the first donation. The following data were gathered: age (continuous, years), university education (yes/no), occupation (unemployed/manual worker/non-manual worker), area of residence (urban/sub-urban/rural), living near agricultural land (yes/no), greenhouse (yes/no) or any industrial activity (yes/no), ex-smoker (yes/no), body mass index (BMI; underweight or normal/overweight or obese); parity (primiparous/multiparous), gestational length (continuous, weeks), birth weight (continuous, g), lifetime duration of breastfeeding (≤6/>6–12/>12–24/>24 months), weight gain during pregnancy (continuous, kg), weight change from before the most recent pregnancy (gain/loss/no change), presence of amalgam dental filling (yes/no), main source of drinking water (tap/bottled water), intake of coffee (1 cup per day/<1 cup per day) and alcoholic drinks (≥1 drink per month/<1 drink per month), average consumption frequency (servings [sv] per day or week) of seafood, fatty and lean fish, dairy products (yoghurt, milk, butter, cheese), red and cold meats, pulses, eggs, bread, chocolate, cereals, rice, pasta, fruit, raw and cooked vegetables, deep-fried food, and canned food (Table S1), and frequency of use of several PCP products (Table S2). PCPs were explored as potential determinants of breast milk metal concentrations because their utilization has been described as a potential source of exposure to toxic metals, including Pb and Cd (Mesko et al., 2020; Vahidinia et al., 2019). Data on dietary intake and the utilization of PCPs referred to the 12 months before the interview. None of the participating donors were pregnant during the period of donation. The post-partum time of the donation was calculated as the period between the date of the donation and the date of the most recent birth, categorized as ≤3, >3–6, >6–9, or >9 months. Cumulative lifetime breastfeeding was calculated by adding the aforementioned period to the lifetime breastfeeding time reported in the research questionnaire. The maternal age was updated at the time of each donation. Given that the transfer of metals into breast milk is produced by binding to proteins, the total protein content of the unpasteurized milk samples was examined as a potential explanatory variable, in addition to the total lipid, lactose, and caloric contents of samples.

2.6. Statistical analysis

In a descriptive analysis, concentrations of metals in individual samples (n = 242) and mean concentrations per donor (n = 83) were reported as medians, and 5, 25, 75, and 95 percentiles. Hg and As were detected in a large proportion of samples, and their values below the LOD were imputed as the LOD divided by the square root of two. Distributions of Hg and As were left-skewed and were therefore natural log (ln)-transformed to normalize data for analyses. Spearman correlation test was used to analyze associations between metal concentrations. Mixed-effect linear regression was used to examine predictors of Hg and As concentrations in breast milk. Given the high percentage of undetected values for Pb and Cd (49.5% and 62.0%, respectively), the odds of breast milk Pb and Cd concentrations above the LOD were assessed by using mixed-effect logistic regression. In mixed-effect models, the donor ID was treated as a random variable (cluster variable) to account for correlation between repeated measurements within subjects. A forward stepwise procedure was used to enter predictors (fixed variables) in the models. All variables listed in Tables 1, 2, S1, and S2 were tested as potential explanatory variables. Sensitivity analysis was performed by excluding outlier concentrations of As (n = 7) and Hg (n = 1) identified with studentized residuals >3. $P < 0.05$ was set to retain variables in the final model. Associations were expressed as exponentiated regression coefficients (exp[β]) or odds ratios (ORs) with 95% confidence intervals (CI). The "nlme" package in the statistical program R v.4.1.0 was used for statistical analyses (The R Project for Statistical Computing, https://www.r-project.org).

3. Results

Participating donors had a mean age of 33 years (range: 19–47 years), 61% had university education, 29% were manual workers, 42% resided in urban areas, 47% were ex-smokers, 31% were overweight or obese, and 10% had an amalgam dental filling (Table 1). At the time of the interview, 46% of donors were multiparous, the mean gestation time and newborn weight in their most recent pregnancy were 38 weeks and 2967 g, respectively, the mean weight gain during the pregnancy was 12 kg, with 53% donors gaining weight since before the pregnancy and 20% losing weight (Table 1). A full description of the dietary habits and PCP utilization of the participants is available in Serrano et al. (2021) and Supplementary material (Tables S1 and S2).

Milk samples were collected at a mean of 238 (range: 20–1513) days after the birth, and most (87%) of them were collected in 2015–2017; 25% of samples were collected after a lifetime breastfeeding time of >24 months and 28% after a time of ≤6 months. The mean protein content of

Table 1
Maternal characteristics and reproductive factors, n = 83 milk donors.

Variables	n (%)	Mean (range)
Maternal characteristics		
Age (years)		33 (19–47)
University education	51 (61.4)	
Occupation		
Unemployed	5 (6.2)	
Manual worker	24 (28.9)	
Non-manual worker	54 (65.1)	
Area of residence		
Rural	24 (28.9)	
Sub-urban	24 (28.9)	
Urban	35 (42.2)	
Living near agricultural land	40 (51.3)	
Living near greenhouse	14 (17.9)	
Living near industrial activity	61 (78.2)	
Ex-smoker	39 (47.0)	
Current BMI (kg/m^2)		23.67 (17.30–36.09)
Overweight/obese	26 (31.3)	
Amalgam dental filling	8 (9.6)	
Reproductive factors[a]		
Multiparous	38 (45.8)	
Length of gestation (weeks)		38 (26–41)
Birth weight (g)		2932 (840–4500)
Weight gain during pregnancy (kg)		12 (1–36)
Weight change from before pregnancy		
Weight loss	17 (20.5)	
Weight gain	44 (53.0)	
No weight change	22 (26.5)	

BMI: Body mass index.
[a] Reproductive data relative to the most recent pregnancy and before the first donation. None of the donors were or became pregnant during subsequent donations.

samples was 2.05 g/100 mL, their mean fat content was 3.83 g/100 mL, mean lactose content was 7.44 g/100 mL, and mean energy content was 68 kcal/100 mL (Table 2).

As was the element most frequently detected in milk samples (97.1%), followed by Hg (81.2%), Pb (50.6%), and Cd (38.0%). The median concentrations (5th-95th percentiles) in breast milk were 1.49 μg/L (0.56–3.50 μg/L) for As, 0.26 μg/L (<0.05–1.17 μg/L) for Hg, 0.14 μg/L (<0.10–6.31 μg/L) for Pb, and <0.04 μg/L (<0.04–0.44 μg/L) for Cd (Table 3). Weak to moderate positive correlations were observed between Pb and Cd (Spearman coefficient, r = 0.52, p < 0.001), between Hg and As (r = 0.27; p < 0.001), between Hg and Cd (r = 0.20, p = 0.001), and between Hg and Pb (r = 0.15, p = 0.02).

Linear regression models showed that concentrations of As in breast milk decreased by 32% ((exp$^{\beta}$-1)*100) (95%CI = 8–49%) in samples collected in 2017 *versus* 2015 and by 22% (95%CI = 1–40%) in samples from multiparous *versus* primiparous donors (Table 4). For Hg, it was found that samples collected at >3–6 *versus* ≤3 months post-partum had 41% (95%CI = 17–58%) lower concentrations; in addition, Hg decreased by 38% (95%CI = 5–59%) per each unit increase in lactose content. In contrast, donors consuming 1 sv/week of fatty fish had 68% (95%CI = 6–166%) higher Hg concentrations compared to <1 sv/week; and those consuming 2 and > 2 sv/week of meat compared to ≤1 sv/week showed a more than 2-fold (95%CI = 34–477%) and 3-fold (95% CI = 87–523%) increase in Hg, respectively (Table 4).

In logistic models (Table 5), the odds of a Pb concentration above the LOD were lower for samples collected in 2017 (OR = 0.38, 95%CI = 0.16; 0.90) and 2018 (OR = 0.15, 95%CI = 0.04; 0.48) compared with 2015, and for samples from donors with an intake of ≥1 sv/week of red meat *versus* rarely/never (OR = 0.23, 95%CI = 0.10–0.50) and from those with an intake of 2 sv/week of eggs *versus* 1 sv/week (OR = 0.38, 95%CI = 0.17–0.84). The odds of Pb detection were higher for multiparous donors (OR = 4.56, 95%CI = 2.09–10.8), those showing an increase in weight since before the pregnancy (OR = 3.15, 95%CI = 1.10–9.54), and ex-smokers (OR = 1.95, 95%CI = 1.05–3.64). The odds of Cd > LOD were higher for donors with university education (OR = 3.27, 95%CI = 1.69–6.56), those with a higher intake of fried food (1 sv/week: OR = 2.94, 95%CI = 1.33–6.76; >1 sv/week: OR = 4.25, 95%CI = 1.85–10.3 *versus* <1 sv/week), those regularly consuming canned food (OR = 3.78, 95%CI = 1.65–9.33), and those using hand cream once a day *versus* less frequently (OR = 2.99, 95%CI = 1.28–7.20); and were lower for those with a greater bread intake (1 sv/day: OR = 0.18, 95%CI = 0.07–0.42; >1 sv/day: OR = 0.21, 95%CI = 0.08–0.51 *versus* <1 sv/day).

Table 2
Characteristics of pooled milk samples (n = 242).

Variables	n (%)	Mean (range)
Year of sample collection		
2015	70 (28.9)	
2016	79 (32.6)	
2017	61 (25.2)	
2018	31 (13.8)	
Post-partum time (days)		238 (20–1513)
Post-partum time (months)		
≤3	60 (24.8)	
>3-6	54 (22.3)	
>6-9	57 (23.6)	
>9	71 (29.3)	
Lifetime duration of breastfeeding (months)		17.2 (0.7–77.3)
≤6	67 (27.7)	
>6-12	68 (28.1)	
>12-24	46 (19.0)	
>24	61 (25.2)	
Nutritional parameters		
Total proteins (g/100 mL)		2.05 (0.20–96.0)
Total lipids (g/100 mL)		3.83 (1.05–33.0)
Lactose (g/100 mL)		7.44 (6.10–8.10)
Calories (kcal/100 mL)		68 (44–117)

Exclusion of As outliers led to an inverse association with lifetime breastfeeding, so that breastfeeding for >6–12 and > 12–24 months *versus* ≤6 months was associated with a significant decrease in As concentration of 21% (95%CI = 6–32%) and 22% (95%CI = 6–36%), respectively, while As concentrations were not influenced by the parity of donors after excluding outliers (Table S3). With respect to Hg, exclusion of one outlier in breast milk concentrations did not change the results (data not shown).

4. Discussion

This study is one of the first to provide information on toxic metal concentrations in breast milk from donors in Spain. As was present in almost all pools of milk, Hg in four out of five, Pb in one out of two, and Cd in one out of three. Results suggest a trend towards a decline in Hg concentrations over the lactation period and a decline in Pb concentrations between 2015 and 2018. In addition, smoking habit, the intake of certain food items, weight change since before the pregnancy, and schooling level, among other factors, emerged as significant predictors of breast milk metal concentrations, which were not associated with the total protein content of samples.

4.1. Arsenic

Concentrations of total As in breast milk from these Spanish donors were higher than concentrations in mature milk obtained from women in Germany (Sternowsky et al., 2002), Italy, Croatia, Slovenia (Miklavčič et al., 2013), Cyprus (Kunter et al., 2017), Sweden (Björklund et al., 2012), Japan, the USA (Carignan et al., 2015), Thailand (Chao et al., 2014), the United Arab Emirates (Abdulrazzaq et al., 2008). Total As concentrations were also higher than those found in 49 pre-concentrated and concentrated breast milk samples from a Brazilian milk bank (Oliveira et al., 2020). They were in the range of concentrations found in the milk of women from India (Samanta et al., 2007) and Bangladesh (Fängström et al., 2008; Islam et al., 2014) exposed to high levels of inorganic As in drinking water (Rebelo and Caldas, 2016), but they were lower than concentrations in primiparas from Lebanon (Bassil et al., 2018). Notably, the maximum As value in Spanish donors (56 μg/L) was higher than the maximum concentration found in milk from the highly exposed women in India (49 μg/L) (Samanta et al., 2007) and Bangladesh (8.9 and 19 μg/L) (Fängström et al., 2008; Islam et al., 2014). In addition, As concentrations in our study population were in the range of those found in the only Spanish study providing data on As in breast milk, which included 70 full-term and 100 preterm mothers recruited in Santiago de Compostela in 2018–2019 (mean = 1.37 μg/L) (Mandiá et al., 2021).

The main sources of As exposure are drinking water contaminated with inorganic As and the intake of seafood and rice, which can contain elevated concentrations of organic and inorganic As, respectively (Hughes et al., 2011). As in breast milk has previously been associated with the intake of fish/seafood (Bassil et al., 2018; Miklavčič et al., 2013) and rice/cereals (Bassil et al., 2018), but these associations were not found in the present study. Organic forms of As such as monomethyl As (MMA) and dimethyl As (DMA) are much less toxic than inorganic forms such as trivalent (AsIII) and pentavalent As (AsV), which have been classified as type 1 carcinogens (IARC, 2016). AsIII is the only form of As transported by the aquaglyceroporins present in mammary glands during lactation (Liu et al., 2004; Matsuzaki et al., 2005). It has been shown that the efficient maternal methylation of inorganic As into MMA and DMA leads to a lower excretion of As *via* breast milk, because MMA and DMA in blood plasma do not easily pass through the mammary glands. For these reasons, breast milk largely contains inorganic As, mainly as AsIII (Rebelo and Caldas, 2016). Rice and seafood consumption has been described as the major source of As exposure in Spain (Signes-Pastor et al., 2017). The specific source of exposure to As in the present donors remains unknown; however, previous studies showed

Table 3
Concentrations of metal(oid)s in breast milk (μg/L).

	LOD	% >LOD	Percentiles					Max.
			5	25	50	75	95	
Individual[a] sample concentrations (n = 242 samples)								
As	0.4	97.1	0.563	1.073	1.494	1.989	3.501	56.52
Hg	0.05	81.2	<0.05†	0.080	0.261	0.538	1.174	2.428
Pb	0.10	50.6	<0.10†	<0.10†	0.138	1.250	6.315	49.32
Cd	0.04	38.0	<0.04†	<0.04†	<0.04†	0.070	0.442	4.936
Mean concentrations per donor (n = 83 donors)								
As	0.4	–	0.596	1.160	1.660	1.981	7.582	52.17
Hg	0.05	–	<0.05†	0.151	0.277	0.529	1.134	1.515
Pb	0.10	–	<0.10†	<0.10†	0.220	1.138	6.013	24.70
Cd	0.04	–	<0.04†	<0.04†	<0.04†	0.093	0.480	1.860

LOD: Limit of detection.
[a] Pools of milk samples.

Table 4
Mixed-effects linear regression models for predictors of concentrations of As and Hg in pooled breast milk.

As (n = 242 samples from 83 donors)			Hg (n = 228 samples from 78 donors)		
Explanatory variables	Exp (β)	95%CI	Explanatory variables	Exp (β)	95%CI
Year of collection (ref: 2015)			Post-partum time (ref: ≤3 months)		
2016	0.78	0.59; 1.02*	>3–6 months	0.59	0.42; 0.83**
2017	0.68	0.51; 0.92**	>6–9 months	0.80	0.55; 1.15
2018	0.96	0.65; 1.42	>9 months	0.86	0.60; 1.23
Multiparous vs. primiparous	0.78	0.60; 0.99**	Lactose content (g/100 mL)	0.62	0.41; 0.95**
			Fatty fish intake (ref: < 1 sv/week)		
			1 sv/week	1.68	1.06; 2.66**
			>1 sv/week	1.40	0.77; 2.53
			Meat intake (ref: ≤1 sv/week)		
			2 sv/week	2.79	1.34; 5.77**
			>2 sv/week	3.42	1.87; 6.23***

***p < 0.001; **p < 0.05; *p < 0.10.

Table 5
Mixed-effects logistic regression models for predictors of Pb and Cd in pooled breast milk (n = 228 from 78 donors).

Detected Pb 113 out of 228 samples (49.6%) >LOD			**Detected Cd 88 out of 228 samples (38.6%) >LOD**		
Explanatory variables	**OR**	**95%CI**	**Explanatory variables**	**OR**	**95%CI**
Year of collection (ref: 2015)			University vs. lower schooling level	3.27	1.69; 6.56***
2016	0.56	0.23; 1.34	Bread intake (ref: <1 sv/day)		
2017	0.38	0.16; 0.90**	1 sv/day	0.18	0.07; 0.42***
2018	0.15	0.04; 0.48**	>1 sv/day	0.21	0.08; 0.51***
Multiparous vs. primiparous	4.56	2.09; 10.8***	Fried food intake (ref: <1 sv/week)		
Weight change (ref: no change)			1 sv/week	2.94	1.33; 6.76**
Weight gain	3.15	1.10; 9.54**	>1 sv/week	4.25	1.85; 10.3***
Weight loss	1.39	0.57; 3.40	Canned food intake (ever vs. never)	3.78	1.65; 9.33**
Ex-smoker vs. never smoker	1.95	1.05; 3.64**	Hand cream (ref: <once a day)		
Red meat intake (ref: never)			once a day	2.99	1.28; 7.20**
<1 sv/week	1.56	0.69; 3.53	>once a day	1.37	0.53; 3.47
≥1 sv/week	0.23	0.10; 0.50***			
Eggs intake (ref: 1 sv/week)					
2 sv/week	0.38	0.17; 0.84**			
>2 sv/week	1.32	0.56; 3.14			

LOD: Limit of detection.
***p < 0.001; **p < 0.05; *p < 0.01.

that the topsoil in Southeastern Spain (including Granada province) contains relatively high concentrations of As (Núñez et al., 2016), likely attributable to past usage of phosphate fertilizers. The high occurrence of As (mainly inorganic As) in the breast milk of donors is of particular concern, given that even low concentrations of As have been shown to impair cognitive function and increase the risk of cancer in infants and young children (Rodríguez-Barranco et al., 2016; Tyler and Allan, 2014). Nonetheless, it has been suggested that exclusively breastfed infants are exposed to lower concentrations of As than are non-breastfed infants (Carignan et al., 2015; Fängström et al., 2008), indicating that exclusive breastfeeding may protect the infant from As exposure. Therefore, it is imperative to implement preventive measures to eliminate or reduce the presence of As in breast milk and to closely monitor its concentration in nursing mothers.

The decrease in As concentrations observed in multiparous donors and those with longer lifetime breastfeeding may suggest the clearance of As during lactation, but As was not associated with the post-partum time. This is consistent with a study in Bangladesh that found no difference in As concentrations in milk samples collected at 1, 6, or 9 months post-partum (Islam et al., 2014). In Portuguese and Taiwanese women, As milk concentrations were significantly higher in colostrum than in mature milk (Almeida et al., 2008; Chao et al., 2014), but these results are not comparable to the present findings because colostrum, which may have a higher concentration of metal-binding proteins, was not collected. Overall, the transport mechanism of As *via* breast milk has not been fully elucidated, and the few available data on postnatal exposure to As from breast milk are not conclusive.

4.2. Mercury

Breast milk concentrations of total Hg reported in the literature vary widely among different regions, with the highest concentrations (up to 59 μg/L) found in the Brazilian Amazon (Rebelo and Caldas, 2016). In general, Hg concentrations in Spanish donors are comparable to those found in women from Austria (Gundacker et al., 2010), Croatia, Greece, Italy, Slovenia (Miklavčič et al., 2013; Valent et al., 2013), Sweden (Björnberg et al., 2005), Brazil (Oliveira et al., 2020), Japan (Iwai-Shimada et al., 2015; Sakamoto et al., 2012) and Iran (Behrooz et al., 2012; Okati et al., 2013), with mean/median concentrations ranging from 0.1 to 0.8 μg/L. The present concentrations are in the range of those observed in mature milk samples collected in 2003–2004 from Spanish women in Madrid (mean = 0.53 μg/L) (García-Esquinas et al., 2011) and more recently in Santiago de Compostela (mean = 0.31 μg/L) (Mandiá et al., 2021). They are lower than concentrations found in breast milk samples from the Faroe Islands (Needham et al., 2011), different Asian regions (China, India, Indonesia, Korea, Taiwan) (Bose-O'Reilly et al., 2008; Chien et al., 2006; Li et al., 2014; Orün et al., 2012; Vahidinia et al., 2019), the Middle East (Saudi Arabia, Iran, Turkey) (Al-Saleh et al., 2013; Orün et al., 2012; Vahidinia et al., 2019), Africa (Tanzania, Zimbabwe) (Bose-O'Reilly et al., 2008), and Latin America (Brazil, Mexico) (Cunha et al., 2013; Gaxiola-Robles et al., 2014; Santos-Silva et al., 2018; Vieira et al., 2013); however, they are higher than concentrations observed in samples from Cyprus (Kunter et al., 2017) and the United Arab Emirates (Abdulrazzaq et al., 2008).

A significant association was observed between the intake of 1 sv/week of fatty fish and a higher Hg concentration in breast milk, consistent with the findings of a large study of samples from Croatia, Greece, Italy, and Slovenia, which found an association between fish consumption and breast milk Hg concentrations (Miklavčič et al., 2013). Other studies of women with a high or relatively high consumption of fish also reported an association of fish/seafood intake with Hg concentrations in breast milk (García-Esquinas et al., 2011; Gaxiola-Robles et al., 2013; Grandjean et al., 1995; Iwai-Shimada et al., 2015; Vollset et al., 2019). In general, the intake of fish, particularly fatty fish, is the main source of exposure to methyl-Hg (MeHg), the most neurotoxic form of Hg (Gil and Gil, 2015). It has also been shown that when fish intake is high, around one-half of breast milk Hg is in the form of MeHg (Islam et al., 2014; Miklavčič et al., 2013; Valent et al., 2013) and the other half is in the form of inorganic Hg (Oskarsson et al., 1996). We were unable to distinguish between organic and inorganic Hg, but a significant amount of MeHg can be expected in the present milk samples because of the relatively frequent consumption of fish by the donors. This is a cause for concern, given that MeHg is almost completely absorbed by the gastrointestinal tract of infants and can readily cross the blood-brain barrier and affect neurological functions (Caserta et al., 2013; Grandjean and Landrigan, 2006), even at low doses (Karagas et al., 2012). The association of meat intake with Hg concentrations is less certain because of the limited information on Hg levels in land animals. Although Hg can also bioaccumulate in this type of animal, the meat is likely to contain low concentrations of Hg (Björnberg et al., 2005; Nawrocka et al., 2020; Vollset et al., 2019). The presence of amalgam fillings, a major source of elemental Hg exposure, was not associated with Hg excretion in these Spanish donors. Studies examining the association of amalgam fillings with Hg in breast milk have yielded conflicting results, with some showing a positive association (Björnberg et al., 2005; Vollset et al., 2019) and others finding no relationship between them (García-Esquinas et al., 2011; Gundacker et al., 2002).

The inverse association observed between post-partum time and Hg concentration suggests a depuration of this metal during lactation, especially in the first few months. However, Hg concentrations in breast milk from Iranian (N = 100) and Swedish (N = 20) women remained unchanged throughout lactation (Björnberg et al., 2005; Vahidinia et al., 2019), while García-Esquinas et al. (2011) reported non-significant decreases in Hg concentrations in milk from older and multiparous women in Spain and in those with a previous history of lactation, suggesting a possible clearance of Hg over their lifetime. A decrease in Hg concentrations over the lactation period can be explained by a reduction in the milk's content of proteins such as albumin and casein, which enable the transport of both inorganic and organic Hg (Sundberg et al., 1999). Indeed, the protein content of the present donor milk samples slightly decreased with longer post-partum time (data not shown). Moreover, MeHg is a lipophilic compound, so that accumulated body stores of MeHg would decline with longer breastfeeding time (Jain, 2013; LaKind et al., 2004). However, the depuration of lipophilic chemicals during lactation may also be influenced by the current exposure of the mother, the volume of milk consumed by the infant, and supplementation with formula or solid food, among other factors (LaKind et al., 2018). In addition, the reason for the inverse association between the lactose and Hg content of samples remains unclear.

4.3. *Lead*

Breast milk Pb concentrations in these Spanish donors are several times lower than concentrations described in studies published over the past two decades in Asia (Chao et al., 2014; Isaac et al., 2012; Li et al., 2000; Sharma and Pervez, 2005), the Middle East (Al-Saleh et al., 2003; Bassil et al., 2018; Vahidinia et al., 2019), South America (Counter et al., 2004; Marques et al., 2013; Oliveira et al., 2020), North America (Hanning et al., 2003; Sowers et al., 2002), and Africa (Adesiyan et al., 2011; Moussa, 2011), and they are similar to or in the lower range of those found in women from Japan (Sakamoto et al., 2012), Australia (Gulson et al., 2003), Mexico (Ettinger et al., 2004, 2006), and various European countries (Abballe et al., 2008; Almeida et al., 2008; Gundacker et al., 2002; Kunter et al., 2017; Leotsinidis et al., 2005). In comparison to other Spanish studies, Pb concentrations in our donors are much lower than concentrations in samples collected in 2003–2004 in Madrid (mean = 15.6 μg/L) (García-Esquinas et al., 2011) but comparable to those in samples recently collected in Galicia (mean = 0.30 μg/L) (Mandiá et al., 2021), indicating a decline in Pb exposure in Spain after the suppression of leaded gasoline in 2001 (RealDecreto403, 2000). In this line, a decreasing trend in breast milk Pb concentrations was observed in the present donors over the period under study (2015–2018).

Diet is considered the major source of Pb exposure for the general population, particularly the intake of vegetables and cereals (Martí-Cid et al., 2008), and breast milk Pb concentrations have been associated with the intake of potatoes in Spanish (García-Esquinas et al., 2011) and Lebanese mothers (Bassil et al., 2018). No food item was found to predict Pb excretion in the present milk samples, probably due to the low Pb concentrations, while the intake of red meat and eggs was associated with lower breast milk Pb. However, these results should be interpreted with caution, given that much of the Pb in breast milk comes from Pb stored in the bones and not from the exposure of mothers during lactation. Pb is also found in cigarette smoke (Bernhard et al., 2005) which may explain the higher odds of detectable Pb in breast milk from former smokers *versus* never smokers. Similar results were reported by the Lebanese and Spanish studies (Bassil et al., 2018; García-Esquinas et al., 2011; Mandiá et al., 2021).

Pb excreted into breast milk is mainly found in the casein fraction (Chao et al., 2014; Ettinger et al., 2006; Leotsinidis et al., 2005; Oskarsson et al., 1996) and, when bone Pb levels are not high, breast milk concentrations generally decline over the course of lactation due to the decrease in casein content (Chao et al., 2014; Ettinger et al., 2006; Leotsinidis et al., 2005). The lack of an association between Pb and post-partum time in the present study may be explained by the low Pb concentrations. The reason for the higher Pb concentrations found in multiparous donors is not clear; while a possible explanation for the higher concentrations in mothers gaining weight since before the pregnancy is that this weight gain would increase the release of Pb from bone deposits, because a low calcium intake has been previously related to obesity and weight gain (Lappe et al., 2017).

4.4. *Cadmium*

Most of the donors in our study had Cd below the LOD, and concentrations were far below the range of those reported for mothers worldwide (Cherkani-Hassani et al., 2017; Rebelo and Caldas, 2016; Oliveira et al., 2020), only being comparable to those described in a few European studies (Björklund et al., 2012; Kantol and Vartiainen, 2001; Vollset et al., 2019) and Iran (Vahidinia et al., 2019). In fact, the concentrations in our donors were several times lower than in previous Spanish studies, which reported mean concentrations of 1.31 μg/L (García-Esquinas et al., 2011) and 0.15 μg/L (Mandiá et al., 2021).

Maternal smoking has been associated with Cd concentrations in breast milk in previous studies (Bassil et al., 2018; García-Esquinas et al., 2011; Gundacker et al., 2007), but no significant association was observed between detectable Cd in breast milk and smoking habit, most likely due to the lack of current smokers and the low Cd concentrations among the donor mothers. Non-smokers are mainly exposed to Cd through their intake of foods such as cereals, tubers, green leafy vegetables, fruit, nuts, pulses, fish, and shellfish (Gundacker et al., 2007; Leotsinidis et al., 2005; Martí-Cid et al., 2008). In the present study, Cd concentrations were not associated with the intake of any of these food items but were higher in mothers with a university education which may be due to a higher intake of foods containing Cd. In line to the present findings, Vahidinia et al. (2019) found no association between low Cd concentrations in breast milk and the intake of vegetable and fruit. The positive associations with the intake of fried and canned food and the inverse association with bread intake should be interpreted with caution, because these novel findings may be affected by imprecision due to the low Cd concentrations. Finally, hand cream use was associated with Cd, and several studies have demonstrated the presence of toxic elements, including Cd, Pb, Hg, and As, in cosmetics products such as lipstick and eye cosmetics (Mesko et al., 2020). Toxic metals may be retained as impurities in the raw materials used in the cosmetics or released from the metallic devices used during their production (Bocca et al., 2014). However, further research is needed to elucidate the potential exposure to toxic metals from cosmetics and other PCPs.

4.5. Limitations and strengths

This study has a number of limitations. First, the milk donors are more homogeneous in terms of socioeconomic profile than are lactating women in general (*e.g.,* most donors had a university education and were non-manual workers); therefore, the study findings cannot be generalized to breastfeeding women in the general population. Second, only total Hg and As were determined, limiting the capacity to identify more specific sources of exposure. Third, bias may have resulted from a misreporting of dietary intake and other factors. It is also possible that their diet might have changed with respect to the 12 months before their first donation, the reference period for the dietary questionnaire. Nevertheless, misclassification is unlikely to be related to breast milk metal concentrations. Another limitation is that the large number of explanatory factors assessed may have generated some spurious statistically significant associations. Finally, the possibility of metal contamination cannot be ruled out, because no special provisions were made during the pooling or processing of the milk to avoid metal contamination.

Current knowledge on the presence of toxic metals in breast milk is generally based on a small number of samples; however, a large number were obtained longitudinally from donors in the present investigation, allowing exploration of the variation in concentrations over the lactation period. A further study strength is the assessment of pooled milk samples (over 1–4 weeks), given that the composition of breast milk and, therefore, its toxic metal concentrations, can change during a feeding session, at different times of day, and from day to day due to variations in the mother's dietary intake, among other factors. Hence, the evaluation of pooled samples may reduce the risk of exposure misclassification in comparison to the measurement of metal concentrations in spot breast milk samples.

It is important to note that the mere presence of the studied metals in breast milk does not necessarily imply a health risk for the breastfeeding infant. Nevertheless, breast milk donated to milk banks is supplied to highly vulnerable preterm infants, and preventive measures are required to avoid their exposure to metals from this source. Recommendations should be especially targeted at ensuring healthy habits in milk donors, including the maintenance of optimal calcium and iron intakes and the limited consumption of fatty fish during pregnancy and breastfeeding.

5. Conclusion

Toxic metals/metalloids such as Pb, Hg, Cd, and As continue to pose a public health threat worldwide. In this study, 97 and 81% of pooled donor breast milk samples had detectable concentrations of As and Hg, respectively, while 51 and 38% had detectable concentrations of Pb and Cd. Given the extreme vulnerability of preterm infants, it is essential to closely monitor concentrations of toxic metals in donor breast milk and to develop appropriate measures to reduce their exposure to these chemicals and avoid unnecessary risks.

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Declaration of competing interest

The authors declare no actual or potential competing financial interests.

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Appendix A. Supplementary data

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