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# Urinary biomarkers of exposure to toxic and essential elements

## *A comparison of infants fed with human milk or formula*

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**Background:** Early-life exposure to nonessential (toxic) and essential trace elements can influence child development. Although infant formula powders and the water used to reconstitute them can contain higher concentrations of many elements compared with human milk, the influence of feeding mode on reliable biomarkers of infant exposure has rarely been demonstrated.

**Methods:** We evaluated associations between urinary biomarkers and feeding mode (exclusively human milk, exclusively formula, or combination-fed) for four toxic (arsenic, cadmium, nickel, and uranium) and three essential elements (cobalt, molybdenum, and selenium) using general linear models.

**Results:** A total of 462 participants from the rural New Hampshire Birth Cohort Study were on average 6 weeks old between July 2012 and March 2019 and had urine samples, 3-day food diaries, and relevant covariate data available. In adjusted models, urinary arsenic was 5.15 (95% confidence interval = 4.04, 6.58), molybdenum was 19.02 (14.13–25.59), and selenium was 1.51 (1.35–1.68) times higher in infants fed exclusively with formula compared with infants fed exclusively with human milk. By contrast, urinary uranium was 0.59 (0.46–0.75) and cobalt was 0.78 (0.65–0.95) times lower with formula feeding than human milk feeding.

**Conclusion:** Our findings suggest that infant exposure to several potentially toxic elements varies by feeding mode, as concentrations of reliable urinary biomarkers were higher with formula or human milk, depending on the element. Importantly, exposure to arsenic increased with household tap water arsenic regardless of feeding mode, suggesting that all infants could be at risk in populations with high concentrations of arsenic in drinking water.

**Keywords:** Arsenic; Cadmium; Uranium; Nickel; Molybdenum; Selenium; Cobalt; Urinary biomarkers

## Introduction

Early life is a critical window of susceptibility to environmental toxicants during which exposure to small amounts of potentially toxic elements can have adverse short-term and long-term effects.[1–3] For example, prenatal exposure to nonessential elements like arsenic,[1,2] nickel,[3] and uranium[4,5] is associated with altered birth outcomes, neurodevelopmental effects, and health complications in adulthood. Similarly, early-life exposure to low levels of arsenic,[6,7] cadmium,[8,9] nickel,[4,8] and uranium[10,11] is associated with adverse neurodevelopmental effects. Micronutrients involved in essential physiological processes—including cobalt,[4,12] molybdenum,[4,6] and selenium[13]—can also be toxic at high doses. While prenatal exposure is relatively well-studied, less is known about exposure to potentially toxic nonessential and essential elements during the immediate postnatal period.

Here, we focus on diet as a source of exposure to potentially toxic elements during the first 6 weeks of life. Although human milk is recommended as the sole source of nutrition for infants during the first 6 months of life,[14] many infants receive other forms of nutrition before that developmental milestone. For example, among infants born in the United States in 2020, more than half (54.7%) consumed substitutes for human milk by 3 months of age.[15]

Although infant formula is intended to be an effective substitute for human milk,[16] both the measured concentrations of

### What this study adds

This study evaluated whether infant exposure to potentially toxic elements, as assessed using reliable urinary biomarkers, differs with consumption of formula or human milk. At approximately 6 weeks of age, the feeding mode associated with higher excretion varied by element: urine concentrations were higher with exclusive formula feeding for arsenic, molybdenum, and selenium, but lower for uranium and cobalt. Our analyses implicate arsenic in the water used to reconstitute infant formula as the source of arsenic for formula-fed infants and suggest that, even among infants fed exclusively with human milk, arsenic in drinking water is correlated with exposure risk.

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*Epidemiologic data are not publicly available due to their sensitive and identifiable nature. Requests to work with data from the New Hampshire Birth Cohort Study should be directed to M.R.K. (Margaret.R.Karagas@Dartmouth.edu). Code for the statistical analyses and the files with analytical QA/QC data are available from Figshare at https://dartgo.org/Pikounis.*

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potentially toxic elements and modeled exposure risks differ between human milk and infant formula.[17–19] Most infant formulas are provided in powdered form and reconstituted with water, leading to two potential routes of exposure: the formula powder and the water used to reconstitute the powder.[20] Formula powder is often fortified with essential elements such as molybdenum, so concentrations of these elements can be substantially higher in reconstituted formula than in human milk.[19] Concentrations of toxic elements, including arsenic[21] and cadmium,[19] can also be higher in some formula powders, while the water used to reconstitute the powder can be high in arsenic[22] or uranium.[23] Moreover, infants tend to have a higher median daily intake of formula (and thus both powder and water) by volume than of human milk beginning at around 6 weeks of age.[24,25] Exposure models that multiply higher elemental concentrations in reconstituted formula times the higher intake volume therefore tend to predict that infants fed exclusively with formula will have a higher intake of potentially toxic elements than infants fed exclusively with human milk.[18,19,24–29]

Biomarkers—elemental concentrations in urine, blood, and hair—provide information about element dose from the environment that can be used to test exposure models.[30] Excretion of elements in urine, for example, indicates processing and removal by the kidneys.[30–32] When urinary concentrations are positively correlated with known exposures, urine is considered a reliable biomarker for that element. Previous studies have established that dietary intake is reflected by urinary concentrations—and thus urine is a useful biomarker—for the nonessential elements arsenic,[32–36] cadmium,[37,38] nickel,[39] and uranium[40,41] and the essential elements cobalt,[42] molybdenum,[30,43] and selenium.[44,45]

Here, we use spot urine samples to test the hypothesis that infants are exposed to higher concentrations of potentially toxic elements via formula as compared with human milk.[19] Although Carignan et al[46] supported this hypothesis for arsenic in a small study of 72 6-week-old infants, to our knowledge it has not yet been tested for multiple potentially toxic elements using reliable urinary biomarkers. We extend their approach to both nonessential (arsenic, cadmium, nickel, and uranium) and essential (cobalt, molybdenum, and selenium) elements with a larger number of 6-week-old infants (including the 72 infants from that paper) who were fed exclusively with human milk, exclusively with formula, or fed both formula and human milk.

## Methods

### *Study participants*

Subjects were part of the New Hampshire Birth Cohort Study (NHBCS), a prospective study of pregnant persons and their children[47] recruited between 2009 and 2019. Pregnant persons between the ages of 18 and 45 years were recruited from prenatal clinics in New Hampshire beginning in January 2009. Initial enrollment criteria included the use of a private unregulated well at home, English literacy, and a singleton pregnancy. Upon enrollment, participants completed a prenatal medical history and lifestyle questionnaire including questions on sociodemographic factors, health history, personal habits, home water source, and home water consumption. They also provided a sample of their home tap water, which was collected and analyzed for potentially toxic elements as described in Gilbert-Diamond et al.[47] This study was reviewed and approved by the Committee for Protection of Human Subjects at Dartmouth College (Study 00020844), and all participants provided written informed consent in accordance with Committee for Protection of Human Subjects guidelines.

Between August 2012 and March 2019, shortly before their infants reached 6 weeks of age, NHBCS parents were asked to complete a 3-day infant food diary and collect a urine sample from their infant as described below.

### *Infant food diaries*

The data collection methods of this study are consistent with previous NHBCS publications on infant feeding.[46,48,49] The 3-day food diary asked the caregivers to record a number of dietary parameters (online supplement, eAppendix 1; http://links.lww.com/EE/A254); we used information about the diet item(s) given at each feeding (e.g., infant formula, human milk via chest feeding, or expressed human milk) to assign each infant to one of three feeding modes: (1) fed exclusively with human milk, including expressed (pumped) milk; (2) fed exclusively with infant formula; or (3) combination-fed, if the infant was given both formula and human milk during the 3-day period recorded in the diary. Because the diary was kept in real time, recall bias should not have been an issue.

We collected additional data from food diaries submitted between June 2013 and November 2016 to learn more about formula consumption patterns and assess the relative exposure via formula versus human milk for combination-fed infants. First, we identified the type of formula consumed (powdered or ready-made) and the water source(s) used to reconstitute powdered formula for all infants who received at least some formula. Though not specifically requested in the diary (eAppendix 1; http://links.lww.com/EE/A254), many families independently identified the use of "filtered tap water" on the food diaries; we recorded these entries separately from "tap water" during data entry, but note that we do not have information about the type of filtration system used from the food diary. Second, for the combination-fed infants only, we calculated the total amount of formula consumed (ounces), total number of formula feedings, and total number of human milk feedings reported on the 3-day food diaries as indicators of dietary exposure via these different pathways.

### *Infant urine collection and analysis*

Spot urine samples were collected on the third day of the food diary using study-provided diapers and pretested cotton pads (Shiseido; Tokyo, Japan) and procedures similar to other studies.[50,51] While cumulative 24-hour urine samples can be better than spot urine samples at capturing overall exposure,[52] such samples would be nearly impossible to collect for diapered infants. Spot samples have been used successfully in infant urine biomarker studies[48,49,51] and in large-scale monitoring programs of older children and adults such as the National Health and Nutrition Examination Survey.[53,54]

As described in Carignan et al,[46] cotton pads were placed in the diaper area where urination was expected. Cotton pads saturated with urine were placed in a collection cup, sealed in a

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polyethylene bag, stored in a cooler with ice packs, and either brought to the birthing parent's 6-week postpartum appointment later that day or shipped to the research lab by next-day mail. Within ~24 hours of sample arrival, the urine was squeezed from the cotton pads, homogenized, divided into aliquots, and frozen at −80 °C until analysis for elemental content. Specific gravity (SG) was measured in one aliquot before freezing using a handheld refractometer with automatic temperature compensation (ATAGO PAL-10S; Atago USA, Inc) to account for variability in elemental concentration due to urinary dilution.[55] Procedural blanks to test for elemental contamination of the cotton pads, collection cups, and freezer vials were prepared and analyzed at intervals throughout the study; these data are provided in the online supplement, eAppendix 2: eTable 1; http://links.lww.com/EE/A254.

Urine samples were analyzed in four batches at the Trace Elements Analysis Core Facility at Dartmouth. Samples were thawed and vortexed before 0.6 ml was pipetted into a preweighed 7 ml polypropylene autosampler vial. Samples were weighed and diluted with 5.4 ml 1% $HNO_3$ (Optima, Fisher Scientific) and a final weight was recorded. Diluted samples were stored at room temperature until analysis by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8900; Agilent Technologies) operated in helium and oxygen modes. We measured concentrations of total arsenic, cadmium, chromium, cobalt, copper, iron, manganese, mercury, molybdenum, nickel, lead, selenium, antimony, uranium, vanadium, and zinc in these solutions. All elements were measured in helium mode; arsenic, selenium, and vanadium were also measured in oxygen mode and concentrations were typically reported for oxygen mode for these elements. Multipoint calibration checks were performed using a custom-multi element ICP-MS standard (Inorganic Ventures, Gaithersburg, VA) and a separate mercury standard. Aliquots of these standards were combined and diluted to create a series of standards covering the expected range of elemental concentrations in the samples. Continuing calibration checks were run after every 10 samples. Every 20 samples, sample duplicates and sample spikes were analyzed. When the sample volume of infant urine was too low to split into two aliquots to perform a true laboratory duplicate, the diluted sample was reanalyzed. QA/QC data are provided in eAppendix 2: eTable 1; http://links.lww.com/EE/A254.

Urine samples were further analyzed for individual arsenic species using liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS) with an Agilent 1260 LC and an 8900 ICP-MS operated in oxygen mode. A 0.3 ml aliquot of urine was centrifuged at 13,300*g* for 15 minutes, then 200 µl mixed with 4 µl of ultrapure $H_2O_2$ and stored at 4 °C until analysis. The LC-ICP-MS system was calibrated with a mixed arsenic species standard containing 50 µg/l arsenobetaine, 50 µg/l dimethylarsinic acid, 10 µg/l monomethylarsonic acid, and 20 µg/l arsenite and arsenate. The LC flow rate was 1 ml/min and the gradient elution was followed with 2.5 mM $(NH_4)_2CO_3$ for 1 minute, 180 mM for 1–4 minutes, and 2.5 mM for 4–7 minutes. QC checks were run every 10 samples, alternating between NIST 2,669 level 1 and level 2. Duplicates and spikes were analyzed once every 20 samples. To assess only the toxic species of arsenic, arsenobetaine (primarily found in fish and seafood) was subtracted from total urinary arsenic because it is thought to be nontoxic and excreted without being metabolized.[56] We refer to this difference as "urinary arsenic" in the results.

### *Urinary biomarker characterization*

Although we measured 16 elements in the infant urine samples, our analyses focused on the seven elements for which urinary excretion has been established as a reliable biomarker of exposure—arsenic,[32–36] cadmium,[37,38] nickel,[39] uranium,[40,41] cobalt,[42] molybdenum,[30,43] and selenium[44,45]—and there was a sufficient fraction of samples with detectable concentrations. The literature supports the use of spot urine samples as biomarkers of exposure to arsenic, cadmium, nickel, uranium, cobalt, and molybdenum; however, the reliability of urinary selenium as an exposure biomarker is established only for integrated 24-hour urine samples.[44,45] We therefore interpret results for selenium with caution.

Three of the other nine elements were excluded because their urinary concentrations did not exceed the limit of detection (LOD) for at least 60% of the infant urine samples, consistent with the criterion used by CDC's National Report on Human Exposure to the Environment:[54] chromium (44% detectable), mercury (20% detectable), and vanadium (57% detectable) (eAppendix 2: eTable 2; http://links.lww.com/EE/A254). The remaining six elements were not included in the primary analyses because urinary excretion is not established as a reliable biomarker of exposure; data and results for these elements are included in the online supplement, eAppendix 3; http://links.lww.com/EE/A254, in case this changes in future. Currently, whole blood is the primary biomarker for lead exposure monitoring,[30,57] and few studies are published assessing general population exposure to antimony using urine.[58] We did not consider urinary copper, iron, manganese, or zinc as viable exposure biomarkers because the kidneys are not involved in maintaining homeostasis of these minerals[31] and urine is not generally used as an exposure biomarker for these elements.

For the seven elements included in our primary statistical analyses, we used the estimate from the standard curve so long as it was higher than the mean calibration blank ICP-MS response; concentrations less than or equal to the mean calibration blank were assigned the LOD divided by the square root of 2 because less than 5% of samples had estimated concentrations below the LOD for any element.[59,60] We then averaged concentrations across samples duplicated for quality control and adjusted urinary concentrations for SG using a ratio approach:[61]

$$C_a = C \times \frac{\mathrm{SG}_{\mathrm{mean}} - 1}{\mathrm{SG} - 1}$$

Here, $C_a$ is the adjusted urinary concentration, $C$ is the original measurement, $\mathrm{SG}_{\mathrm{mean}}$ is the mean SG across all participating infants, and SG is the SG for the infant from whom the sample was collected.[62] One urine sample had a SG of exactly 1.000 and so was excluded from statistical analyses.

### *Statistical analysis*

All statistical analyses were performed using R version 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Linear models were fit using base R and the package "car"[63] and graphs were created using "ggplot2,"[64] "ggthemes,"[65] and "patchwork."[66]

We analyzed associations between each urinary biomarker and feeding mode separately. We first calculated geometric mean, SG-adjusted urinary concentrations stratified by feeding mode to evaluate patterns, then $\log_{10}$-transformed the adjusted urinary element concentrations to improve normality. Several general linear models (GLMs) quantified associations with feeding mode, including a parsimonious model with only feeding mode and a fully adjusted model that also included a linear term for infant age and categorical variables for infant sex, analytical batch, and maternal smoking. We evaluated the association with feeding mode using indicator variables that compared the mean $\log_{10}$-transformed, SG-adjusted urinary element concentrations for formula- and combination-fed infants to the mean for infants fed exclusively with human milk. If the effect of feeding mode was statistically significant at $\alpha = 0.05$ based on a type-III analysis of variance (ANOVA), we interpreted the back-transformed

parameters for the indicator variables, together with their 95% and 99.5% confidence intervals (CIs), as estimates of the fold-change in the predicted mean urinary concentration in formula- or combination-fed infants relative to infants fed exclusively with human milk. The 99.5% CIs are included as a conservative, Bonferroni-style correction for multiple testing; our initial analyses included 11 elements, so a Bonferroni-corrected alpha would be 0.05/11 = 0.0045. For the fully adjusted model, we estimated the partial $R^2$ for feeding mode by comparing models with and without the indicator variables. We also assessed associations without adjustment for analytic batch in secondary analyses because of the observed variability in element concentrations by batch.

We explored associations between urinary element concentrations and the household tap water concentration by adding $\log_{10}$-transformed tap water concentration of that element and the interaction between feeding mode and $\log_{10}$-transformed tap water concentration to the fully adjusted GLM for each element. These models allowed us to determine whether there was a linear association between $\log_{10}$-transformed, SG-adjusted urinary element concentrations and the $\log_{10}$-transformed concentration of the corresponding element in the household tap water sample provided at enrollment in the NHBCS and if this association changed with feeding mode for the 456 infants with household tap water data available.

Finally, we quantified the relative exposure to each element via human milk and formula by leveraging the variability among infants in the degree of combination feeding experienced for the subset of infants for which the appropriate data were available. Specifically, we used simple linear regression to evaluate associations between $\log_{10}$-transformed, SG-adjusted urinary element concentrations and each of the three quantitative dietary parameters calculated from food diaries for combination-fed infants submitted from June 2013 to November 2016: the total ounces of formula consumed, the total number of formula feedings, and the total number of human milk feedings. The number of formula and human milk feedings was available for 54 infants, while the volume of formula consumed was available for 42 infants.

## Results

### *Participant characteristics and exposure distributions*

A total of 2,410 dyads were recruited into the NHBCS, 1,238 of which were age-eligible to participate in this substudy. Of the age-eligible dyads, 569 (46%) provided infant urine samples and 511 (41%) provided both a urine sample and an infant feeding diary. After applying our exclusion criteria (Figure 1), the primary analyses in this study included 462 infants. Table 1 summarizes selected demographic characteristics of the substudy population and the full NHBCS.

On the day of urine collection, infants in this substudy had a mean age of 44.6 days (range: 17–90 days). Based on the food diaries, 73% were fed exclusively with human milk, 17% were fed with both formula and human milk (hereafter, "combination-fed"), and 10% were exclusively formula-fed (hereafter, "formula-fed"). Nearly all (98.2%) of the combination- and formula-fed infants with fully analyzed food diaries consumed at least some reconstituted formula powder, and 87% of the known water sources were either filtered household tap water or bottled water (eAppendix 2: eTable 3; http://links.lww.com/EE/A254).

The infant urine samples were dilute (specific gravities <1.02, Table 1). After adjustment for SG, geometric mean urinary concentrations of the four focal nonessential elements were all <2 µg/l (Table 2A), while geometric mean urinary concentrations of the essential elements varied widely, from 0.2 µg/l for cobalt to 16 µg/l for selenium (Table 2B).

### *Associations with feeding mode*

In the fully adjusted GLMs, urinary concentrations differed by feeding mode for arsenic, uranium, cobalt, molybdenum, and selenium (see feeding mode effect columns in Table 3 and Figure 2). Infant age, infant sex, and maternal smoking during pregnancy did not explain meaningful amounts of variability in urinary element concentrations. Urinary arsenic concentrations were highest in formula-fed infants, intermediate in combination-fed infants, and lowest in infants fed exclusively with human milk (Figure 2A). Predicted mean urinary arsenic was 2.51 times (95% CI = 2.09, 3.03) higher in combination-fed infants and 5.15 times (95% CI = 4.04, 6.58) higher in formula-fed infants relative to infants fed exclusively with human milk (Table 3A). In contrast, the predicted mean urinary concentrations for uranium were 0.81 times (95% CI = 0.67, 0.98) lower in combination-fed infants and 0.59 times (95% CI = 0.46, 0.75) lower in formula-fed infants than in infants fed exclusively with human milk (Table 3A; Figure 2D). Trends in urinary cadmium and nickel were similar to urinary uranium in being highest in infants fed exclusively with human milk (Figure 2B, C), but the association with feeding mode was not statistically significant at the 5% level (Table 3A).

Urinary element concentrations of all three essential elements differed by feeding mode, with smaller differences for cobalt than for selenium and molybdenum (Table 3B, Figure 2). Formula-fed infants had 19.02 times (95% CI = 14.13, 25.59) higher predicted urinary molybdenum and 1.51 times (95% CI = 1.35, 1.68) higher selenium compared with infants fed exclusively with human milk, while combination-fed infants had intermediate urinary concentrations (Table 3B; Figure 2E, F). By contrast, the mean urinary cobalt was 0.78 times (95% CI = 0.65, 0.95) lower in formula-fed infants than in infants fed exclusively with human milk; the cobalt results were not statistically significant after correction for multiple testing (Table 3B).

These findings were generally robust in simpler statistical models without adjustment for covariates (eAppendix 2: eTable 4; http://links.lww.com/EE/A254), including analytical batch (eAppendix 2: eTable 5; http://links.lww.com/EE/A254).

### *Associations with household tap water as a function of feeding mode*

There were positive associations between SG-adjusted urinary element concentrations and tap water concentrations only for arsenic; these associations differed by feeding mode (Figure 3; eAppendix 2: eFigure 1; http://links.lww.com/EE/A254, eTable 6; http://links.lww.com/EE/A254). Consistent with the main analyses (Table 3), the estimated Y-intercepts differed among feeding modes, increasing from infants fed exclusively with human milk to combination-fed infants to exclusively formula-fed infants. Notably, urinary arsenic increased with household tap water arsenic for all three feeding modes, with steeper slopes for formula-fed infants than for infants fed exclusively with human milk (eAppendix 2: eTable 6; http://links.lww.com/EE/A254).

### *Exposure via human milk versus formula in the combination-fed infants*

Consistent with the analyses based on feeding mode for all infants, urinary arsenic, molybdenum, and selenium increased with the reported ounces of the formula used for the combination-fed infants for which this information was available (Figure 4A, E, F). There were no significant associations for urinary cadmium, nickel, uranium, or cobalt (Figure 4B, C, D, G). Urinary molybdenum also increased with the number of formula feedings and both urinary arsenic and urinary molybdenum

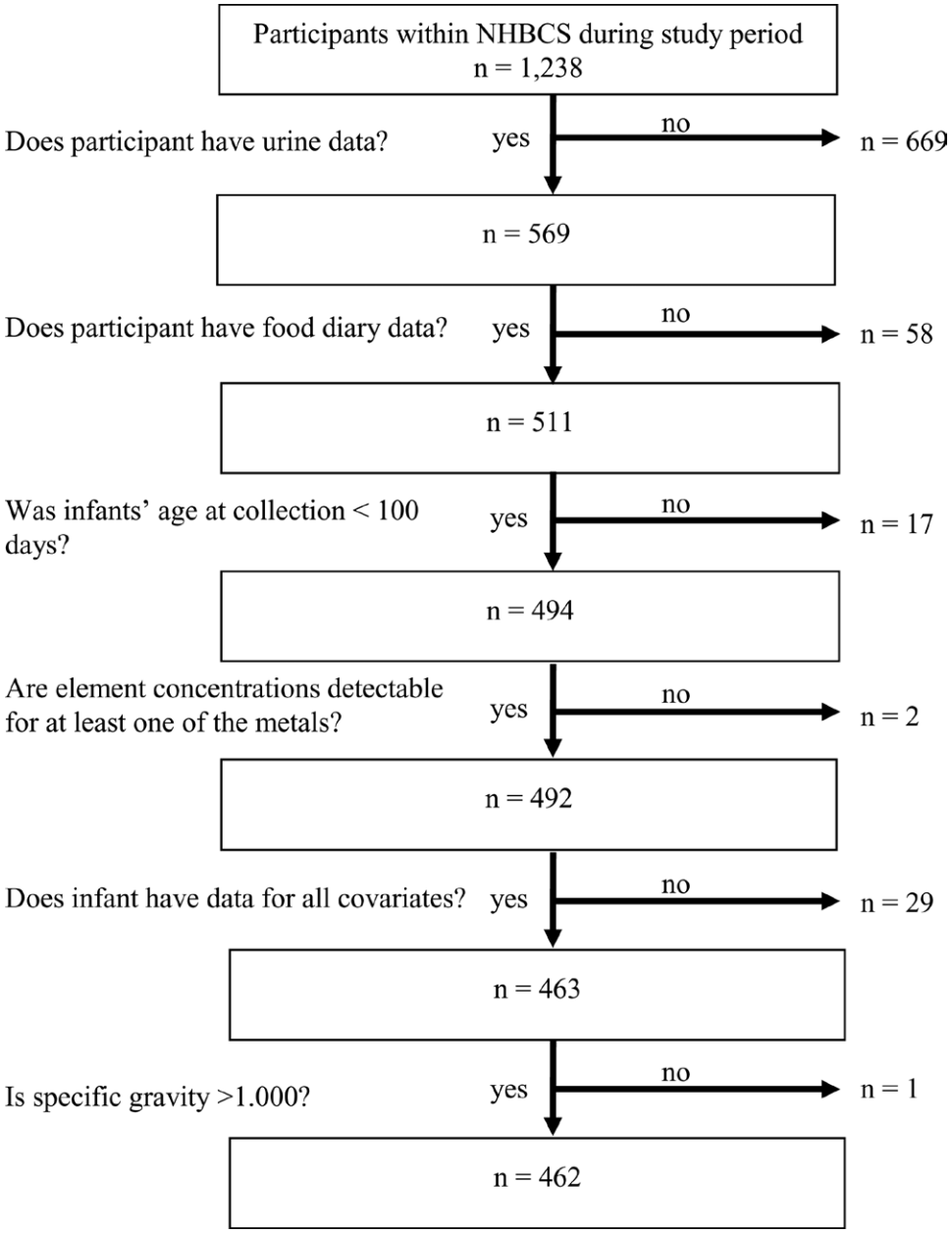


**Figure 1.** Flow chart for inclusion/exclusion of study participants in the primary analyses. The final exclusion step was applied before statistical analyses that required adjustment for specific gravity.

decreased with the number of human milk feedings (eAppendix 2: eFigures 2, 3; http://links.lww.com/EE/A254).

## Discussion

This study provides insight into exposure to potentially toxic, essential and nonessential elements via human milk and formula during a critical window that has rarely been investigated: early infancy. Prior studies have illustrated that concentrations in spot urine samples reliably reflect dietary intake of arsenic,[32–36] cadmium,[37,38] nickel,[39] uranium,[40,41] cobalt,[42] and molybdenum;[30,43] urinary selenium is a reliable biomarker in integrated 24-hour samples.[44,45] As biomarkers of exposure for these elements, urine concentrations provide a unique opportunity to investigate relative exposures from human milk compared with formula. Past exposure models and laboratory data for formula reconstituted with deionized water[19,26,68] had suggested that infants fed with formula would be exposed to higher concentrations of most elements than infants fed exclusively with human milk. Our study of urinary excretion in young infants from the NHBCS supports this hypothesis only for arsenic, molybdenum, and selenium; by contrast, urinary uranium and cobalt were lower in formula-fed infants (and trended lower for cadmium and nickel as well).

### *Arsenic*

Consistent with prior work with a smaller set of NHBCS infants,[46] feeding mode was a predictor of urinary arsenic, suggesting a >five-fold greater dietary exposure to arsenic for infants fed exclusively with formula comparedto infants fed exclusively with human milk; exposure was intermediate for combination-fed infants. Importantly, both formula powder and the water used to reconstitute the powder can be sources of exposure to arsenic.[19,69] In the United States, private groundwater wells are not subject to regulation and, depending on local geology and past land use, can contain high concentrations of arsenic[70–72] and uranium.[23] While arsenic

**Table 1.**

**Selected characteristics for pregnant persons and infants in New Hampshire Birth Cohort Study included in this urinary biomarker and feeding mode substudy (n = 463; includes the infant with specific gravity = 1.000) and for the cohort as a whole (n = 2,410)**

| Characteristics | This substudy mean (range) or n (%) | NHBC as a whole mean (range) or n (%) |
|---|---|---|
| **A. Pregnant persons** | | |
| Age at enrollment, years | 32.06 (19.28–43.66) | 31.34 (18–46.18) |
| <20 | 2 (0%) | 33 (1%) |
| 20–25 | 31 (7%) | 208 (9%) |
| 25–30 | 111 (24%) | 694 (29%) |
| 30–35 | 201 (44%) | 917 (38%) |
| 35–40 | 99 (21%) | 473 (20%) |
| >40 | 19 (4%) | 84 (3%) |
| Parity | | |
| 0 | 202 (44%) | 992 (43%) |
| ≥1 | 257 (56%) | 1,310 (57%) |
| Relationship status | | |
| Married | 393 (89%) | 1,681 (85%) |
| Single and never married | 36 (8%) | 234 (12%) |
| Separated or divorced | 13 (3%) | 57 (3%) |
| Widowed | 0 (0%) | 2 (0%) |
| Level of education | | |
| <11th grade | 2 (0%) | 22 (1%) |
| High school graduate or GED | 39 (9%) | 227 (12%) |
| Junior college graduate | 61 (14%) | 328 (17%) |
| College graduate | 170 (39%) | 758 (40%) |
| Any postgraduate schooling | 167 (38%) | 559 (30%) |
| Ever smoker | | |
| Yes | 47 (6%) | 245 (12%) |
| No | 400 (94%) | 1,774 (88%) |
| Smoked cigarettes during pregnancy | | |
| Yes | 28 (6%) | 134 (6%) |
| No | 435 (94%) | 1,933 (94%) |
| **B. Infant** | | |
| Sex | | |
| Female | 210 (45%) | 1,184 (50%) |
| Male | 253 (55%) | 1,196 (50%) |
| Race | | |
| White | 439 (95%) | 2,255 (95%) |
| Other | 24 (5%) | 118 (5%) |
| Year of birth | | |
| 2009–2011 | 0 (0%) | 508 (21%) |
| 2012–2013 | 112 (24%) | 522 (22%) |
| 2014–2016 | 231 (50%) | 547 (23%) |
| 2017–2019 | 120 (26%) | 473 (20%) |
| 2020–2021 | 0 (0%) | 334 (14%) |
| Birth weight, g | 3,437 (1,320–5,400) | 3,409 (357–5,400) |
| Age at sample collection, days | 44.6 (17–90) | 51.4 (17–225) |
| Urinary specific gravity | 1.00 (1.00–1.02) | 1.00 (1.00–1.02) |
| Feeding mode at 6 weeks[a] | | |
| Exclusively human milk | 339 (73%) | |
| Combination-fed | 80 (17%) | |
| Exclusively formula-fed | 44 (10%) | |
| Feeding mode at 4 months[b] | | |
| Exclusively human milk | 243 (60%) | 871 (53%) |
| Combination-fed | 149 (37%) | 692 (42%) |
| Exclusively formula-fed | 13 (3%) | 83 (5%) |

[a]Food diaries were available only for the infants in this substudy.

[b]Families were asked about their child's diet at 4 months of age in a telephone questionnaire; see Farzan et al[67] for details.

concentrations in public drinking water supplies are regulated at the state and national levels to be <5 and <10 µg/l, respectively, all participants in the NHBCS lived in households with private drinking water wells for which water testing must be arranged by the homeowner.[73] As part of the NHBCS, we provided participants with the results of our household tap water testing for arsenic. In a previous report,[74] we found that those who had higher concentrations of arsenic in their tap water (>10 µg/l) were less likely to use their tap water for reconstituting formula powder. Indeed, for the current study, 87% of the formula- and combination-fed infants with known water sources were fed with formula reconstituted with either filtered household tap water or bottled water (eAppendix 2: eTable 3; http://links.lww.com/EE/A254). It is also notable that participants reported the use of filters with their household tap water without being prompted for this information. Nevertheless, the urinary biomarker results suggest that infants fed wholly or partially with formula were exposed to more arsenic than those fed wholly with human milk (Figure 2A) and that combination-fed infants who consumed more formula (Figure 4A) and had fewer human milk feedings (eAppendix 2: eFigure 3; http://links.lww.com/EE/A254) during the 3-day study period were exposed to more arsenic. That this exposure increased with home tap water arsenic in a dose-dependent manner (Figure 3) implicates arsenic in the water used to reconstitute the formula as the source

**Table 2.**

**Geometric mean urinary concentrations (with 95% confidence intervals) measured in the substudy of 6-week-old infants with a urine sample, 3-day food diary, and measurable concentrations of at least one targeted element**

| Element (µg/l) | All infants (n = 462) | | Fed exclusively with human milk (n = 338) | | Combination-fed (n = 80) | | Exclusively formula-fed (n = 44) | |
|---|---|---|---|---|---|---|---|---|
| **A. Non-essential elements** | | | | | | | | |
| As[a] | 0.23 | (0.21, 0.25) | 0.17 | (0.15, 0.18) | 0.42 | (0.35, 0.5) | 0.85 | (0.66, 1.09) |
| Cd | 0.14 | (0.13, 0.15) | 0.15 | (0.13, 0.16) | 0.14 | (0.12, 0.16) | 0.12 | (0.09, 0.16) |
| Ni | 1.82 | (1.70, 1.94) | 1.88 | (1.74, 2.04) | 1.75 | (1.49, 2.05) | 1.49 | (1.19, 1.86) |
| U | 1.20 | (1.12, 1.29) | 1.32 | (1.22, 1.42) | 1.05 | (0.87, 1.27) | 0.74 | (0.55, 0.99) |
| **B. Essential elements** | | | | | | | | |
| Co | 0.20 | (0.19, 0.21) | 0.2 | (0.19, 0.22) | 0.2 | (0.17, 0.23) | 0.16 | (0.13, 0.19) |
| Mo | 2.26 | (1.99, 2.57) | 1.21 | (1.10, 1.34) | 9.07 | (7.13, 11.54) | 21.99 | (17.44, 27.74) |
| Se | 16.47 | (15.90, 17.06) | 15.19 | (14.62, 15.79) | 19.61 | (18.17, 21.17) | 22.26 | (19.63, 25.24) |

Data are provided for the full substudy and then for each of the three infant feeding modes. All values were adjusted for specific gravity.
[a]As refers to total urinary As minus arsenobetaine.

**Table 3.**

**Association between specific gravity-adjusted urinary element concentrations and feeding mode in general linear models that also included infant sex, infant age at collection, maternal smoking, and analytical batch as covariates**

| | | Feeding mode effect | | | Combination-fed vs. human milk | | | Formula-fed vs. human milk | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Element** | **Model $R^2$** | **Partial $R^2$** | **$F_{2,453}$** | ***P*** | $10^{\hat{\beta}}$ | **95% CI** | **99.5% CI** | $10^{\hat{\beta}}$ | **95% CI** | **99.5% CI** |
| **A. Non-essential elements** | | | | | | | | | | |
| As[a] | 0.36 | 0.33 | 116.0 | <0.0001 | 2.51 | (2.09, 3.03) | (1.92, 3.28) | 5.15 | (4.04, 6.58) | (3.63, 7.32) |
| Cd | 0.11 | 0.01 | 2.7 | 0.065 | 0.93 | (0.79, 1.11) | (0.73, 1.20) | 0.77 | (0.61, 0.96) | (0.55, 1.06) |
| Ni | 0.14 | 0.01 | 2.2 | 0.108 | 0.93 | (0.78, 1.10) | (0.73, 1.18) | 0.79 | (0.64, 0.99) | (0.58, 1.09) |
| U | 0.08 | 0.04 | 10.3 | <0.0001 | 0.81 | (0.67, 0.98) | (0.62, 1.06) | 0.59 | (0.46, 0.75) | (0.41, 0.83) |
| **B. Essential elements** | | | | | | | | | | |
| Co | 0.20 | 0.01 | 3.3 | 0.037 | 1.00 | (0.86, 1.15) | (0.81, 1.23) | 0.78 | (0.65, 0.95) | (0.60, 1.03) |
| Mo | 0.58 | 0.54 | 295.7 | <0.0001 | 7.59 | (6.05, 9.51) | (5.48, 10.50) | 19.02 | (14.13, 25.59) | (12.42, 29.12) |
| Se | 0.27 | 0.14 | 40.7 | <0.0001 | 1.30 | (1.20, 1.41) | (1.16, 1.46) | 1.51 | (1.35, 1.68) | (1.29, 1.76) |

Model $R^2$ is for the fully adjusted model. The feeding mode effect columns include the partial $R^2$ for this factor together with the *F*-statistic, degrees of freedom, and *P* value for a type-III ANOVA, signaling when it is appropriate to look at the back-transformed parameters ($10^{\hat{\beta}}$) and confidence intervals (CIs) that represent the estimated fold-change in each urinary element for combination-fed and formula-fed infants compared with infants that were fed exclusively with human milk. Bold font is used when the effect of feeding mode was significant and 95% parameter confidence limits do not overlap 1.0; gray font is used when the type-III ANOVA test was not significant at $\alpha = 0.05$. The 99.5% CI is included as a conservative check on multiple testing; see text for details.
[a]As refers to total arsenic minus arsenobetaine.

of arsenic, though we cannot rule out further contributions from the formula powder from the data we have in hand.

Surprisingly, urinary arsenic concentrations increased with household tap water arsenic even in infants fed exclusively with human milk (Figure 3). These increases were modest but unexpected given prior studies in populations with very high drinking water arsenic that have shown that the arsenic mothers consume from water is generally not passed through human milk to infants.[46,50,75,76] Additional work on populations with low to moderate levels of exposure via tap water is needed to better understand this result, including consideration of other pathways of exposure such as dermal exposure during bathing.[71]

Given that there are no known "safe" levels of arsenic exposure, investigation of human milk, formula powder, and water used to reconstitute formula should continue to help guide parents in their feeding choices.

### *Other nonessential, toxic elements: uranium, cadmium, nickel*

Contrary to our general expectation of increased exposure in formula-fed infants, urinary concentrations of uranium were lower in infants fed exclusively with formula than exclusively with human milk. The biological significance of these statistical differences is at present unclear, because feeding mode explains only 4% of the variability in urinary uranium concentrations (Table 3A) and there is limited literature on the relative exposure to uranium via formula versus human milk.[77] Although uranium in unregulated tap water can, like arsenic, be of public health concern,[23] urinary uranium did not increase with household water concentration in the NHBCS (eAppendix 2: eFigure 1; http://links.lww.com/EE/A254). The source of uranium exposure in infants fed with human milk may warrant further attention if other studies show similar increases relative to formula-fed infants.

Urinary cadmium and nickel did not vary by feeding mode, which differs from the projections of previously published exposure models.[18,19] Formula-fed infants had been projected to have greater intake of cadmium,[19] although there is also evidence that maternal cadmium exposure can be passed to infants through human milk.[78] In our cohort, urinary cadmium suggests that exposure via human milk was not significantly different from exposure via formula. By contrast, exposure modeling from southern China projected that formula-fed infants would have lower intake of nickel,[18] although authors noted that their infant formula samples had lower levels of nickel than reported values from other countries. These differences by feeding mode were not reflected by urinary nickel in our study. The reasons for these null findings are unclear and bear follow-up investigation to see if they are unique to this study or hold more broadly.

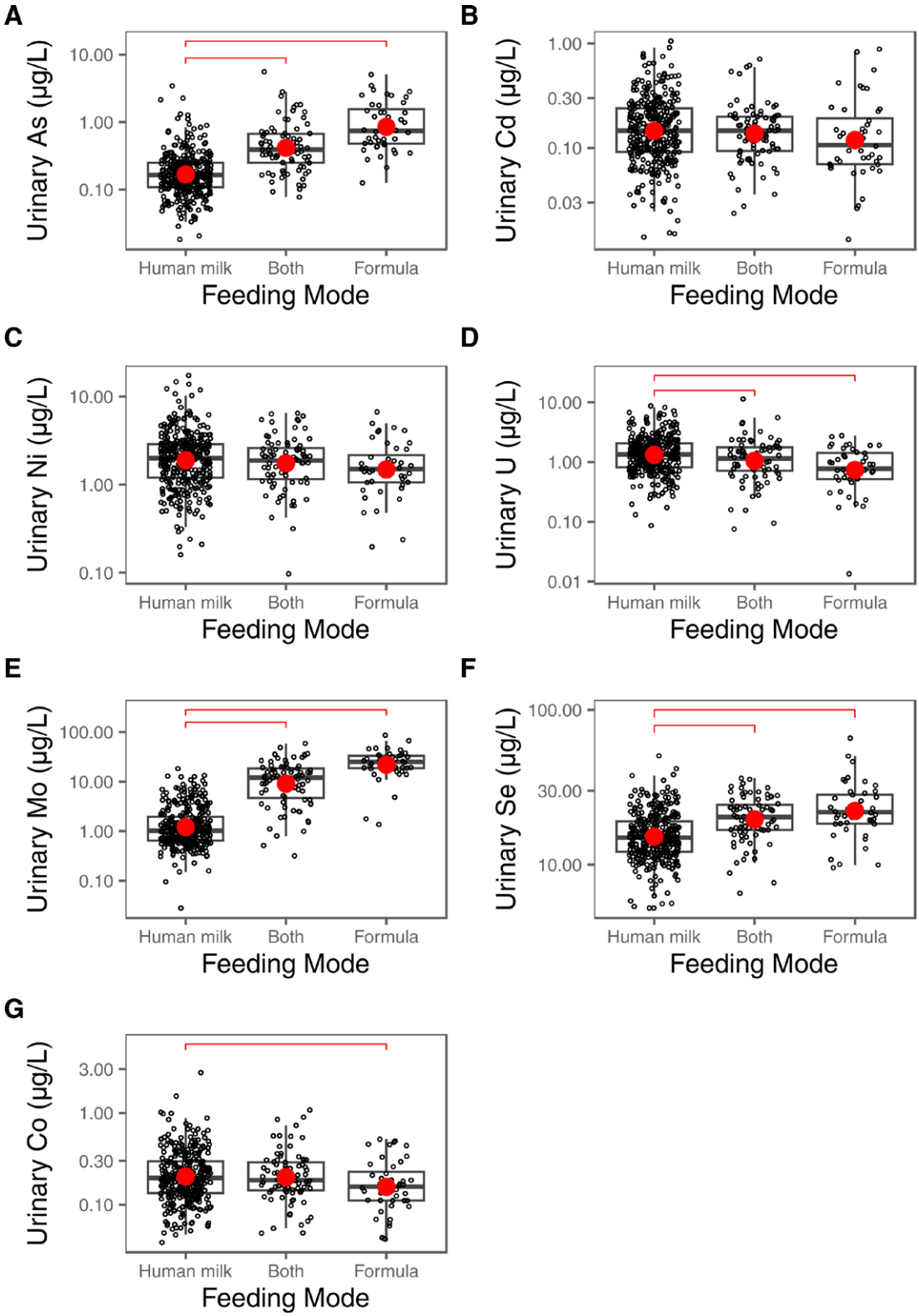


**Figure 2.** Box plot distributions of nonessential (toxic) and essential elements in urine by feeding mode during early infancy. Urinary arsenic (total arsenic minus arsenobetaine) (panel A), cadmium (B), nickel (C), uranium (D), molybdenum (E), selenium (F), and cobalt (G) concentrations were adjusted for specific gravity and measured in units of µg/L. *Y* axis is on the $\log_{10}$ scale, adjusted among panels to reflect the varying concentrations of elements in urine. Small dots represent individual results; the larger red circle is the mean. Red brackets above the boxplots indicate statistically significant differences from exclusive feeding with human milk, consistent with the 95% CIs for effect sizes in Table 3.

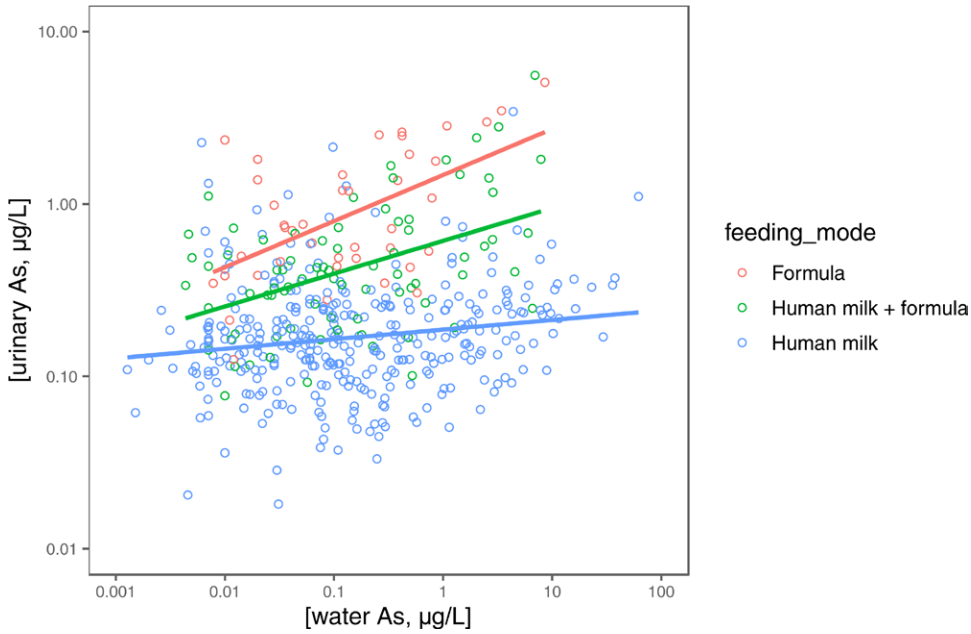


**Figure 3.** Specific gravity-adjusted infant urinary arsenic concentrations as a function of unfiltered household tap water arsenic concentrations and infant feeding mode (N = 456). The fitted lines and their 95% confidence bands illustrate simple linear regression models fit to each feeding mode separately and without adjustment for other covariates (see eAppendix 2: eTable 6; http://links.lww.com/EE/A254 for the fully adjusted indicator variables model). Note the $\log_{10}$-transformed axes.

### *Essential elements*

Interpretation of urinary biomarkers for the three essential elements is more complicated than the known toxic elements. Molybdenum, selenium, and cobalt are all essential for normal growth and development, but it is currently unknown whether the observed urinary concentrations represent a health risk given the nonlinear toxicity curve and lack of reference values for urinary biomarkers during early infancy.

Consistent with published exposure models,[19,28] urinary molybdenum was substantially higher in infants fed exclusively with formula than exclusively with human milk; it also increased with formula consumption and decreased with human milk consumption in combination-fed infants. As urinary molybdenum concentrations have been found to be directly related to dietary intake,[43] our results suggest that consumption of infant formula increases molybdenum intake compared with human milk. We hypothesize that molybdenum exposure was due to the formula powder, not water, because there was no association between urinary molybdenum and household tap water concentration when tap water molybdenum concentration was added to the fully adjusted GLMs (eAppendix 2: eFigure 1; http://links.lww.com/EE/A254). Molybdenum content in formula could be a matter of concern if neonatal exposure is too high; high prenatal exposure to molybdenum has been associated with elevated blood pressure during childhood, negative developmental and behavioral outcomes, and impaired psychomotor development.[4,79,80]

NHBCS infants fed exclusively with formula also had higher urinary selenium than infants fed exclusively with human milk. We interpret this finding cautiously, as the reliability of urinary selenium as a biomarker of selenium exposure has not been established for spot urine samples, only integrated 24-hour samples.[44,45] However, the higher urinary excretion of selenium contrasts with expectations from previous exposure modeling for selenium in Sweden[19] and Spain[29] that suggested selenium intake would be lower in formula-fed infants than those fed exclusively with human milk. This difference could be due to issues with spot urine samples as a biomarker, or to differences in maternal diet,[81] the type of formula powder used,[19] or geographic variation in powdered formula concentrations (selenium levels in formula from the United States can be higher than in European countries[82]).

In contrast, the differences in urinary cobalt between infants fed with human milk versus formula in the NHBCS were much smaller than for molybdenum and selenium (Figure 2E, F, G) and not statistically significant after correction for multiple testing (Table 3). Here, we had expected higher exposure in formula-fed infants because there were higher concentrations of cobalt in formula compared with human milk in a Chinese study,[18] but found the opposite pattern. Further work is needed to reconcile these results and establish whether they hold more broadly.

## Conclusions

We conducted this study to assess urinary biomarkers of exposure to potentially toxic elements in the immediate postnatal period, focusing on differences due to consumption of human milk, formula, or both. We acknowledge that feeding exclusively with human milk was more prevalent among NHBCS participants than in the United States as a whole: 73% of the infants in this study were fed only human milk at approximately 6 weeks of age, compared with 44%–47% of US infants born between 2013 and 2020 at 3 months of age based on the National Immunization Survey.[15] This difference in feeding mode was likely due to the combined effects of geographic location and cohort demographics. First, New Hampshire had higher rates of exclusive feeding with human milk during the first 3 months of infancy than the national average, ranging from 51.8% to 63% from 2012 to 2019.[83] Additionally, NHBCS demographics (Table 1) are consistent with populations expected to engage more in exclusive feeding with human milk: nationally, being older than 30 years of age and being college-educated are both associated with higher exclusive feeding with human milk at 3 months of age.[83] Nevertheless, we did observe sufficient combination feeding and exclusive formula feeding to make quantitative comparisons of urinary concentrations among feeding modes and water sources.

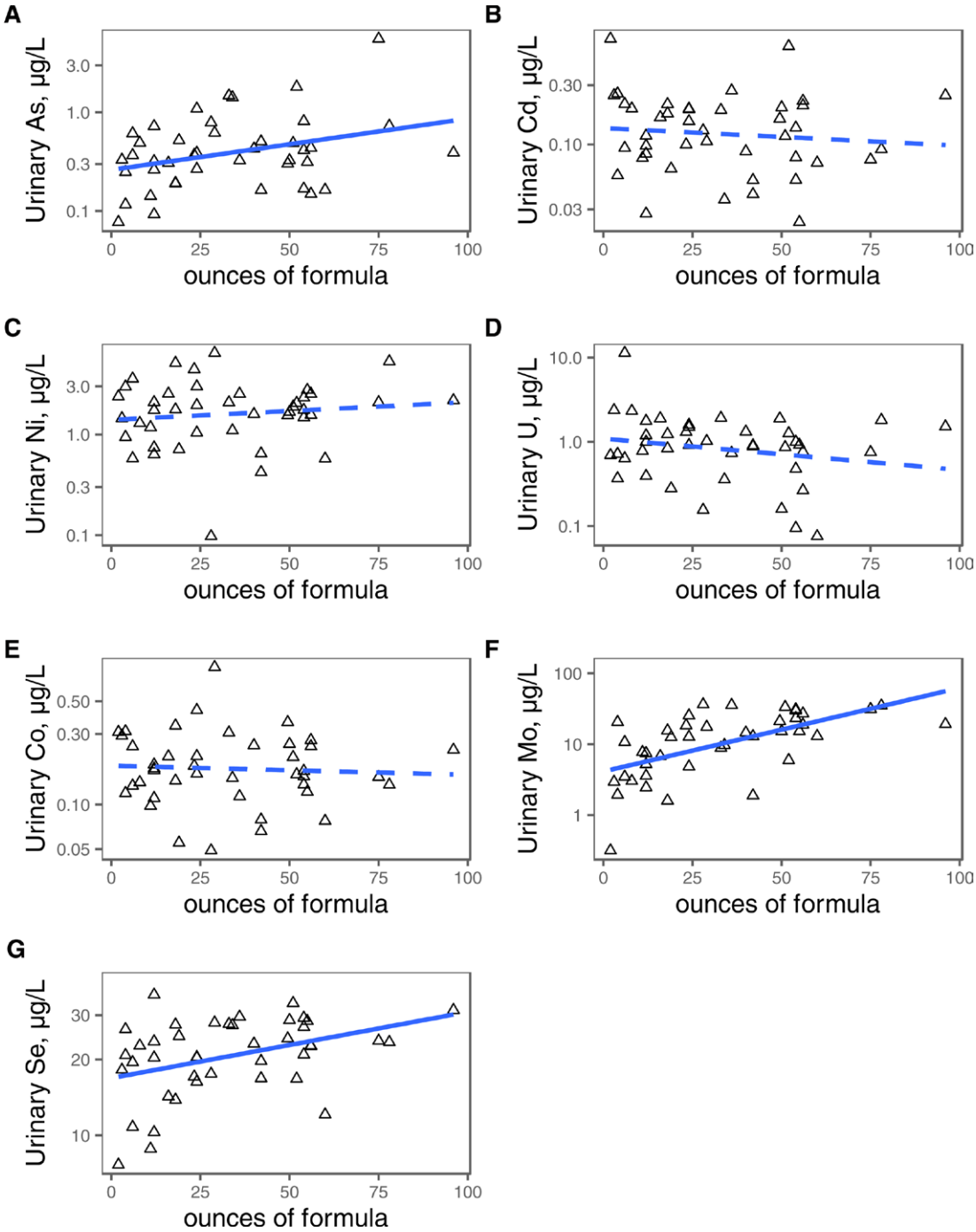


**Figure 4.** Specific gravity-adjusted infant urinary element concentrations as a function of the total number of ounces of formula reported on the 3-day food diaries for the subset of combination-fed infants with this information available, N = 42. Panels indicate urinary (A) arsenic (total arsenic minus arsenobetaine), cadmium (B), nickel (C), uranium (D), cobalt (E), molybdenum (F), and selenium (G) concentrations. Solid lines and 95% confidence intervals indicate associations that are significant at the 5% level. Dotted lines indicate nonsignificant associations.

Previous work had suggested that exposure would generally be higher in formula-fed infants than in infants fed with human milk, but our results suggest that exposure patterns—as inferred from urinary excretion, for both the whole substudy and just the combination-fed infants—are more nuanced and vary by element. We speculate that exposure also varies geographically and through time with the composition of both formula and human milk. For example, human milk varies with the maternal diet and as infants develop.[48,84,85] Moreover, the processes used to manufacture infant formula powders vary by product (e.g., derived from cow's milk vs. plant sources such as soy[20,86]), by region (as noted above), and through time (e.g., arsenic in certain toddler formulas made with organic brown rice syrup was reduced[87,88] following the publication of Jackson et al[21]). Generalizations about exposure to all potentially toxic elements may therefore not be possible, and while the greater nutritional and immunological value of human milk is well-established,[14] not all parents have the option to feed with human milk. Ensuring the safe production of formula powder and reconstitution with water free of contaminants should therefore remain a priority to protect infant health.

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