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Exposure to melamine and its derivatives and aromatic amines among pregnant women in the United States: The ECHO Program

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Abbreviations: EPA, United States Environmental Protection Agency; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MS/MS, tandem mass spectrometry; NHANES, National Health and Nutrition Examination Survey; NIH, National Institutes of Health; PR, prevalence ratio; RPD, relative percent difference; U.S., United States.

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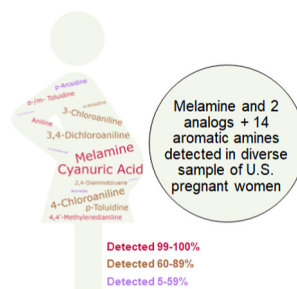
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HIGHLIGHTS

- Measured four melamine derivatives and 41 aromatic amines in ECHO pregnant women.
- Melamine, cyanuric acid, and ten aromatic amines detected in >60% of women.
- Concentrations higher in non-Hispanic Black or Hispanic women for several compounds.
- Most chemicals positively associated with the tobacco biomarker cotinine.
- Annual, within-day, seasonal, and geographic variability observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Melamine, melamine derivatives, and aromatic amines are nitrogen-containing compounds with known toxicity and widespread commercial uses. Nevertheless, biomonitoring of these chemicals is lacking, particularly during pregnancy, a period of increased susceptibility to adverse health effects.

Objectives: We aimed to measure melamine, melamine derivatives, and aromatic amine exposure in pregnant women across the United States (U.S.) and evaluate associations with participant and urine sample collection characteristics.

Methods: We measured 43 analytes, representing 45 chemicals (i.e., melamine, three melamine derivatives, and 41 aromatic amines), in urine from pregnant women in nine diverse ECHO cohorts during 2008–2020 (N = 171). To assess relations with participant and urine sample collection characteristics, we used generalized estimating equations to estimate prevalence ratios (PRs) for analytes dichotomized at the detection limit, % differences (%Δ) for continuous analytes, and 95% confidence intervals. Multivariable models included age, race/ethnicity, marital status, urinary cotinine, and year of sample collection.

Results: Twelve chemicals were detected in >60% of samples, with near ubiquitous detection of cyanuric acid, melamine, aniline, 4,4'-methylenedianiline, and a composite of o-/m-toluidine and m-toluidine (99–100%). In multivariable adjusted models, most chemicals were associated with higher exposures among Hispanic and non-Hispanic Black participants. For example, concentrations of 3,4-dichloroaniline were higher among Hispanic (%Δ: +149, 95% CI: +17, +431) and non-Hispanic Black (%Δ: +136, 95% CI: +35, +311) women compared with non-Hispanic White women. We observed similar results for ammelide, o-/m-toluidine, 4,4'-methylenedianiline, and 4-chloroaniline. Most chemicals were positively associated with urinary cotinine, with strongest associations observed for o-/m-toluidine (%Δ: +23; 95% CI: +16, +31) and 3,4-dichloroaniline (%Δ: +25; 95% CI: +17, +33). Some chemicals exhibited annual trends (e.g., %Δ in melamine per year: –11; 95% CI: –19, –1) or time of day, seasonal, and geographic variability.

Discussion: Exposure to melamine, cyanuric acid, and some aromatic amines was ubiquitous in this first investigation of these analytes in pregnant women. Future research should expand biomonitoring, identify sources of exposure disparities by race/ethnicity, and evaluate potential adverse health effects.

1. Introduction

Tens of thousands of chemicals are used in the United States (U.S.) (Pool and Rusch, 2014), with a small fraction biomonitoring in the general population (CDC, 2021). Pregnancy exposures are concerning as susceptibility is heightened for pregnant women (Varshavsky et al., 2020) and developing fetuses (Andersen, 2003). The Environmental influences on Child Health Outcomes (ECHO) Program is a collaborative study of over 50,000 children from 69 cohorts across the U.S., including diverse racial/ethnic and sociodemographic groups (Romano et al., 2021). ECHO is uniquely poised to address gaps in exposure assessment and risk characterization of chemical exposures during pregnancy and childhood (Buckley et al., 2020). ECHO investigators previously identified understudied priority chemicals for biomonitoring, including melamine and three aromatic amines, based on likelihood of exposure during pregnancy, potential toxicity, and the existence of a biomarker (Pellizzari et al., 2019).

Melamine is a nitrogen-rich industrial chemical widely used in adhesives, plastics, flame retardants, fertilizers, and a degradation product of the pesticide cyromazine (NLM, 2021c). Melamine derivatives often accompany melamine exposure, as they are manufacturing impurities

and degradation byproducts of melamine (Gong et al., 2016). One melamine derivative, cyanuric acid, has additional applications as a disinfectant, plastic additive, chlorine stabilizer in swimming pools, and nitrogen source in animal feed (NLM, 2021b). Melamine and cyanuric acid are high production volume chemicals with aggregated annual production volumes surpassing 100 million pounds (EPA, 2020). While melamine and melamine derivatives are not approved for direct addition to human food or animal feeds marketed in the U.S. (FDA, 2021a), suggested exposure pathways involve contaminated food (NLM, 2021c) and house dust (Shin et al., 2020; Zheng et al., 2020; Zhu and Kannan, 2018b). They are commonly detected in foodstuffs in the U.S. (Zhu and Kannan, 2018a, 2019b), possibly due to leaching from tableware (Takazawa et al., 2020), food packaging (Zhu and Kannan, 2019b), contaminated water (Zhu and Kannan, 2020b), animals exposed to melamine-containing fertilizers and animal feed, and/or illegal addition. In 2007–2008, illegal addition in infant formula and pet feed resulted in urolithiasis (stone in the urinary system) and kidney failure incidents, and in extreme cases, death (Gossner et al., 2009; He et al., 2009). Melamine is also classified as a carcinogen (WHO, 2019). Still, studies characterizing exposures or assessing relations with cancer or other health endpoints in adult human populations are limited.

Aromatic amines are used in dyes and pigments, oil refining, rubber, explosives, pesticides, and pharmaceuticals (WHO, 2010). Tobacco smoke, diesel exhaust, and cooking meat can also result in exposure (Manabe et al., 1991; Stabbert et al., 2003; Turesky et al., 2005) and universal detection in the environment (Lee et al., 1997; Nelson and Hites, 1980; Palmioto et al., 2001; Tkaczyk et al., 2020). Several aromatic amines are classified as carcinogenic (WHO, 2010; OEHHA, 2021), particularly to bladder (Talaska, 2003). Aromatic amines are also suggested developmental toxicants, some of which (anisidine, o-toluidine, and 3,4-dichloroaniline) have previously been recommended as high priority for biomonitoring in ECHO (Pellizzari et al., 2019). An additional 25 aromatic amines were deferred for prioritization, pending more research on non-occupational exposure levels, appropriate biomarker development, and/or toxicity (Pellizzari et al., 2019).

Despite the toxicities of melamine, melamine derivatives, and some aromatic amines (Pellizzari et al., 2019), many are still produced in large volumes and ubiquitously detected in the environment in the U.S. (Zhu et al., 2019; Zhu and Kannan, 2018b). Exposures are not well-characterized during pregnancy and they are not routinely biomonitoring. In order to understand the extent of exposures among US pregnant women, we measured 43 analytes that represented 45 chemicals (i.e., melamine, three melamine derivatives, and 41 aromatic amines) in the urine of a diverse population of pregnant women from nine ECHO cohorts and explored differences by participant and urine sample collection characteristics.

2. Materials and methods

2.1. Study population

Each of the nine cohorts from the ECHO Program contributed between 14 and 20 banked urine samples collected between 2008 and 2020 from cohort participants (Supplemental Table S1). The only criterion for inclusion was the availability of 6 mL of urine collected during pregnancy. These nine cohorts include pregnant women from California, Georgia, Illinois, New Hampshire, New York, and Puerto Rico, with residences across the rural-urban spectrum and reflecting socio-demographic diversity (Supplemental Table S1).

The study protocol was approved by the local (or central ECHO) Institutional Review Board (IRB). We obtained written informed consent for participants in cohort-specific research and/or the ECHO-wide Cohort Data Collection Protocol. The work of the ECHO Data Analysis Center is approved through the Johns Hopkins Bloomberg School of Public Health IRB.

2.2. Analyses of urine samples

Cohorts shipped urine samples on dry ice to the Wadsworth Human Health Exposure Analysis Resource (HHEAR) laboratory at New York University for analysis. Supplemental Excel S1 provides details on the full list of measured analytes.

Melamine, cyanuric acid, ammelide, and ammeline in urine were analyzed following a method described previously (Zhu and Kannan, 2019a). Briefly, two 250 μ L aliquots of urine were transferred into 15 mL polypropylene tubes. One aliquot was acidified with 50 μ L of 1% (v/v) formic acid for cyanuric acid analysis; the other aliquot was alkalinized with 50 μ L of 5% (v/v) ammonium hydroxide for the analysis of melamine, ammelide, and ammeline. The samples were then fortified with 5.0 ng of isotopically labeled internal standard (IS) mixtures and extracted twice with 2 mL of ethyl acetate/isopropanol (95:5, v/v). The combined supernatant was concentrated to near dryness under a gentle nitrogen stream, reconstituted in 250 μ L of acetonitrile/5 mM ammonium formate buffer (pH = 4.0) (9:1, v/v), and filtered through a 0.2 μ m nylon filter (Corning, NY) into glass vials prior to high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. Melamine, ammelide, ammeline, and cyanuric acid were

analyzed using a Shimadzu LC-30 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan), connected to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Chromatographic separations were performed using a Luna hydrophilic-lipophilic interaction liquid chromatography column (100 mm \times 3.0 mm, 3.0 μ m; Phenomenex, Torrance, CA), serially connected to a Betasil C18 guard column (20 mm \times 2.1 mm, 5 μ m; Thermo Scientific, Waltham, MA) with acetonitrile (A) and 5 mM ammonium formate buffer (pH = 4.0; B) as mobile phases. Two acquisition modes were used in the MS/MS analysis. The negative mode was applied from 0 to 4.2 min for ammelide and cyanuric acid detection, and the positive mode was applied from 4.2 to 18 min for melamine and ammeline detection. Recoveries of target analytes through the analytical method ranged from 68 to 82%, and the limits of detection (LODs) were 0.08 ng/mL for melamine, 0.10 ng/mL for ammelide, 0.15 ng/mL for ammeline, and 0.20 ng/mL for cyanuric acid.

Thirty-nine analytes representing 41 aromatic amines (37 individual aromatic amines, a composite of o-toluidine and m-toluidine, and a composite of 3,4- and 2,4- diaminoanisole) were measured in aliquots of urine samples following the HPLC-MS/MS method described elsewhere (Chinthakindi and Kannan, 2021). Urine samples (2 mL) were fortified with IS and hydrolyzed using 50 μ L of 10 M sodium hydroxide solution at 95 °C for 15 h, and target analytes were extracted using methyl-tert-butyl ether. Next, 15 μ L of 0.25 M HCl was added to the extract. Extracts were evaporated to near-dryness, and the residue was reconstituted in 200 μ L of water:methanol (9:1, v/v) and transferred into a vial with a 300 μ L glass insert for HPLC-MS/MS analysis. Chromatographic separation of the analytes was achieved on an Ultra Biphenyl column (100 mm \times 2.1 mm, 5 μ m, Restek, Bellefonte, PA, USA) connected to a Betasil C18 guard column with 0.1% formic acid in water: methanol mixture (95:5, v/v; A) and 0.1% formic acid in methanol (B) as mobile phases. MS was operated in electrospray ionization positive ion multi-reaction monitoring mode. Since this analytic method was originally developed to simultaneously measure aromatic amines and tobacco exposure, we measured nicotine and cotinine along with the 39 analytes that represented 41 aromatic amines. Of the 39 aromatic amine analytes and 2 tobacco exposure analytes fortified in quality control (QC) samples, recoveries (%) were in the range of 75–114% for 37 analytes and lower for the other four analytes (16–74%). Using the isotopic-dilution method for quantification, the concentrations were adjusted for lower recoveries of nine analytes. The LODs and limits of quantification (LOQs) of target analytes were in the range of 0.025–0.200 ng/mL and 0.1–0.5 ng/mL, respectively. Details of the QC procedures are provided in the Supplemental Materials.

2.3. Covariates

We examined several participant and urine sample collection characteristics as potential predictors of exposure. We selected characteristics *a priori* based on prior literature describing predictors of non-persistent chemical exposures and data availability within all nine participating cohorts. All covariates were ascertained by the individual cohort research teams and subsequently harmonized by the ECHO Data Analysis Center. Participant characteristics included age at prenatal urine sample collection (years; continuous), self-identified race/ethnicity (non-Hispanic White race; non-Hispanic Black race; Hispanic ethnicity, any race; non-Hispanic Asian; non-Hispanic other or multiple race), pre-pregnancy and early pregnancy body mass index (BMI, measured from preconception to 16 completed weeks of gestation; continuous), highest educational attainment (high school diploma, general educational development [GED], or less; some college, Associate's degree, or trade/vocational school; Bachelor's degree or higher), and marital status (single, separated, divorced, widowed; married or partnered and living together). Given that three of the nine cohorts were located in California, we assessed residence as a binary variable (California; other state/territory). We assessed associations with tobacco

related exposure during pregnancy using log₂-transformed creatinine-standardized urinary cotinine concentrations (ng/mL). Although nicotine concentrations were also measured, cotinine has a longer half-life than nicotine and is the preferred biomarker of tobacco smoke exposure in urine (Avila-Tang et al., 2013). Finally, we assessed urine sample collection characteristics, including trimester (first, second, or third), time of day (morning [2am-9:59am], midday [10am-3:59pm], evening [4pm-10pm]), calendar season (autumn [September–November], winter [December–February], spring [March–May], summer [June–August]), and year of collection (continuous).

2.4. Statistical analysis

2.4.1. Descriptive statistics

To describe the demographic characteristics of our study sample and all participants enrolled in the nine participating ECHO cohorts, we calculated the mean and standard deviation (SD) or geometric mean (GM) and geometric standard deviation (GSD) of the continuous variables or sample size (%) of the categorical variables. We calculated the detection frequency, geometric mean, minimum and maximum, and 25th, 50th, and 75th percentiles for all analytes. We excluded 27 analytes (one melamine derivative, 25 individual aromatic amines, and a composite of 3,4- and 2,4- diaminoanisole) from subsequent descriptive analyses since they were not detected in any sample (Supplemental Excel S2). For the remaining analytes, we calculated descriptive statistics (median, interquartile range [IQR]) by cohort and categories of age, race/ethnicity, highest educational attainment, marital status, and urinary cotinine. For descriptive analysis, we used machine-read values (if available and non-zero) or singly imputed values < LOD with the LOD/√2 (Hornung and Reed, 1990; Lubin et al., 2004) and calculated log 2-transformed concentrations. For machine-read values reported as zero because no signal was detected (n = 63 values for nine analytes and cotinine), we added 0.0001 to allow for log₂ transformation. Lastly, we calculated Spearman's correlations for analytes detected in ≥70% of the study sample and visualized them with a correlation heat map (Hornung and Reed, 1990).

2.4.2. Predictors of analyte concentrations

We restricted analyses of predictors to 13 analytes representing 14 chemicals, since they were detected in at least 10% of the overall study sample (all were detected in participants from more than two cohorts). We modeled analytes as either dichotomous or continuous depending on their detection frequency. For the five analytes detected in <70% of participants (i.e., ammelide, *o*-anisidine, 3-chloroaniline, *p*-toluidine, *p*-anisidine), we created dichotomized variables based on each analyte's LOD. For the eight analytes of nine chemicals detected in ≥70% of participants (i.e., cyanuric acid, melamine, aniline, 4,4'-methylenedianiline, *o*/*m*-toluidine, 2,4-diaminotoluene, 4-chloroaniline, 3,4-dichloroaniline), we used the log 2-transformed concentrations as previously described. Prior to analyses, we accounted for urinary dilution by calculating creatinine-standardized concentrations ($E_{corrected}$) using the following formula: $E_{corrected} = E_{observed} * \frac{Cr_{cohortmedian}}{Cr_{observed}}$, where we multiplied observed urinary concentrations ($E_{observed}$) by the ratio of the cohort-specific median creatinine concentration ($Cr_{cohortmedian}$) and sample-specific creatinine concentration ($Cr_{observed}$) (Boeniger et al., 1993; Kuiper et al., 2021).

We first modeled associations between each analyte and predictor using generalized estimating equations with a working correlation matrix to account for clustering by cohort. All covariates previously described were considered as predictors. We estimated percent differences for continuously modeled analytes using an identity link and Gaussian family. We estimated prevalence ratios (PR) for analytes dichotomized at LOD using a log-link and Poisson family. We also estimated corresponding 95% confidence intervals (CIs) with robust Huber-White sandwich estimation of variance and standard errors. Next, we

conducted a multivariable analysis that included age, race/ethnicity, marital status, cotinine, and calendar year in the model. All analyses of predictors were conducted in a sub-sample that was restricted to non-Hispanic White, non-Hispanic Black, and Hispanic ethnicity of any race (n = 160) due to concerns about sparse data for other racial/ethnic groups.

We also conducted two sensitivity analyses. First, we repeated the analysis while excluding eight women with cotinine >27 ng/mL, a threshold previously identified to differentiate occasional smoking during pregnancy (Aurrekoetxea et al., 2013). Second, we explored the influence of creatinine standardization on our estimates given that some of the study chemicals are known or suspected kidney toxicants (WHO, 2010; Peerakietkhajorn et al., 2019) and thus could affect creatinine excretion (O'Brien et al., 2017). For this analysis, we repeated our multivariable model using unstandardized concentrations that did not account for creatinine.

We used a complete-case approach for all analyses and did not report cell sizes less than five. All statistical analyses were conducted using Stata v16.1 (StataCorp, College Station, Texas) or R v4.0.2 Statistical Software (Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Participant demographics

The study population consisted of 171 participants with an average age of 29.5 years at the time of urine sample collection (Table 1). The majority of women self-identified as Hispanic ethnicity (40%) followed by non-Hispanic White race (34%), non-Hispanic Black/African American race (20%), and non-Hispanic other or multiple races that included Asian, Native Hawaiian/Pacific Islander, multiple races, or "other" (Table 1). Sixty-eight percent of women were either married or living with a partner, and 46% of women had attained at least a Bachelor's degree (Table 1). The characteristics of pregnant women in our sample were similar to all pregnant women enrolled in the nine participating ECHO cohorts although a lower proportion of participants in our sample were non-Hispanic White or married/living with a partner (Table 1). The collection of urine samples occurred during all three trimesters and all four seasons, and the majority were collected between 2017 and 2020 (77%; Table 1). Ninety-two percent of samples were spot samples (Table 1). Most of the samples were collected between 10 a.m. and 3:59 p.m. (69%), and 82% had undergone one freeze-thaw cycle prior to assay (Table 1).

3.2. Analyte concentrations and correlations

Sixteen analytes representing 17 chemicals (15 individual chemicals and a composite of *o*-toluidine and *m*-toluidine) were detected in at least one sample, with near ubiquitous detection of 5 analytes - cyanuric acid (100%), melamine (99%), aniline (100%), a composite of *o*-toluidine and *m*-toluidine (99%), and 4,4'-methylenedianiline (99%) (Table 2). The following aromatic amines were also detected in over 60% of samples: 2,4-diaminotoluene (84%), 4-chloroaniline (79%), 3,4-dichloroaniline (70%), *o*-anisidine (67%), 3-chloroaniline (63%), and *p*-toluidine (63%) (Table 2). Geometric mean concentrations of all analytes were <1 ng/mL, except for melamine (1.6 ng/mL) and cyanuric acid (27 ng/mL; Table 2). Cotinine and nicotine were detected in 91% and 74% of samples, respectively (Table 2). The distribution of several analytes varied by cohort (Supplemental Excel S3) and participant characteristics (Supplemental Excel S4). Median raw concentrations were generally higher in non-Hispanic Black or non-Hispanic other/multiple race women although Hispanic women also had elevated levels of cyanuric acid. After creatinine standardization, Non-Hispanic Black women still had elevated median concentrations of most analytes. Certain creatinine-standardized concentrations were higher among non-Hispanic White (aniline), Hispanic (cyanuric acid, ammelide, aniline,

Table 1

Demographic and urine specimen collection characteristics of pregnant women in the study sample and all pregnant women in the nine participating ECHO cohorts. All statistics are sample size (%) unless noted otherwise.

Demographic characteristics	Study sample (n = 171)	Participating cohorts (n = 7420)
Age (years) ^a ; mean (SD)	29.5 (5.3)	30.7 (5.5)
Missing		1192
Age category (years) ^a		
<25	35 (20)	945 (15)
25 to < 30	51 (30)	1572 (25)
30 to < 35	47 (28)	2096 (34)
≥35	38 (22)	1615 (26)
Missing		1192
Race/ethnicity		
Non-Hispanic White	57 (34)	3300 (46)
Non-Hispanic Black/African American	34 (20)	786 (11)
Non-Hispanic Other or multiple race	11 (6)	436 (6)
Hispanic	68 (40)	2599 (37)
Missing	1	299
Highest educational attainment		
Less than high school	16 (10)	413 (7)
High school degree, GED, or equivalent	28 (17)	972 (17)
Some college, Associate's degree, or trade/vocational school	45 (27)	1293 (23)
Bachelor's degree	36 (22)	1638 (29)
Master's, professional, or doctorate degree	39 (24)	1393 (24)
Missing	7	1711
Marital status (missing: n = 6)		
Single; partnered, not living together	45 (27)	1045 (18)
Widowed; separated; divorced	8 (5)	325 (6)
Married or living with a partner	112 (68)	4388 (76)
Missing		1662
Pre-pregnancy or early pregnancy BMI (kg/m ²); mean (SD)	26.4 (6.5)	26.4 (6.4)
Missing	12	1667
California residence	54 (32)	2114 (28)
Urine specimen collection characteristics^b		
Creatinine (mg/dL); geometric mean (GSD)	61.4 (1.7)	
Time of day (missing: n = 9)		
Morning (2 a.m.–9:59 a.m.)	40 (25)	
Midday (10 a.m.–3:59 p.m.)	112 (69)	
Evening (4 p.m.–10 p.m.)	10 (6)	
Trimester (missing: n = 2)		
1 (0–13 completed weeks' gestation)	19 (11)	
2 (14–26 completed weeks' gestation)	82 (49)	
3 (27+ completed weeks' gestation)	68 (40)	
Calendar season		
Winter (December–February)	37 (22)	
Spring (March–May)	39 (23)	
Summer (June–August)	52 (30)	
Autumn (September–November)	43 (25)	
Calendar year		
2008–2015	19 (11)	
2016	20 (12)	
2017	40 (23)	
2018	46 (27)	
2019–2020	43 (25)	
Collection type (missing: n = 3)		
Spot	154 (92)	
First morning void	14 (8)	
Freeze-thaw cycles		
1	140 (82)	
2	31 (18)	

^a Age at specimen collection for study sample; age at delivery for participating cohorts.

^b Urine collection characteristics are provided for the study sample only.

2,4-diaminotoluene, and para-anisidine), non-Hispanic Asian (melamine, ammelide, and aniline), or non-Hispanic other or multiple race (anisidine and 2,4-diaminotoluene) women (**Supplemental Excel S4**). We also observed differences by cotinine for most chemicals, with higher

concentrations of chemicals among individuals with higher urinary cotinine concentrations (**Supplemental Excel S4**). Some chemicals were elevated in the youngest age group and unmarried/partnered women (e.g., 2,4-diaminotoluene, 3,4-dichloroaniline, and 3-chloroaniline) and in samples collected during earlier years (e.g., melamine, cyanuric acid, and 3-chloroaniline; **Supplemental Excel S4**).

Among the eight continuously modeled analytes (seven individual chemicals and o-/m-toluidine), Spearman's correlations (ρ) between creatinine-standardized concentrations ranged between -0.14 (2,4-DAT and melamine) and 0.62 (3,4-DCA and 4-CA), with most analytes being weakly correlated ($|\rho| < 0.3$) with one another (**Supplemental Fig. S1**). The Spearman's correlation between cotinine and melamine was lower ($\rho = 0.06$) than between cotinine and other analytes ($0.14 < \rho \leq 0.35$; **Supplemental Fig. S1**). Correlations of melamine, melamine derivative, and aromatic amine analytes with nicotine were generally similar to correlations with cotinine ($0.1 < \rho \leq 0.4$; **Supplemental Fig. S1**).

3.3. Predictors of analytes

Thirteen analytes met our criteria for assessment of predictors (i.e., detected in at least 10% of the overall study sample). Of these, eight analytes (seven individual chemicals and o-/m-toluidine) were modeled continuously, and five analytes were modeled dichotomously. Generally, most analytes exhibited variation by race/ethnicity, urinary cotinine, and marital status in the unadjusted models (**Supplemental Excel S5**) although associations for marital status were attenuated in the adjusted models (**Fig. 1**; **Supplemental Excel S6**). We observed differences by race/ethnicity for the majority of analytes ($n = 11$) after adjusting for age, marital status, cotinine, and calendar year (**Fig. 1**; **Supplemental Excel S6**). Ten of the analytes were elevated in non-Hispanic Black ($n = 6$) or Hispanic ($n = 9$) women as compared to non-Hispanic White women, while o-anisidine was lower in Hispanic women as compared to non-Hispanic White women. Creatinine-standardized concentrations of 3,4-dichloroaniline were elevated among Hispanic (% difference: 149, 95% CI: 17, 431) and non-Hispanic Black (% difference: 136, 95% CI: 35, 311) women compared with non-Hispanic White women, with similar trends observed for 4-chloroaniline, 4,4'-methylenedianiline, o-/m-toluidine, and ammelide. Some analytes were elevated only among Hispanics (2,4-diaminotoluene % difference: 58; 95% CI: 16, 115; cyanuric acid % difference: 30; 95% CI: $-6, 80$; aniline % difference: 17; 95% CI: $-10, 51$) or among non-Hispanic Black women (3-chloroaniline PR: 1.5; 95% CI: 1.1, 2.2; **Supplemental Excel S6**).

Cotinine was positively associated with most ($n = 9$) analytes in the unadjusted analyses (**Supplemental Excel S5**) and in the analyses adjusted for age, race/ethnicity, marital status, and year (**Fig. 1**; **Supplemental Excel S6**). Associations were strongest for 2,4-diaminotoluene (% difference: 38; 95% CI: 23, 55), 3,4-dichloroaniline (% difference: 28; 95% CI: 8, 51), and o-/m-toluidine (% difference: 25; 95% CI: 17, 33; **Supplemental Excel S6**). In the sensitivity analyses of unadjusted associations that excluded eight women with urinary cotinine concentrations > 27 ng/mL (i.e., potential active smokers or those with high secondhand smoke exposure), associations became stronger for most analytes but were closer to the null for others, including o-/m-toluidine (% difference: 13; 95% CI: 5, 21; **Supplemental Excel S7**).

Calendar year was associated with concentrations of some analytes in both the unadjusted (**Supplemental Excel S8**) and adjusted analyses (**Fig. 1**; **Supplemental Excel S6**). Creatinine-standardized concentrations decreased by calendar year for melamine (% difference: -11 ; 95% CI: $-19, -1$), p-anisidine (PR: 0.9; 95% CI: 0.8, 0.9), and o-anisidine (PR: 0.97; 95% CI: 0.96, 0.98), whereas the concentrations of aniline (% difference: 6.79; 95% CI: 4.32, 9.33), 4,4'-methylenedianiline (% difference: 5.55; 95% CI: 1.69, 9.55), and o-/m-toluidine (% difference: 2.34; 95% CI: $-0.084, 4.83$) increased with calendar year (**Supplemental Excel S6**).

In the unadjusted models, variability in exposure was also observed

Table 2

Descriptive statistics of urinary analytes of melamine, melamine analogs, aromatic amines, and tobacco exposure measured among 171 pregnant women from nine participating ECHO cohorts.

Full analyte name	Abbreviation	LOD	N (%) > LOD	GM	Minimum	25th percentile	Median	75th percentile	Maximum
Melamine and melamine analogs									
Cyanuric acid	CYNA	0.08	171 (100)	27	0.12	17	28	45	280
Melamine	MEL	0.03	170 (99)	1.6	< LOD	0.78	1.6	2.8	351
Ammelide	AMD	0.05	22 (13)	0.069	< LOD	< LOD	< LOD	< LOD	36
Aromatic amines									
Aniline	ANI	0.1	171 (100)	0.81	0.15	0.52	0.74	1.1	34
4,4'-Methylenedianiline	MDA	0.025	169 (99)	0.13	< LOD	0.076	0.12	0.20	1.3
Ortho/meta-Toluidine	o-TD/m-TD	0.05	169 (99)	0.75	< LOD	0.45	0.77	1.3	7.1
2,4-Diaminotoluene	2,4-DAT	0.1	144 (84)	0.64	< LOD	0.20	0.69	3.2	21
4-Chloroaniline	4-CA	0.05	135 (79)	0.14	< LOD	0.058	0.12	0.30	67
3,4-Dichloroaniline	3,4-DCA	0.1	120 (70)	0.16	< LOD	< LOD	0.20	0.57	5.7
Ortho-Anisidine	o-ANSID	0.025	115 (67)	0.033	< LOD	< LOD	0.039	0.086	0.48
3-Chloroaniline	3-CA	0.05	109 (64)	0.083	< LOD	< LOD	0.09	0.31	8.6
Para-Toluidine	p-TD	0.025	108 (63)	0.031	< LOD	< LOD	0.037	0.067	0.38
Para-Anisidine	p-ANSID	0.05	39 (23)	< LOD	< LOD	< LOD	< LOD	< LOD	29
2,4-Dimethylaniline	2,4-DMA	0.05	13 (8)	< LOD	< LOD	< LOD	< LOD	< LOD	0.31
2,4,5-Trimethylaniline	2,4,5-TMA	0.05	11 (6)	< LOD	< LOD	< LOD	< LOD	< LOD	0.18
Para-Cresidine	p-CD	0.025	9 (5)	< LOD	< LOD	< LOD	< LOD	< LOD	0.66
Tobacco exposure									
Cotinine	COTT	0.1	156 (91)	0.52	< LOD	0.24	0.40	0.73	446
Nicotine	NICT	0.025	127 (74)	0.23	< LOD	< LOD	0.26	0.78	1890

Note: Concentration units are ng/mL.

Abbreviations: GM, geometric mean; L, liter; LOD, limit of detection; mL, milliliter; ng, nanogram.

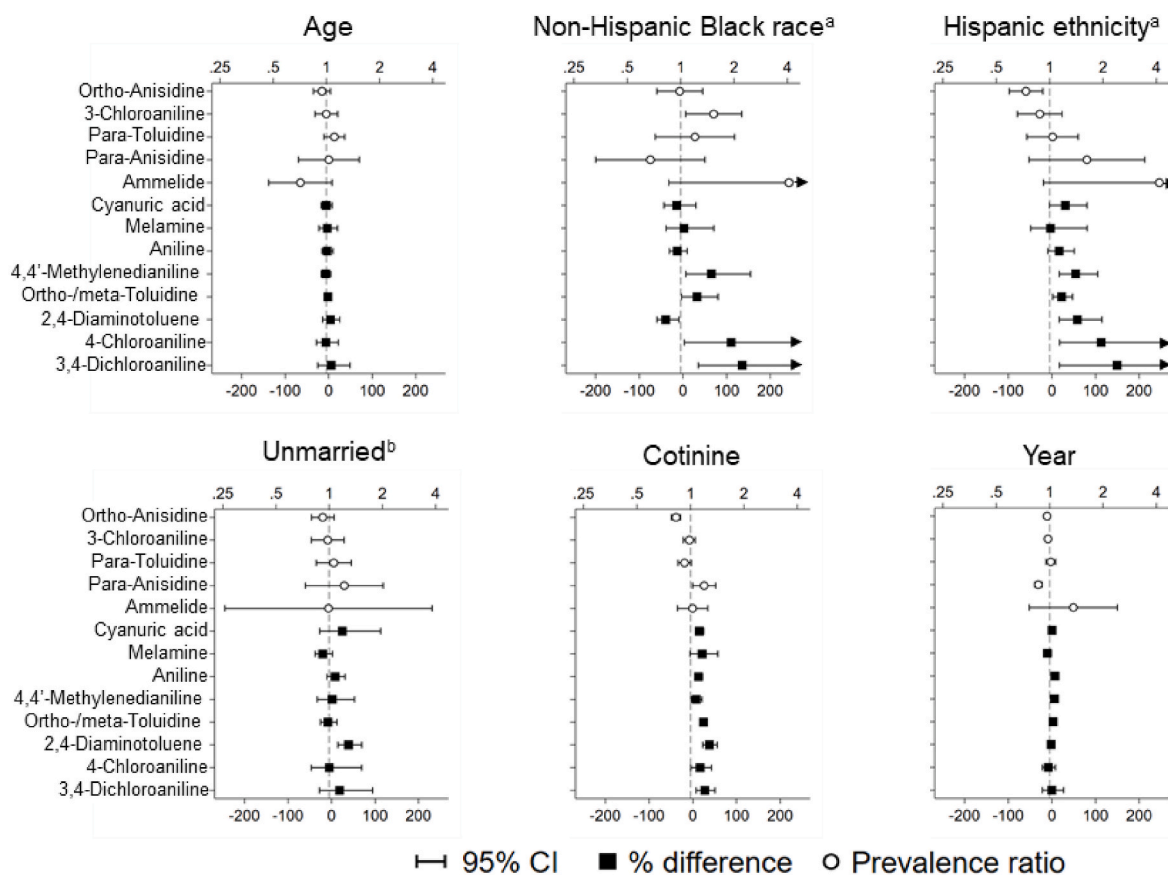


Figure 1. Adjusted associations of sociodemographic and sample collection variables with concentration or detection prevalence of urinary melamine, cyanuric acid, and select aromatic amines in pregnant women (n = 171). Reference categories: ^anon-Hispanic White, ^bmarried/partnered and living together.

for California state residence and season/time of sample collection although the direction of associations varied by specific chemicals (**Supplemental Excel S5; Supplemental Excel S8**). Pregnant women residing in California had elevated creatinine-standardized concentrations of melamine, para-anisidine, and ortho-anisidine. Afternoon/evening samples contained elevated creatinine-standardized concentrations of melamine, cyanuric acid, and aniline, whereas morning samples contained higher levels of *o*-anisidine and 4-chloroaniline. Seasonal variability was observed for melamine, 4-chloroaniline, and 3,4-dichloroaniline. Melamine and chloroanilines were higher in summer and fall months.

In a sensitivity analysis using unstandardized, rather than creatinine-standardized, concentrations in our multivariable model, we observed a similar direction of associations for most predictors (**Supplemental Excel S9**). The strength of association, however, varied for some analytes. Point estimates for aniline were attenuated consistently for all predictors, with the largest difference observed for calendar year (creatinine-standardized % difference: 7, 95% CI: 4, 9; unstandardized % difference: 4, 95% CI: 2, 6). Similar trends were observed for cyanuric acid. Strengths of association were inconsistently affected for some analytes; for example, the *o*-*m*-toluidine % difference between non-Hispanic Black and non-Hispanic White women became larger (creatinine-standardized: 32, 95% CI: -3, 80; unstandardized: 130, 95% CI: 96, 169), but the % difference associated with cotinine became weaker (creatinine-standardized: 25, 95% CI: 17, 32.9; unstandardized: 16; 95% CI: 9, 24).

4. Discussion

This is the largest U.S. study to date of melamine, melamine derivatives, and aromatic amines in a geographically and demographically diverse population of pregnant women. Our findings provide important public health implications by estimating exposure levels of widespread chemicals that are not routinely biomonitoring and exploring their socio-demographic predictors. Although none of the chemicals measured in our study are routinely included in the National Health and Nutrition Examination Survey (NHANES), 11 analytes of 12 chemicals were detectable in over 60% of the samples, and five of these were detected in nearly every sample. Of the frequently detected analytes, melamine and four aromatic amines were those we had previously recommended for biomonitoring in the ECHO Program (i.e., *o*/*m*-toluidine, 3,4-dichloroaniline, and *o*-anisidine) and six additional aromatic amines we had recommended deferred due to the need for more research on toxicity, non-occupational exposure levels, and/or biomarker development (i.e., aniline, 4,4'-methylenedianiline, 2,4-diaminotoluene, 4-chloroaniline, 2,4-diaminotoluene, and 4-chloroaniline) (Pellizzari et al., 2019). Our study included nine cohorts within ECHO, which enabled us to identify differences in exposure concentrations by participant and urine sample collection characteristics.

All but one urine sample had detectable concentrations of both melamine and cyanuric acid, whereas ammeline was infrequently detected (13%) and ammelide was not detected. Melamine concentrations in our study were slightly lower than that reported in pregnant women in East Asian countries (Tsai et al., 2021; Wu et al., 2020) and U.S. children (Melough et al., 2022; Sathyanarayana et al., 2019) but comparable to previous studies of non-occupationally exposed U.S. adults and females (Guo et al., 2020; Panuwet et al., 2012; Zhu and Kannan, 2019a) (**Supplemental Table S3**). We observed relatively high concentrations of cyanuric acid compared with melamine (15-fold difference), whereas previous studies have generally reported cyanuric acid concentrations up to 5-fold of melamine (Zhu and Kannan, 2019a). Cyanuric acid concentrations of our population were notably higher than among U.S. adults (Zhu and Kannan, 2019a) but comparable to U.S. children (Sathyanarayana et al., 2019). The only other study that measured cyanuric acid in U.S. adults used 213 repeat samples provided by 19 healthy Asian or Caucasian volunteers in New York in 2018 (Zhu

and Kannan, 2019a). The median cyanuric acid concentrations in a subset of Caucasian females in the Zhu and Kannan study (8.1 ng/mL) were considerably lower than the median among non-Hispanic White participants in our study (24 ng/mL). While such differences in concentrations may reflect a true exposure contrast, there may be other contributing factors as well. Our study population differed from Zhu and Kannan's in terms of pregnancy status, geographic location, and calendar year; however, geographic location and calendar year were not strong predictors of cyanuric acid concentrations in our study. Time of day of urine collection may explain study heterogeneity in exposure levels since we observed lower cyanuric acid concentrations in morning samples and the previous study used repeated first morning voids. It is also possible that higher levels in pregnant women compared with the general population reflect more frequent exposure to certain source products, such as disinfectants (Patel and Jones, 2007) and drinking (tap/bottled) water (Zhu and Kannan, 2020b).

The ubiquitous detection of both melamine and cyanuric acid in our pregnant population is concerning since kidney toxicities have been reported at varying exposure levels and there are potentials for developmental effects. Kidney effects, ranging from symptomatic or asymptomatic urolithiasis to acute renal failure and urinary tract obstruction, have been observed in children with chronic histories of melamine-contaminated formula consumption (He et al., 2009; Lam et al., 2009). Multiple agencies derived tolerable daily intake to address melamine kidney toxicity (EFSA, 2010; FDA, 2021a; World Health Organization, 2009), however, risk assessments extrapolated rat experiments of bladder stone formation using an adjustment factor and did not fully consider other health endpoints or the effects of prenatal exposures. In humans, there are no established minimal risk levels for internal melamine concentrations. High urinary melamine concentrations have been linked with calcium urolithiasis in children (8.2–73 µg/mmol creatinine (Lam et al., 2009)) and adults (0.48–3.29 µg/mmol creatinine (Liu et al., 2017)); lower levels in the general population (NHANES, 2003–2004 geometric mean: 1.51 ng/mL, 1.4 µg/g creatinine, equivalent to 0.16 µg/mmol creatinine) may also affect kidney function as indicated by lower glomerular filtration rate (Guo et al., 2020). Our pregnant study population's range of melamine exposure is below those in some studies (Lam et al., 2009; Liu et al., 2017) but similar to those found in NHANES (Guo et al., 2020). Additionally, our population is exposed during the prenatal period which could increase the risk for developmental effects in the children due to unique or heightened periods of developmental susceptibility and the potential for maternal-fetal transfer via the placenta (Jingbin et al., 2010; Wu et al., 2020) and breastmilk (Chan et al., 2011; Yalcin et al., 2020). Developmental exposure to melamine and melamine analogs could additionally affect other health endpoints as supported by animal studies reporting fetal growth restriction, incomplete ossification, and spatial cognitive impairments in animal studies (An and Sun, 2017; Kim et al., 2011). Finally, synergistic effects are also possible when exposed to both melamine and melamine analogs (Jacob et al., 2011). Epidemiologic investigations of pregnancy exposures are limited, calling for assessments of individual or combined exposures to melamine and melamine analogs in the pregnant population, and their potential links to adverse health outcomes in children.

Analytes of 10 aromatic amines (eight individual analytes and a composite of *o*-*m*-toluidine) were detectable in over 60% of our samples. Among these is *o*-anisidine, which was measured with similar concentrations among non-smoking females in NHANES 2013–2014 (unstandardized median: 0.041 ng/mL; creatinine-standardized median: 0.052 ng/mL; **Supplemental Table S4**). Such prevalent detection may be partially due to the widespread application of *o*-anisidine to produce herbicides, rubber, and dyes/pigments, and related high production volumes (NLM, 2021d). Similar to *o*-anisidine, most aromatic amines commonly detected in our study are classified as high production volume chemicals (**Supplemental Excel S1**), except for 4-chloroaniline and 3-chloroaniline.

Not all high production volume aromatic amines were frequently

detected in our study population. This was expected because some high production volume aromatic amines are not used directly in commercial materials but rather as starting products to synthesize other nitrogen-containing chemicals. We did not detect 2,6-dimethylaniline, a high production volume chemical used to manufacture pesticides, dyes, pharmaceuticals, and fragrances (NLM, 2021a), which was detected in over half of non-smoking females in NHANES 2013–2014 (CDC, 2021). Notably, the 2,6-dimethylaniline concentrations observed in NHANES were low (median: 0.0185 ng/mL; Q1-Q3: <LOD - 0.0438 ng/mL; Supplemental Table S4). Such differences in detection frequencies are in part due to the higher LOD in our study (0.05 ng/mL) compared with that of NHANES (0.0157 ng/mL). Additionally, short half-lives of aromatic amines and stability in urine may have contributed to such differences in detection frequencies.

Certain aromatic amines that no longer have large-scale production in the U.S. (i.e., 4-aminobiphenyl: 1950s; benzidine and 2-naphthylamine: 1970s (NTP, 2016)) were below the LOD in all our study samples. While 2-naphthylamine and 4-aminobiphenyl were prevalently detected in non-smoking females in NHANES 2013–2014 (Supplemental Table S4), LODs were lower than in our study. Our LODs for 2-naphthylamine and 4-aminobiphenyl were 0.025 ng/mL and 0.05 ng/mL, whereas the CDC method LODs were 0.00279 ng/mL and 0.00175 ng/mL, respectively (CDC, 2021). Since their biological half-lives range from hours to days (WHO, 2010), the detection of these aromatic amines at low levels in NHANES may reflect low-level continuing exposures rather than high-level exposure from dyes. Benzidine, 4-aminobiphenyl, and 2-naphthylamine were historically produced in large volumes by dye industries but are now produced in small amounts for limited purposes (NTP, 2016). Contemporary exposure may be attributable to tobacco smoke (Saha et al., 2009) or certain imported or contaminated dyes (Choudhary et al., 2001). Populations residing within proximity to hazardous waste sites may also be exposed to 4-aminobiphenyl and benzidine, which have been identified in groundwater and soil samples collected from Superfund sites and are included in the Substance Priority List (ATSDR, 2021; Choudhary et al., 2001). Additionally, benzidine can form from bodily metabolism of benzidine-based dyes, some of which are currently listed under TSCA Significant New Use Rules (EPA, 2021) and could be regulated (EPA, 2010).

The levels of most common-detect chemicals varied by race/ethnicity, even after adjusting for age, marital status, cotinine, and year. Compared with non-Hispanic White women, Hispanic and non-Hispanic Black women had elevated levels of aromatic amines often used as dyes, pesticides, and/or in polyurethane. Some chemical concentrations were elevated only among Hispanic women (i.e., 2,4-diaminotoluene, cyanuric acid, aniline) or among non-Hispanic Black women (i.e., 3-chloroaniline), while both Hispanic and non-Hispanic Black women had greater concentrations of four analytes representing five aromatic amines (i.e., 4,4'-methylenedianiline, o/m-toluidine, 4-chloroaniline, and 3,4-dichloroaniline) and a higher detection of ammelide as compared to non-Hispanic White women. We were unable to assess specific exposure factors that may help to explain such differences and could not fully disentangle race/ethnicity from cohort-level characteristics. Still, our findings are in line with other studies reporting racial and ethnic disparities in relation to sources of chemical exposures, such as phthalates (Chan et al., 2021). Further research is needed to replicate our findings and examine individual, socio-economic, neighborhood, and other structural factors that may contribute to such exposure differences.

We also observed higher concentrations among women who were not married or had higher concentrations of cotinine. While differences by marital status, which could be a proxy for some aspects of socio-economic status, were attenuated in our multivariable model, associations with cotinine remained. Relationships between cotinine and o-/m-toluidine may reflect exposure through active smoking since 1) their positive association was attenuated in our sensitivity analysis excluding

eight individuals with high cotinine concentrations and 2) o-/m-toluidine has been previously detected in tobacco smoke (Goniewicz and Czogala, 2005; Stabbert et al., 2003). However, not all aromatic amines previously identified in tobacco smoke (Goniewicz and Czogala, 2005; Stabbert et al., 2003) showed similar trends: associations between cotinine and certain aromatic amines did not attenuate when restricted to individuals with low-level cotinine. Persistent, positive associations between cotinine and aromatic amines after removal of active smokers suggests environmental tobacco smoke (Aurrekoetxea et al., 2013) as a potential source of aromatic amine exposure. Alternatively, there may be residual confounding through unmeasured sources, such as occupation or dietary nicotine (Davis et al., 1991; Shrivastava and Patel, 2010), as we included only a limited number of variables in our multivariable models due to a small sample size.

Some commonly detected chemicals varied by time of day, month, and year of urine sample collection. Variability by time of day of sample collection may reflect the timing of encounters with source products, given their short biological half-lives ranging from minutes to days (WHO, 2010). Afternoon/evening samples contained elevated concentrations of melamine, cyanuric acid, and aniline, whereas morning samples contained higher levels of o-anisidine and 4-chloroaniline. Seasonal variability was observed for melamine, 4-chloroaniline, and 3,4-dichloroaniline. Melamine was higher in summer and fall months, possibly due to greater leaching from melamine-containing products due to higher temperature (Takazawa et al., 2020); we observed similar patterns for the two chloroanilines. We also observed annual trends for chemicals, including melamine, which may in part be due to increased regulatory actions by international organizations and governmental agencies, prompted by poisoning incidents worldwide. For example, tolerable daily intakes have been derived by the World Health Organization (World Health Organization, 2009) and the United States Food and Drug Administration (FDA) (FDA, 2021b) and are currently in use by the European Food Safety Authority (EFSA, 2010), in Canada. Government Of Canada (2021), and in the U.S. (FDA, 2021b). Additionally, FDA issued a letter in 2008 to alert the food manufacturing industry, increased surveillance in food products, and developed analytic methods to better determine melamine and its derivatives (Kuehn, 2009; FDA 2021bb). However, such regulations are limited to food items and human exposure is still possible through contact with consumer products and the residues in the ambient environment (Shin et al., 2020; Takazawa et al., 2020; Zheng et al., 2020; Zhu et al., 2019; Zhu and Kannan, 2018a; b, 2019b, 2020a; b). Other chemicals, such as aniline, o-/m-toluidine, p-anisidine, and 3-chloroaniline, were also associated with calendar year although the underlying reason is unclear.

Our findings are based on measurements of 45 target chemicals, using 43 analytes, from a single urine sample using previously validated analytical methods with low CVs. The widespread detection of melamine, cyanuric acid, and 10 aromatic amines in our samples indicate a need to expand biomonitoring to include other aromatic amines that were previously detected in human populations or have high production volume. One limitation of our study is that approximately half of the measured aromatic amines were below the LOD. While concentrations below the LOD in our samples could reflect rare exposures, our higher LOD could have contributed to such low level of detection compared with other studies. While our LODs are higher for the small number of aromatic amines measured in NHANES (CDC, 2021) and some previous studies (WHO, 2010), we used a different instrumental technique that allowed us to simultaneously measure 40 aromatic amines. Low detection, in general, may also be due to short biological half-lives, excretion as unmeasured metabolites, or other urine sample collection and storage conditions. Accounting for urinary dilution is both necessary and potentially problematic if the chemicals under study affect kidney function (O'Brien et al., 2017). We did not have measures of specific gravity, another proxy for urinary dilution that may be somewhat less affected by kidney function and other participant characteristics than creatinine (Kuiper et al., 2021; O'Brien et al., 2017). Although our

findings were similar when using either unstandardized or creatinine-standardized concentrations, future studies should consider alternative approaches for urine dilution. While the characteristics of our study population were similar to those of the nine participating ECHO cohorts, our findings may not be fully representative of pregnant women in the greater ECHO Program or the general U.S. population. Further biomonitoring is needed to characterize exposure variability across time and in populations under-represented in our study, such as residents of Southwest/Midwest/Southeast states and Asians. For the chemicals frequently detected in our study sample, additional studies are needed to identify major predictors of exposure and associated health effects.

In this first comprehensive study of exposures among a diverse sample of U.S. pregnant women, we detected melamine, cyanuric acid, and nine aromatic amines in more than half of our participants. Many of these compounds with widespread exposures also have known toxicities, suggesting the critical need to identify intervention approaches in addition to monitoring exposures and health effects in the population. Further, our data indicate important differences in exposures by race and ethnicity; evaluating potential sources of exposure that may contribute to these inequities is needed. Our larger follow up study will allow us to better characterize exposures across the U.S. during a critical period of development and further assess influential predictors and demographic differences that we characterized in this initial study. Finally, our study demonstrates the importance of continuous identification of environmental factors that can play an important role in maternal and child health.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data sets for this manuscript are not publicly available because, per the NIH-approved ECHO Data Sharing Policy, ECHO-wide data have not yet been made available to the public for review/analysis. Requests to access the data sets or code should be directed to the ECHO Data Analysis Center, ECHO-DAC@rti.org.

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Appendix A. Supplementary data

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