Gestational Cd Exposure in the CD-1 Mouse Induces Sex-Specific Hepatic Insulin Insensitivity, Obesity, and Metabolic Syndrome in Adult Female Offspring

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ABSTRACT

There is compelling evidence that developmental exposure to toxic metals increases risk for obesity and obesity-related morbidity including cardiovascular disease and type 2 diabetes. To explore the hypothesis that developmental Cd exposure increases risk of obesity later in life, male, and female CD-1 mice were maternally exposed to 500 ppb CdCl₂ in drinking water during a human gestational equivalent period (gestational day 0–postnatal day 10 [GD0–PND10]). Hallmark indicators of metabolic disruption, hepatic steatosis, and metabolic syndrome were evaluated prior to birth through adulthood. Maternal blood Cd levels were similar to those observed in human pregnancy cohorts, and Cd was undetected in adult offspring. There were no observed impacts of exposure on dams or pregnancy-related outcomes. Results of glucose and insulin tolerance testing revealed that Cd exposure impaired offspring glucose homeostasis on PND42. Exposure-related increases in circulating triglycerides and hepatic steatosis were apparent only in females. By PND120, Cd-exposed females were 30% heavier with 700% more perigonadal fat than unexposed control females. There was no evidence of dyslipidemia, steatosis, increased weight gain, nor increased adiposity in Cd-exposed male offspring. Hepatic transcriptome analysis on PND1, PND21, and PND42 revealed evidence for female-specific increases in oxidative stress and mitochondrial dysfunction with significant early disruption of retinoic acid signaling and altered insulin receptor signaling consistent with hepatic insulin sensitivity in adult females. The observed steatosis and metabolic syndrome-like phenotypes resulting from exposure to 500 ppb CdCl₂ during the pre- and perinatal period of development equivalent to human gestation indicate that Cd acts developmentally as a sex-specific delayed obesogen.

Key words: cadmium; diabetes mellitus type 2; endocrine disruption; insulin resistance; metabolic; nonalcoholic fatty liver disease; retinoic acid.

Globally, more than 1.9 billion adults are overweight; of those, 650 million are considered obese (Ward et al., 2019; World Health Organization, 2020). In the United States, obesity affects >30% of the population and is projected to increase to approximately 50% by 2030 (Andolfi and Fisichella, 2018). Cardiovascular disease and type 2 diabetes (T2D) are principal

comorbidities of obesity and account for \$500 billion in annual health care costs, which represents almost 14% of the US health care expenditures (Heron, 2019). The underlying cause of obesity is an imbalance between energy consumed and energy expended. There is, however, compelling evidence that exposure to some xenobiotic and toxic metal pollutants during

development increases risk for obesity and obesity-related morbidity including cardiovascular disease and T2D (Heindel et al., 2017). Epidemiologic data from different worldwide populations demonstrate that prenatal exposure to the toxic metal cadmium (Cd) is associated with harmful effects on fetal development, with especially well-described impacts on birth weight (Al-Saleh et al., 2014; Luo et al., 2017), Apgar score (Al-Saleh et al., 2014), cognition (Sanders et al., 2015), and epigenetic status (Appleton et al., 2017; Joubert et al., 2012, 2016; Park et al., 2017; Vilahur et al., 2015).

Experimental and epidemiologic analysis indicate that chronic Cd exposure adversely affects metabolism and increase risk factors for metabolic disease. For example, chronic exposure of adult mice to CdCl₂ decreases expression of lipid synthesis genes and insulin secretion (Kawakami et al., 2010; Li et al., 2019). In adult humans, increases in blood Cd concentrations are associated with elevated blood glucose and hypertension (Gallagher and Meliker, 2010; Huang et al., 2013; Kuo et al., 2013; Tellez-Plaza et al., 2013; Wallia et al., 2014). Numerous epidemiological studies have also linked elevated Cd exposure with increased weight gain, T2D, and cardiovascular disease (Anetor et al., 2016; Edwards and Prozialeck, 2009; Schwartz et al., 2003; Wu et al., 2017). However, the link between Cd exposure and T2D is not clear cut, as some studies fail to identify associations between increased Cd exposure and increased risk of T2D (Borné et al., 2014; Moon, 2013; Shapiro et al., 2015). Recent meta-analysis has also concluded that T2D risk in the general population is not significantly associated with urinary or blood Cd concentrations, but significant sources of study heterogeneity, including failing to consider sex differences, limited detection of associations across studies (Wu et al., 2017).

Sex differences are an especially important consideration in the evaluation of the effects of Cd exposure because females are more sensitive to the adverse effects of Cd, in part due to the longer biological half-life of Cd in females (Shimada et al., 2012; Suwazono et al., 2009; Taguchi and Suzuki, 1981). Developmentally, sex differences in programming of the placental epigenome and sex-specific patterns of gene expression within the placenta may play a role in sexually dimorphic responsiveness to Cd toxicity *in utero* (Everson et al., 2019; Martin et al., 2017).

Despite decades of research evaluating toxicity and carcinogenicity of Cd, mechanisms of fetal and placental Cd toxicity remain uncertain. Results from human and animal studies have revealed that maternal Cd levels, rate of maternal fetal transfer of Cd, timing and route of exposure can influence the nature and magnitude of toxicity in exposed offspring (Bhattacharyya, 1983; Christley and Webster, 1983; Lau et al., 1998; Sonawane et al., 1975). Because gestation is a critical developmental period for later in life development of obesity, diabetes, and liver disease, the impacts of maternal Cd exposures may have especially profound effects on offspring metabolism (Heindel and Vandenberg, 2015; Schug et al., 2015). During gestation, the placenta regulates fetal nutrition and oxygen transfer in response to maternal nutritional and fetal-demand signals (Jansson, 2016). Therefore, maternal exposure to Cd could adversely alter hormonal and nutritional signaling of the placental unit. For example, gestational exposure to toxic levels of CdCl₂ have been shown increase placental corticosterone levels that are associated with intrauterine growth restriction, and alter the maternal/fetal balance of monovalent and divalent cations (Jacobo-Estrada et al., 2017; Ronco et al., 2009; Wang et al., 2014).

Cadmium, a cumulative toxicant that accumulates primarily in the liver and kidney, with lower levels accumulating in

pancreas, heart, testis, bone, and neural tissues (Agency for Toxic Substances and Disease Registry [ATSDR], 2012). The organ-specific pattern of uptake and resulting toxicity of Cd is mediated by differential expression of SLC39 transporters, SLC30 efflux transporters, and the divalent metal transporter-1 which together mediate intracellular Cd-uptake (Fujishiro et al., 2012). The mechanism of cumulative Cd toxicity results from high-affinity binding of Cd by metallothioneins and a resulting dose-dependent loss of protective antioxidant mechanisms leading to oxidative damage (Nair et al., 2013; Nemmiche, 2016; Sabolić et al., 2010). The functional roles of the Zip8 (SLC39A8) and Zip14 (SLC39A14) Zn and Mn transporter proteins in regulating the absorption, intracellular uptake, accumulation, and toxicity of Cd in the testis, kidney and liver are well established (Aydemir and Cousins, 2018; Fujishiro et al., 2012; Fujishiro and Himeno, 2019; Sabolić et al., 2010). In *Slc39a14*-knockout mice, loss of Zip14 function alters glucose homeostasis and gluconeogenesis resulting in increased adiposity (Jenkitkasemwong et al., 2018; Xin et al., 2017). Additional studies suggest a mechanistic role for Zip14 in preadipocyte differentiation and adipose metabolism (Tominaga et al., 2005; Troche et al., 2016). However, the impacts of gestational Cd exposure and mechanisms of resultant metabolic disruption and later in life pathology are unknown.

To evaluate the physiological impact of gestational-only Cd exposure on offspring metabolic health, pregnant female CD-1 mice were exposed to 500 ppb CdCl₂ in drinking water throughout a developmental period equivalent to human gestation as defined by brain and retina development (Leasure et al., 2008; Semple et al., 2013). This exposure paradigm resulted in a maternal body burden for dams that was approximately equivalent to the geometric mean blood Cd level for women of childbearing age in the US population (Mijal and Holzman, 2010). The impacts of Cd exposure in male and female offspring were comprehensively evaluated for indications of metabolic disruption, hepatic steatosis, and metabolic syndrome. RNA sequence (RNAseq), functional genomic and pathway analysis was performed on offspring livers on postnatal day 1 (PND1), PND21, and PND42 to describe the mechanistic progression of observed hepatic steatosis and metabolic syndrome-like phenotypes resulting from gestational Cd exposure.

MATERIALS AND METHODS

Animal husbandry. All animal procedures were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee and followed recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Procedures and study reporting follow the ARRIVE guidelines (Supplementary material). Study animals were housed in single-use polyethylene cages (Innovive, San Diego, California) with Sanichip bedding (PJ Murphy Forest Products Corp, Montville, New Jersey) and pulped virgin cotton fiber nestlets (Ancare, Bellmore, New York) on a 12:12 light cycle at 25°C and 45%–60% average relative humidity in an AAALAC accredited animal facility. Defined AIN-93G diet (D10012G; lot: 17010510A6, Research Diets, New Brunswick, New Jersey) and sterile drinking water produced from a reverse osmosis water purification system (Millipore Rios with ELIX UV/Progard 2, Billerica, Massachusetts) was supplied *ad libitum*.

Strain CRL: CD-1(ICR) (CD-1) male and female breeder mice were obtained from Charles River Laboratories (Raleigh, North Carolina). At the time of arrival, mice were assigned a coded study identification number that was used to randomly assign

each animal to control or Cd-exposed study group (Random# Generator v1.2 for Apple iPhone iOS10) and housed separately by sex and exposure group for 2 weeks prior to mating. The study was independently replicated in 2 cohorts, with 1 beginning in March 2017 ($n=20$ dams) and the second initiated March 2019 ($n=18$ dams). Because no significant differences in water or food consumption, Cd exposure, gestational weight gain, litter size, sex ratio, or body weights from PND1 to PND42 were identified, data were merged for analysis.

Exposure, mating, and endpoint analysis. The experimental protocol used is shown in Figure 1A. Beginning 2 weeks prior to mating and ending on PND10, drinking water of dams in the Cd-exposed group was supplemented with a final concentration of $0.5 \mu\text{g/l}$ (500 ppb) Cd chloride (CdCl_2 ; CAS 10108-64-2; 99.99% purity, Lot MKBM1769V, Sigma Aldrich). Beginning on PND10, drinking water for both control and exposed study groups for the remainder of the study was Cd-free. During the 2-week acclimation exposure period, general health, body weight, and food and water consumption were monitored. Following acclimation, 2 dams were paired with each male and monitored daily for presence of a copulation plug no more than 3 h into the light-on cycle. Identified plug-positive females (gestational day 0 [GD0]) were single-housed and monitored for general health, food and water consumption, body weight and parturition.

At the time of pairing, blood was collected from a subset of unanesthetized control and Cd-exposed dams by saphenous venipuncture ($n=3$ per group) and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using an Elan DRCII ICP-MS (Perkin Elmer). Blood hemoglobin levels were measured using a handheld AimStrip Hemoglobin Meter (Germaine Laboratories, San Antonio, Texas) in a subset of dams ($n=10$ per group) at study onset and again at breeding and in a subset of offspring on PND1 and PND21.

A subset of dams ($n=3$ per group) were euthanized by CO_2 asphyxiation and rapid decapitation on GD18 and fetuses isolated 2–3 h after lights on. Dams and fetuses were weighed, and tissues collected. The intrauterine position of each fetus was recorded and fetal genetic sex determined by Sry-specific PCR as previously described (Lambert et al., 2000). For the remaining litters, litter size, pup sex, and pup weight were recorded on the day following parturition (PND1). Offspring were separated from dams on PND21–23 and housed 2–4 per cage separated by sex and study group. For all study animals, food and water consumption and body weights were measured at least weekly until necropsy. From each litter, 1 male and 1 female representative of the mean litter body weight was euthanized on PND1, PND21, PND42, PND90, and at study termination on PND120. Study animals remained in their home cage, with food and water available, until euthanized by carbon dioxide asphyxiation or transcardiac perfusion under isoflurane anesthesia. Liver Cd levels in tissue isolated at necropsy on PND42 were determined using an ICAP RQ ICP-MS (Thermo Fisher) at the NCSU Molecular Education, Technology and Research Innovation Center as previously described (Hudson et al., 2019). All study animals were necropsied with study samples and tissues isolated at the time of sacrifice. Systematic bias was avoided by housing animals randomly on cage racks and ensuring the timing and order of measurements, data collection, and experimental manipulations were the same. Necropsy was performed at the same approximate time of day, independent of study group, and dependent on date of birth. For adult females, experimental manipulations were performed in estrus.

Intraperitoneal glucose and insulin tolerance tests. For the glucose tolerance test (GTT), mice were fasted overnight (16 h) and blood glucose was determined with a handheld glucometer (Truetrack by Nipro Diagnostics, Inc, Fort Lauderdale, Florida). Blood from the tail vein ($5 \mu\text{l}$) was applied directly to the glucose strip to measure fasting blood glucose (T_0). Glucose ($1.5 \text{ mg glucose/g body weight}$) was administered by intraperitoneal injection and blood glucose measured at 15, 30, 60, 90, and 120 min after injection. For the insulin tolerance test (ITT), the same method as GTT was used with the following modifications. Mice were fasted for 4 h beginning at the start of the light cycle. Fasting glucose was measured, then $0.75 \text{ U insulin Aspart/kg body weight}$ (Novo Nordisk Inc, Plainsboro, New Jersey) was administered by intraperitoneal injection. Blood glucose was measured at 0, 15, 30, 45, 60, and 120 min after injection.

Tissue isolation and histology. At necropsy, tissues were dissected, frozen on powdered dry ice, and stored at -80°C until prepared for analysis. Livers were blocked and embedded in tissue freezing media (OCT, Electron Microscopy Sciences, Hatfield, Pennsylvania). Cryosections ($12 \mu\text{m}$) were cut at -18°C and collected onto slides (SuperFrost Plus, Fisher Scientific) and stored at -80°C until stained.

For Oil Red O staining, sections were brought to room temperature and immersion fixed in 10% neutral buffered formalin (NBF) for 5 min. Fixed sections were washed in running tap water for 5 min, transferred to 60% isopropanol/deionized water for 30 s with gentle agitation, stained for 15 min in freshly prepared and filtered 0.3% (wt/vol) Oil Red O (CAS 1320-06-5, Lot: S20D046, Alfa Aesar, Haverhill, Maryland) in 60% isopropanol, washed in running tap water for 15 min, and then cover-slipped with aqueous mounting media (Crystal Mount, Biomedica, Foster City, California).

For glycogen staining, slides were brought to room temperature, dehydrated through a series of ethanol (70%–95%), fixed with Carnoy's fixative (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid), rinsed and rehydrated through 95%–70% series of ethanol, and 2 changes of water. Sections were immersed in 0.5% periodic acid for 5 min, rinsed with 5 changes of water. Sections were then covered with Lille's "cold Schiff" reagent (1% basic fuchsin, 1.9% $\text{Na}_2\text{S}_2\text{O}_5$ in 0.15 N HCl) for 15 min in a humidified chamber, and then immersed in sulfurous rinse (300 ml distilled water, 15 ml 1 N HCl, 18 ml 10% $\text{Na}_2\text{S}_2\text{O}_5$) 3 times. Slides were counterstained in Harris's hematoxylin for 1 min, washed in running tap water for 10 min, dehydrated, and cover slipped with Permunt mounting medium (Fisher Scientific, Hampton, New Hampshire). Sections incubated at 37°C with amylase (Fisher Scientific) were used as negative controls for staining.

For Masson's trichrome staining, tissues were isolated following transcardiac perfusion with potassium phosphate buffered saline. Tissues were fixed for 24 h in 10% NBF, post-fixed in fresh NBF for 24 h, and then transferred to 70% ethanol. Specimens were prepared by automated tissue processing for 40–45 min each in 7 changes of graded alcohols followed by embedding with 3 changes in paraffin at 58°C with applied vacuum (Tissue-Tek VIP 3000; Sakura Torrence, California). Serial $5 \mu\text{m}$ microtome liver sections on positively charged slides were Masson's trichrome stained (Thermo Scientific Cat No. 87019 Richard-Allan, Kalamazoo, Michigan) using standard protocols.

Stained sections were examined on a Nikon Eclipse 80i microscope using a DSFi1 CCD camera controlled with Digital Sight Software (Nikon, Melville, New York) by an investigator blind to exposure group. Digital bright field photomicrographs

Figure 1. Experimental timeline and effects of gestational exposure to CdCl₂ on total body weight, perigonadal fat weight, and circulating triglycerides in female CD-1 mice. Experimental timeline showing that 500 ppb CdCl₂ was administered to pregnant dams beginning 2 weeks prior to mating and extending through postnatal day 10. Offspring were sacrificed and tissues were collected at gestational day 18 and postnatal days 1, 21, 42, 90, and 120 (A). Growth trajectories showing body weight of control and CdCl₂-exposed F1 female (B) and male (C) CD-1 mice are shown (geometric mean of litters $\pm$ SEM) plotted against postnatal day. In CdCl₂-exposed offspring, body weight was increased at birth in females (A). Perigonadal fat weight is shown normalized to control female fat weight on PND90 (D) and PND120 (E). Plasma total triglycerides (mean $\pm$ SEM) were increased in CdCl₂ exposed female offspring but not males (F). Serum thyroxine showed no difference between control and exposure groups in either sex (G) (mean $\pm$ SEM.). Growth trajectory: Females: control, $n = 11$; CdCl₂, $n = 12$. Males: control, $n = 10$; CdCl₂, $n = 11$. Perigonadal fat weight (PND90): Females: control, $n = 6$; CdCl₂, $n = 9$. Males: control, $n = 4$; CdCl₂, $n = 8$; (PND120): Females: control, $n = 5$; CdCl₂, $n = 8$. Males: control, $n = 3$; CdCl₂, $n = 7$. Triglycerides: Females: control, $n = 11$; CdCl₂, $n = 10$. Males: control, $n = 7$; CdCl₂, $n = 9$. Thyroxine: Females: control, $n = 7$; CdCl₂, $n = 7$. Males: control, $n = 6$; CdCl₂, $n = 7$.

(3–5 per section from 3 sections per liver) representative of each section were captured. Acquired images for Oil Red O and glycogen were converted to 8-bit gray scale and thresholded to background staining intensity using ImageJ (Schneider et al., 2012). Total stained area and intensity were calculated with neutral lipid or glycogen staining reported for each sample as the mean percent of image area stained and staining intensity. Pathology of PND90 and PND120 deidentified liver specimens was evaluated by examination of Masson's trichrome stained sections. Non- and preneoplastic hepatic lesions were scored according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (Thoolen et al., 2010). No threshold for morphological changes was applied to the analysis, and any lesion consistent with each pathology was scored positive. All histology and pathology were assessed by multiple observers blinded to study group, with differences in scoring reviewed by all investigators and resolved by consensus. Final figures were representative and generated using Adobe Photoshop (San Jose, California).

Triglyceride and total thyroxine assay. Total plasma triglycerides and total thyroxine were determined using a colorimetric triglyceride assay (Cat. No. 10010303; Cayman Chemical, Ann Arbor, Michigan) or thyroxine ELISA (Cat no. 3149-18; Diagnostic Automation/Cortez Diagnostics, Woodland Hills, California) according to the manufacturer's protocols. For triglyceride analysis, individual samples were analyzed in duplicate, with 4 replicate quality control samples included throughout the assay. The intra-assay coefficient of variation of the replicate quality control samples was 1.6%, and the coefficient of variation for all duplicate samples <4.0%; the thyroxine samples were run in triplicate, and the coefficient of variation for all samples ≤7.2%.

RNA sequence analysis of livers. Liver RNA was extracted from 30 to 50 mg of tissue using the RNEasy miniprep kit, according to manufacturer protocols (Qiagen, Valencia, California). The RNA integrity number for all samples was ≥7 (Agilent 2100 Bioanalyzer). Library creation and sequencing were performed using a single-end 125 bp protocol on the Illumina HiSeq2500 sequencer, with approximately 33 million uniquely mapped reads generated per sample. Quality control of read data was evaluated using FastQC and STAR short read aligner was used to align reads to *Mus musculus* (mm10) reference genome. Mapped read number was determined using featureCounts software, with data normalized for distortion and depth. The DESeq2 Bioconductor package in R normalized count data by fitting a generalized linear model for each gene, taking into account sex on PND42 and exposure group on PND1, PND21, and PND42 (CdCl₂, control). No samples were excluded from differential expression based on QC analysis. Differential expression analysis of 14 259 genes (PND1 and PND21) and 13 092 genes (PND42) between sex and exposure was set to adjusted $p = .05$ cutoff to identify differences between control and CdCl₂ exposure. A sample heat map of overall mapped reads, volcano plot, and hierarchical cluster diagrams are included for each time point (Supplementary Figs. 1–3). Identification of counts in a minimum of 75% of samples was required for further analysis of differential expression between control and CdCl₂ exposure groups or between female and male mice. For all samples, the overall mean of the normalized counts, the log₂ (fold-change), and the adjusted p value (p_{adj} , adjusted for multiple testing using the Benjamini–Hochberg false discovery rate, FDR) were determined and analyzed for gene pathway enrichment using the Ingenuity Pathway Analysis (IPA) software platform (Spring

2020 version, Qiagen). For PND21 and PND42, FDR was set to 5%, and for PND1 an FDR of 15% was used because of known increased gene expression variability at birth (Doktor et al., 2017). Along with IPA, GOTERMFINDER (Boyle et al., 2004) and WebGestalt (Liao et al., 2019) were used to identify ontology, KEGG pathways, and molecular pathways associated with differentially expressed gene (DEG) responses to CdCl₂ exposure. The RNAseq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE150679 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150679>).

Quantitative RT-PCR analysis. Total RNA was isolated using the RNEasy Mini Kit (Qiagen). One microgram of RNA was reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY) following the manufacturer's recommendations. Standard PCR amplification was performed in triplicate on a Step One Plus Real-Time PCR System (Applied Biosystems) in a final volume of 20 µl containing approximately 10 ng of cDNA (1.5 µl of RT product), 1× Universal Master Mix and TaqMan expression assay primers (Supplementary Table 1; Applied Biosystems). Relative expression was quantified using the 2^{−ΔΔCt} method, in which ΔΔCt is the normalized value. Five independent reference genes, ribosomal protein S18 (Rps18), beta-actin (Actb), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and beta-glucuronidase (Gusb) were used for normalization (Vandesompele et al., 2002).

Data and statistical analysis. All procedures, measurements, and endpoint assessments were made by an observer blinded to treatments, litter, and sex when appropriate. To avoid the influence of extreme litter size on endpoint sensitivity, litters with fewer than 6 pups were excluded from analysis and litters with greater than 14 pups were culled to a maximum of 12 (Palmer and Ulbrich, 1997). All animal assignments and litter information are provided (Supplementary Table 2).

Comparison between cohorts was performed using 2-way analysis of variance (ANOVA) (sex, exposure) for each endpoint measured. Growth curve analysis was performed using a repeated measure (RM) 1-way ANOVA within sex with postnatal day as the repeated measure. Body weight and adipose weight differences at each time point were analyzed using 2-way ANOVA (sex, exposure), with litter size included as a covariate. For GTT/ITT an RM 2-way ANOVA (sex, exposure) was run with time after injection as the repeated measure. Results of Oil Red O and glycogen staining were analyzed using a Mann-Whitney U (based on normality tests). If overall effects were significant, a Fisher's least significant differences post hoc test was performed to evaluate pair-wise differences. A Fisher's exact test was used to evaluate incidence of pre-neoplastic and non-neoplastic lesions, and multinomial logistic regression was used to evaluate lesion severity. Effect sizes were calculated depending on the statistical test, values for “ η^2 ” for ANOVA, “Cohen's d ” for t-tests, and “ r ” for Mann-Whitney U are reported. Student's t-test was used for analysis of total plasma triglycerides, plasma thyroxine, total hepatic lipids, and glycogen content. Unless specifically noted, the experimental unit for analysis was the litter; when necessary, litter and litter size were included in statistical models as covariates. Significance between differences in values were defined as $p < .05$. All data were analyzed using Prism v8 (GraphPad, La Jolla, California) and SPSS V.25 (IBM, California).

Table 1. Gestational Exposure, Food and Water consumption, Dam and Litter Characteristics

	Control (0 Cd)	500 ppb CdCl ₂
Water consumption (ml/day, GD0-PND0)	5.6 ± 0.9 (16)	5.3 ± 1.0 (18)
Cd exposure (mg/kg/day)	0	0.050 ± 0.003
Gestational food consumption (g/day) ^a	4.58 ± 0.4 (15)	4.42 ± 0.4 (17)
Gestational food consumption (kcal/day)	18.3 ± 1.6 (15)	17.7 ± 1.6 (17)
Perinatal (PND1–10) food consumption (g/day)	9.0 ± 0.7 (15)	9.4 ± 1.6 (17)
Perinatal (PND1–10) food consumption (kcal/day)	35.7 ± 2.6 (15)	36.9 ± 7.8 (17)
Juvenile (PND21–42) food consumption	4.21 ± 0.5 (15)	4.14 ± 0.9 (17)
Female	4.42 ± 1.12	3.86 ± 0.21
Male	4.01 ± 1.02	4.48 ± 0.65
Young adult (PND42–90) food consumption	4.52 ± 0.4 (6)	4.32 ± 0.4 (9)
Female	4.74 ± 1.19	4.45 ± 0.16
Male	4.3 ± 1.09	4.18 ± 0.25
Adult (PND90–120) food consumption	5.88 ± 0.7 (5)	5.32 ± 0.6 (8)
Female	5.52 ± 1.41	5.31 ± 0.34
Male	6.23 ± 1.57	5.33 ± 0.22
Gestational weight gain (g)	23.8 ± 4.0 (16)	24.6 ± 5.2 (18)
Number of pups per litter	11.4 ± 1.8 (16)	11.7 ± 1.7 (18)
Sex ratio	54% Female 46% Male	48% Female 52% Male

Values represent group mean ± SD; the number of samples analyzed is shown in parenthesis. One dam in each group was excluded from all analyses because of death prior to parturition, and 2 litters were excluded from the control group due to small litter size.

^aOne dam in each group was excluded because they ground food onto cage bottom. kcal = grams × 4.

Abbreviations: GD, gestational day; PND, postnatal day.

RESULTS

Maternal Weight Gain, Water Consumption, and Food Consumption Was Unaffected

Analysis of maternal water and food consumption indicated no significant differences in water consumption ($t(26) = .83$, $p = .41$, $d = .32$), food consumption during gestation ($t(24) = .78$, $p = .44$, $d = .31$), or perinatal food consumption ($t(18) = .72$, $p = .48$, $d = .32$) (Table 1). Mean daily intake of Cd in the CdCl₂ exposure group during acclimation and gestational exposure period (GD0-PND10) was 0.050 ± 0.003 mg/kg/day, an exposure equivalent to the ATSDR intermediate duration minimal risk level (ATSDR, 2012). At mating, blood Cd concentrations in control dams was below the limit of quantification ($0.1 \mu\text{g/l}$), and $0.42 \pm 0.04 \mu\text{g/l}$ ($n = 3$) in CdCl₂-exposed dams. Tissue Cd levels in female offspring from both control and exposure groups on PND42 were below the limit of detection ($0.08 \mu\text{g/l}$; $n = 3$ per group).

There were no changes in maternal weight gain during gestation ($t(18) = .37$, $p = .72$, $d = .16$), and there was no effect of exposure on litter size ($t(18) = .38$, $p = .71$, $d = .17$). Male and female offspring consumed similar amounts of food, and no exposure-related changes in food consumption were observed in either sex during the juvenile period of growth (PND21–42) ($t(18) = .18$, $p = .86$, $d = .10$), or in adults (PND42–90: $t(13) = .88$, $p = .40$, $d = .46$; PND90–120: $t(13) = 1.7$, $p = .12$, $d = .87$) (Table 1).

Maternal Cd Exposure Decreased Offspring Hemoglobin Levels at Birth

Because gestational Cd exposure is associated with anemia and iron deficiency, and adult Cd exposure induces anemia and iron deficiency, blood hemoglobin was assessed in dams and offspring (Akesson et al., 2002; Horiguchi et al., 2011). Maternal blood hemoglobin levels were unaffected by CdCl₂ exposure. Offspring hemoglobin levels at birth were significantly lower in both male ($t(10) = 3.5$, $p = .006$, $d = 2.18$) and female offspring ($t(11) = 2.9$, $p = .01$, $d = 1.65$) in the CdCl₂ exposure groups compared with same sex controls. This effect was transient, with no

differences between control and Cd-exposed groups detected at weaning male: ($t(7) = 1.1$, $p = .30$, $d = .74$); female: ($t(8) = .66$, $p = .53$, $d = .42$) (Supplementary Table 3).

Gestational Cd Exposure Increases Female Body Weight and Adiposity

Gestational Cd exposure increased body weight and perigonadal fat weight in female offspring (Figs. 1B–E). On GD18, exposed offspring were heavier ($F(1, 72) = 4.73$, $p = .03$, $\eta^2 = .17$), with post hoc analysis indicating that only CdCl₂-exposed female offspring were significantly heavier than unexposed controls (females: 6% increase, $p = .02$; males: $p = .48$). On PND1, Cd-exposed offspring were heavier ($F(1, 307) = 20.0$, $p < .001$, $\eta^2 = .12$); both males and females were significantly heavier than same sex controls on PND1 (females: 8% increase, $p < .001$; males: 5% increase, $p < .001$). Differences in weight during perinatal development were transient and not observed after PND1. The growth curve for Cd-exposed female offspring was significantly different from control females ($F(1, 7) = 101$, $p < .001$, $\eta^2 = .94$), with the mean weight of females in the exposed group becoming significantly heavier after PND90 ($p = .003$) (Figure 1B). Compared with control females, the Cd-exposed females were 23% heavier at PND90 and 28% heavier PND120. After PND1, there were no differences in weight or growth trajectory of the exposed male offspring ($F(1, 5) = .303$, $p = .61$, $\eta^2 = .06$) (Figure 1C).

Perigonadal fat weight was increased 2.5-fold in exposed females on PND90 and 7-fold on PND120 (Figs. 1D and E). Two-way ANOVA found a significant interaction of sex and exposure on fat weight (PND90: ($F(1, 26) = 4.7$, $p = .04$, $\eta^2 = .18$); PND120: ($F(1, 23) = 5.0$, $p = .04$, $\eta^2 = .22$)). At each time point, post hoc analysis using Fisher's LSD found statistically increased fat pad weight only in Cd-exposed females (PND90: females, $p = .003$; males, $p = .33$; PND120: females, $p = .001$; males, $p = .20$).

Along with increased adiposity, dyslipidemia was evident in Cd-exposed females with increased circulating triglycerides ($t(19) = 2.1$, $p = .047$, $d = .91$) (Figure 1F). No differences were detected in males ($t(14) = .65$, $p = .53$, $d = .33$). There were no

Figure 2. Effects of gestational exposure to CdCl₂ on glucose (PND42) and insulin tolerance (PND49). Following a 16-h fast, a glucose bolus was administered, and glucose measurements were taken at regular intervals. On PND42, peak glucose was increased, and glucose clearance was decreased in female (A) and male (B) offspring gestationally exposed to 500 ppb CdCl₂. Glucose AUC for the GTT is depicted (C). Following a 4-h fast, an insulin bolus was administered, and glucose measurements were taken at regular intervals. On PND49, glucose was delayed in returning to basal levels in female (D) and male (E) Cd-exposed offspring. Glucose AUC for the ITT is depicted (F). Females: control, n = 3; CdCl₂, n = 5. Males: control, n = 3; CdCl₂, n = 5.

differences in serum thyroxine levels in either sex (Figure 1G; female: $t(12) = .68$, $p = .51$, $d = .36$; male: $t(11) = 1.3$, $p = .22$, $d = .70$)).

Cd Exposure Differentially Alters Glucose and Insulin Responses

Glucose tolerance and insulin tolerance testing of 6- to 7-week-old offspring revealed impaired responses in both males and females. Repeated measures 2-way ANOVA (sex, exposure) revealed a significant ($F(1, 11) = 26.8$, $p < .001$, $\eta^2 = .69$) effect of exposure on peak glucose levels during GTT (Figs. 2A and B). Post hoc analysis revealed that the effect of gestational exposure was significant in both sexes (females: $p = .02$; males: $p = .03$). Repeated measures 2-way ANOVA (sex, exposure) indicated a significant effect of Cd exposure on glucose clearance during GTT ($F(1, 11) = 17.8$, $p = .001$, $\eta^2 = .62$) in both sexes (females: $p = .004$; males: $p = .007$). Area under the curve (AUC) analysis for both males and females also indicated that the effect of gestational Cd exposure significantly altered the response to glucose challenge ($F(3, 11) = 12.3$, $p = .0006$, $\eta^2 = .75$) in both sexes (females: $p = .005$; males: $p = .006$) (Figure 2C). Two-way ANOVA (sex, exposure) revealed a significant ($F(1, 14) = 17.8$, $p = .001$, $\eta^2 = .62$) effect of exposure on return to basal glucose levels following an insulin bolus during ITT (Figs. 2D and E). Post hoc analysis revealed the effect of gestational exposure was significant in both sexes (females: $p = .001$; males: $p = .01$), but the effect was more pronounced in females, with exposed males only significantly different at the 15 and 30-min timepoints. Analysis of AUC indicated the effect of gestational Cd exposure significantly altered the insulin response only in females ($F(3, 11) = 4.7$, $p = .02$, $\eta^2 = .56$; females: $p = .01$; males: $p = .17$; Figure 2F).

Hepatic Pathology

Oil Red O and periodic acid Schiff staining was performed to assess neutral lipid accumulation and glycogen content on PND42. A 3.3-fold increase in lipid droplet staining was observed

in livers of Cd-exposed female offspring (Figure 3A, Control: Mdn = 5.1; Figure 3C, Cd-exposed: Mdn = 15.5, $U = 0$, $p = .02$, $r = .69$) demonstrating female-specific hepatic steatosis at this early age (Figure 3E). There were no significant changes in males (Figure 3B, control: Mdn = 6.9; Figure 3D, Cd-exposed: Mdn = 8.7, $U = 16$, $p = .82$, $r = .19$). No differences in hepatic glycogen storage were observed in either females (Control: Mdn = 13.4; Cd-exposed: Mdn = 34.2, $U = 15$, $p = .70$, $r = .28$) or males (control: Mdn = 21.2; Cd-exposed: Mdn = 28.5, $U = 16$, $p = .82$, $r = .19$). Masson's trichrome staining at PND90 and PND120 revealed a significant and progressive increases in incidence and severity of both preneoplastic and non-neoplastic hepatic lesions in adult female livers (Table 2; Figs. 3F–H). Minimal age-dependent increases in hepatic fibrosis unrelated to exposure were notable in livers of each sex.

Hepatic Transcriptome Analysis

Transcriptome analysis was initially performed on PND42 to evaluate the sex-specific differences in hepatic metabolic responses to gestational Cd exposure and the mechanisms underlying the female-specific steatosis. The hepatic transcriptome of males was essentially unchanged by exposure with only 11 DEGs identified ($\text{padj} < .05$; Geo accession number GSE150679). By contrast, in livers from Cd-exposed females on PND42, there were 5789 DEGs; of those, 3103 (54%) were downregulated and 2686 (46%) were upregulated in livers of the Cd-exposed females (Supplementary Figure 1; Geo accession number GSE150679). Consistent with those findings, principal components analysis showed segregation by sex and exposure with sex accounting for 14.9% of the variance and gestational Cd exposure accounting for 38.8% of the observed variance (Figure 4A).

Gene ontology (GO) and IPA network analysis identified significantly enriched signaling pathways related to stress-responses (EIF2 signaling, NRF2 signaling, and protein

Figure 3. Effects of gestational exposure to CdCl₂ on hepatic morphology and pathology in CD-1 mice. Shown are representative photomicrographs of Oil red O stained liver sections from a PND42 control female (A) and male (B) CD-1 mouse and from a female (C) and male (D) exposed to 500ppb CdCl₂ (during gestation). Quantitative analysis of the percent staining intensity relative to controls is presented for females and males (E). Representative photomicrographs of Masson's trichrome stained liver sections from female CD-1 mice exposed to 500ppb CdCl₂ (during gestation) collected on PND90 and PND120. Images from PND90 show fatty change (F), hypertrophy (G, H), and inflammation (F, H) throughout. Black arrows indicate mononuclear infiltration (F, H). Black arrowheads indicate multinucleated cells (G). Scale bars represents 100μm. Oil red O: PND 42 Females: control, n = 6; CdCl₂, n = 6. Males: control, n = 6; CdCl₂, n = 6. Masson's trichrome: PND90 Females: control, n = 6; CdCl₂, n = 7. PND120 Females: control, n = 5; CdCl₂, n = 8.

ubiquitination), mitochondrial dysfunction, sirtuin signaling and senescence, regulation of eIF4 and P70S6K and mTOR signaling, cancer mechanisms, and estrogen receptor signaling

pathways in PND42 female liver transcriptome (Figure 4B; Geo accession number GSE150679). Significant ($\text{padj} < .01$) enrichment for 658 GO molecular functions were identified (Supplementary Table 4). Many (>18%) of those processes are involved in metabolism or energy balance, suggesting a generalized disruption of mitochondrial functions and metabolic processes. The 10 most significantly enriched signaling pathways are related to mitochondrial dysfunction, sirtuin signaling, retinoic acid (RA) biosynthesis, and retinoic acid receptor (RAR) activity (Figure 4B). The alterations in RA-related canonical pathways are striking, wherein 13/34 genes in the RA biosynthesis pathway ($z\text{-score} = -2.3$, $p = .03$) and 70/194 genes in the RAR activation pathway were differentially expressed ($p = .00002$; Figure 4C).

There was also significant enrichment in 124 biological processes including ubiquinone activity, and cation, metal, zinc, and iron ion binding (Supplementary Table 4). Consistent with the notable hepatic insulin insensitivity, the canonical insulin signaling pathway was significantly downregulated ($z = -2.1$, $p < .0001$) with differential expression in 47/137 genes (Figure 4D). The *Insr* upstream regulator was predicted to be significantly inhibited with 43 of 83 DEGs in the insulin receptor (*Insr*) signaling pathway ($z\text{-score} = -4.604$, $p = .0002$). Glycolysis (9/23 genes, $z\text{-score} = -2.3$, $p = .03$) and *Igf1* signaling pathways (31/102, $z\text{-score} = -0.85$, $p = .01$) were also downregulated in livers of the Cd-exposed females. Pathway and upstream regulator analysis also revealed changes in protein folding related to endoplasmic reticulum (ER) stress, oxidative stress, lipid metabolism, and glucose metabolism that were consistent with the observed hepatic insulin insensitivity and steatoses phenotypes in the Cd-exposed female offspring.

Comparative differential gene expression and functional genomic analysis of Cd-induced modifications of the liver transcriptome in female offspring on PND1, PND21, and PND42 were used to evaluate the developmental progression and mechanism of Cd-induced of hepatic steatosis. Principal components analysis of the hepatic gene expression data sets on PND1, PND21, and PND42 indicated that postnatal day accounted for 64.3% of the observed variance (Figure 5A), with a minimal overlap of 13 common DEGs shared across all time points (Supplementary Table 5). There were 20 DEGs shared between PND1 and PND21 (Figure 5B; Supplementary Table 6). Pathway over-representation analysis identified significant over enrichment of shared DEGs involved in retinol metabolism, fatty acid metabolism, and nonalcoholic fatty liver disease (NAFLD). Significant over-representation of multiple pathways involved with ER stress and misfolded protein responses in the ER were identified in the 150 overlapping DEGs shared between the PND21 and PND42 time points.

In CdCl₂-exposed females on PND1, there were 278 genes differentially expressed in liver tissue compared with controls (Supplementary Figure 2; Geo accession number GSE150679). Gene ontology group analysis identified significant ($\text{padj} < .01$) enrichment for 219 GO molecular functions and 48 biological processes, primarily related to dysregulation of cellular metabolism, mitochondrial function, and stress-related functions (Supplementary Table 7). The 10 most significantly enriched signaling pathways were related to mitochondrial dysfunction, sirtuin signaling, RA biosynthesis, and RAR activity (Figure 5C). On PND21, 446 DEGs were identified (Supplementary Figure 3; Geo accession number GSE150679), with GO group analysis identifying significant ($\text{padj} < .01$) enrichment for 41 GO molecular functions and 15 biological processes. Many identified functional groups (>37%) were involved in metabolic processes with

Table 2. Preneoplastic and Non-neoplastic Lesions

Preneoplastic Lesion	Control	Gestational CdCl ₂	Control	Gestational CdCl ₂
	PND90		PND120	
Clear cell focus	1 (.16)	5 (2.0) [#]	2 (.40)	7* (1.8)
Eosinophilic focus	0 (0)	1 (.14)	0 (0)	1 (.25)
Basophilic focus	0 (0)	0 (0)	0 (0)	0 (0)
Mononuclear infiltration	2 (.83)	7* (2.0)	5 (2.4)	8* (3.5)
Neutrophil infiltration	0 (0)	0 (0)	0 (0)	0 (0)
Multinucleated cells	0 (0)	5* (.86)	1 (.20)	8* (1.38)
Lesion	Control	Gestational CdCl ₂	Control	Gestational CdCl ₂
	PND90		PND120	
Fatty change, diffuse	4 (.89)	7 (2.4) [#]	2 (.60)	8* (3.3)
Inflammation	1 (.17)	7* (1.86) [#]	2 (.60)	8* (2)
Fibrosis	0 (0)	0 (0)	4 (1.2)	3 (1)
Hyperplasia	0 (0)	5* (1)	2 (.40)	8* (1.38)
Hypertrophy	1 (.17)	4 (.71)	0 (0)	6* (1.5)
Number of Animals	6	7	5	6

Values represent incidence with severity score in parenthesis.

*Significant differences in incidences relative to control are indicated in the CdCl₂ column, $p < .05$. [#]Significant differences in severities relative to control are indicated in the CdCl₂ column, $p < .05$.

evident enrichment of processes and functions related to oxidative and ER stress (Supplementary Table 8). There was also over-representation of functions related to metal ion binding including ion binding and calcium ion binding, findings that suggest compensatory changes in metal ion homeostasis have occurred. The top 10 canonical pathways altered by Cd exposure on PND21 were unfolded protein response, BAG2 signaling pathway, pregnolone biosynthesis, histidine degradation VI, ubiquinol-10 biosynthesis, ER stress pathway, Huntington's disease signaling, Nrf2-mediated oxidative stress response, aldosterone signaling in epithelial cells, and acetone degradation (Figure 5D). On PND21, the PPAR α signaling pathway (z -score = -0.816, $p = .009$) and other related canonical pathways including PPAR α /RXR α (z -score = -0.333, $p = .002$) were downregulated.

Quantitative RT-PCR Validation

Quantitative RT-PCR was used to validate the subset of DEGs identified by RNAseq and comprehensively evaluate dysregulation of insulin signaling pathway and the genes encoding the Zn/Mn transporters, Zip 8 (Slc39a8), and Zip 14 (Slc39a14), in female livers (Figure 6). On PND42, there were significant effects of exposure on expression of 13/14 analyzed mRNA transcripts from the insulin-signaling pathway (Figure 6A; Supplementary Table 1). In Cd-exposed females, Slc39a8 mRNA expression was significantly decreased on PND42, and Slc39a14, which is expressed at much higher levels, was also significantly downregulated compared with unexposed females on PND21 and 42 in Cd-exposed females (Figure 6B; Supplementary Table 1).

DISCUSSION

To explore the hypothesis that developmental Cd exposure increased risk of obesity in later in life, *in utero* and perinatal exposure of CD-1 mice from GD0-PND10 to CdCl₂ was used to model Cd exposure during a human gestational equivalent period (Semple et al., 2013). Cadmium body burden of dams at mating was between the 50th and 75th percentile of women of

childbearing age in the United States (Mijal and Holzman, 2010). Because of the short duration of exposure and the relatively low concentration of Cd in drinking water, the resulting Cd exposure was lower and of much shorter duration than those causing acute or chronic toxicity (ATSDR, 2012; Hudson et al., 2019; Park et al., 2017). Overall gestational exposure to offspring is expected to be low, because metallothioneins in the placenta sequester a significant portion of Cd before it reaches the fetus. Subsequent Cd exposure occurred from birth through weaning via breast milk (Jacobo-Estrada et al., 2017). Although not a toxic exposure, maternally administered 500 ppb CdCl₂ resulted in a remarkable metabolic syndrome-like phenotype in adult female offspring as evidenced by early onset insulin insensitivity, increased circulating triglycerides, NAFLD-like pathology with excessive adiposity, and weight gain. The observed exposure-related effects were sexually dimorphic. Adult male body weight, adiposity, and hepatic lipid accumulation were unchanged by gestational CdCl₂ exposures. In both sexes, Cd exposure decreased glucose clearance and insulin sensitivity relative to same-sex controls; however, the insulin insensitivity of females was more severe. Further evidence of major metabolic changes in Cd-exposed females included progressive increases in hepatic pathology and increased incidence and severity of preneoplastic lesions associated with development of hepatocellular carcinoma (Thoolen et al., 2010). Together, the findings of this study provide evidence that exposure to Cd during gestation results in disruption of hepatic lipid and carbohydrate metabolism that induces metabolic syndrome and progressive obesity in female adult mice.

Gestational CdCl₂ Induces Obesity in Adult Female Offspring

Exposure to 500 ppb CdCl₂ during the pre- and perinatal period of development equivalent to human gestation acted as a delayed obesogen in the exposed female offspring. From adolescence until PND90, there were no differences in body weight of either sex. However, by PND90, female body weight was increased 23% and perigonadal fat weight was more than 220%

Figure 4. Hepatic transcriptome changes measured by RNAseq analysis on PND42. RNA isolated from the liver of control and gestationally CdCl₂-exposed offspring on PND42 was used for RNA-sequencing analysis. On PND42, principal components analysis shows segregation by exposure in females but not males (A). The top 10 canonical pathways altered by Cd exposure are shown for females on PND42 (B) with 5789 genes differentially expressed in females and only 11 in males. The percentage of upregulated genes are denoted on the right and downregulated on the left of each bar. Ingenuity pathway analysis of the hepatic transcriptome of PND42 CdCl₂-exposed female mice revealed significant changes in the retinoate biosynthesis, RAR activation, and insulin signaling canonical pathways. Differentially expressed genes in RA signaling (C) and insulin signaling pathways (D) are shown. Processes predicted to be affected altered based on gene expression analysis are shown in gray. Females: control, *n* = 4; CdCl₂, *n* = 4. Males: control, *n* = 4; CdCl₂, *n* = 4.

Figure 5. Hepatic transcriptome changes measured by RNAseq analysis from birth through adulthood. RNA isolated from the liver of control and gestationally CdCl₂-exposed offspring on PND1, PND21, and PND42 was used for RNA-sequencing analysis. Principal components analysis shows segregation by timepoint of analysis (A). Overlap between differentially expressed genes is shown in a Venn diagram (B). The top 10 canonical pathways altered by Cd exposure are shown for females on PND1 (C) and PND21 (D). The percentage of upregulated genes are denoted by the bars. PND1: Females: control, *n* = 4; CdCl₂, *n* = 4. PND21: Females: control, *n* = 3; CdCl₂, *n* = 3. PND42: Females: control, *n* = 4; CdCl₂, *n* = 4.

Figure 6. Quantitative reverse transcription PCR (qRT-PCR) analysis of insulin signaling transcriptomic signature genes and *Slc39a8* and *Slc39a14* gene expression. Quantitative RT-PCR analysis comparison of unexposed and CdCl₂-exposed (500 ppb) CD-1 female mice on PND42 for selected genes related to insulin signaling are shown (A). Differential expression of the genes encoding the metal ion transporters *Zip8* and *Zip14* on PND1, PND21, and PND42 are shown (B). Relative differences in gene expression between control and exposed groups were determined by the $\Delta\Delta$ cycle threshold method with specific TaqMan assays and TaqMan Custom Format Array (Supplementary Table 1). Results are expressed as mean of group $\pm$ SEM with significantly upregulated and downregulated mRNAs indicated. $N \geq 3$ animals. The level of statistical significance for differences between mean values of control and CdCl₂-exposed groups was determined by Student's *t* test for all experiments and is indicated by * $p < 0.05$.

greater than age matched control females. On PND120, Cd-exposed females were ~30% heavier and had 700% more perigonadal fat than unexposed control females. Those findings are concordant with accumulating evidence linking elevated Cd exposure during gestation with increased risk of obesity and T2D later in life (Ettinger et al., 2014; Green et al., 2018; Kelishadi et al., 2013; Wu et al., 2017; Young et al., 2019; Zhou et al., 2016). In humans, prenatal Cd exposure is associated with lower birth weight followed by a steep rise in adiposity and increased obesity by age 5 years (Green et al., 2018). Steep growth trajectories in early life, like those caused by prenatal Cd exposure, are a consistent risk factor for metabolic disease in adulthood (Boyer et al., 2015). However, there is little understanding of the ways in which gestational Cd exposure alters metabolic function in humans. Our RNAseq analysis identified mitochondrial dysfunction and oxidative stress as key mechanisms involved with the etiology and progression of adverse metabolic outcomes. Mitochondrial dysfunction and oxidative stress are also hallmark mechanisms of chronic Cd toxicity. The finding that low concentrations of Cd during critical periods of gestational development have adverse impacts on metabolic function involving these well-defined mechanisms of Cd toxicity, sets the stage for rational public health policy aimed at protecting against later in life adverse effects of developmental Cd exposure. It is notable that at the end of gestation, offspring mice were heavier than same-sex controls whereas human studies have observed associations between elevated Cd levels and low birth weight followed by steep gains in weight and adiposity (Park et al., 2017). The observed differences between humans and mice are likely attributable to differences in levels or impacts of exposure occurring during later periods of human gestational development and the corresponding perinatal development in the mouse offspring.

Gestational CdCl₂ Dysregulates Glucose Metabolism in Adult Offspring of Both Sexes

In humans, insulin resistance is a hallmark feature of metabolic syndrome and T2D (Asrih and Jornayvaz, 2015). Gestational Cd exposure resulted in impaired glucose homeostasis in young adult mouse offspring of both sexes. The exposure-related changes in response to glucose in Cd-exposed male and female offspring were similar; compared with same sex controls, peak glucose concentrations were increased, and the rate of glucose

clearance was decreased. Those results are similar to a previous study that found exposure to 500 ppb Cd during gestation decreased glucose clearance at weaning, that resulted in insulin resistance in adult Wistar rats (Jacquet et al., 2019).

The significant peripheral insulin resistance, hepatic lipid accumulation, and steatosis observed in the Cd-exposed female offspring is constant with a pathophysiological mechanism mediated by decreased hepatic insulin clearance resulting in hyperinsulinemia, leading to lipotoxicity, with subsequent weight gain and increased adiposity (Al-Share et al., 2015; Moore, 2012). This interpretation is supported by the observed female-specific changes in the hepatic transcriptome on PND42 that revealed significant impacts on insulin-signaling pathway, including upregulation of *Insr* and the insulin-like growth factor receptor (*Igf1r*) effects that are indicative of hepatic insulin resistance (Kluth et al., 2014; Marchand et al., 2016). In Cd-exposed males, the observed decrease in glucose clearance without pronounced insulin insensitivity, and the lack of exposure-induced changes in hepatic gene expression, suggest that altered glucose metabolism in exposed males was independent of altered hepatic functions and hepatic insulin insensitivity. Rather, insulin resistance in males may involve pancreatic insulin secretion impairment, an interpretation consistent with previous studies in males and male-derived cell lines that found Cd can cause pancreatic β -cell toxicity leading to decreased insulin secretion (Huang et al., 2019; Shimada et al., 2000; Tinkov et al., 2017). Defining the specific nature of the Cd-induced changes of glucose metabolism in males will require additional study focusing on sex differences of gestational Cd and pancreatic functions.

Gestational CdCl₂ Induces Hepatic Steatosis in Female Offspring

Although Cd exposure ended on PND10 and Cd was not detectable in the liver on PND42, gestational exposure to 500 ppb CdCl₂ caused dyslipidemia, increased hepatic lipid deposition, and altered expression of NAFLD-related genes in the livers of adult females. On PND42, a 400% increase in hepatic lipid accumulation and a 33% increase in circulating triglycerides was observed in females. This robust steatosis phenotype in female offspring contrasts with the lack of observed effects in male offspring, consistent with a previous 20-week chronic exposure study in adult male C57BL/6J mice exposed to 10 000ppb CdCl₂, where only a modest 4% increase to hepatic lipid deposition

was detected (Go et al., 2015). These findings suggest that along with sex, exposure timing, dose, and duration of Cd exposure are important determinants of the resulting adverse metabolic effects. In addition to impacts of sex and strain on Cd-sensitivity, it is likely that Cd exposure during critical periods of development cause more severe metabolic phenotypes than those resulting from adult exposures. Differential impacts of Cd on RA signaling or RAR activities during development maybe a key mechanism responsible for those differences. Because gestational Cd exposure caused female-specific obesity, fatty liver disease, and hepatic insulin resistance, and no changes in lipid deposition, hepatic gene expression, or adiposity in age-matched male offspring, it is evident that sex is a major modifier of the developmental effects of Cd-mediated metabolic disruption.

Gestational CdCl₂ Increased Preneoplastic Lesions in Adult Female Liver

Cadmium is a known carcinogen wherein environmental Cd exposure increases risk for development of hepatocellular carcinoma (Hyder et al., 2013; Satarug, 2012). In humans, nearly 50% of hepatocellular carcinoma arise in the absence of cirrhosis or fibrosis, but are instead highly associated with NAFLD and metabolic syndrome (Olofson et al., 2018; Salomao et al., 2010; Yasui et al., 2011). Here, the comparative analysis of hepatic pathology revealed progressive impacts of gestational Cd exposure in females. Age-related increases in body weight and adiposity were associated with progressive increases in inflammation, cellular hyperplasia, and hypertrophy. In addition, there was a significant and progressive increase in incidence and severity of preneoplastic lesion in the livers of the exposed female offspring. To our knowledge, this is the first analysis to find evidence for gestational-only Cd exposure increasing preneoplastic lesions later in life. Although hepatocellular carcinoma was not detected here, likely due to the duration of the study, the observed increases in preneoplastic lesions are consistent with 16-week chronic exposure studies in adult mice that found 1500 ppb Cd increased the incidence of hepatocellular carcinoma and hepatic adenomas (Waalkes and Rehm, 1994). Although increased incidence was a secondary endpoint of the current study, the decreased dose and short duration of exposure employed here suggests that the carcinogenic effects of Cd may be increased with gestational exposure relative to adult-only exposure. Additional longer duration analysis will be necessary to define the carcinogenic potency of gestational Cd exposures.

Link Between Cd-Induced Metabolic-Disruption and Preneoplastic Liver Lesions

In humans, NAFLD and T2D are associated with insulin resistance, inflammation, and oxidative stress that could promote the progression of hepatocellular carcinoma; incidence of hepatocellular carcinoma was 2–3 times higher in patients presenting with T2D (Li et al., 2017; Singh et al., 2018). The changing DEG profiles from birth through adulthood in Cd-exposed female offspring observed here were consistent with the observed progression of metabolic changes and increases in hepatic pathology. Gestational Cd exposure altered EIF2 signaling, unfolded protein response, ER stress, sterol biosynthesis, and mitochondrial dysfunction signaling pathways as early as PND1, changes that were persistent in adult females. On PND21, gestational Cd exposure was associated with transient downregulation of PPAR α -related pathways that was not evident on PND1 or 42. Compared with control, DEGs involved in fatty acid β -

oxidation and FXR-RXR signaling were downregulated at birth and again on PND42, but not on PND21. Consistent with no detectable increases in pathologic fibrosis at any timepoint analyzed, there was no enrichment of DEGs in pathways associated with the inflammasome and fibrosis. These findings are similar to those found in diet-induced animal model studies of NAFLD where similar transcriptomic profiles of altered lipid metabolism, cell stress, and liver inflammation corresponded with metabolic disease progression from NAFLD to steatohepatitis, fibrosis, and ultimately hepatocellular carcinoma later in life occur (Cazanave et al., 2017). As described above, hepatic insulin resistance and alterations in the hepatic insulin-signaling pathways were concomitant with hepatic lipid accumulation and appeared before progression to preneoplastic hepatic lesions. The time course and progression of metabolic pathology observed here suggests that gestational Cd exposure is causing peripheral insulin resistance and hepatic insulin insensitivity which are driving development of NAFLD, effects that are priming offspring for increased hepatocellular carcinoma risk later in life.

Gestational Cd Exposure Disrupts RA Signaling

Pathway and upstream regulator analysis of DEGs in the livers of the Cd-exposed females revealed exposure-related changes in RA metabolism and RAR signaling pathways at every timepoint analyzed. Overall, those analyses suggest that the adverse metabolic actions of gestational Cd are mediated by developmental or metabolic disruption of RA signaling. During embryogenesis, RA levels regulate anteroposterior patterning through RAR/RXR-mediated induction of Hox gene expression (Langston and Gudas, 1994). Gestational exposure to excessive levels of either retinoids or high concentrations of Cd can alter developmental patterning to cause a similar teratogenic spectrum of embryonic malformations (Hen Chow and Cheng, 2003; Messerle and Webster, 1982; Padmanabhan and Hameed, 1990; Scott et al., 2005). Combined exposure to Cd and RA can induce synergistic effects on these fetal malformations, findings that suggest mechanistic convergence between the RA and Cd toxicity pathways (Lee et al., 2006).

Retinoids also have well-defined roles in regulating lipid and carbohydrate metabolism in the liver. Metabolic activity of RAR/RXR signaling by RA influences hepatic lipid metabolism by increasing lipolysis and the breakdown of triglycerides, whereas loss of RXR function increases hepatic cholesterol and circulating triglyceride levels (He et al., 2013; Wan et al., 2000). Retinoic acid signaling is also necessary for maintenance of glucose-stimulated insulin secretion and can repress the development of obesity and insulin resistance (Brun et al., 2015). Conversely, vitamin A deficiency can induce hyperglycemia and reduce insulin secretion, leading to T2D (Trasino et al., 2015). Previous analysis has also demonstrated that Cd alters RA signaling by upregulating genes involved in RA metabolism and inhibiting enzymes involved in RA degradation (Cui and Freedman, 2009). The dysregulated expression of retinoid signaling pathways identified in the livers of Cd-exposed female-offspring provides further evidence that gestational Cd acts as an obesogenic RA disruptor that acts by altering RA signaling to induce metabolic disease in adulthood.

Gestational Cd Exposure Induces Oxidative and ER Stress

In sensitive tissues including the liver, chronic uptake, and intracellular accumulation of excessive levels of Cd induce toxicity by inhibiting antioxidant activity, causing increased production of mitochondrial reactive oxygen species that

induce oxidative stress (Shaikh et al., 1999; Cuypers et al., 2010). It is notable that even at the relatively low 500 ppb concentration used here, gestational Cd exposure induced DEG patterns indicative of the hallmark molecular phenotypes of Cd toxicity that occur with longer duration exposures to higher concentrations of Cd (Biagioli et al., 2008; Hiramatsu et al., 2007; Liu et al., 2006; Tamás et al., 2014; Yokouchi et al., 2007). The patterns of DEGs identified in liver of Cd-exposed female offspring at each analyzed timepoint revealed the involvement of oxidative stress, mitochondrial dysfunction, and ER stress in the observed mechanism of metabolic dysfunction. Along with being a fundamental component of the mechanism of Cd toxicity, mitochondrial dysfunction also has a critical role in dysregulation of energy balance and metabolic processes associated with insulin resistance and T2D pathogenesis (Patti and Corvera, 2010; Rueggsegger et al., 2018). The link between Cd-related toxicity and gestational Cd-induced metabolic disruption is further supported by the clear changes in DEGs associated with ER stress and unfolded protein response that have well-defined functions in abnormal lipid and energy metabolism and cause increased hepatic lipid accumulation and liver diseases including NAFLD and hepatocellular carcinoma (Henkel and Green, 2013; Nouredin and Rinella, 2015).

Zinc Transporters and Cd-Induced Metabolic Disruption

The molecular initiating event of Cd-induced toxicity is the cellular uptake of Cd by divalent cation transporters (He et al., 2009). The Zn/Mn transporter Zip14 encoded by the Slc39a14 gene is primarily responsible for intestinal uptake of ingested Cd and mediates intracellular Cd accumulation in liver, pancreas, and heart where it is expressed at the highest levels (Aydemir and Cousins, 2018; Jeong and Eide, 2013; Sabolić et al., 2010; Taylor et al., 2005). The expression of Slc39a14 was decreased in livers of exposed females at weaning following gestational Cd exposure, and this effect persisted into adulthood concordant with a decrease in expression of Slc39a8, the gene encoding the Zip8 Zn transporter. A direct mechanistic connection of the observed changes in metal ion transporter gene expression and the metabolic effects in exposed female offspring is not possible. It is, however, noteworthy that Slc39a14 knockout mice display a spectrum of age-related metabolic phenotypes including increased adiposity, altered glucose homeostasis, and impaired gluconeogenesis (Aydemir et al., 2012, 2016; Hojyo et al., 2011). Alterations in Zn homeostasis are also associated with insulin resistance and T2D, wherein Zn is protective against insulin resistance and Zn deficiency is associated with impaired insulin secretion and action (Cruz et al., 2017; Faure et al., 1992; Maret, 2017). Defining the role of alterations in metal ion transporters in gestationally induced Cd metabolic disruption will require further analysis aimed at defining the role of dysregulated metal ion homeostasis during the developmental of metabolic disease.

Sex-Specific Responsiveness to Cd Toxicity

The mechanistic basis of the observed sexually dimorphic metabolic responses to developmental Cd exposure is unknown. In both human and experimental rodent models, females are more sensitive to the toxic effects of Cd than males. This increase in sensitivity has been attributed to the longer biological half-life of Cd in females (Suwazono et al., 2009; Taguchi and Suzuki, 1981). Because of the relatively low dose-duration of the gestational exposure, and the lack of evidence for Cd accumulation in liver, sex differences in Cd half-life are considered unlikely to have played a major role in mediating the markedly

different metabolic impacts and hepatic pathology observed. Because the placenta is a critical site of Cd sequestration by metallothionein and placental expression of metallothionein can be induced by oral Cd exposure, sexually dimorphic placental epigenetic programming and gene expression could be responsible for the observed sex-specific Cd toxicity (Everson et al., 2019; Martin et al., 2017). Although studies examining the sex-related effects of environmental exposures on glucose metabolism exist for other toxicants, there is limited analysis of gestational Cd exposure on metabolic endpoints (Naville et al., 2013). At high concentrations, Cd has multiple activities that can alter steroid hormone concentrations and activities. These include alteration of steroid binding globulins concentrations, dysregulation of steroidogenesis, with some evidence suggesting that Cd can act as a metalloestrogen (Aquino et al., 2012; Chedrese et al., 2006; Kawai et al., 2002). However, there was a paucity of evidence from this analysis for direct involvement of steroid hormone-related endocrine disruption in the etiology of the metabolic impacts observed. Considering the well-documented interactions of sex hormones, glucose metabolism, and insulin sensitivity, ongoing analysis of Cd toxicity must consider sex as an important modifier of Cd-mediated effects on insulin resistance and metabolic syndrome.

CONCLUSION

Gestational Cd exposure in mice, at body burdens equivalent to those found in many women of childbearing age, caused hepatic steatosis, dyslipidemia, glucose intolerance, and insulin resistance in young female offspring. In adult females, gestational Cd increased body and perigonadal fat weight and liver pathology that indicates increased risk of hepatocellular carcinoma. Those findings extend previous work demonstrating the carcinogenic potential of prolonged exposures, or exposures at higher concentrations, and find that female, but not male, CD-1 mice are differentially susceptible to toxicity following gestational Cd exposure. Analysis of the hepatic transcriptome revealed mechanistic insight into the etiology of Cd-induced metabolic disease. Oxidative stress, ER stress, and mitochondrial dysfunction were notable at birth and progressed to pathologic metabolic disease in early adulthood, all of which point to alterations in the developmental programming, potentially involving disruptions in timing or levels of RA signaling. The severe liver damage and obesity induced in female offspring by relatively low levels of Cd during gestation is consistent with Cd acting as a developmental RA disruptor, obesogen, and potential carcinogen.

DATA AVAILABILITY

Data available.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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