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ARTICLE

Animal Models

Nonnutritive sweetener consumption during pregnancy, adiposity, and adipocyte differentiation in offspring: evidence from humans, mice, and cells

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Abstract

Background Obesity often originates in early life, and is linked to excess sugar intake. Nonnutritive sweeteners (NNS) are widely consumed as "healthier" alternatives to sugar, yet recent evidence suggests NNS may adversely influence weight gain and metabolic health. The impact of NNS during critical periods of early development has rarely been studied. We investigated the effect of prenatal NNS exposure on postnatal adiposity and adipocyte development.

Methods In the CHILD birth cohort ($N = 2298$), we assessed maternal NNS beverage intake during pregnancy and child body composition at 3 years, controlling for maternal BMI and other potential confounders. To investigate causal mechanisms, we fed NNS to pregnant C57BL6J mice at doses relevant to human consumption (42 mg/kg/day aspartame or 6.3 mg/kg/day sucralose), and assessed offspring until 12 weeks of age for: body weight, adiposity, adipose tissue morphology and gene expression, glucose and insulin tolerance. We also studied the effect of sucralose on lipid accumulation and gene expression in cultured 3T3-L1 pre-adipocyte cells.

Results In the CHILD cohort, children born to mothers who regularly consumed NNS beverages had elevated body mass index (mean z-score difference +0.23, 95% CI 0.05–0.42 for daily vs. no consumption, adjusted for maternal BMI). In mice, maternal NNS caused elevated body weight, adiposity, and insulin resistance in offspring, especially in males (e.g., 47% and 15% increase in body fat for aspartame and sucralose vs. controls, $p < 0.001$). In cultured adipocytes, sucralose exposure at early stages of differentiation caused increased lipid accumulation and expression of adipocyte differentiation genes (e.g., C/EBP-α, FABP4, and FASN). These genes were also upregulated in adipose tissue of male mouse offspring born to sucralose-fed dams.

Conclusion By triangulating evidence from humans, mice, and cultured adipocytes, this study provides new evidence that maternal NNS consumption during pregnancy may program obesity risk in offspring through effects on adiposity and adipocyte differentiation.

Introduction

Globally, over 20% of children are overweight or obese [1]. Mounting evidence shows that obesity originates early in life, perhaps even *in utero*. The Developmental Origins of Health and Disease hypothesis postulates that prenatal and early postnatal exposures can "program" lifelong metabolism, weight gain, and other endocrine pathways [2]. Moreover, in early life, environmental exposures can stimulate adipocyte precursor cells to induce the process of adipocyte differentiation, creating a large reservoir of adipocytes to support the development of obesity in response to over-nutrition later in life [3–7].

Supplementary information The online version of this article (https://doi.org/10.1038/s41366-020-0575-x) contains supplementary material, which is available to authorized users.

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Excess energy intake from sugar, especially sugar-sweetened beverages, is strongly associated with obesity [8–11]. Sugar substitutes or "nonnutritive sweeteners" (NNS) including aspartame and sucralose are marketed as healthier alternatives [12, 13]. NNS are widely consumed, including by pregnant women. Almost 30% of mothers in the Canadian CHILD cohort consumed NNS during pregnancy [14], and similar rates have been reported in the USA (24%) [15] and Denmark (45%) [16]. Contrary to their intended benefits, NNS have been inconsistently associated with metabolic derangements and adverse effects on cardiometabolic health in adults [17–19] and children [20]; however, few studies have investigated NNS exposure *in utero*. In the CHILD cohort, we found that daily NNS beverage consumption during pregnancy was associated with a twofold higher risk of infant overweight at 1 year of age [14]. Similar results were observed among older children in the Danish National Birth Cohort [16]. Interestingly, both studies observed stronger effects in males, although a third study found no association in children of either sex [21].

Limited evidence from animal studies also suggests that NNS consumption during pregnancy and lactation may predispose offspring to develop obesity and metabolic syndrome [22]. However, most studies have used doses that exceed the human acceptable daily intake (ADI), equivalent to 20 packets of NNS or 12 cans of diet soda per day [13]. In mice, chronic lifetime exposure to NNS (55 mg/kg/day aspartame; exceeding the ADI by 1.4-fold), commencing *in utero*, has been associated with increased weight gain and decreased insulin sensitivity in adulthood [23], but the impact of maternal NNS intake was unclear because exposure was maintained in the offspring after weaning. A recent study found that maternal NNS intake (a combination of sucralose and acesulfame-K at twofold ADI) altered the microbiome and metabolism of young offspring and reduced their body weight [24], although adiposity was not assessed and the offspring were not followed after weaning. Another study found that rats exposed to very high doses of NNS (343 mg/kg/day aspartame; 8.6-fold ADI) during gestation gain more weight and have altered lipid profiles during adulthood [25], yet other studies reported no difference in weight gain following prenatal NNS exposure [26]. Similarly, conflicting evidence from in vitro studies suggests that NNS can either stimulate [27] or downregulate [28, 29] adipocyte differentiation.

Overall there is a paucity of evidence from human and experimental studies on the potential impact of prenatal NNS exposure on the development of obesity and metabolic health. Here, we extend our previous findings on maternal NNS consumption and infant body composition in the longitudinal CHILD cohort [14] by reassessing this relationship at 3 years of age. Further, we used experimental model systems to examine mechanisms in mice, using physiologically relevant doses of aspartame and sucralose. Finally, we characterized these mechanisms using an in vitro model of adipocyte differentiation. The combination of clinical and experimental findings provides new evidence that maternal NNS consumption conditions obesity risk in the offspring.

Methods

CHILD birth cohort

We accessed data from the observational CHILD Cohort Study, a longitudinal pregnancy cohort study of 3455 families across four sites in Canada, enrolled between 2008 and 2012 [30]. This study was approved by the Human Research Ethics Boards at the Hospital for Sick Children, McMaster University and the Universities of Manitoba, Alberta, and British Columbia. All mothers provided written informed consent. For the current secondary analysis, we included 2298 mother–child dyads with complete data on maternal NNS consumption and child BMI at 3 years. Maternal sweetened beverage consumption during pregnancy, total energy intake, and Healthy Eating Index were documented in the CHILD study using a food frequency questionnaire [31–34] during the second or (usually) third trimester of pregnancy, as described previously [14] (Supplementary Methods). NNS beverages included "diet soft drinks or pop" (1 serving = 12 oz. or 1 can) and "artificial sweetener added to tea or coffee" (1 serving = 1 packet). At 3 years of age, child height, weight, and subscapular skin folds were measured by trained CHILD study staff following a standardized protocol. Age- and sex-specific *z*-scores were calculated against the 2006 World Health Organization reference. Child sex, birth weight, gestational age, gestational diabetes, and maternal age were collected from hospital records. Maternal BMI was calculated from measured height and self-reported prepregnancy weight [14]. Mothers reported their education, smoking, and breastfeeding duration, and their child's screen time (indicator of physical inactivity) and fresh and frozen food consumption (indicator of diet quality).

Experimental mouse model

All procedures were approved by the Animal Welfare Committee of the University of Manitoba, which adheres to the principles developed by the Canadian Council on Animal Care and the Council for International Organizations of Medical Sciences. Male and female C57BL6J mice were obtained and mated at 8 weeks. Dams were randomly assigned to drinking water (control), sucrose (45 g/L,

~7.2 g/kg body weight/day anticipating 4 mL water intake, and 25 g body weight), aspartame (0.2 g/L, ~32 mg/kg/day) or sucralose (0.04 g/L, ~6.4 mg/kg/day) throughout pregnancy, and lactation ($n = 6$ females/litters per group). Based on previous metabolic studies, this sample size is sufficient to determine effects [35]. In a preliminary dose-finding study, dams received low (0.05 g/L), medium (0.1 g/L), and high (0.2 g/L) levels of aspartame (Table S6) or low (0.01 g/L), medium (0.02 g/L), and high levels (0.04 g/L) of sucralose (Table S7). These NNS concentrations are relevant to human consumption, translating to doses near or below the ADI limits for humans (40 mg/kg for aspartame and 5 mg/kg for sucralose). Dams delivered naturally and when necessary, litters were reduced to eight pups (four males and four females) to avoid competition for food. Beginning at 3 weeks of age (the usual weaning age for mice), offspring were fed regular chow and tap water.

Mouse offspring

Food and water intake and body weight were measured weekly for all offspring. At 11 weeks, body composition of mouse offspring was assessed by Dual-Energy X-ray Absorptiometry (DEXA) by a technician blinded to the offspring experimental groups. Glucose tolerance and insulin tolerance were tested as described previously [35] (Supplementary Methods). At 12 weeks, offspring were euthanized by intraperitoneal injection of sodium pentobarbital and blood was collected by cardiac puncture. Tissues were dissected, rinsed in PBS, weighed, and either fixed in 10% formalin or freeze clamped in liquid nitrogen and stored at −80 °C. Histopathological preparations and hematoxylin/eosin (HE) staining were performed according to standard procedures. For the analysis of adipocyte size and number, the internal diameters of 80 consecutive adipocytes from two randomly selected fields on each HE stained slide were measured under light microscopy at ×20 magnification using a digital micrometer and averaged. For all analyses, data from cage mates were averaged and the litter was used as the unit of analysis.

Cell culture experiments

3T3-L1 pre-adipocyte cells were differentiated as described previously [36]. Three separate cell preparations from American Type Culture Collection (Manassas, Virginia) were used (passage 10) for differentiation and each sample was performed with three technical replicates. Two days post confluency (Day 0), the cells were stimulated with 1 µM dexamethasone, 1 µg/ml insulin, and 0.5 mM methylisobutyl-xanthine in Dulbecco's Modified Eagle's Medium with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Oakville, ON). Cells were fed with fresh media every 2 days, with insulin and FBS on Day 2 and FBS alone from Day 4 until Day 8. Throughout adipocyte differentiation, 200 nM sucralose was added at different stages of cellular development (Fig. 4a) until Day 8. At Day 8, the cells reached full differentiation and were collected for analysis. After fixation with 10% formaldehyde for 2 h at room temperature, cells were washed with 60% isopropanol and lipid accumulation was evaluated by oil red O staining for 1 h at room temperature followed by washing twice with distilled water. An EVOS digital inverted microscope (AMG, Bothel, WA) was used to capture 12 images for every 100 mm plate, with seven pictures taken around the outside and five taken around the center of the plate. The image analyst was blinded to the identity of the cell conditions.

Gene expression

RNA was isolated from tissues and cells using a QIAshredder column and further purified using the RNeasy kit (Qiagen, Valencia, CA). For qPCR analysis, cDNA was synthesized using the Protoscript kit (NEB, Ipswich, MA). The QuantiTect SYBR Green PCR kit (Qiagen) was used to monitor amplification of cDNA on a CFX96 thermocycler (Bio-Rad, Hercules, CA). Expression of genes was assessed in duplicate using $2^{-\Delta\Delta CT}$ and data normalized by geometric averaging of multiple control genes that were constant across all groups of offspring [37], including eukaryotic initiation factor 2a (eIF2a) and cyclophilin A, using validated primer sequences (Table S9).

Statistical analyses

Statistical analyses are described in the Supplementary Methods.

Results

Maternal NNS intake during pregnancy is associated with higher BMI and adiposity in children

Building on our previous findings at 1 year of age in the CHILD cohort [14], we reexamined the association of maternal NNS consumption and child body composition at 3 years of age among 2298 mother–child dyads. During pregnancy, 29.9% of mothers reported consuming any NNS beverages and 5.2% consumed them daily (Table S1). Consistent with our previous results, children born to mothers reporting daily NNS beverage consumption had

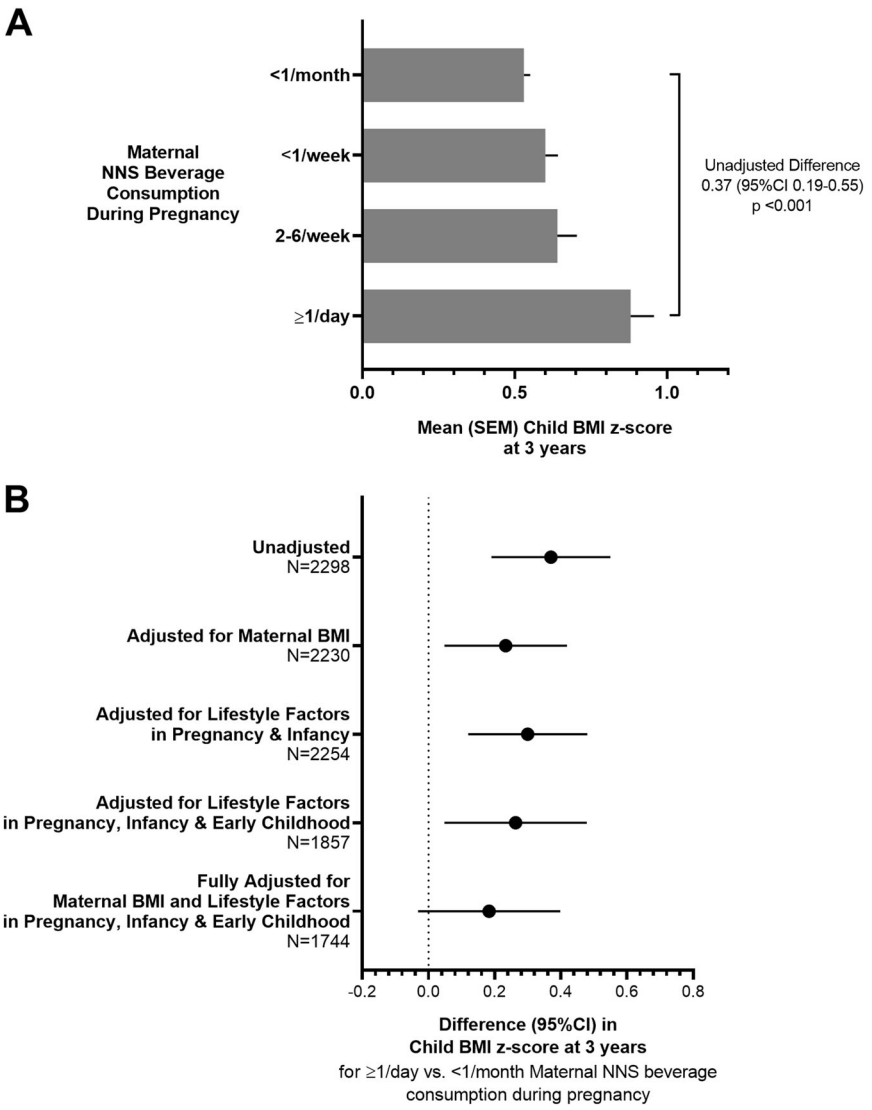

Fig. 1 Maternal consumption of NNS-sweetened beverages and child body mass index (BMI) at 3 years of age in the CHILD cohort. A Child BMI *z*-score at 3 years according to maternal NNS beverage consumption during pregnancy. Values are means ± SEM. BMI *z*-scores were calculated against the World Health Organization reference standard; a *z*-score of 0 indicates a normal "healthy" BMI, a *z*-score of +1 indicates a BMI 1 standard deviation higher than normal. **B** Difference in BMI *z*-score for highest consumption group (≥1 beverage per day) vs. no consumption. Values are unadjusted and adjusted beta estimates and 95% confidence intervals from multiple linear regression models (N's for each model are noted; see Table S2 for full results). Lifestyle factors in pregnancy and infancy include: maternal total energy intake, Healthy Eating Index score, sugar-sweetened beverage intake, postsecondary education, smoking, and diabetes during pregnancy; breastfeeding duration; child sex. Lifestyle factors in early childhood include: screen time, fresh, and frozen food intake (as an indicator of diet quality) at 3 years. CI confidence intervals, NN nonnutritive sweetener.

significantly higher BMI at 3 years of age than children born to mothers who did not consume NNS beverages (mean BMI *z*-score 0.88 vs. 0.53; unadjusted difference: 0.37, 95% CI 0.19–0.55). This difference was reduced after adjusting for potential confounders including maternal BMI and lifestyle factors in pregnancy (diabetes, smoking, and overall diet quality), infancy (breastfeeding duration), and early life (child diet quality and screen time) (adjusted difference: 0.18, 95% CI −0.03, 0.40) (Fig. 1 and Table S2). No sex differences were observed (not shown), and results were similar for the adiposity outcome of subscapular skin folds (Table S3). Together, these results suggest that maternal NNS consumption during pregnancy may promote excessive weight gain or adiposity in offspring, although confounding by maternal BMI and lifestyle factors appears to partially explain this relationship. To eliminate the possibility of confounding, determine causality, and investigate biological mechanisms, we undertook mechanistic studies exposing pregnant mice and cultured adipoctyes to NNS at doses relevant to human consumption.

Sucrose and NNS variably impact weight gain and energy intake in pregnant mice

Although not statistically significant, pregnant mice receiving sucrose in their drinking water weighed more than control dams at e−18.5 (Table S4). Dams receiving sucrose consumed more sweetened water and more food, thus their average daily energy intake was ~1.4-fold greater than controls (Table S4; $p < 0.01$). Dams receiving aspartame or sucralose increased their food intake by a lesser degree (1.1- and 1.2-fold, respectively; $p < 0.01$), but their body weight was not affected. Notably, maternal sucrose and aspartame consumption increased the number of pups in the litters (Table S4; $p < 0.01$) whereas sucralose did not significantly affect the litter size.

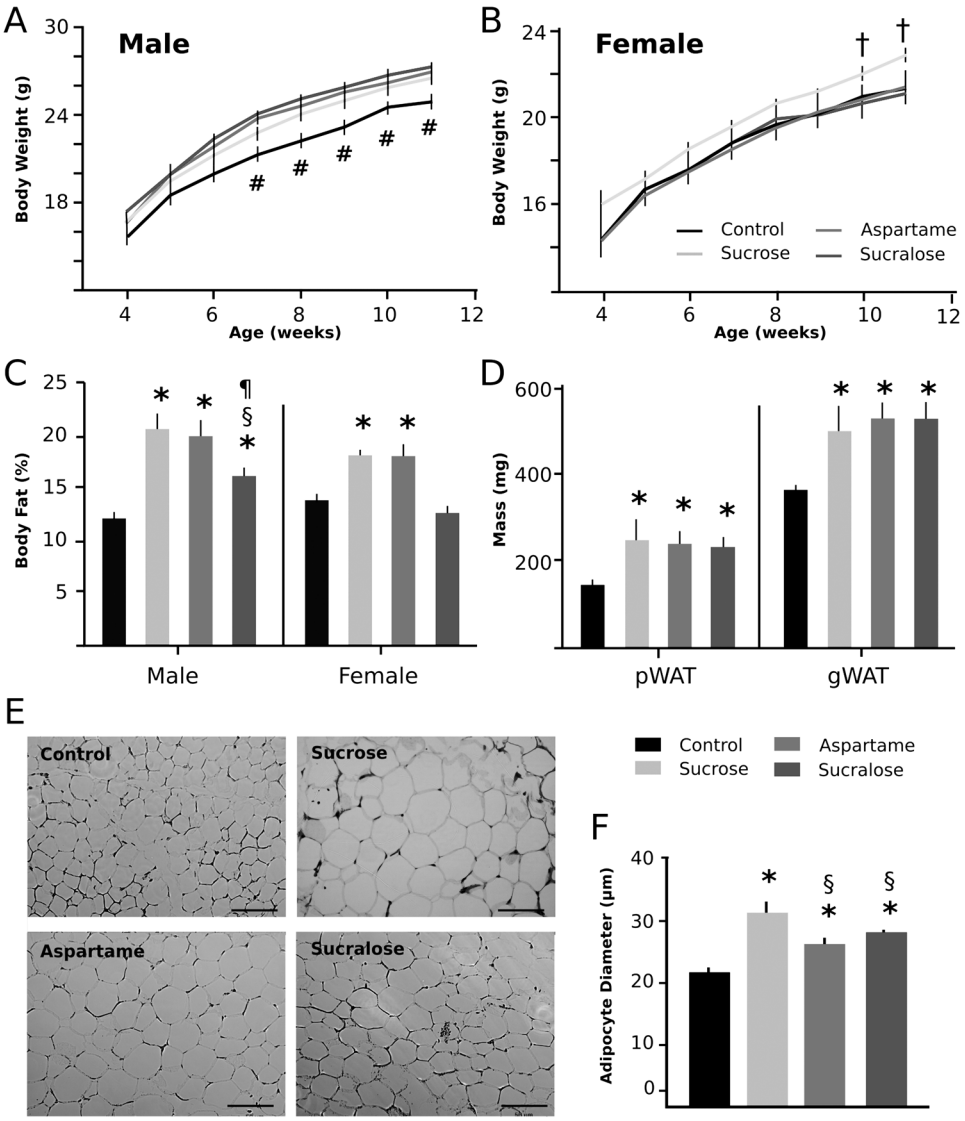

Fig. 2 Body composition in male and female mouse offspring of dams fed sucrose, aspartame or sucralose during pregnancy and lactation. Body weight trajectory of (**A**) male and (**B**) female offspring; **C** Percent body fat of male and female offspring at 11 weeks of age; **D** pWAT and gWAT weights of male offspring; **E** Representative images of H&E stained sections of pWAT adipocytes at ×20 magnification; **F** Adipocyte diameter, values represent mean ± SEM, $n = 6$. p values represent significance after two-way repeated measures ANOVA with Bonferroni post hoc tests: $^{\#}p < 0.05$ between control male offspring vs. the male offspring of sucrose-, aspartame- and sucralose-fed dams. $^{\dagger}p < 0.05$ between female offspring of sucrose-fed dams vs. the offspring of control, aspartame- and sucralose-fed dams. p values represent significance after one-way ANOVA with Bonferroni post hoc tests: $^{*}p < 0.05$ vs. offspring of control dams, $^{\S}p < 0.05$ vs. offspring of sucrose-fed dams and $^{\P}p < 0.05$ vs. offspring of aspartame-fed dams. pWAT perirenal white adipose tissue, gWAT gonadal white adipose tissue.

Maternal NNS intake has sex-specific effects on adiposity in mouse offspring

Next, we investigated the influence of maternal NNS intake on weight gain in male and female mouse offspring. Maternal sucrose, aspartame and sucralose consumption all conditioned increased body weight in male offspring by 7 weeks of age compared with the male offspring of control dams (Fig. 2a; all $p < 0.001$). The elevated body weight in these male offspring persisted until sacrifice at 11 weeks of age. Conversely in female offspring, only maternal sucrose consumption (not aspartame or sucralose) induced elevated body weight at 10 and 11 weeks of age (Fig. 2b; $p < 0.05$). Interestingly, the elevated body weight did not appear to be due to differences in energy intake because average daily food intake was similar across all offspring groups (Table S5).

To determine whether increased body weight was related to alterations in lean and/or fat mass in the offspring, we performed dual-energy X-ray absorptiometry (DXA). This analysis showed that maternal sucrose, aspartame, and sucralose all markedly increase the percent body fat (50%, 47%, and 15% increases, respectively) in male offspring, compared to controls (Fig. 2c; $p < 0.0001$). Maternal sucrose and aspartame also increased the percent body fat in female offspring (Fig. 2c; $p < 0.05$); however, sucralose had no effect on percent body fat in females. Consistent with these observations, maternal consumption of sucrose, aspartame, and sucralose all increased the weight of perirenal white adipose tissue (pWAT) and gonadal white adipose tissue (gWAT) fat pads of the male offspring, compared with controls (Fig. 2d; $p < 0.05$). Notably, the effect of sucralose was dose dependent, as lower levels of sucralose administration to dams did not induce elevated body weight and fat pad mass in offspring (Tables S7 and S8). H&E staining of perirenal adipose tissue (Fig. 2e) revealed that maternal aspartame and sucralose consumption increased the mean adipocyte diameter of the

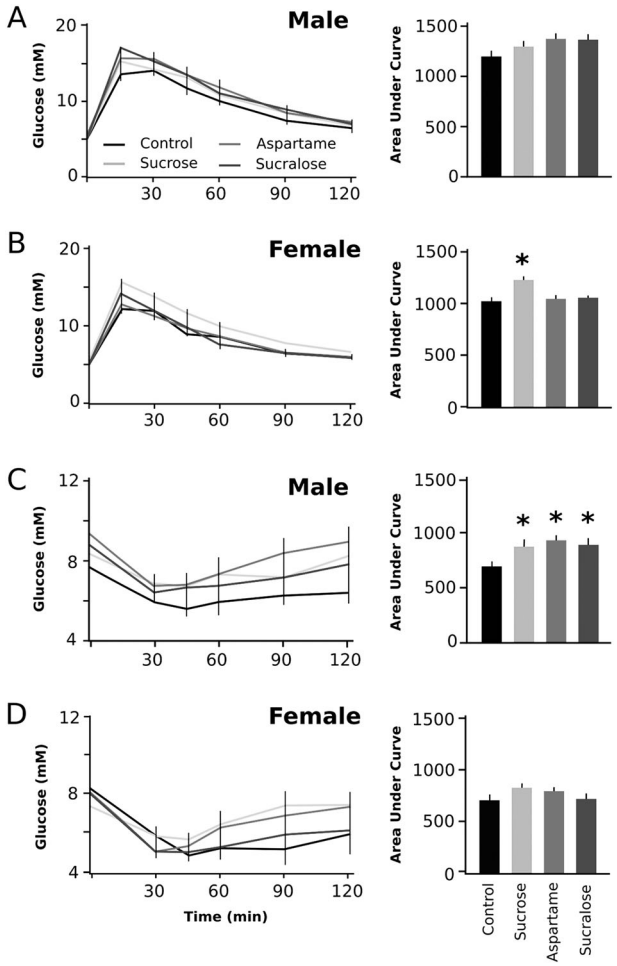

Fig. 3 Glucose tolerance and insulin sensitivity in 10-week-old male and female mouse offspring of dams fed sucrose, aspartame or sucralose during pregnancy and lactation. A GTT (left) and area under the curve (right) in male offspring; **B** GTT and area under the curve in female offspring; **C** ITT and area under the curve in male offspring; **D** ITT and area under the curve in female offspring. GTT glucose tolerance test, ITT insulin tolerance test. Values represent the mean ± SEM, $n = 6$. p values represent significance after one-way ANOVA with Bonferroni post hoc tests: $^*p < 0.05$ vs. control offspring. No significant differences were observed in Bonferroni post hoc testing at each time point whereas some differences were detected in the overall area under the curve.

male offspring by 22% and 30%, respectively (Fig. 2f, $p < 0.05$). Adipose tissue was the only major organ system that increased in weight; the liver, heart, kidney, and spleen of the offspring were generally similar between all groups (Table S8). One notable exception was increased liver mass in female offspring of sucrose-fed dams (Table S8).

Maternal NNS intake has sex-specific effects on insulin sensitivity in mouse offspring

Next, since maternal NNS consumption increased body fat accumulation in offspring, we examined whether glucose tolerance and insulin sensitivity were also affected (Fig. 3). In cross-sectional analyses, these measures did not differ between groups at any individual time point during the 120 min challenge. However, using the area under the curve to assess overall glucose tolerance and insulin sensitivity throughout the whole challenge, we found that maternal sucrose consumption induced significant glucose intolerance in female offspring, while maternal aspartame and sucralose consumption had no effect (Fig. 3b). Glucose tolerance was similar in all groups of male offspring (Fig. 3a). By contrast, insulin tolerance tests revealed that the male offspring of sucrose, aspartame, and sucralose-fed dams were more insulin resistant than the male offspring of control dams (Fig. 3c), whereas insulin sensitivity of the female offspring was similar across all groups (Fig. 3d).

Sucralose has pro-adipogenic effects on 3T3-L1 pre-adipocytes in vitro

Since maternal NNS influenced body fat accumulation in male mouse offspring, and early life is a critical stage that determines stem cell fate, we examined the effects of sucralose in cultured cells using the well-established male 3T3-L1 pre-adipocyte cell line. Previous research has shown that aspartame affects lipid accumulation and adipocyte differentiation in 3T3-L1 cells [29]. Therefore, we examined the stage(s) of adipocyte differentiation affected by sucralose. The 3T3-L1 adipocytes were incubated with induction medium in the presence or absence of sucralose (200 nM) for the indicated periods of time (illustrated in Fig. 4a). As expected, control cells incubated with induction medium for 8 days differentiated into adipocytes, with lipid accumulation visualized by oil red staining (Fig. 4b). Cells treated with sucralose from d0 to d2 (treatment b, modeling germline exposure) or d0 to d8 (treatment e, throughout differentiation) exhibited the highest accumulation of lipid (Fig. 4b). Of note, lipid accumulation was not significantly affected by sucralose treatment in other time windows, including d2–d4 (treatment c, modeling fetal exposure) or d4–d8 (treatment d, modeling postnatal exposure) (Fig. 4b). These results collectively suggest that sucralose administration enhances adipogenesis at an early phase of differentiation, consistent with the effects of prenatal NNS exposure on body fat accumulation observed in mice (Fig. 2) and 3-year-old participants in the CHILD study (Fig. 1).

Sucralose stimulates pro-adipogenic regulators and enzymes in vitro and in vivo

Since adipocyte differentiation is a complex process that can be modulated by multiple stimuli including transcription factors, we examined how sucralose affected the gene expression of regulators of the adipocyte phenotype.

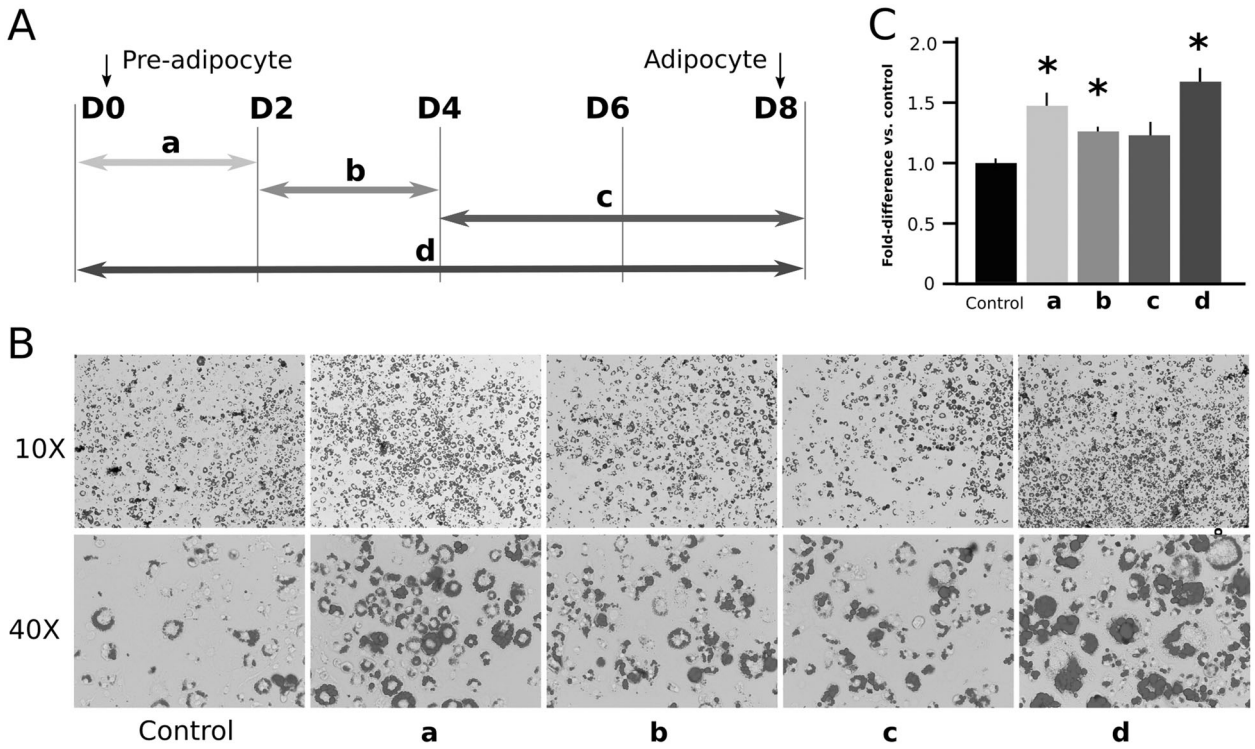

Fig. 4 Effect of sucralose on 3T3-L1 adipocyte differentiation in vitro. A Schematic outline of the experimental design: double-headed arrows indicate the length of treatment. 3T3-L1 cells were treated with 200 nM sucralose for the indicated periods of time. **B** Oil red O staining to measure cellular lipid content assessed 8 days following induction of 3T3-L1 pre-adipocyte differentiation with induction medium containing MDI, insulin, and fetal bovine serum in the presence or absence of 200 nM sucralose. **C** Quantification of cellular lipid content. Values represent the mean ± SEM of data from three independent experiments with three replicates. *p* values represent significance after one-way ANOVA with Bonferroni post hoc tests: $*p < 0.05$ vs. control (no sucralose treatment).

The addition of sucralose to the culture media from d0 to d8 (treatment d) induced a small but significant increase in the expression of the peroxisomal proliferator activated receptor (PPAR)-γ transcription factor at d8 of adipocyte differentiation (*Pparg*; Fig. 5a). On the other hand, the addition of sucralose earlier in the adipocyte differentiation program as well as throughout (treatments b and d), induced marked increases in the expression of the adipogenesis-dependent transcription factor, CCAT enhancer binding protein (C/EBP)-α by d8 of adipocyte differentiation (*Cebpa*; Fig. 5b). Moreover, the addition of sucralose to the media at the early stages of differentiation (treatments a and b) as well as throughout (treatment d), induced 1.5–2-fold increases in the mRNA expression of the adipocyte marker genes, adiponectin (*Adipoq*; Fig. 5c) and fatty acid binding protein (*Fabp4*; Fig. 5d). Consistent with these findings, sucralose also increased the expression of the lipid droplet coat protein, perilipin (*Plin2*; Fig. 5e). Sucralose did not affect the expression of the adipogenesis inhibitory factor, *Pref-1* (Fig. 5f), suggesting that most of the effects of sucralose are driven by promoting adipogenesis rather than removing factors that maintain the undifferentiated state. Overall, treatment of the cells with sucralose at earlier stages of adipocyte differentiation (treatments a, b and d) had remarkable effects on regulators of the adipocyte phenotype whereas treatment of the cells with sucralose at later stages (treatment c) had no effect.

Next, we examined whether sucralose also impacted the expression of genes encoding metabolic enzymes involved in fat storage and mobilization during adipocyte differentiation. Indeed, sucralose administration early in the adipocyte differentiation program increased the expression of fatty acid synthase (*Fasn*; Fig. 5g) as well as glycerol phosphate acyltransferase (*Gpam*; Fig. 5h). Sucralose also significantly increased the expression of hormone sensitive lipase (*Lipe*; Fig. 5i) and adipose tissue triglyceride lipase (*Atgl*; Fig. 5j). These findings suggest that sucralose promotes fatty acid and triacylglycerol synthesis as well as its mobilization in differentiating 3T3-L1 adipocytes.

Finally, we assessed whether maternal sucralose consumption also affected the expression of several of these genes in the pWAT of male mouse offspring. Interestingly, in the offspring of sucralose-fed dams, as well as sucrose-fed and aspartame-fed dams, a ~1.5-fold increase in *Cebpa* and *Fabp4* mRNA expression in pWAT was observed compared with the offspring of control dams (Fig. 6a, b). In addition, sucralose (but not sucrose or aspartame) increased *Fasn* and *Gpam* mRNA expression ~7-fold and ~3-fold,

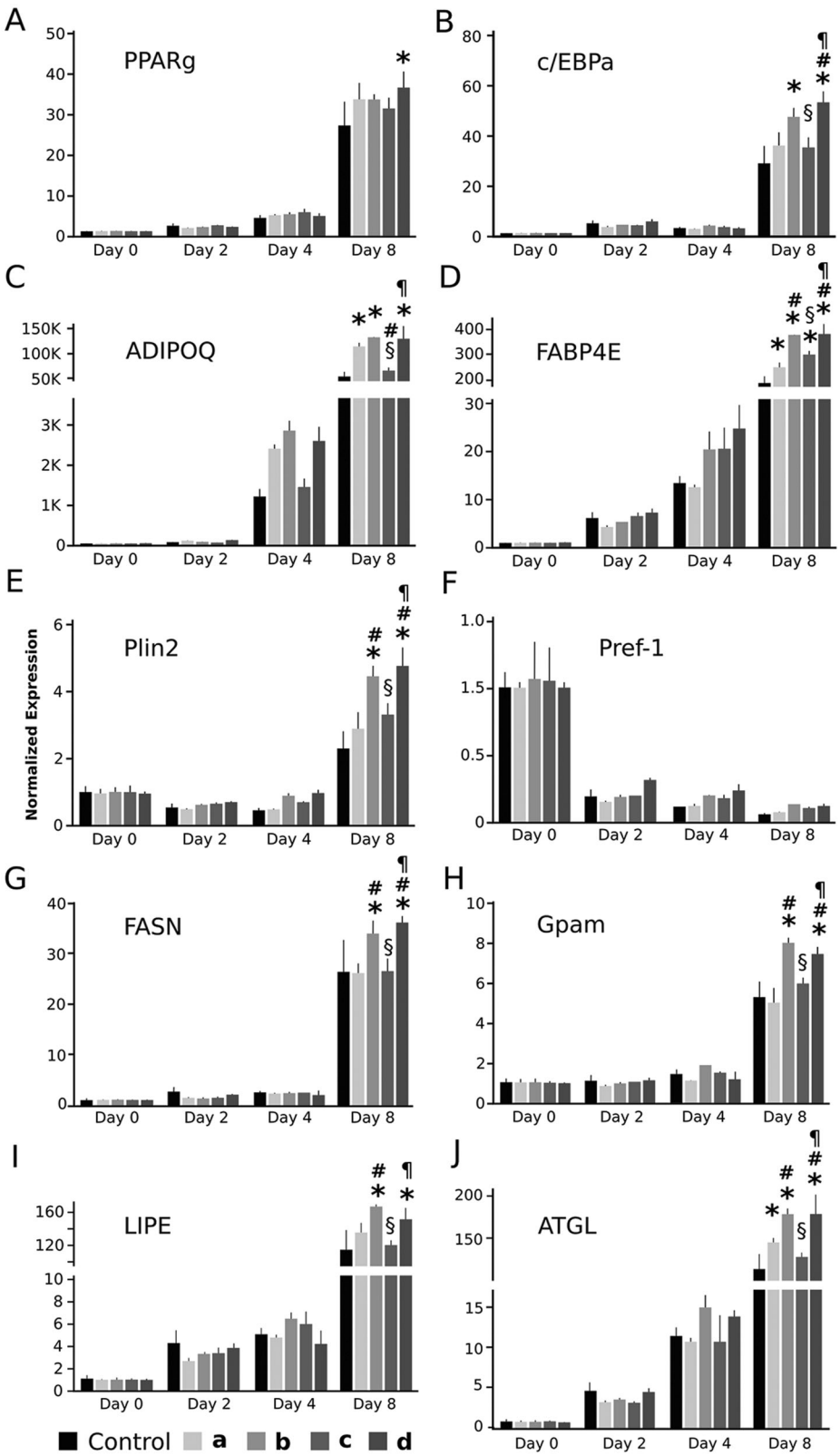

Fig. 5 Sucralose increases the expression of pro-adipogenic regulators, fat storage, and mobilization genes in 3T3-L1 cells in vitro. A *PPAR-γ* gene expression, **B** *Cebpa* gene expression, and **C** *Adipoq* gene expression. **D** *Fabp4* gene expression, **E** *Plin1* gene expression, and **F** *Pref1* gene expression. **G** *Fasn* gene expression, **H** *Gpam* gene expression, **I** *Lipe* gene expression, and **J** *Atgl* gene expression. Values represent the mean ± SEM of data from three independent experiments with three replicates. qPCR gene expression is relative to the geomean of *Eif2a* and *CycA* and normalized the control group. *p* values represent significance after two-way ANOVA with Bonferroni post hoc tests: $^{*}p < 0.05$ vs. control (no sucralose treatment), $^{\#}p < 0.05$ vs. **b**, $^{\S}p < 0.05$ vs. **c**, $^{\P}p < 0.05$ vs. **d**. Treatments a, b, c, d refer to the sucralose treatments depicted in Fig 4a: a = sucralose on days 0–2, b = days 2–4, c = days 4–8, d = days 0–8.

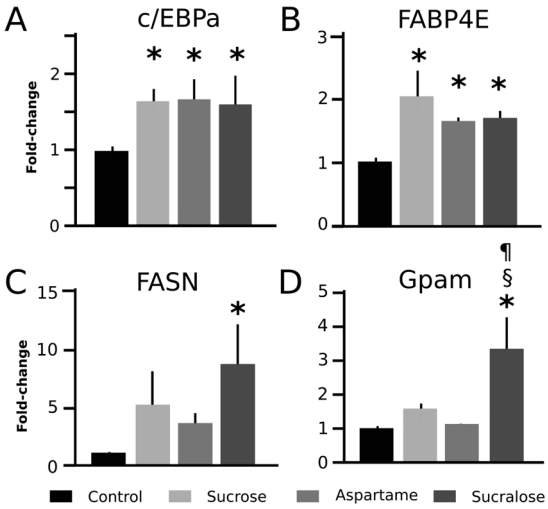

Fig. 6 Sucralose increases the expression of pro-adipogenic regulators and fat storage and mobilization genes in mouse offspring adipose tissue in vivo. A *Cebpa* gene expression, **B** *Fabp4* gene expression, and **C** *Fasn* gene expression. **D** *Gpam* gene expression. Values represent the mean ± SEM, $n = 6$. qPCR gene expression is relative to the geomean of *Eif2a* and *CycA* and normalized to the control group. p values represent significance after one-way ANOVA with Bonferroni post hoc tests: $^{*}p < 0.05$ vs. control offspring dams, $^{\S}p < 0.05$ vs. offspring of sucrose dams, $^{\P}p < 0.05$ vs. offspring of aspartame dams.

respectively, compared with the offspring of control dams (Fig. 6c, d).

Discussion

Our study provides new evidence on the potential adverse effects of NNS, which are typically marketed as "healthier" alternatives to caloric sweeteners, especially for the purposes of weight management and diabetes control. Given that maternal obesity and gestational diabetes are on the rise [38], NNS may be especially appealing to pregnant women, yet very few studies have explored the long-term impact of NNS exposure *in utero*. Here, we used a translational approach to triangulate evidence from a human cohort, a mouse model, and cell culture experiments to show that prenatal NNS exposure influences adipocyte differentiation, fat mass accumulation, and adiposity in offspring.

In the prospective CHILD cohort, we found that children born to mothers who regularly consumed NNS-sweetened beverages had higher BMI and adiposity by 3 years of age. This association was partially explained by differences in maternal BMI and other confounders, which cannot be fully disentangled in an observational study. Thus, to establish causality and investigate biological mechanisms, we undertook experiments in mice, finding that offspring exposed to NNS *in utero* had increased adiposity compared to controls, consistent with our observation in the CHILD cohort.

Our results add to an emerging body evidence from rodent studies examining early-life NNS exposure. Collison et al. [23] showed that exposing mice to aspartame *in utero* and throughout life (55 mg/kg/day, 1.4-fold ADI) resulted in increased body weight, visceral fat deposition, and fasting glucose levels, while von Poser Toigo et al. [25] found that male rat offspring exposed to high levels of aspartame (343 mg/kg/day, 8.6-fold ADI) during gestation had increased weight gain. In contrast, Olivier-Van Stichelen et al. found that maternal NNS throughout pregnancy and lactation either had no impact (for sucralose combined with acesulfame-K at levels approximating the ADI) or reduced offspring body weight (for higher doses of ~2-fold ADI) [24], although adiposity was not measured and the offspring were not followed beyond weaning. Here, we separately assessed physiologically relevant doses of aspartame and sucralose consumption. We showed that exposures approximating the human ADI of these NNSs during pregnancy and lactation increased body weight in male offspring, primarily due to an increase in their adiposity. Interestingly, and similar to sex-specific findings by Collison et al. [23], female offspring did not experience these effects. These findings are also consistent with sex differences observed in the CHILD infants at 1 year of age [14], although not replicated in our current analysis at 3 years of age. Further research is needed to understand the potentially sex-specific effects of NNS during critical periods of development.

We also uniquely evaluated the impact of maternal NNS intake on glucose and insulin tolerance in the offspring. Previously, Collison et al. found that exposure to 55 mg/kg/day of aspartame throughout gestation and postnatally increased fasting blood glucose levels in both male and female offspring and decreased insulin sensitivity in male offspring only [23]. While we did not detect differences in fasting blood glucose in NNS-exposed offspring, we did observe greater insulin resistance in the male offspring, which was consistent with their obesity. Since insulin resistance typically precedes the development of glucose intolerance and hyperglycemia, it is possible that these phenotypes could develop with advanced age or the addition of a high calorie diet.

Finally, we used a cell culture model of adipocyte differentiation to further explore the mechanisms of NNS-induced adiposity observed in the CHILD cohort and mouse offspring. Previously it was reported that saccharine and aspartame affected adipocyte differentiation and lipid metabolism [27–29], but these studies used extremely high millimolar dosages. Since we observed the greatest effects of sucralose on male mouse offspring, we treated male 3T3-L1 pre-adipocyte cells with 200 nM sucralose at different stages of the differentiation process. We found that sucralose exposure very early in the differentiation program had

the greatest effect on increasing lipid accumulation within the cells. In addition, this treatment increased the expression of several transcription factors that convert pre-adipocytes into adipocytes and have key roles in the regulation of lipid and glucose metabolism by adipocytes [39]. These include *PPAR-γ* and *C/EBP-α*, as well their downstream target genes *Adipoq*, *Fabp4*, and *Plin2*. Moreover, sucralose stimulated the expression of several genes involved in lipid metabolism, including *Fasn*, *Gpam*, *Lipe*, and *Atgl*. Importantly, we confirmed that these changes in gene expression were also present in adipose tissues isolated from male offspring exposed to sucralose *in utero*. Together, these findings suggest that sucralose can directly induce a pro-adipogenic gene expression program at doses that approximate human consumption.

The major strength of this study is our translational approach. We used data from a large, longitudinal national birth cohort that collected objective measures of body composition and accounted for many possible confounders. We performed complementary mechanistic studies in mice and cultured adipocytes, and assessed two different NNS at physiologically relevant doses. Limitations of the CHILD cohort study include the limited assessment of NNS in beverages during pregnancy, without details on NNS consumption during lactation, type of NNS, or NNS in foods, which are an increasingly common source of NNS exposure. As in all observational studies, residual confounding is also possible, although we accounted for key factors including maternal BMI, diabetes, and diet quality. To overcome these limitations, we used experimental models to address causality and examine mechanisms. A limitation of our mouse study is that we did not separate the effects of NNS during pregnancy and lactation. A limitation of our adipocyte differentiation study is that although we used a dose that is relevant to human consumption, sucralose is not fully absorbed from the gut [40]; therefore, the dose we applied to our cell culture system might be higher than what is achieved in vivo. However, our in vitro results were confirmed in mouse adipose tissue, demonstrating the compatibility of these model systems. Overall, our findings from the CHILD cohort and the experimental model systems are complementary and provide new insights into the biological impact of prenatal NNS exposure.

Further research is needed to confirm and characterize the potentially sex-specific biological mechanisms by which prenatal NNS exposure influences postnatal weight gain and adiposity. In addition to stimulating adipocyte differentiation, NNS may alter the maternal microbiome [41, 42], which is transmitted to the offspring during birth and postnatal interactions [43, 44], and contributes to host metabolism and weight gain [45–47]. Future studies should also assess other types and sources of NNS, such as plant-derived NNS and NNS in foods, and distinguish between NNS exposure during pregnancy versus lactation. Finally, it will be important to study and model the maternal conditions that motivate NNS use, notably obesity and gestational diabetes, to clearly establish and disentangle their independent effects on offspring development. This research will be important for establishing the long-term safety of prenatal NNS exposure and informing recommendations for pregnant women.

In summary, our translational research provides new evidence that exposure to NNS *in utero* stimulates postnatal weight gain, insulin resistance, and adiposity. Associations observed in the CHILD cohort were investigated in experimental model systems, revealing a previously unknown mechanism involving altered expression of pro-adipogenic (e.g., *Cebpa*) and lipid metabolism genes (e.g., *Gpam*, *Fasn*). Collectively, these results suggest that maternal NNS consumption is a modifiable obesogenic exposure that may be contributing to the global obesity epidemic, and call for further research on the long-term metabolic effects of NNS exposure in early life.

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Author contributions MBA and VWD conceived of the study design, obtained funding for this research, and drafted the paper. MRS, PS, TJM, SET, PJM, and ABB obtained funding for and oversaw recruitment of the CHILD cohort and data collection. AA performed the statistical analysis of clinical data from the CHILD cohort under the supervision of MBA. RJS contributed nutritional expertise. MMT, AH, and KGC performed mouse and cell culture experiments under the supervision of VWD. All authors critically reviewed and approved the paper. MBA had full access to the human data and VWD had full access to the mouse and adipocyte data, and take final responsibility for the decision to submit for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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