Endocrinology, 2025, **166**, bqaf063
https://doi.org/10.1210/endocr/bqaf063
Advance access publication 3 April 2025
Research Article

Transgenerational Effects of the Obesogen Tributyltin on Metabolic Health in Mice: Interactions With a Western Diet

Richard C. Chang,[1] Yikai Huang,[1] Kaitlin To,[1] Ryan Scott Whitlock,[1] Katelyn Uyen Nguyen,[1] Michelle Clara Joemon,[1] Miranda Lopez,[1] Kritin Guy Deeprompt,[1] Toshi Shioda,[2] and Bruce Blumberg[1,3]

[1]Department of Developmental and Cell Biology, University of California, Irvine, CA 92697-2300, USA
[2]Center for Cancer Research, Massachusetts General Hospital, Charlestown, MA 02129, USA
[3]Department of Biomedical Engineering, University of California, Irvine, CA 92697-2300, USA

Correspondence: Bruce Blumberg, PhD, Department of Developmental and Cell Biology, 2011 Biological Sciences 3, University of California Irvine, Irvine, CA 92697, USA. Email: blumberg@uci.edu; or Toshi Shioda, PhD, Krantz Family Center for Cancer Research, Massachusetts General Hospital, Bldg 149, 13th St, Charlestown, MA 02129, USA. Email: tshioda@mgh.harvard.edu.

Abstract

Obesity is a global health crisis, with increasing evidence linking environmental factors such as exposure to endocrine-disrupting chemicals (EDCs) to its development. This study examines the transgenerational effects of exposure to the model obesogen, tributyltin (TBT), on obesity and metabolic health, specifically focusing on how these effects interact with a diet modeling the 50th percentile of US dietary consumption [the Total Western Diet (TWD)]. Pregnant F0 dams were exposed to TBT, and their offspring were subjected at adulthood to different diets, including a high-fat diet and TWD, across multiple subsequent generations (F1-F3). We found that TBT exposure predisposed male offspring to increased fat accumulation, insulin resistance, and metabolic dysfunction, effects that were exacerbated by the TWD. Notably, male offspring displayed elevated leptin levels, hepatic fibrosis, and inflammatory responses under TWD exposure, suggesting an additive or synergistic relationship between obesogen exposure and dietary fat intake. These transgenerational effects were largely absent in female offspring, underscoring sex-specific vulnerabilities to environmental and dietary factors. Our results demonstrated that the combination of prenatal TBT exposure and TWD amplifies metabolic disturbances across generations, highlighting the need to consider both environmental chemicals and dietary patterns in addressing the obesity pandemic. This study underscores the critical role of early-life EDC exposures and dietary factors in shaping long-term metabolic health and the potential for transgenerational programming of susceptibility to obesity and metabolic disorders.

Key Words: obesogen, tributyltin, TBT, transgenerational inheritance, metabolic dysfunction, Western diet diet-induced obesity

Obesity is a significant public health challenge, adding more than $200 billion annually to healthcare costs in the United States (1). Over 42% of US adults were classified as obese, and the pandemic disproportionately affects certain populations, including African Americans, Hispanics, and women (1, 2). The prevalence of obesity has continued to rise despite increased physical activity levels and stable caloric intake, indicating that additional factors beyond diet and exercise are contributing to the obesity epidemic (3, 4). One emerging factor is the role of early-life exposure to endocrine-disrupting chemicals, which can reprogram metabolic pathways and predispose individuals to obesity and related disorders (5, 6).

The obesogen hypothesis posits that specific endocrine-disrupting chemicals, such as tributyltin (TBT), promote adipogenesis and disrupt metabolic homeostasis, contributing to obesity (7, 8). Due to its toxic effects on marine ecosystems and potential health risks, TBT was globally banned in 2008 under the International Maritime Organization Anti-Fouling Systems Convention. However, TBT remains approved for other uses, and its persistence in the environment and continued bioaccumulation pose ongoing exposure risks (9). Previous research has demonstrated that prenatal TBT exposure biases mesenchymal stem cells toward the adipose lineage, leading to increased fat accumulation and metabolic dysfunction in multiple generations (10, 11). However, the interaction between TBT-induced metabolic reprogramming and dietary factors, particularly the high-fat, energy-dense Western diet, remains poorly understood.

The Total Western Diet (TWD) models the 50th percentile of macro- and micronutrient composition in the US diet, providing a relevant model for studying how diet modifies the effects of environmental obesogens (12). Emerging evidence suggests that the combination of obesogen exposure and a Western diet could have additive or synergistic effects, promoting greater fat accumulation and metabolic disturbances than either factor alone. Our recent findings indicated that TBT exposure not only increased adiposity but also led to hepatic fat accumulation, disturbances in brown adipose tissue, and a "thrifty phenotype" in which calories were more readily stored than expended, even during fasting conditions (10, 11).

This study aimed to investigate the molecular mechanisms underlying the interaction between transgenerational effects

Received: 4 November 2024. **Editorial Decision:** 28 March 2025. **Corrected and Typeset:** 11 April 2025

of prenatal TBT exposure and the TWD. Specifically, we hypothesized that TBT exposure would sensitize animals to increased dietary fat, exacerbating metabolic dysfunction across generations. By focusing on this critical interplay between obesogen exposure and diet, we aim to uncover novel insights into the mechanisms driving transgenerational obesity and metabolic health risks. Understanding the potentially interacting effects of environmental chemicals and poor dietary patterns is essential for addressing the obesity pandemic.

Here, we investigate the transgenerational impacts of prenatal and early-life TBT exposure on obesity and metabolic health in mice subjected to a human-relevant diet, the TWD. Given concerns about environmental obesogens and their interaction with diet, this study aims to determine how ancestral TBT exposure modulates metabolic outcomes across generations. We also explore potential sex-specific differences and alterations in metabolic hormone and cytokine regulation. Understanding these interactions is critical for developing comprehensive prevention strategies that address both environmental and lifestyle factors.

Materials and Methods

Chemicals and Reagents

TBT, dexamethasone, and insulin were obtained from Sigma-Aldrich (St. Louis, MO). Blood glucose meter kits (BG1000) were procured from Clarity Diagnostics (Boca Raton, FL). Mouse Leptin ELISA Kit (#90030, RRID: AB_2722664), mouse C-peptide ELISA kit (#80954, RRID: AB_3677465), and mouse insulin ELISA kit were sourced from Crystal Chem (Elk Grove Village, IL).

Animal Maintenance and Exposure

C57BL/6J mice were acquired from the Jackson Laboratory (Sacramento, CA) and housed in micro-isolator cages under controlled environmental conditions. The housing facility maintained a temperature-controlled environment (23-24 °C) with a 12-hour light/dark cycle. Mice were provided ad libitum access to water and food unless specified otherwise. All procedures involving animal subjects were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Measures were taken to ensure humane treatment and minimize suffering throughout the study.

For this transgenerational experiment, designated as T5, a total of 65 male and 185 female C57BL/6J mice (5 weeks of age) were procured. Female mice (92 per treatment group) were randomly allocated to different F0 treatment groups and exposed to 50 nM TBT or 0.1% dimethyl sulfoxide (DMSO) vehicle (both diluted in 0.5% carboxymethyl cellulose in water to maximize solubility) for 7 days prior to mating as we have described previously (11). This dose is within the range of environmentally relevant exposures and has been widely used in previous metabolic studies of TBT. Breeding was facilitated by housing 1 male with 2 vehicle or TBT-exposed F0 females per cage during the dark cycle. Vaginal plug appearance was defined as embryonic day (E) 0.5. Chemical treatment was removed during mating then resumed for F0 females after copulation plugs were detected (and males removed), then maintained until the pups were weaned at postnatal day 21. The selected TBT concentration was based on prior studies (10, 11); notably, this is 5-fold lower than the established no observed adverse effect level for rodents (13).

Chemical exposures during gestation in multiparous animals can be confounded by litter effects. These are controlled by using the entire litter as the "n" or by selecting 1 male and 1 female per litter as representative. Litter size and sex ratio can affect growth trajectories and subsequent body composition leading to litter effects when assessing metabolic endpoints such as obesity (14). We avoid this by controlling litter sizes and rejecting those with fewer than 6 or more than 8 pups and with fewer than 2 members of 1 sex. We tested litter size and sex distribution in each generation to identify potential litter effects and found none. From each generation, we randomly chose only 1 male and 1 female per litter for endpoint analysis and another 1 male and 1 female per litter for breeding to produce the next generation. To randomize the breeding process and avoid selection bias, we did not breed siblings and never bred females from the same litter with the same male. Control animals were bred within the control group, and TBT-exposed animals were paired within the treatment group.

Diet Challenge and Body Composition Analysis

Animals from control and treatment groups were initially maintained on a standard diet (PicoLab 5053, 24.5% Kcal from protein, 13.1% Kcal from fat, and 62.3% Kcal from carbohydrates) from weaning onward, with body weight and composition monitored weekly using EchoMRI™ Whole Body Composition Analyzer. Daily food intake was recorded for all groups to assess potential differences in caloric intake. No significant differences were observed between TBT-exposed and control groups. Subsequently, F1, F2, and F3 descendants underwent a diet challenge in 2 separate arms. In the first, animals were transitioned to a higher-fat diet (PicoLab 5058, 20.3% Kcal from protein, 21.6% Kcal from fat, and 58.1% Kcal from carbohydrates) for 6 weeks while controls were maintained with ad libitum food access to a standard diet. In the second arm, the animals were transitioned to fully synthetic diets. Half of the animals were fed with the TWD (Envigo TD.160422; 17.3% Kcal from protein, 40.4% Kcal from fat, and 42.3% Kcal from carbohydrates) for 6 weeks while control groups were maintained on ad libitum food access to AIN-93M (Envigo TD.94048; 14% Kcal from protein, 10% Kcal from fat, and 76% Kcal from carbohydrates). TWD models the 50th percentile of the US dietary intake of micro- and macronutrients (12), whereas AIN-93M is a standard synthetic rodent diet. Weekly assessments continued to track changes in body composition, with 12-hour fasting conducted prior to euthanasia and tissue collection. To minimize variability, all animals were housed under controlled conditions, with males and females housed separately. Tissue collection was performed between 8:00 and 10:00 AM for all animals to reduce potential circadian influences on metabolic parameters. While we did not monitor the estrous cycle in female mice, prior studies suggest that standardizing collection time mitigates fluctuations in metabolic and hormonal endpoints. Blood was collected via the saphenous vein at week 12 and week 20 (before and after diet challenge) for F1 and at weeks 12 and 18 for F2, F3, and F4. Blood was collected into heparinized tubes and then centrifuged for 15 minutes at 5000 RPM at 4 °C. Resulting plasma was transferred to a clean tube and preserved at −80 °C.

Tissue Histology

Samples of gonadal white adipose tissue (gWAT) and liver of male mice were sent to the University of California Irvine Experimental Tissue Resources for processing. The tissues underwent paraffin embedding, sectioning, and staining with Masson's trichrome or hematoxylin and eosin. Paraffin-embedded tissues were sectioned at 5 μM, and after standard processing, the sections were stained with Masson's trichrome for visualizing collagen fibers, nuclei, and cytoplasm. Examination under a light microscope at 20× magnification allowed for the documentation of adipocyte droplet size, potential fibrosis, and other histological features. This standardized methodology ensured the acquisition of high-quality tissue sections suitable for detailed histological analysis, enabling investigation into the transgenerational effects of TBT exposure on adipose tissue dynamics.

Scoring of Liver and Fat Histology for Lipid Accumulation, Fibrosis and Inflammation

Liver tissues were sectioned and stained for histological examination to assess liver fibrosis and injury. An experienced pathologist (R. Edwards), blinded to the treatment groups, performed the scoring to ensure unbiased evaluation. Histological scoring remains the gold standard in metabolic disease and toxicology research, particularly in assessing fibrosis and lipid accumulation in liver tissues (15, 16). Each slide was evaluated on a semiquantitative scale from 0 to 3, where 0 indicated no injury or fibrosis, 1 represented mild injury or fibrosis, 2 indicated moderate injury or fibrosis, and 3 denoted severe injury or fibrosis. The parameters scored included steatosis, cytoplasmic vacuolization, and fibrosis in intraportal/periportal, perivascular, and intralobular/intercellular regions. Both the F1 and F2 generation slides were scored using the same criteria. Data were collated into 2 sheets, Raw Data and Summary, ensuring a comprehensive analysis of liver histopathology across generations.

Glucose and Insulin Tolerance Tests

Glucose and insulin tolerance assessments were conducted following published guidelines (17). In brief, during the intraperitoneal glucose tolerance test, animals were subjected to a 12-hour fasting period overnight in unused cages to minimize feces reuptake. Blood samples were collected from the tail vein. Subsequently, glucose was administered intraperitoneally at 2 g/kg body weight; then blood samples were obtained at specified time intervals and measured by glucometer.

In the intraperitoneal insulin tolerance test, animals were tested in a fed state. Soluble insulin was intraperitoneally injected at a dosage of 0.75 IU/kg body weight, followed by blood sample collection from the tail vein. Blood glucose levels were determined using a glucometer. Notably, the phase of the estrous cycle was not monitored during the execution of the intraperitoneal glucose tolerance test or intraperitoneal insulin tolerance test.

Quantitative Real-time RT-PCR

Tissue samples, previously snap-frozen in liquid nitrogen, were cut into approximately 20 mg pieces and lysed using Trizol following the manufacturer's recommended protocol (Thermo Fisher Scientific, MA). Total RNA was recovered after isopropanol precipitation (Fisher Chemical, PA). Complementary DNA was synthesized from 5 μg of total RNA utilizing the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) following the manufacturer-recommended instructions. Gene expression analysis was conducted via real-time quantitative PCR (qPCR) employing SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) on a Roche LightCycler 480 II system (Roche, Switzerland). Primer sequences for the target genes are listed in Supplementary Fig. S1 (18). Cycle threshold values were determined as the second derivative maximum utilizing LightCycler software (Roche, Switzerland). Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method (19), adjusted for primer efficiency (20). The expression of genes studied in this article was normalized to the housekeeping gene *GAPDH* and compared to the DMSO descendant group. Error bars indicate the SEM calculated from 12 biological replicates, employing standard propagation of error (21).

Measurement of Blood Cytokine Levels

Serum levels of leptin were quantified using enzyme immunoassay (Crystal Chem #90030; Elk Grove Village, IL) at 2 distinct time points (pre- and postdiet challenge) in plasma derived from blood samples collected after a 12-hour overnight fast. Similarly, insulin levels were assessed via enzyme immunoassay (Crystal Chem #90080) in plasma obtained from blood samples collected following a 12-hour overnight fast. To further study the profile of serum cytokine, a MILLIPLEX® Multiplex Assays customized kit was used and ghrelin, glucan-like peptide-1 (GLP-1), glucagon, IL-6, resistin, TNF, leptin, and insulin were measured using 50 uL serum collected at the end point of the diet challenge.

Results

Transgenerational Effects of TBT Exposure on Body Fat Accumulation and Interaction With Diet

Pregnant F0 dams were exposed to 50 nM TBT or DMSO vehicle via drinking water throughout pregnancy and lactation (Fig. 1A). We selected 50 nM TBT based on previous studies demonstrating its obesogenic effects in mammalian models (10, 22, 23). Offspring were divided into 2 experimental arms to assess body fat accumulation in response to different diets. In the first arm, mice were placed on either a control chow diet (5053) or a higher-fat chow diet (5058) at 12 weeks of age, and body composition was measured weekly using EchoMRI. TBT-exposed males exhibited significant increases in body fat under the high-fat diet, while DMSO controls showed no such effect (Fig. 1B–1D). These findings confirm and extend our previous studies demonstrating male-specific susceptibility to obesity following gestational and lactational TBT exposure across multiple transgenerational experiments (10, 11, 24).

In the second arm, we examined the impact of a synthetic, human-relevant diet, the TWD, compared to the synthetic AIN93M control diet. A priori power analysis determined a group size of 16 per condition. TBT-exposed males on the TWD exhibited significantly greater fat accumulation (~28% body fat) than those on the high-fat chow diet (~20%) (Fig. 1E–1G). Notably, even DMSO control males displayed increased adiposity on TWD compared to AIN93M, indicating a dietary effect independent of TBT exposure. Food intake did not differ significantly between groups, suggesting that the

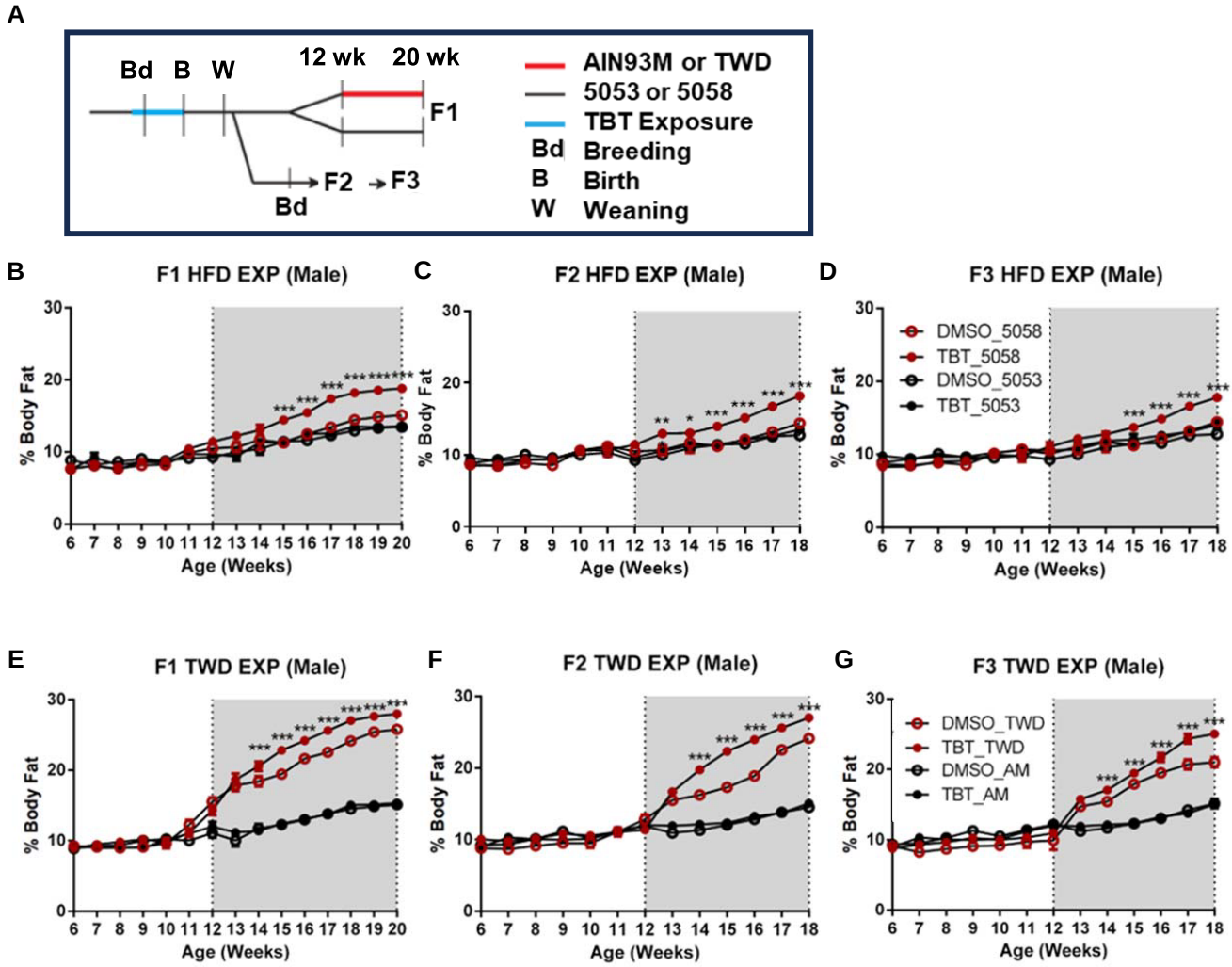

Figure 1. The consistent increase in body fat among male offspring exposed to TBT following different types of diet challenge. (A) Pregnant F0 dams received either DMSO vehicle or 50 nM TBT throughout pregnancy and lactation. Offspring were divided into 8 groups, with 1 male and 1 female per litter subjected to the dietary challenge. (B-D) Regular chow diet (5053) vs higher-fat diet (5058) and (E-G) control diet (AIN93M) vs TWD. Only male data are presented because female offspring did not exhibit significant changes in body fat across any diet condition, irrespective of ancestral TBT exposure. Panels B-D depict 12 animals per sex exposed to either the control or high-fat diet from 12 weeks of age, while panels E-G show 16 animals per group exposed to control or TWD high-fat diet. Weekly measurements of body composition and weight were conducted, and body fat was normalized to body weight for each animal. Statistical significance was determined using 2-way ANOVA, with pair-wise Bonferroni post hoc tests applied to compare different groups across all panels. Data are presented as mean ± SEM, with $*P < .05$, $**P < .01$, and $***P < .001$ indicating significance. Error bars are shown for all data points but may not be visible at certain time points due to minimal variation in body weight measurements among individual animals.

Abbreviations: DMSO, dimethyl sulfoxide; TBT, tributyltin; TWD, Total Western Diet.

observed increases in body fat in TBT-exposed males were not due to increased caloric intake. No differences in body fat accumulation were observed in females across any conditions (Supplementary Fig. S2) (18). Given its relevance to human dietary patterns, subsequent experiments focused on TWD vs AIN93M comparisons.

Impact of TBT Exposure and Dietary Patterns on Leptin Levels and Metabolic Phenotypes

In previous studies, we demonstrated that TBT-exposed animals exhibited elevated circulating leptin levels when subjected to a higher-fat chow diet (~21% Kcal from fat) (10, 11, 24). This increase in leptin levels in the presence of obesity is commonly considered to be indicative of leptin resistance in clinical settings (25). Leptin resistance, characterized by diminished response to leptin signaling, is associated with various metabolic disorders (26). To investigate the potential presence of leptin resistance in TBT-exposed animals exposed to the TWD, we assessed leptin levels before and after the diet challenge (Fig. 2). Under the AIN diet, there were no significant differences in plasma leptin levels between the DMSO and TBT groups. However, in the TWD group, males exhibited markedly elevated plasma leptin levels, with significant differences observed between the DMSO and TBT offspring (Fig. 2A–2C). Plasma leptin levels were higher in DMSO group animals on the TWD diet compared to the AIN diet. Interestingly, despite the increased leptin levels elicited by the diet challenge, we did not observe significant differences in food intake between the DMSO and TBT groups in any generation throughout the diet challenge period (Fig. 2D–2F). In contrast to the situation in males, no significant differences in plasma leptin levels or food intake were observed in females across the same conditions and generations (Supplementary Fig. S3) (18).

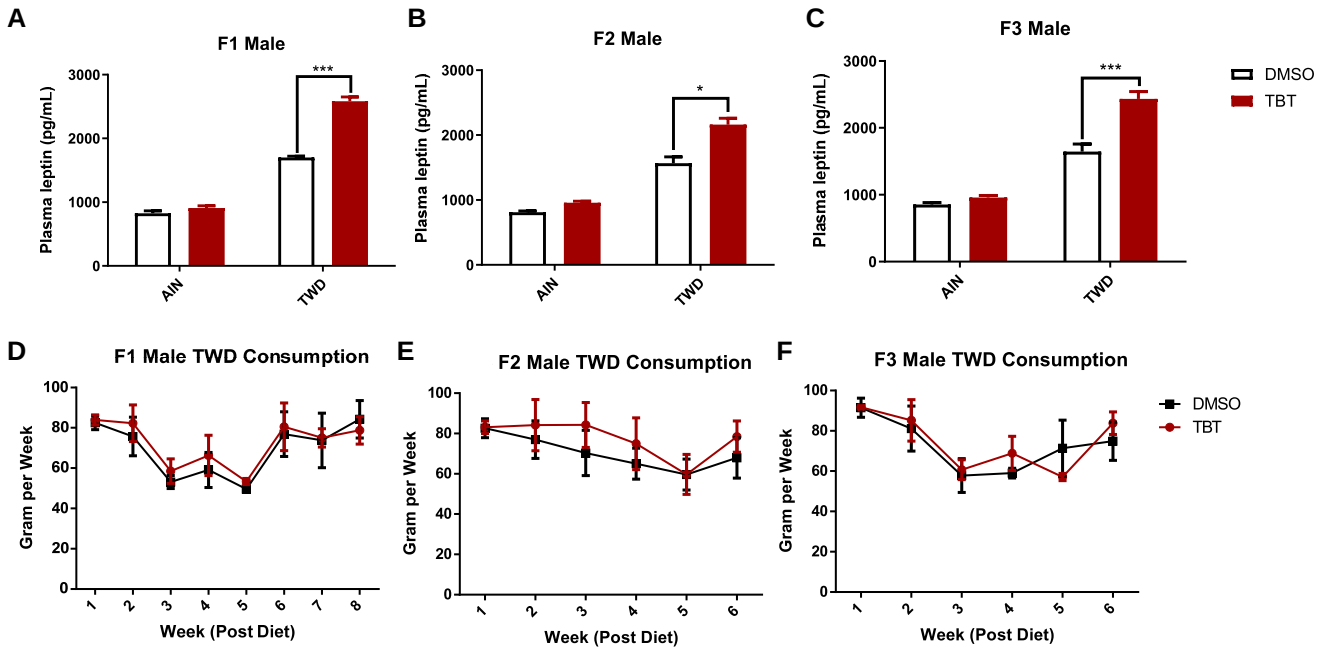

Figure 2. TBT increased plasma leptin levels in T5 male mice, despite no significant change in food intake. Plasma samples were collected before and after the diet challenge at different time points (F1: 8 weeks, F2 and F3: 6 weeks), and leptin levels were measured using enzyme immunoassay for (A) F1, (B) F2, and (C) F3. Weekly food intake was assessed for (D) F1, (E) F2, and (F) F3, calculated twice per week and presented as the weekly intake per animal throughout the entire diet challenge period. Statistical analysis was conducted using 2-way ANOVA for panels A and B. Pair-wise Bonferroni posttests were employed to compare different groups across all panels. The data are depicted as mean ± SEM, with significance denoted as $*P < .05$, $**P < .01$.

Abbreviation: TBT, tributyltin.

Transgenerational Impact of TBT Exposure and TWD on Hepatic Fat Accumulation, Fibrosis and Inflammation

Obesity is strongly linked to an elevated risk of metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease (27). MASLD is characterized by hepatic steatosis resulting from excessive fatty acid uptake and de novo fatty acid synthesis. We assessed liver phenotypes to examine the impact of TBT and TWD on hepatic fibrosis (Fig. 3A–3D), lipid accumulation (Fig. 3E–3H), expression of inflammatory TNF (Fig. 3I, 3J), and expression of mRNAs encoding various markers of fibrosis, adipogenesis, and inflammation (Fig. 3K–3M).

Fibrosis was evaluated using Masson's trichrome staining (Fig. 3A) Histologically, animals exposed to the AIN93M diet exhibited minimal to no detectable fibrosis in the liver in either vehicle or TBT groups. In contrast, TWD-treated animals displayed increased fibrosis in both vehicle and TBT groups, with increased fibrosis in TBT compared with vehicle animals (Fig. 3B–3D). Additionally, the TWD diet caused detectable fibrosis in the liver of DMSO-treated animals compared to those on the AIN diet. Hepatic lipid accumulation was evaluated using hematoxylin and eosin and Masson's trichrome staining (Fig. 3E). Livers were scored as 1 = no obvious lipid vesicles, 2 = small vesicles, 3 = many vesicles but equal to or smaller than nuclei, and 4 = many vesicles larger than nuclei (Fig. 3E). There were no detectable lipid vesicles in vehicle or TBT groups in the AIN group, whereas both vehicle and TBT groups showed a significant increase in lipid vesicle size and number in TWD group male animals. TBT exposure significantly exacerbated both the number and size of lipid droplets, many of which were substantially larger than the nuclei in the TWD-treated animals (Fig. 3F–3H). Thus, we inferred that TWD exposure exacerbated hepatic lipid accumulation promoted by TBT exposure, consistent with our previous findings on a chow diet (10). We measured hepatic TNF protein levels to evaluate liver inflammation and found increased TNF levels in TBT F1, F2, and F3 offspring challenged by the TWD (Fig. 3J) but not the AIN93M control diet (Fig. 3I). Gene expression analysis revealed significant upregulation of fibrotic genes including collagen 1A1 (*Col1a1*), collagen 3A1 (*Col3a1*), collagen 5A1 (*Col5a1*), and collagen 6A1 (*Col6a1*) and lipogenic genes including lipase E (*Lipe*), lipoprotein lipase (*Lpl*), fatty acid binding protein 4 (*Fabp4*), and peroxisome proliferator activated receptor gamma (*Pparg2*), as well as inflammatory genes including TNF (*Tnf*) and IL-1 β (*Il1b*) in TBT animals treated with TWD compared to controls in F1 (Fig. 3K), F2 (Fig. 3L), and F3 (Fig. 3M) generation male animals. There were no such effects in females (data not shown). Collectively, these results indicate that direct or ancestral TBT exposure can elicit liver damage when animals are exposed to TWD. This damage is reflected by increased fibrosis, larger lipid droplet size and number, and elevated inflammatory gene expression and cytokine levels. This effect was male-specific, as no differences were detected in the female groups.

TBT Exposure and TWD Intervention Altered Expression of Adipose Tissue Marker Genes

To characterize the impact of TBT exposure on adipocyte gene expression, we utilized qPCR to analyze mRNA levels

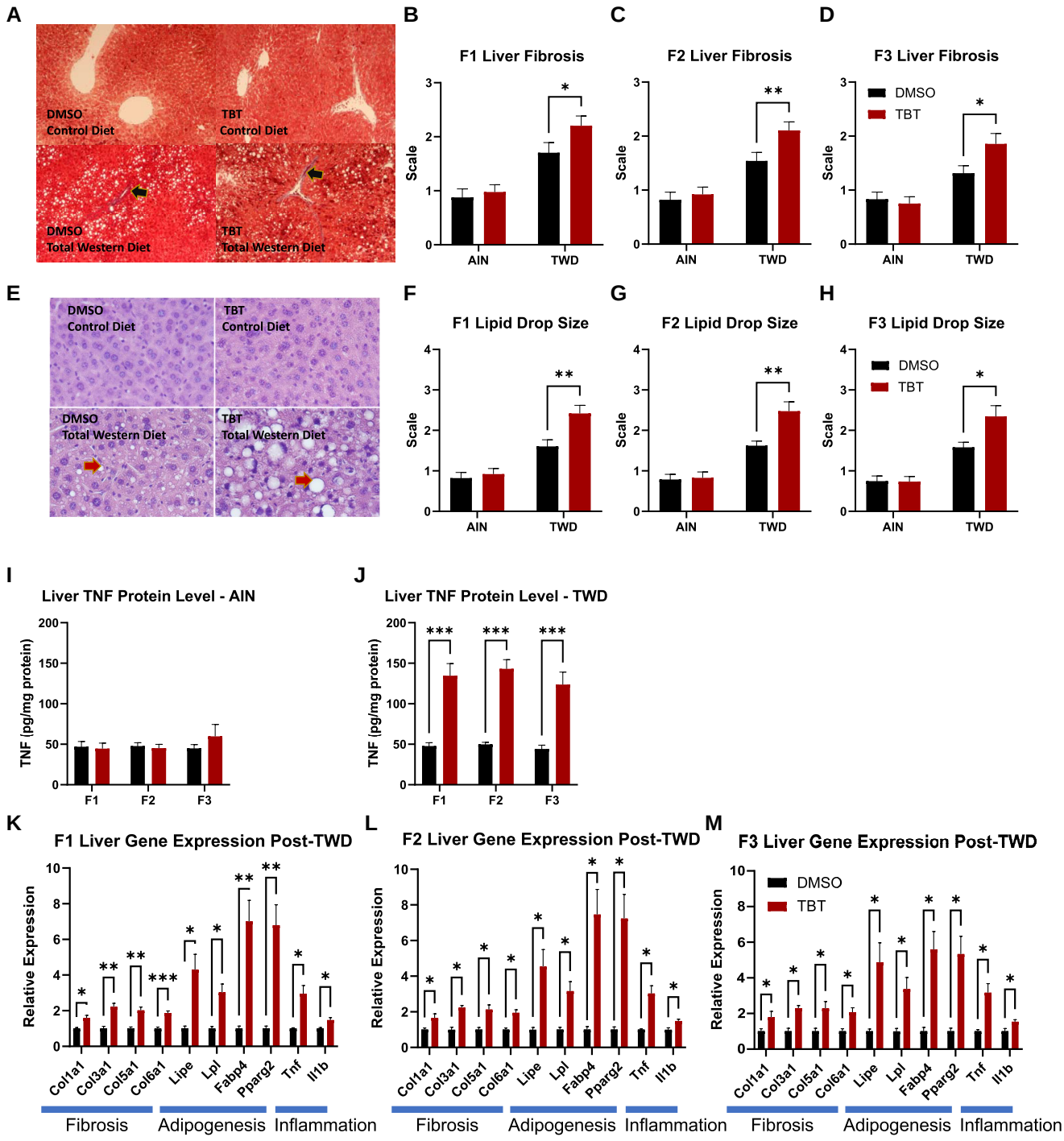

Figure 3. The impact of the TWD diet challenge on hepatic stress, which was exacerbated by TBT exposure. (A-D) Effect on hepatic fibrosis. (A) Masson's trichrome staining highlighted fibrosis, with blue staining indicating fibrotic regions. (B-D) Quantitative analysis of fibrosis in F1-F3 male animals as analyzed by an experienced histologist blinded to the experimental groups. (E-H) Effects on hepatic lipid storage. (E) H&E staining revealed lipid droplets, indicated by white holes (red arrows). TBT + TWD treatment notably increased the size and quantity of lipid droplets compared to DMSO + TWD and the control diet (AIN93M). (F-H) The size and number of droplets were evaluated by 3 blinded investigators, showing statistically significant differences. (I, J) ELISA analysis of TNF protein levels. (I) Expression of hepatic TNF protein levels in AIN-93M group animals. (J) Expression of hepatic TNF protein levels in TWD group animals. (K-M) mRNA expression of marker genes for fibrosis (*Col1a1*, *Col3a1*, *Col5a1*, *Col6a1*), adipogenesis (*Lipe*, *Lpl*, *Fabp4*, *Pparg2*), and inflammation (*Tnf*, *Il1b*). Statistical significance was determined using a *t*-test. Data are presented as mean ± SEM, with significance denoted as $*P < .05$, $**P < .01$, $***P < .001$.

Abbreviations: DMSO, dimethyl sulfoxide; H&E, hematoxylin and eosin; TBT, tributyltin.

of marker genes specific for beige (*Teme26*, *Cd40*, *Cd137*), white (*Fabp4*, *Lpl*, *Fsp27*), and brown (*Ebf2*, *Pdk4*, *Zic1*) adipocytes (Fig. 4A) across multiple generations (28).

In gWAT from F1, F2, and F3 mice, qPCR analysis revealed a significant upregulation of white adipocyte-specific genes (*Fabp4*, *Lpl*, *Fsp27*) in the TBT-exposed groups compared

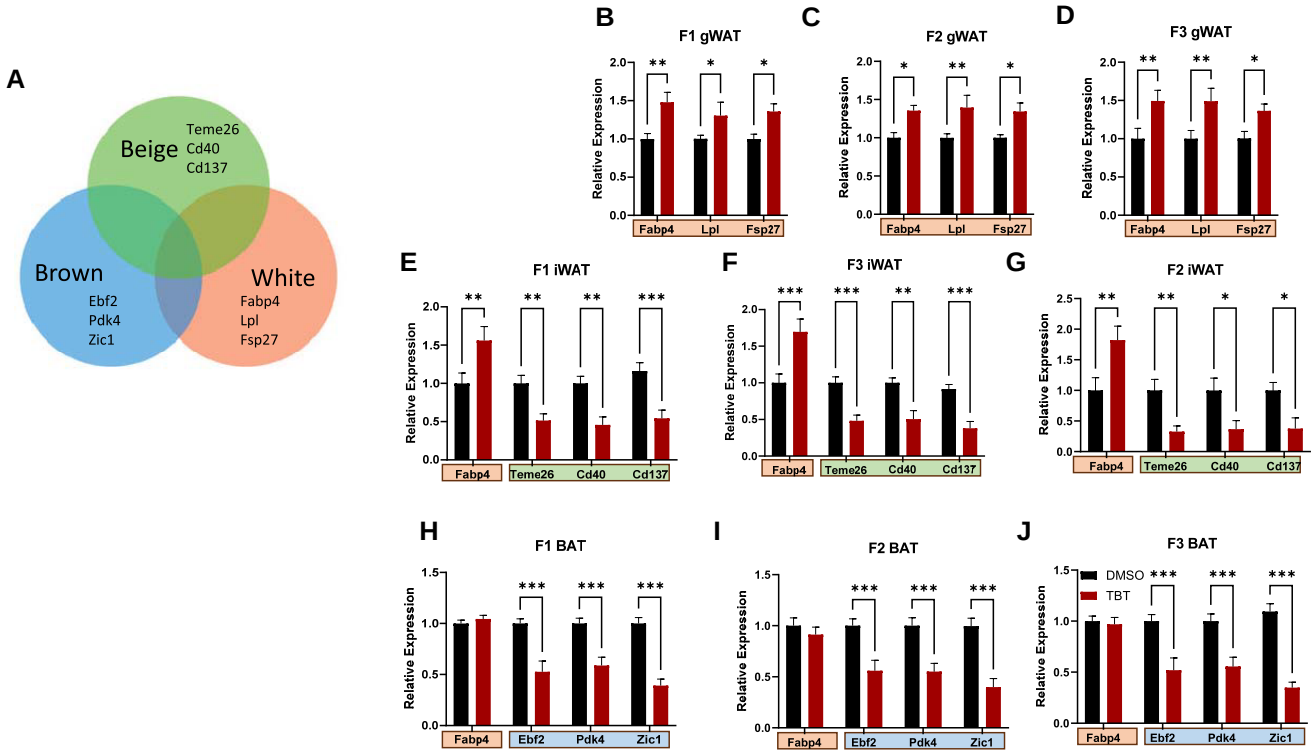

Figure 4. Impact of TBT in combination with different diets on marker gene expression in different adipose tissue types. (A) Venn diagram showing the overlap of differentially expressed genes in beige, brown, and white adipose tissues, identifying unique and shared gene sets between the groups. (B-D) qPCR analysis of WAT genes: *Fabp4*, *Lpl*, and *Fsp27* in gWAT of F1, F2, and F3 offspring, respectively, comparing DMSO and TBT-exposed groups. (E-G) qPCR analysis of beige genes (*Teme26*, *Cd40*, *Cd137*) and the white gene *Fabp4* in iWAT of F1, F2, and F3 offspring, respectively, comparing DMSO and TBT-exposed groups. (H-J) qPCR analysis of BAT genes (*Ebf2*, *Pdk4*, *Zic1*) and the white adipose gene *Fabp4* in BAT of F1, F2, and F3 offspring, respectively, comparing DMSO and TBT-exposed groups. Data are expressed as mean ± SEM. Significant differences between DMSO and TBT groups are indicated ($P < .05$, $P < .01$, $^*P < .001$).

Abbreviations: BAT, brown adipose tissue; DMSO, dimethyl sulfoxide; gWAT, gonadal white adipose tissue; iWAT, inguinal white adipose tissue; TBT, tributyltin; WAT, white adipose tissue.

to controls (Fig. 4B–4D). This consistent increase suggests that the number of white adipocytes has increased in gWAT, consistent with our previous results (10). Beige adipocytes are typically found in the inguinal white adipose tissue (WAT); therefore, we assessed the expression of beige markers in inguinal WAT, together with *FAB4* as a WAT control (Fig. 4E–4G). As was the case for gWAT, *FABP4* expression increased, indicating that the number of white adipocytes was likely increased. However, beige-specific markers (*Teme26*, *Cd40*, *Cd137*) decreased in the TBT group, indicating that the development of beige adipocytes was likely to be impaired.

Lastly, analysis of brown adipose tissue from F1, F2, and F3 animals (Fig. 4H–4J) demonstrated a sharp reduction in levels of mRNA encoding brown adipocyte markers (*Ebf2*, *Pdk4*, *Zic1*) in TBT-exposed offspring, while *Fabp4* expression levels were low and remained unchanged. This indicates that TBT exposure adversely affects brown adipocyte gene expression. Overall, our findings suggest that TBT exposure promotes increased WAT depot size, accompanied by a decrease in the size, number, and/or function of thermogenic beige and brown adipocytes.

Assessment of Glucose Tolerance and Insulin Sensitivity in TBT-exposed Offspring

Obesity is closely linked to the development of insulin resistance and type 2 diabetes. To evaluate potential glucose intolerance or insulin resistance in these animals, we conducted glucose tolerance tests and insulin tolerance tests. These well-established assays assess insulin receptor sensitivity by monitoring changes in blood glucose levels before and after glucose or insulin administration. Our findings revealed no significant differences in glucose tolerance between the vehicle control (DMSO) or TBT offspring from F1 to F3 following glucose administration (Fig. 5A–5C). Despite optimizing the insulin dose in preliminary experiments, analysis of F1 blood glucose data during the insulin tolerance test revealed that both DMSO and TBT F1 offspring maintained blood glucose levels at approximately 80% of fasting glucose levels after insulin administration (Fig. 5D). This indicated the need for further insulin dose optimization. Subsequently, we adjusted the insulin dose, standardizing it to an increase from 1 U/kg to 1.5 U/kg starting with the F2 animals. Administering 1.5 U/kg resulted in a considerable reduction in blood glucose levels after insulin administration (Fig. 5E and 5F). The F2 and F3 TBT group offspring exhibited a weaker decrease in blood glucose levels after insulin administration, suggesting insulin resistance (Fig. 5E and 5F). This observation was further supported by calculating the area under the curve, which demonstrated significantly reduced insulin sensitivity in both F2 and F3 males (Fig. 5E and 5F). These findings aligned with our previous report of elevated blood insulin levels due to impaired insulin clearance

in animals from a previous transgenerational experiment. Considering that F3 male mice exhibited decreased insulin sensitivity together with significant increases in body fat, it is plausible that insulin insensitivity may be associated with obesity in these animals. This may be due, in part, to decreased expression of insulin-degrading enzymes.

We next studied insulin clearance by measuring plasma insulin levels after either glucose or insulin injection. Glucose was injected to stimulate intrinsic insulin secretion, and the results showed that elevated intrinsic insulin levels were detected at 15 minutes post-glucose injection (Fig. 5G–5I). The TBT group maintained significantly increased plasma insulin levels even after 120 minutes post-glucose injection (Fig. 5G–5I for F1, F2, F3 mice, respectively). Following the injection of recombinant insulin, we observed an increase in plasma insulin at 15 minutes postinjection, with the TBT animals maintaining higher plasma insulin levels after 120 minutes (Fig. 5J–5L for F1, F2, F3 offspring, respectively). These results suggest a decrease in insulin clearance of both intrinsic and injected insulin, consistent with impaired expression of insulin-degrading enzyme and reduced insulin clearance. Overall, the data showed that TBT-exposed animals exhibited impaired insulin clearance after stimulation with either glucose or insulin.

Transgenerational Effects of TBT Exposure: Altered Expression of Metabolic Hormones and Cytokines in F3 Offspring

We examined the impact of ancestral TBT exposure on F3 offspring, which were derived from F0 dams exposed to TBT throughout pregnancy and lactation. F1 animals were exposed to TBT in utero and while nursing. There was no subsequent TBT exposure in the F2 or F3 generations. The serum samples were collected and analyzed using the Millipore multiplex kit to assess levels of various metabolic hormones and cytokines. These included ghrelin, GLP-1, glucagon, IL-6, TNF, leptin, insulin, and resistin (Fig. 6). Comparisons between the TBT-exposed and control groups revealed significant alterations in hormone and cytokine levels. Specifically, mice in the TBT group exhibited significantly decreased levels of ghrelin and glucagon compared to the control group (Fig. 6A and 6B). Levels of TNF, leptin, IL-6, insulin, and resistin were significantly increased in the TBT-exposed mice compared to controls (Fig. 6C–6G), whereas GLP-1 levels did not change significantly (Fig. 6H). These findings suggest that transgenerational TBT exposure may induce substantial changes in circulating levels of key metabolic hormones and cytokines, potentially contributing to metabolic dysregulation in the exposed offspring (Fig. 7). In contrast, there were no detectable changes in any of these hormones or cytokines in the female groups (Supplementary Fig. S4) (18).

Discussion

Our study delved into the transgenerational effects of TBT exposure on body fat accumulation and its interaction with a human-relevant diet, the TWD. We exposed pregnant F0 dams to either DMSO vehicle or 50 nM TBT during pregnancy and lactation and observed notable alterations in body fat levels in subsequent generations. Although we did not directly measure serum TBT concentrations in pregnant animals, prior studies demonstrate that TBT bioaccumulates in tissues and is transferred to offspring via the placenta and lactation (9). Male offspring exhibited significant increases in body fat upon exposure to a modestly increased fat diet (high-fat diet) in the TBT groups, while the DMSO vehicle group did not. These findings confirm and extend our earlier studies and underscore the persistent predisposition to increased body fat induced by F0 TBT exposure across multiple generations in repeated experiments. Moreover, we found that exposure to the TWD, which modeled the 50th percentile of US dietary macro- and micronutrient intake, substantially elevated body fat levels in both control and exposed groups, with TBT exposure exacerbating these effects. This indicates that a Western dietary pattern exacerbates the effects of exposure to obesogens such as TBT.

Leptin is a key hormone involved in appetite regulation and energy balance that plays a pivotal role in metabolic homeostasis (26). Our investigation into the impact of TBT exposure and dietary patterns on leptin levels and metabolic phenotypes revealed intriguing findings. While both DMSO and TBT groups exhibited markedly elevated plasma leptin levels following exposure to the TWD, the TBT group displayed particularly heightened leptin levels. These observations suggest a potential link among TBT exposure, dietary patterns, and alterations in leptin signaling pathways. Despite the significant increase in leptin levels in TBT group animals, there were no corresponding differences in food intake between the DMSO and TBT groups. This reveals the complexity of leptin regulation in the context of obesogen exposure and that further exploration into the mechanisms underlying leptin resistance and its association with transgenerational obesogen exposure and dietary patterns will be important.

Leptin levels and fibrosis markers were significantly increased in TBT-exposed males, whereas no substantial changes were observed in females. This sex-specific effect is consistent with prior studies suggesting differential metabolic responses to endocrine disruptors. Although we did not directly assess insulin or leptin receptor expression, previous research indicates that TBT exposure can disrupt insulin signaling and leptin regulation, potentially contributing to the metabolic dysfunction observed in our study (8, 10). Further investigations are needed to determine the precise mechanisms by which TBT influences these pathways.

MASLD represents a significant health concern, with obesity serving as a primary risk factor for its development (27). Our investigation into the hepatic phenotypes revealed substantial alterations induced by TBT exposure and the TWD. TBT exposure exacerbated hepatic lipid accumulation, inflammation, and fibrosis, and this effect was elevated in response to the TWD. These findings demonstrate the combined effects of obesogen exposure and the Western dietary pattern on impaired hepatic health and emphasize the importance of comprehensive interventions targeting both factors in combatting MASLD and related metabolic disorders.

Histological examination of gWAT revealed that while TBT exposure and dietary patterns influenced adipocyte morphology in male mice, with significant increases observed in adipocyte size in response to the TWD, no discernible changes were noted in female mice. Additionally, quantitative analysis of fibrosis ranking revealed consistent trends across treatment groups, suggesting minimal fibrotic alterations in the gWAT sections. These findings show the sex-specific effects of TBT exposure and dietary interventions on adipose tissue morphology and fibrosis severity.

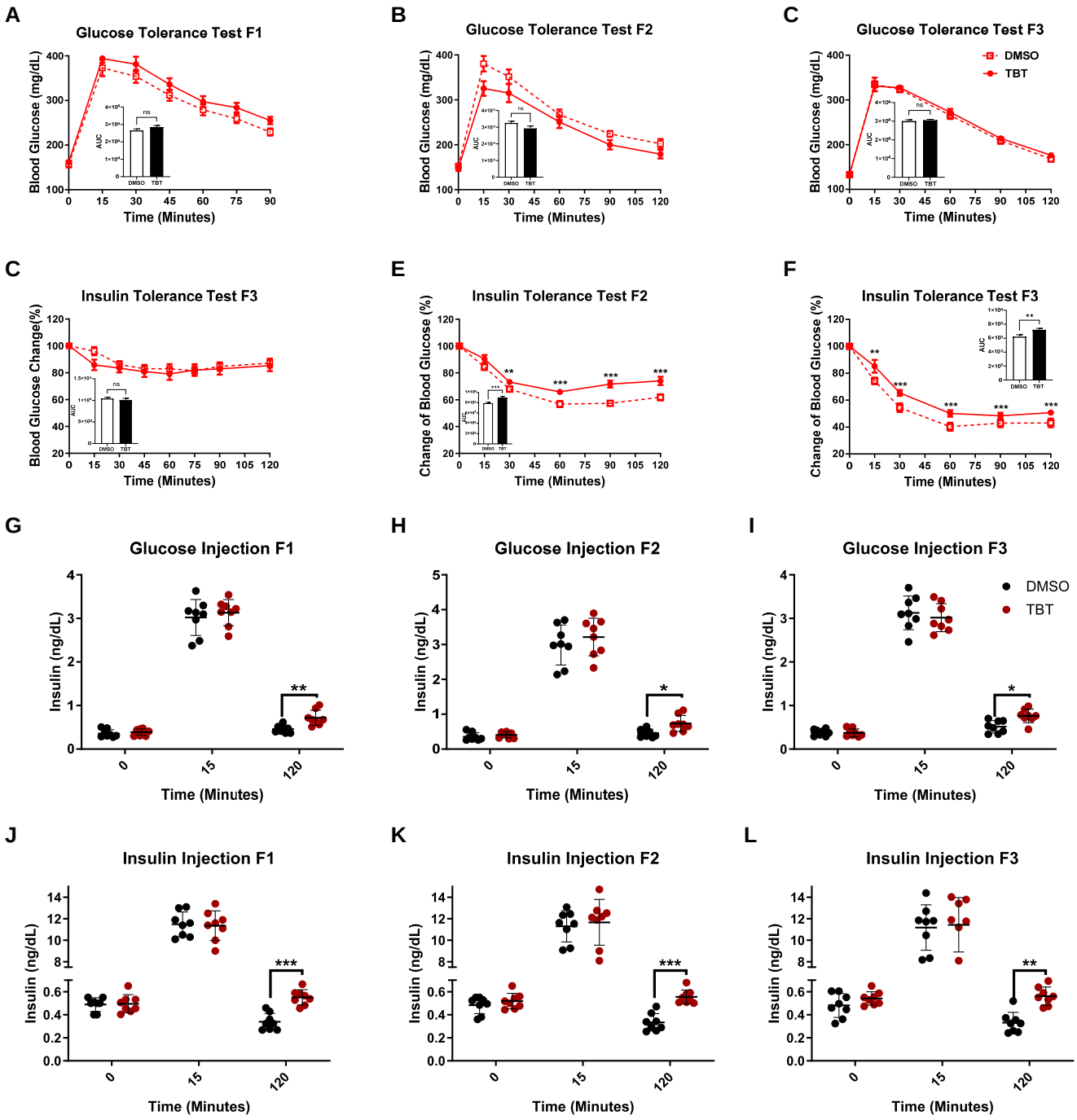

Figure 5. Assessment of insulin sensitivity and glucose tolerance in F1-F3 mice following TWD challenge. Prior to endpoint dissection, (A) F1, (B) F2, and (C) F3 animals underwent overnight fasting, followed by administration of 1.5 g of glucose/kg body mass to conduct the glucose tolerance test, with blood glucose levels measured over time. For the insulin tolerance test, (D) F1 received an injection of 1 U insulin/kg body weight, while (E) F2 and (F) F3 received 1.5 U insulin/kg body weight. The area under the curve was computed and is depicted as mean ± SEM. Statistical significance was evaluated using *t*-tests. (G-I) Insulin levels measured at 0, 15, and 120 minutes following glucose injection in F1, F2, and F3 offspring, respectively. TBT-exposed mice showed significantly lower insulin levels at the 120-minute time point compared to DMSO controls in all generations ($P < .05$, $P < .01$). (J-L) Insulin levels measured at 0, 15, and 120 minutes following insulin injection in F1, F2, and F3 offspring, respectively. TBT-exposed mice exhibited significantly reduced insulin clearance at the 120-minute time point compared to DMSO controls, particularly in F1 and F2 offspring ($P < .001$, $P < .01$). Data are expressed as mean ± SEM, with significant differences between DMSO and TBT groups indicated.

Abbreviations: DMSO, dimethyl sulfoxide; TWD, Total Western Diet.

Insulin resistance is typically coincident with obesity and metabolic dysregulation, with significant implications for the development of type 2 diabetes (29). Our evaluation of glucose tolerance and insulin sensitivity in TBT-exposed offspring revealed that while no significant differences in glucose tolerance were observed between the DMSO and TBT offspring, alterations in insulin sensitivity were noted, particularly in response to TWD exposure. These results support a potential link between TBT exposure, dietary patterns, and insulin resistance (30) and highlight the need for

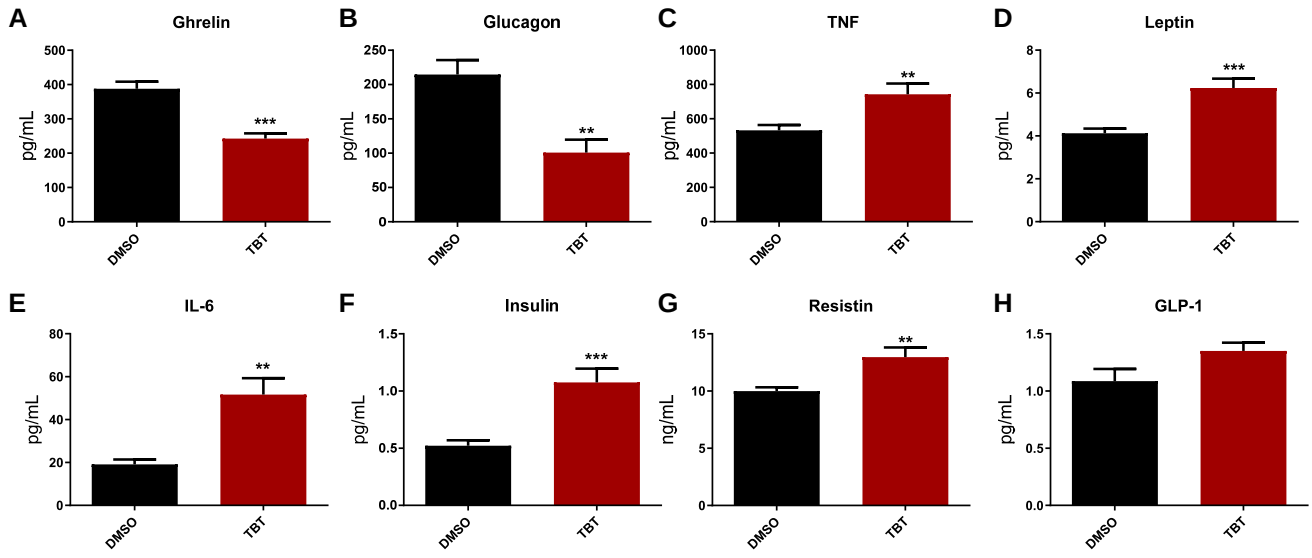

Figure 6. Impact of transgenerational TBT exposure on metabolic hormones and cytokines in F3 male offspring. Serum samples from F3 offspring were collected and analyzed using the Millipore multiplex kit to assess levels of metabolic hormones and cytokines, including (A) ghrelin, (B) glucagon, (C) TNF, (D) leptin, (E) IL-6, (F) insulin, (G) resistin, and (H) glucagon. Comparative analysis between the TBT-exposed group and the control group revealed significant alterations in hormone and cytokine levels. TBT-exposed mice exhibited significantly decreased levels of ghrelin and glucagon compared to controls (A, B), while TNF, leptin, IL-6, insulin, and resistin levels were notably increased in the TBT-exposed mice compared to controls (C-G). These findings suggest that transgenerational TBT exposure may induce substantial changes in the secretion of key metabolic hormones and cytokines, potentially contributing to metabolic dysregulation in the exposed offspring. Statistical significance was evaluated using *t*-tests. The data are depicted as mean ± SEM, with significance denoted as $*P < .05$, $**P < .01$.

Abbreviation: TBT, tributyltin.

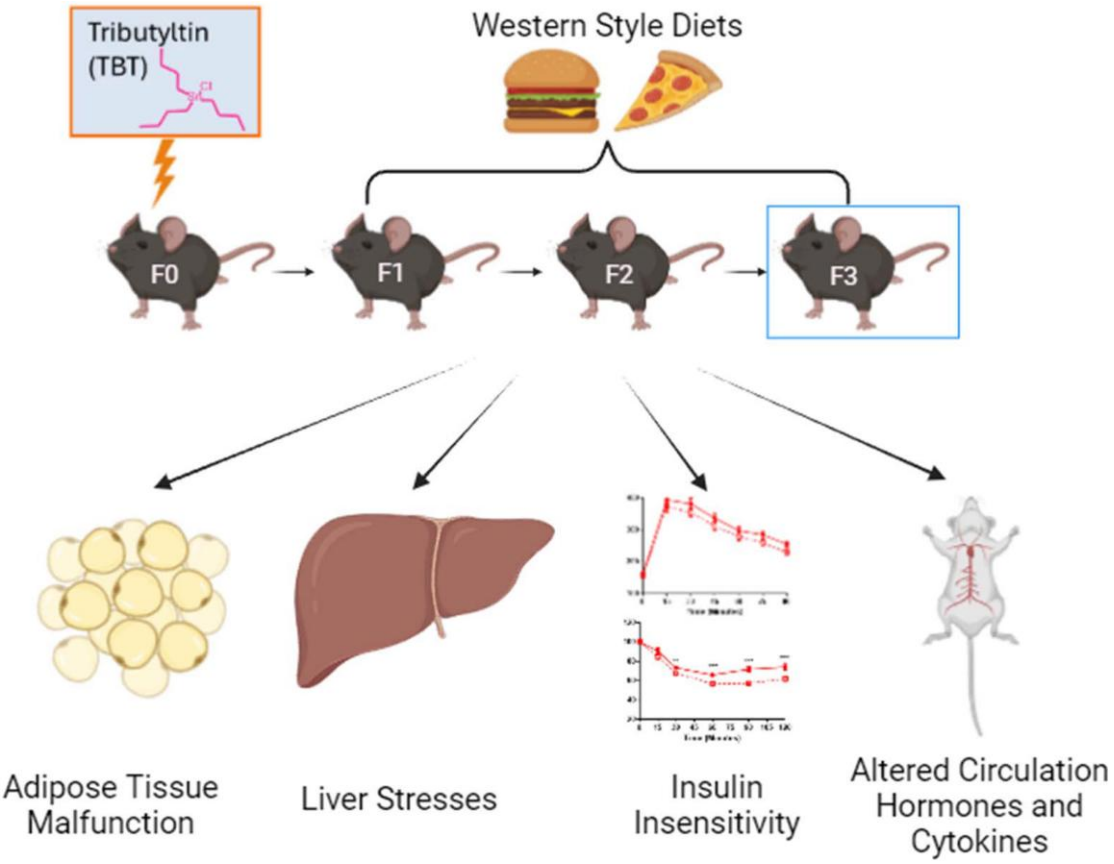

Figure 7. Comprehensive summary of transgenerational effects of TBT exposure on metabolic health across generations.

Abbreviation: TBT, tributyltin.

further investigation into the underlying mechanisms driving these metabolic alterations.

Our examination of metabolic hormones and cytokines in F3 offspring exposed to TBT revealed significant alterations, indicative of metabolic dysregulation (31). Specifically, the observed decrease in ghrelin and glucagon levels may reflect a disruption in hunger signaling and glucose homeostasis. Ghrelin is primarily known for its role in stimulating appetite and has been linked to energy balance and adiposity. Reduced ghrelin levels in TBT-exposed mice could suggest a shift in energy regulation and/or energy storage (32, 33). Similarly, glucagon plays a crucial role in maintaining glucose levels by promoting gluconeogenesis and glycogenolysis (34); its decreased levels might indicate impaired glucose mobilization, potentially leading to hypoglycemic states or altered glucose dynamics in these animals (35). Increased levels of TNF, leptin, IL-6, and insulin suggest a state of chronic low-grade inflammation and insulin resistance (36). Elevated TNF and IL-6 are well-known markers of inflammation and have been implicated in the development of obesity-related insulin resistance (37). The rise in leptin levels, often associated with leptin resistance, further supports the notion of disrupted energy homeostasis and adiposity regulation in TBT-exposed mice (38). The concomitant increase in insulin levels indicates a compensatory response to insulin resistance, which may predispose these animals to metabolic disorders such as type 2 diabetes (39). These findings highlight the transgenerational impact of TBT exposure on metabolic health and the intricate interplay between obesogen exposure, metabolic hormones, and cytokines in shaping metabolic phenotypes across generations (40). Understanding these hormonal and cytokine alterations provides crucial insights into how environmental factors like TBT contribute to the metabolic dysfunction observed in descendants, potentially setting the stage for obesity and related diseases.

While we did not assess genital alterations (eg, imposex) in newborns in this study, previous research has shown that TBT can interfere with sexual differentiation and aromatase activity, potentially affecting fat metabolism and reproductive development (41, 42). The extent to which these effects contribute to the metabolic differences observed in our study remains an open question and warrants further investigation. Sex steroid hormones play a critical role in metabolic regulation, and disruptions in steroidogenesis have been linked to obesity and metabolic dysfunction. While we did not observe significant differences in testosterone or estradiol levels before dietary intervention, prior studies suggest that TBT can interfere with steroidogenesis and aromatase activity, potentially modulating metabolic pathways (41, 42). These findings highlight the need for further investigation into whether sex steroid alterations contribute to the sex-specific metabolic effects observed in response to TBT exposure, particularly under dietary challenges. However, in our study, no differences in fecundity were observed between groups, and the sex ratio of offspring remained unchanged, suggesting that TBT exposure did not overtly affect reproductive outcomes in our model. Given the established links between steroid hormones and metabolic health, future studies should examine whether TBT-induced metabolic effects are mediated, at least in part, through disruptions in sex hormone signaling.

The consistent absence of phenotypic changes in female offspring following TBT exposure could be attributed to several factors that differentiate male and female responses to environmental stressors. One possibility is the role of sex hormones, such as estrogen, which may provide a protective effect against the obesogenic and metabolic disruptions observed in males (43). Estrogens have been shown to modulate energy balance, glucose metabolism, and lipid storage, potentially buffering females from the effects of endocrine disruptors like TBT (43). Additionally, sex-specific differences in gene expression and epigenetic regulation may contribute to the observed discrepancy (44). For instance, females may activate compensatory mechanisms that mitigate the impact of TBT on metabolic pathways, which are not as robust in males (45). The timing and dosage of exposure, as well as differences in the metabolism of TBT between sexes, could influence the degree of susceptibility to obesogenic effects (46). Lastly, variations in the distribution and function of adipose tissues between males and females might also play a role, as females tend to store fat subcutaneously, which is less metabolically active than visceral fat typically accumulated by males (47). These factors, alone or in combination, might underlie the lack of observable phenotypic changes in female offspring within the context of this study.

In summary, our study provides compelling evidence that exposure of pregnant F0 dams to TBT has lasting and profound transgenerational effects on metabolic health and susceptibility to diet-induced metabolic disturbances. We demonstrated that TBT exposure leads to significant alterations in metabolic hormone and cytokine levels, resulting in disrupted energy balance, increased adiposity, and markers of chronic inflammation and insulin resistance across multiple generations. This was accompanied by increased hepatic lipid storage and fibrosis together with WAT inflammation in males of F1-F3 generations. These findings are particularly concerning given the widespread presence of metabolism disruptors such as TBT in the environment, highlighting their potential role in the global obesity pandemic and related metabolic disorders. By elucidating the specific metabolic pathways impacted by TBT, our research advances the understanding of how environmental factors contribute to metabolic dysfunction and emphasizes the urgent need for strategies to mitigate these effects and protect future generations from the burden of metabolism disrupting agent-induced metabolic diseases.

Acknowledgments

The authors wish to acknowledge the support of the Chao Family Comprehensive Cancer Center Experimental Tissue Resource Shared Resource, supported by the National Cancer Institute of the National Institutes of Health under award number P30CA062203. We especially thank Dr. R. Edwards, who, under this support, contributed his expertise by scoring liver and fat histology for lipid accumulation, fibrosis, and inflammation in a blinded manner. We thank Dr. S. Phatak for suggesting the use of the Total Western Diet and for helpful discussions in the planning phase of this experiment.

Funding

This work was supported by grants from the U.S. Public Health Services, National Institutes of Health (R01ES023316 and R01ES031139) to B.B. and T.S.

Disclosures

B.B. is a named inventor on US patents 5,861,274; 6,200,802; 6,815,168; and 7,250,273 related to PPARγ. All other authors declare they have no actual or potential competing financial interests.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References. Supplementary Figs. S1-S4 can be found online at Figshare (18).

References

1. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of obesity and severe obesity among adults: United States, 2017–2018. *NCHS Data Brief*. 2020;(360):1-8.
2. Bluher M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol*. 2019;15(5):288-298.
3. Church TS, Thomas DM, Tudor-Locke C, *et al*. Trends over 5 decades in U.S. occupation-related physical activity and their associations with obesity. *PLoS One*. 2011;6(5):e19657.
4. Füzéki E, Banzer W. Physical activity recommendations for health and beyond in currently inactive populations. *Int J Environ Res Public Health*. 2018;15(5).
5. Hales CN, Barker DJ. The thrifty phenotype hypothesis. *Br Med Bull*. 2001;60(1):5-20.
6. Heindel JJ, Alvarez JA, Atlas E, *et al*. Obesogens and obesity: state-of-the-science and future directions summary from a healthy environment and endocrine disruptors strategies workshop. *Am J Clin Nutr*. 2023;118(1):329-337.
7. Grün F, Blumberg B. Endocrine disrupters as obesogens. *Mol Cell Endocrinol*. 2009;304(1-2):19-29.
8. Janesick AS, Blumberg B. Obesogens: an emerging threat to public health. *Am J Obstet Gynecol*. 2016;214(5):559-565.
9. Antizar-Ladislao B. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. a review. *Environ Int*. 2008;34(2):292-308.
10. Chamorro-García R, Sahu M, Abbey RJ, Laude J, Pham N, Blumberg B. Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. *Environ Health Perspect*. 2013;121(3):359-366.
11. Chamorro-Garcia R, Diaz-Castillo C, Shoucri BM, *et al*. Ancestral perinatal obesogen exposure results in a transgenerational thrifty phenotype in mice. *Nat Commun*. 2017;8(1):2012.
12. Hintze KJ, Benninghoff AD, Ward RE. Formulation of the total western diet (TWD) as a basal diet for rodent cancer studies. *J Agric Food Chem*. 2012;60(27):6736-6742.
13. Vos JG, De Klerk A, Krajnc EI, Van Loveren H, Rozing J. Immunotoxicity of bis(tri-n-butyltin)oxide in the rat: effects on thymus-dependent immunity and on nonspecific resistance following long-term exposure in young versus aged rats. *Toxicol Appl Pharmacol*. 1990;105(1):144-155.
14. Suvorov A, Vandenberg LN. To cull or not to cull? Considerations for studies of endocrine-disrupting chemicals. *Endocrinology*. 2016;157(7):2586-2594.
15. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-2474.
16. Kleiner DE, Brunt EM, Van Natta M, *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41(6):1313-1321.
17. Benedé-Ubieto R, Estévez-Vázquez O, Ramadori P, Cubero FJ, Nevzorova YA. Guidelines and considerations for metabolic tolerance tests in mice. *Diabetes Metab Syndr Obes*. 2020;13:439-450.
18. Chang RC, Kaitlin To YH, Whitlock RS, *et al*. Transgenerational impacts of obesogen exposure: insights into obesity, metabolic health, and dietary interactions in mice. *figshare*. 2024. doi: 10.1210/endocr/bqaf063.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25(4):402-408.
20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
21. Bevington PR, Robinson DK. *Data Reduction and Error Analysis for the Physical Sciences. 3rd ed*. McGraw-Hill; 2003. xi, 320 p.
22. Grün F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology*. 2006;147(6):S50-S55.
23. Shioda K, Odajima J, Blumberg B, Shioda T. Transgenerational transcriptomic and DNA methylome profiling of mouse fetal testicular germline and somatic cells after exposure of pregnant mothers to tributyltin, a potent obesogen. *Metabolites*. 2022;12(2):95.
24. Chamorro-García R, Poupin N, Tremblay-Franco M, *et al*. Transgenerational metabolomic fingerprints in mice ancestrally exposed to the obesogen TBT. *Environ Int*. 2021;157:106822.
25. Farr OM, Tsoukas MA, Mantzoros CS. Leptin and the brain: influences on brain development, cognitive functioning and psychiatric disorders. *Metabolism*. 2015;64(1):114-130.
26. Friedman JM. Leptin and the regulation of body weigh. *Keio J Med*. 2011;60(1):1-9.
27. Hassan K, Bhalla V, El Regal ME, A-Kader HH. Nonalcoholic fatty liver disease: a comprehensive review of a growing epidemic. *World J Gastroenterol*. 2014;20(34):12082-12101.
28. Pilkington AC, Paz HA, Wankhade UD. Beige adipose tissue identification and marker specificity-overview. *Front Endocrinol (Lausanne)*. 2021;12:599134.
29. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 2006;444(7121):840-846.
30. Defronzo RA. Banting lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes*. 2009;58(4):773-795.
31. Berthoud HR, Morrison C. The brain, appetite, and obesity. *Annu Rev Psychol*. 2008;59(1):55-92.
32. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;402(6762):656-660.
33. Wren AM, Seal LJ, Cohen MA, *et al*. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab*. 2001;86(12):5992-5992.
34. Sprague JE, Arbelaez AM. Glucose counterregulatory responses to hypoglycemia. *Pediatr Endocrinol Rev*. 2011;9(1):463-473. quiz 474–5.
35. Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. *J Clin Invest*. 2017;127(1):1-4.
36. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444(7121):860-867.
37. Myers MG Jr, Olson DP. Central nervous system control of metabolism. *Nature*. 2012;491(7424):357-363.
38. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev*. 2000;21(6):697-738.
39. Taylor R. Insulin resistance and type 2 diabetes. *Diabetes*. 2012;61(4):778-779.
40. Chamorro-García R, Blumberg B. Transgenerational effects of obesogens and the obesity epidemic. *Curr Opin Pharmacol*. 2014;19:153-158.
41. Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environ Sci Technol*. 2004;38(23):6271-6276.

42. McAllister BG, Kime DE. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). *Aquat Toxicol*. 2003;65(3):309-316.
43. Barros RP, Gustafsson JA. Estrogen receptors and the metabolic network. *Cell Metab*. 2011;14(3):289-299.
44. Arnold AP, Chen X. What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? *Front Neuroendocrinol*. 2009;30(1):1-9.
45. Nilsson EE, Skinner MK. Environmentally induced epigenetic transgenerational inheritance of disease susceptibility. *Transl Res*. 2015;165(1):12-17.
46. Rubin BS. Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *J Steroid Biochem Mol Biol*. 2011; 127(1-2):27-34.
47. Karastergiou K, Smith SR, Greenberg AS, Fried SK. Sex differences in human adipose tissues—the biology of pear shape. *Biol Sex Differ*. 2012;3(1):13.