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Assessing Metabolic and Reproductive Consequences of Prenatal Paracetamol Exposure in Female CD1 Mice

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ABSTRACT

Paracetamol is a widely used analgesic during pregnancy; however, emerging evidence suggests it may act as an endocrine/metabolism disrupting compound. Using an F1 female CD1 mouse model, this study scrutinized the long-term metabolic and reproductive consequences linked to intrauterine paracetamol exposure. Pregnant dams were administered paracetamol at three dose levels (Cmax/10, Cmax, and Cmax × 10) during a critical window of organogenesis (gestational days 7.5–16.5). Offspring were monitored until 17 weeks of age to assess growth, glucose homeostasis, lipidomic profiles, and ovarian histology. Prenatal exposure did not significantly affect total body weight but induced a dose-dependent increase in peritoneal adipose tissue mass, particularly in the Cmax × 10 group. Metabolic assessments revealed impaired glucose tolerance in the Cmax/10 group and increased insulin sensitivity across all dose groups. Adipose tissue gene expression analysis demonstrated upregulation of adipogenic markers (*Pparγ*, *Lpl*, *Fasn*), while untargeted lipidomics revealed consistent dysregulation of lipid species, including lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), sphingomyelins (SMs), and triacylglycerols (TGs), indicating altered lipid storage and inflammatory signaling. Histological evaluation showed a marked reduction in total follicle number and increased follicular atresia following paracetamol exposure. Degenerative changes in oocytes and granulosa cells were observed, and multi-oocyte follicles (MOFs) were detected exclusively in the Cmax/10. Collectively, these findings demonstrate that intrauterine paracetamol exposure induces persistent developmental reprogramming in female offspring, characterized by visceral adiposity, disrupted lipid homeostasis, and depletion of the ovarian reserve. Notably, several endpoints exhibited non-monotonic dose–response relationships, underscoring the sensitivity of developing systems to even subtherapeutic paracetamol exposure.

1 | Introduction

Characterized by a high BMI, obesity is a leading global health threat that severely elevates the risk of stroke, diabetes, heart disease, and various cancers (Ritchie and Roser 2017; WHO 2026). According to the World Health Organization, obesity ranks among the leading contributors to global mortality and is associated with approximately 3.68 million deaths annually (Ritchie and Roser 2017). Alongside adults, the prevalence of obesity among children has also increased dramatically, contributing to the earlier onset of metabolic disorders (Liu and Peterson 2015). Although caloric imbalance and

genetic predisposition are recognized as key drivers of weight gain and obesity, these factors alone cannot fully explain the rapid and dramatic global increase in obesity prevalence (Amato et al. 2021). Consequently, growing evidence suggests the contribution of environmental factors to the pathogenesis of obesity.

Since the early 2000s, research has increasingly focused on the impact of environmental exposures on obesity risk. In particular, endocrine disrupting chemicals—often referred to as "metabolism disrupting compounds (MDCs)"—have been implicated in the disruption of hormonal and metabolic pathways involved in energy balance, adipogenesis, and lipid metabolism, predisposing individuals to increased adiposity (Heindel et al. 2015). Numerous MDCs, including bisphenol A, phthalates, organotins, and polychlorinated biphenyls, have been shown to alter adipocyte differentiation, lipid storage, and metabolic regulation (Darbre 2017; Entezari et al. 2022). Many of these chemicals are highly lipophilic and resistant to metabolic degradation, allowing them to accumulate in adipose tissue and potentially interfere with pathways regulating energy homeostasis (Alonso-Magdalena et al. 2011; Elobeid and Allison 2008). Adipogenesis involves the differentiation of preadipocytes into mature adipocytes, governed by the synchronized regulation of core transcription factors, specifically peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) family. During this process, adipocytes acquire the ability to accumulate and store lipids. Lipogenesis, which refers to the synthesis of fatty acids and triglycerides, is closely associated with adipocyte differentiation and is regulated by key lipogenic factors such as sterol regulatory element-binding protein 1 (SREBP1), lipoprotein lipase (LPL), and fatty acid–binding protein 4 (FABP4). Increasing evidence suggests that exposure to MDCs can impair the regulation of adipogenesis and lipogenesis by altering adipogenic signaling pathways and lipid metabolism, thereby contributing to obesity and metabolic disorders (Lefterova and Lazar 2009; Song et al. 2020).

Many such chemicals can cross the placental barrier, thereby exposing the developing fetus during sensitive periods of organogenesis and metabolic programming (Braun 2017; Liu and Peterson 2015). Accumulating evidence indicates that exposure to MDCs during critical developmental windows may have long-lasting metabolic consequences (de Cock and van de Bor 2014). According to the developmental origins of health and disease (DOHaD) concept, environmental exposures during fetal development, even at low doses, can permanently alter tissue structure, metabolic pathways, and gene regulation, potentially increasing susceptibility to obesity and related metabolic disorders later in life. Experimental evidence supporting this concept has been demonstrated for several endocrine/MDCs, including diethylstilbestrol, one of the most well-known examples (Hao et al. 2012; Newbold et al. 2007). Therefore, elucidating the association between prenatal exposure to such compounds and the subsequent development of obesity later in life is of particular importance.

Alongside environmental and industrial chemicals, certain pharmaceuticals have also been suggested to cause endocrine and/or metabolic disruption independent of their primary therapeutic effects (Baillie-Hamilton 2002; Entezari et al. 2022, 2026). Paracetamol is one of the most commonly used analgesics during pregnancy and is generally considered safe. However, emerging evidence suggests that prenatal exposure to paracetamol may influence endocrine function and developmental processes in the offspring (Arendrup et al. 2018; Bauer et al. 2021; Kristensen et al. 2016). Several epidemiological studies have explored the potential relationship between in utero paracetamol exposure and childhood metabolic outcomes. While a cohort study by Liew et al. did not find a strong association between prenatal paracetamol exposure and body mass index, it suggested an increased risk of overweight in adolescent girls, indicating that possible metabolic effects may be sex-specific (Liew et al. 2019). Other epidemiological studies have also investigated associations between prenatal paracetamol exposure and childhood obesity or metabolic alterations, although the available evidence remains limited and inconsistent (Liew et al. 2019; Murphy et al. 2015; Sorrow et al. 2019).

Beyond potential metabolic outcomes, prenatal paracetamol exposure has also been linked to reproductive alterations in the offspring. Epidemiological studies have reported associations between in utero paracetamol exposure and reproductive abnormalities, including reduced anogenital distance, genital malformations, and earlier onset of puberty (Arendrup et al. 2018; Bauer et al. 2021). Experimental studies in female mice have further demonstrated that in utero paracetamol exposure can reduce ovarian follicle reserves and impair fertility in adulthood (Arendrup et al. 2018; Holm et al. 2016; Wu et al. 2024). Similarly, Dean et al. have shown that prenatal paracetamol exposure can disrupt germ cell development and reproductive function in F1 female rat offspring (Dean et al. 2016).

Considering these findings, this study aimed to characterize the lifelong metabolic and reproductive impacts of gestational paracetamol exposure on F1 female CD1 mice. Following the exposure of pregnant dams to physiologically relevant doses of paracetamol during critical embryonic windows, F1 female offspring were assessed for metabolic parameters—including adipose tissue distribution, glucose regulation, and adipogenic signaling—as well as ovarian follicle counts as a key indicator of reproductive function.

2 | Materials and Methods

2.1 | Animal Caring and Paracetamol Exposure

Six-week-old CD1 mice were obtained from the TÜBİTAK Marmara Research Center (TÜBİTAK MAM) and housed at the Ege University Laboratory Animal Research and Application Center under a 12-h light/dark cycle, with ad libitum access to standard chow and water. Followed by a 7-day acclimation period, 7-week-old mice were paired for mating (one male and one female per cage). The presence of a vaginal plug was checked daily, and the day on which a plug was detected was marked as gestational day 1 (GD1), indicating successful mating. Pregnant dams were randomly assigned to control or treatment groups. Starting from GD1, the dams' body weights were monitored daily and individualized doses were calculated according to the formula described by Krishnan (2019).

Paracetamol is commonly administered during pregnancy, with a standard single therapeutic dose of 500–1000 mg in adults. A oral single dose of 1000 mg in a 70-kg adult results in an estimated maternal maximum plasma concentration (Cmax) of approximately 100–200 μM (Rayburn et al. 1986), with fetal exposure occurring through transplacental transfer. To investigate the long-term effects of prenatal low-dose paracetamol exposure, pregnant dams received paracetamol by oral gavage at doses of 0.55, 5.5, and 55 mg/kg, representing a prenatal low-dose exposure range spanning approximately 3–300 mg in humans, based on the allometric scaling formula described below. For consistency throughout the manuscript, these experimental groups are referred to as Cmax/10 (0.55 mg/kg), Cmax (5.5 mg/kg), and Cmax × 10 (55 mg/kg), respectively. Fresh paracetamol dosing solutions were prepared daily in sterile distilled water, and serial dilutions were performed immediately before administration to ensure accurate dose delivery. The final administration volume for each dam was individually adjusted according to its daily body weight (targeting approximately 200 μL per animal) to deliver the calculated dose via oral gavage.

$$\text{Dose (human)}\left(\frac{\frac{\text{mg}}{\text{kg}}}{\text{day}}\right) = \text{Dose (animal)}\left(\frac{\frac{\text{mg}}{\text{kg}}}{\text{day}}\right) \times \left(\frac{\text{BWanimal}}{\text{BWhuman}}\right) \times \wedge 0.33$$

To account for pregnancy-associated weight changes, body weight was measured daily and doses were adjusted individually throughout GD 7.5–16.5. To target the critical window of early-to-mid organogenesis (including primordial germ cell migration, genital system differentiation, and mammary gland development), pregnant dams were treated orally by gavage with paracetamol or distilled water. Following the dosing period, dams were individually housed and monitored until delivery. A total of three pregnant dams were included in the control group, while five pregnant dams were included in the dose groups. After weaning at postnatal day 21 (PND21), female offspring within each treatment group were pooled and randomly reallocated into cages prior to assignment to the different experimental endpoints in order to minimize potential postnatal maternal or cage-related effects (Table 1).

TABLE 1 | Number of female pups in in vivo study.

Groups	Control	Cmax/10	Cmax	Cmax × 10
Dams (*n*)	3	5	5	5
Female pups (*n*)	14	11	16	11

2.2 | Body Weight, Organ, and Local Fat Pad Weights

To evaluate potential metabolic effects of paracetamol in female mice, food intake, water consumption, and body weight of the F1 offspring were measured weekly from PND21 until Week 17 and were compared with those of the control group. Following euthanasia, liver, pancreas, ovaries, and uterus were excised and weighed. To further assess the localized fat distribution, peritoneal and ovarian fat pads were excised, weighed, and recorded. All organ and fat tissue weights were normalized to the body weight of each animal.

2.3 | Metabolic Tolerance Tests

To evaluate glucose homeostasis, metabolic tolerance tests were performed on the F1 offspring. At postnatal week 15, a glucose tolerance test (GTT) was conducted following an 18-h overnight fast. After recording baseline blood glucose levels (time 0) using a glucometer (Accu-Chek, Roche Diagnostics), D-glucose was injected intraperitoneally at 2 g/kg body weight, and blood glucose levels were measured at 15, 60, and 120 min postinjection from the tail vein. Moreover, an insulin tolerance test (ITT) was performed in 16-week-old F1 offspring after a 4-h fasting period. Following baseline glucose measurement, human insulin (0.5 IU/kg body weight) was administered via intraperitoneal injection, and blood glucose concentrations were subsequently determined at 15, 30, and 60 min following injection.

2.4 | Adipogenic and Lipogenic Gene Expression Analysis

Adipogenesis is initiated with an increase in pro-adipogenic transcription factors (CAAT/enhancer-binding proteins alpha, beta (C/EBPα, C/EBPβ), and SREBP1), followed by an increase in master regulator, PPARγ. Activated PPARγ stimulates other transcription factors, leading to increased transcription of adipocyte genes (such as fatty acid synthase (FASN), LPL) and subsequent adipocyte differentiation (Janesick and Blumberg 2012; Lefterova and Lazar 2009). The expression levels of *Pparγ* and its downstream target genes in peritoneal adipose tissue were determined by RT-qPCR. Total RNA was isolated using the Aurum Total RNA Mini Kit, and cDNA was synthesized using the iScript cDNA Synthesis Kit according to the manufacturers' instructions. Quantitative real-time PCR was performed using BlasTaq 2× qPCR MasterMix. The RT-qPCR conditions were used as described in our previous study (Entezari et al. 2024). Primer sequences of adipogenic and lipogenic genes used in this study are demonstrated in Table 2.

TABLE 2 | Primer sequences of adipogenic and lipogenic genes.

Genes	Forward	Reverse
Pparγ	TGGGTGAAACTCTGGGAGATTC	AATTTCTTGTGAAGTGCTCATAGGC
Fasn	TCGGGTGTGGTGGGTTTGGTGAAT	ACTTGGGGGCGTGAGATGTGTTGC
Lpl	GTGTTGCTTGCCATTCTC	TCTCCTGATGACGCTGAT
Srebp1	GCCCCTGCCCACCTCAAACCT	ACTGGCACGGGCATCCTTCCTC

2.5 | Adipogenic and Lipogenic Protein Expression Analysis

Protein expression levels of PPARγ and its downstream adipogenic and lipogenic targets in peritoneal adipose tissue were determined by Western blot analysis in 17-week-old F1 female CD1 mice. To assess these effects, lysates were prepared from peritoneal adipose tissues as described before (An and Scherer 2020). Protein concentrations of lysates were measured by Bradford method (Bradford 1976). Equal amounts of denatured lysates (95°C) were loaded for SDS-PAGE separation and electrotransferred to PVDF membranes. Following the blocking, the membranes underwent overnight incubation at 4°C with primary antibodies (Proteintech). Next, target proteins were incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies for 1 h at room temperature to facilitate chemiluminescent detection. For protein visualization, membranes were incubated with an enhanced chemiluminescence (ECL) substrate and imaged on a Fusion X7 system. The band intensities were analyzed and quantified through the ImageJ software.

2.6 | Untargeted Lipidomic Analysis

To evaluate the alterations in the lipid profiles of F1 female mice after gestational paracetamol exposure, an untargeted lipidomic analysis was conducted. Frozen plasma samples were brought to room temperature and thoroughly vortexed. Next, a 200-μL aliquot from each sample was mixed with a methanol:water:chloroform solution (3:1:3, v/v/v) to extract total lipids. The mixture was then centrifuged at 10,000 rpm for 10 min at 4°C to induce phase separation. The lower organic layer was collected, evaporated to dryness under a vacuum, and dissolved the residue in an isopropanol:acetonitrile mixture (7:3, v/v). To monitor instrument stability and data reproducibility, a quality control (QC) sample was generated by pooling equal volumes from each extracted specimen.

Chromatographic separation was achieved on an LC–qTOF–MS system using a C18 reverse-phase column (2.1 × 100 mm, 2.7 μm) maintained at 60°C. The mobile phase comprised binary solvents: Solvent A (water: acetonitrile, 6:4, v/v) and Solvent B (isopropanol:acetonitrile, 9:1, v/v), both supplemented with 10 mM ammonium formate and 0.05% formic acid. The elution followed a 26-min gradient method at a constant flow rate of 0.25 mL/min, with a sample injection volume of 10 μL. Mass spectrometric detection spanned across a predefined mass-to-charge (m/z) range in full-scan mode. We acquired data in both positive and negative electrospray ionization (ESI) modes to broaden the coverage of distinct lipid classes. Additionally, the pooled QC samples were subjected to tandem MS/MS analysis at various collision energies to facilitate structural identification.

The MS-DIAL software (v4.60) was utilized to process the raw spectral data for peak detection, retention time alignment, and metabolite annotation against public databases. Total ion chromatogram (TIC) normalization was applied to the dataset. To ensure robust data quality, we filtered out features presenting a relative standard deviation (RSD) greater than 30% in QC runs or missing values exceeding 50%. The filtered dataset was then exported to SIMCA software for multivariate modeling, including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA). Discriminatory lipid species separating the experimental cohorts were selected based on a variable importance in projection (VIP) score greater than 1.0 combined with a p-value less than 0.05. Finally, biological pathway enrichment analysis was performed using the LION/web platform to contextualize the metabolic shifts.

2.7 | Histological Analysis

Immediately prior to euthanasia, vaginal cytology was performed in all F1 female mice to determine the estrous stage at the time of tissue collection. Vaginal smears were collected, spread onto glass slides, fixed in methanol, stained with Giemsa, and evaluated under a light microscope according to the criteria described by (Cora et al. 2015). The identified estrous stage was taken into consideration during the histopathological evaluation of ovarian and uterine tissues.

Reproductive organs (ovaries and uterus) were isolated from 17-week-old F1 female offspring following euthanasia. After recording the organ weights, the samples were transferred to a 4% formaldehyde solution for 24–48 h of routine fixation at room temperature. The tissue pieces were then bathed overnight in PBS (pH 7.4) to wash away excess fixative. Subsequently, specimens were processed through graded ethanol solutions (80% to 100%; 1 h each) to remove water, cleared using xylene, and blocked in paraffin. Finally, serial microtome sections were prepared and subjected to routine hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining protocol.

2.8 | Statistical Analysis

Offspring from different litters within each treatment group were pooled at weaning (PND21) and randomly reallocated into cages to minimize potential litter-related bias. As the study aimed to evaluate individual metabolic outcomes in adulthood, individual pups were treated as the experimental unit. The sample sizes for each group are summarized in Table 1. Data analysis was performed with GraphPad Prism (version 8), employing the Shapiro–Wilk normality test to determine the appropriate testing strategy. Quantitative differences among groups presenting normal distributions were calculated via one-way ANOVA followed by post hoc analysis (Tukey's or Dunnett's tests). Where data did not meet parametric assumptions, the Kruskal–Wallis test with a post-estimation Dunn's test was substituted. The confidence level for significance was set at 95% ($p < 0.05$); levels of significance are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3 | Results

3.1 | Effects on Body Weight, Food and Water Consumption, Organ, and Fat Pad Weights

Following weaning at 3 weeks of age, pups were weighed weekly until week 17. Body weights remained comparable between the control and all treatment groups throughout the

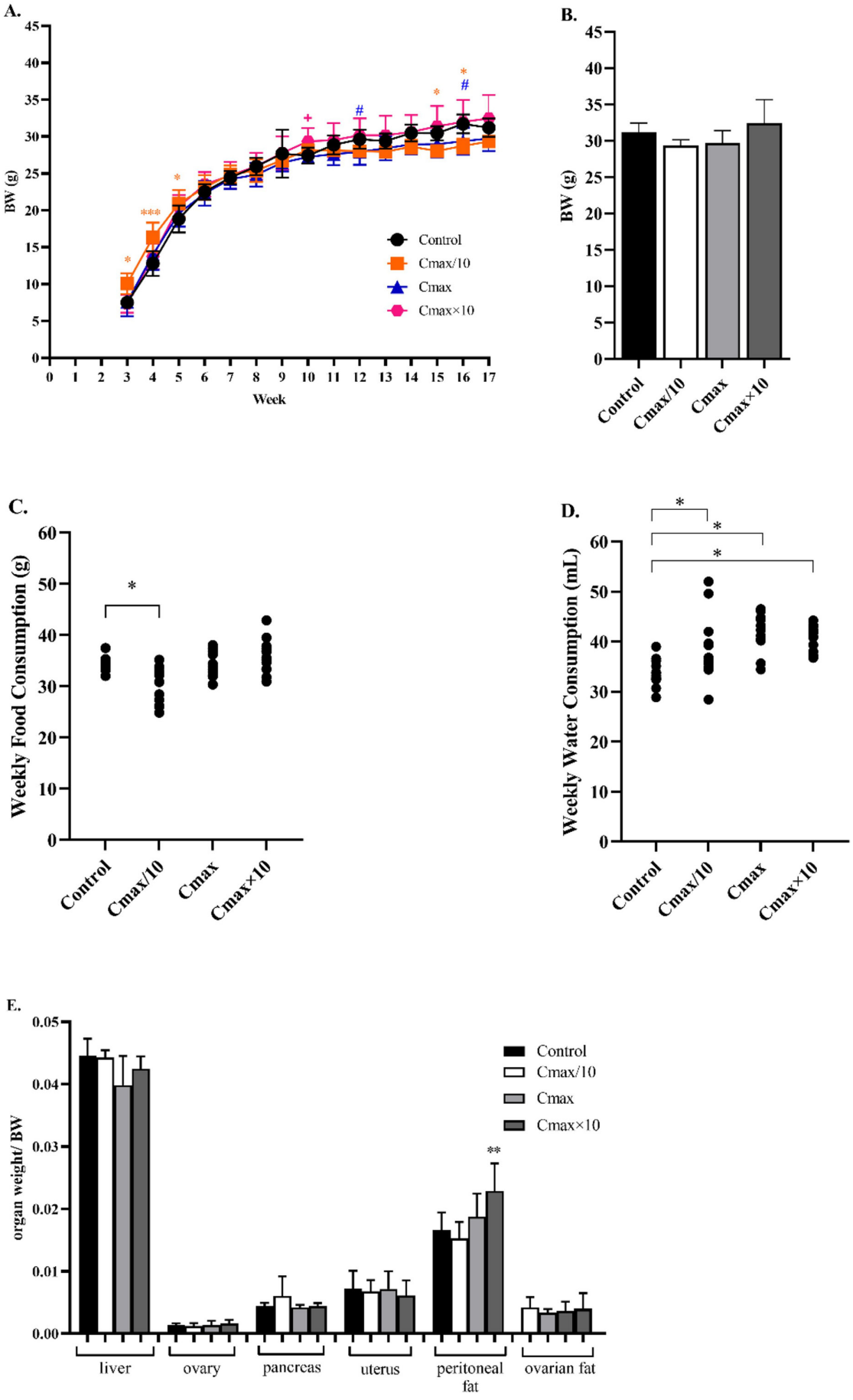

FIGURE 1 | Legend on next page.

FIGURE 1 | Effect of prenatal paracetamol exposure on body weight, food, and water consumption. Weekly body weight (A), body weight of 17-week-old F1 CD1 female mice (B), food consumption (C), water consumption (D), and organ and fat pad weights (E). Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with control group.

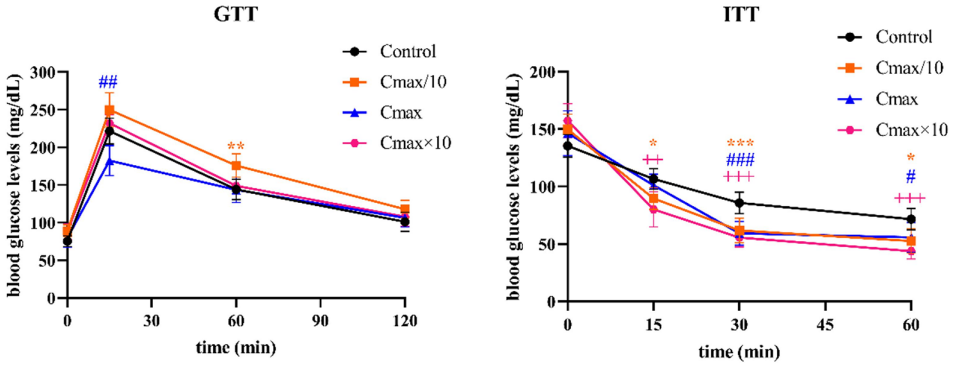

FIGURE 2 | Metabolic tolerance tests in F1 CD1 female mice. GTT: Glucose tolerance test; ITT: Insulin tolerance test. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the control group.

study (Figure 1A,B). Food and water intake were also recorded weekly from Week 5 onward. The Cmax/10 group showed a sustained reduction in food intake at all time points, whereas water consumption significantly increased across all dose groups. (Figure 1C,D). Moreover, the effect of prenatal paracetamol exposure on organ (liver, pancreas, gonads) and local fat pad weights was further investigated in 17-week-old F1 female pups. No significant effect was observed in liver, pancreas, ovary, or uterus weights. However, a dose-dependent increase in peritoneal fat weight was observed, and this effect was statistically significant in the Cmax×10 group (Figure 1E).

3.2 | Effects on Metabolic Tolerance Tests

For the GTT and ITT, blood glucose levels were measured at multiple time points after an intraperitoneal injection of 2 g/kg glucose or 0.5 IU/kg insulin, respectively. During the GTT, blood glucose levels in Cmax/10 female F1 mice remained higher than the control at all time points. In the Cmax group, glucose levels were significantly lower than the control at the 15th minute but returned to control levels afterward. Glucose levels in the Cmax×10 group were similar to those observed in the control group, and no significant differences were detected. During the ITT, glucose levels declined more rapidly in all three dose groups and remained lower at all time points (15-, 30-, and 60-min), compared with the control group (Figure 2).

3.3 | Effects on Expression of Adipogenic/Lipogenic Factors

To assess transcriptional changes, the expression levels of selected adipogenic and lipogenic genes in the peritoneal fat pad were quantified via RT-qPCR. *Lpl* was the most prominently affected gene, showing 6.6-, 4.98-, and 5.2-fold increases in the Cmax/10, Cmax, and Cmax×10 groups, respectively. Moreover, *Pparγ* expression was significantly upregulated by 3.5-, 2.5-, and 3-fold in the respective dose groups. Although *Fasn* expression did not change significantly in the Cmax group, it was elevated by 2.8- and 3.1-fold in the Cmax/10 and Cmax×10 groups, respectively. In contrast, *Srebp1* expression was consistently downregulated across all dose groups (Figure 3A).

Analysis of adipogenic and lipogenic protein expression demonstrated a 1.4-fold increase in LPL levels in both the Cmax/10 and Cmax groups. In addition, SREBP1 expression was significantly elevated by 1.45-fold in the Cmax group. No significant changes were observed in PPARγ or FASN expression (Figure 3B,C).

3.4 | Lipidomics

Untargeted lipidomic analysis demonstrated a clear separation between the control and paracetamol-exposed groups in PLS-DA score plots, indicating distinct dose-dependent lipidomic signatures (Figure 4A–D). The separation was particularly pronounced in the Cmax and Cmax×10 groups, suggesting a stronger metabolic shift at higher exposure levels.

VIP score analysis identified multiple discriminant lipid species, predominantly belonging to glycerophospholipids, sphingolipids, fatty acids, and triacylglycerols (TGs). Across all exposure groups, lysophosphatidylcholine (LPC) species (including LPC 18:0, LPC 18:2, LPC 20:4, LPC 22:6) were among the most significantly altered lipids. In addition, several PC species (e.g., PC 36:1, PC 38:4) and sphingomyelin (SM) species (e.g., SM 42:2, SM 42:3) contributed strongly to group discrimination. In the Cmax/10 group, lipid alterations were characterized by a mixed pattern, with several LPC and FA species increased, while certain PC and TG species were reduced compared with controls. In contrast, the Cmax group exhibited a more pronounced shift, with increased levels of fatty acids (FA 16:1, FA 22:3, FA 22:4) and LPC species, alongside decreases in selected PC species. The Cmax×10 group demonstrated the most distinct lipidomic

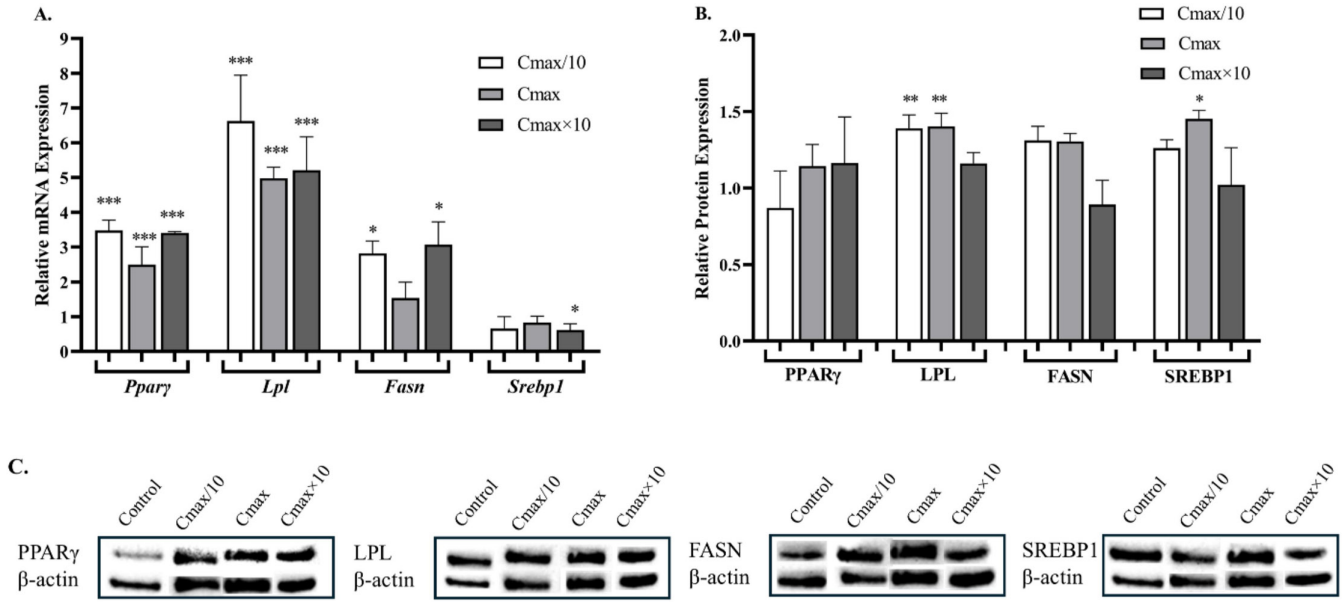

FIGURE 3 | Effect of prenatal paracetamol exposure on adipogenic/lipogenic gene (A) and protein (B–C) expressions in peritoneal adipose tissue, in F1 CD1 females. Statistical significance is indicated as $*p<0.05$, $**p<0.01$, and $***p<0.001$ compared with the control group.

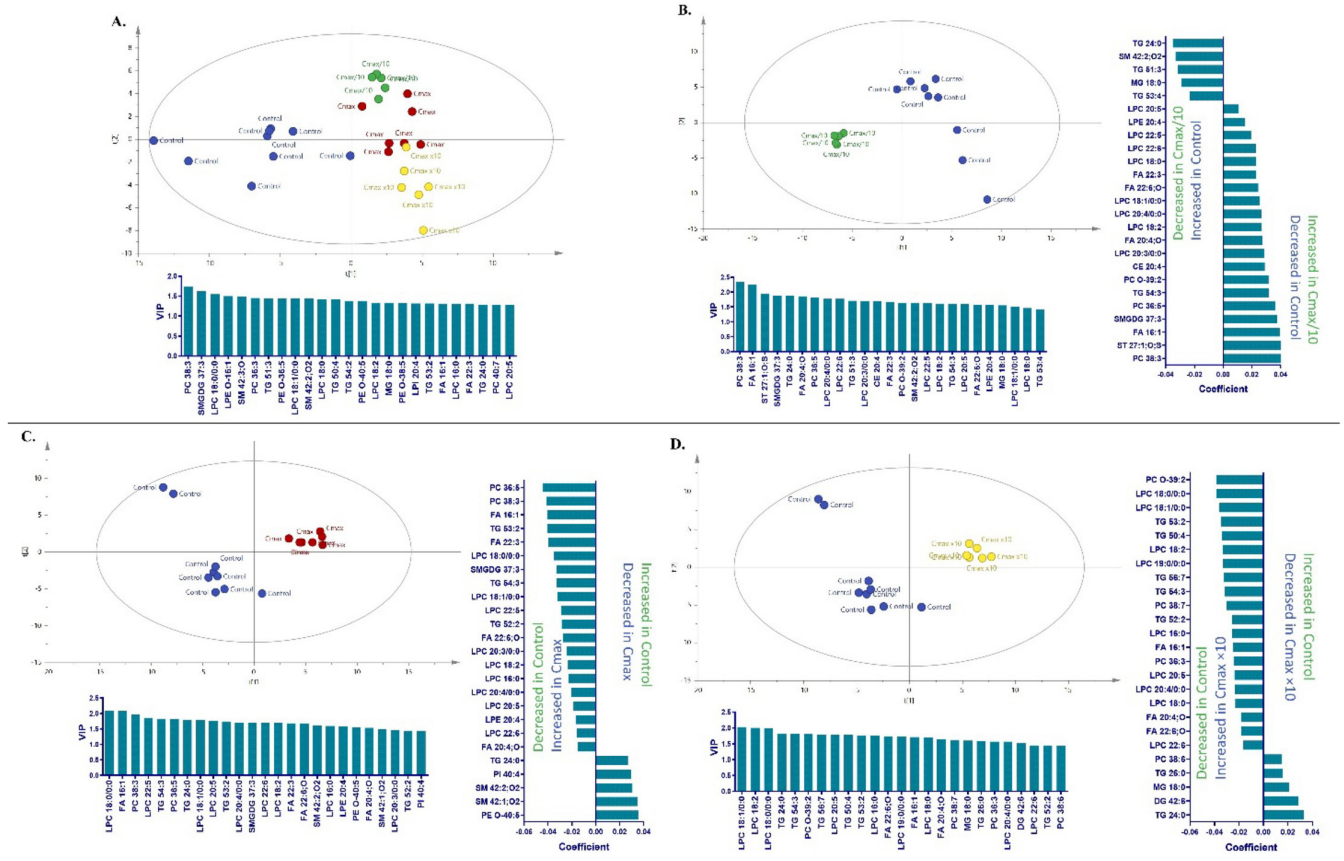

FIGURE 4 | Multivariate data analysis of lipidomics data from prenatally paracetamol-exposed female F1 mice. (A) PLS-DA score plot of all dose groups. (B–D) Comparison of Cmax/10, Cmax, and Cmax × 10 versus control in female mice, respectively.

profile, with marked alterations in TG species (e.g., TG 52:2, TG 56:3) and continued dysregulation of LPC and PC species. Notably, several TGs and LPCs were increased, indicating enhanced lipid storage and remodeling processes.

Based on the lipidomic analysis results, pathway enrichment analysis was performed using the significantly altered lipid metabolites ($p<0.05$) (Figure 5A–C). The results showed that prenatal paracetamol exposure affected fatty acid metabolism,

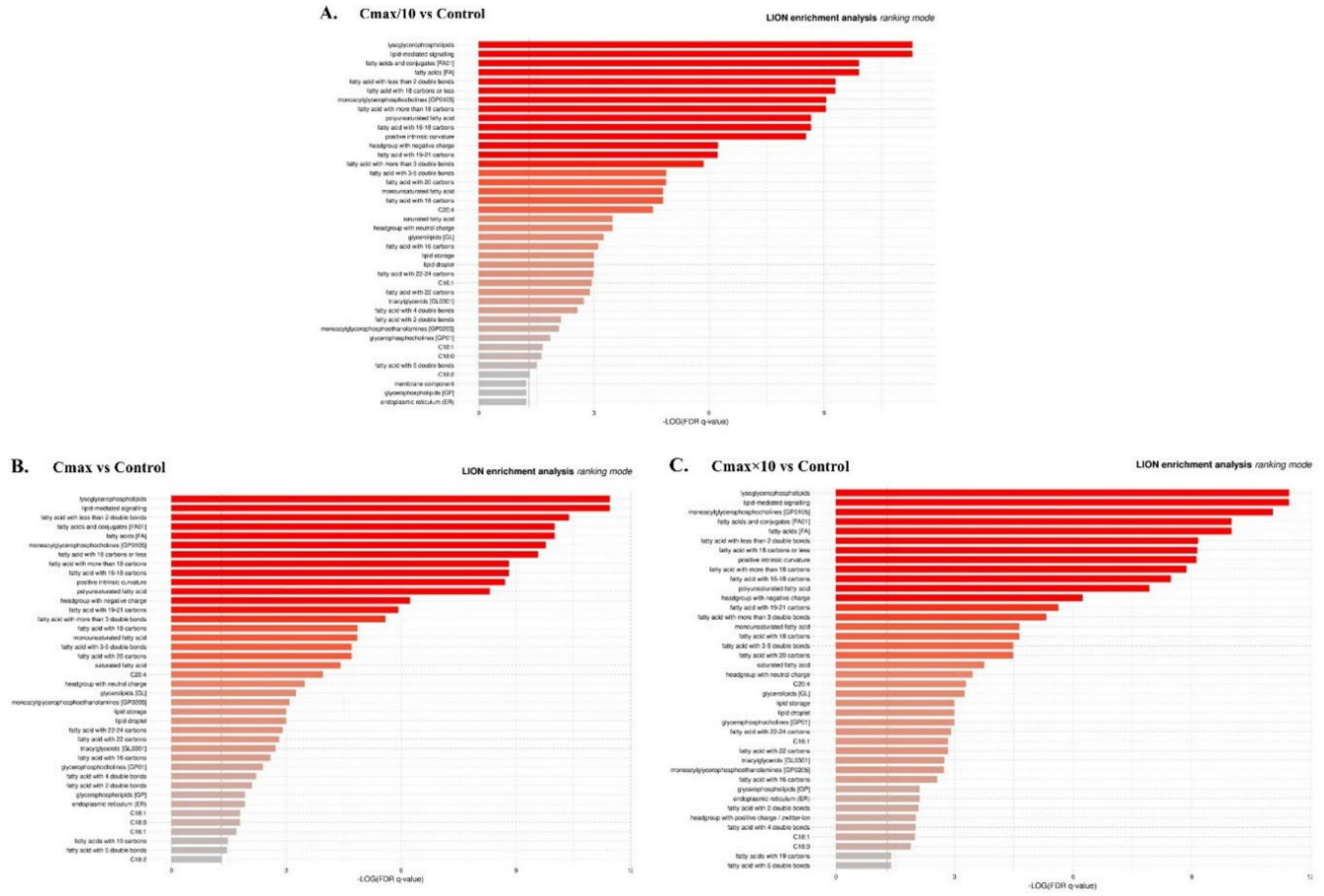

FIGURE 5 | Pathway enrichment analysis of statistically significant ($p < 0.05$) altered lipid metabolites in paracetamol-treated female mice. (A–C) Pathway analysis results for Cmax/10, Cmax, and Cmax × 10 groups in F1 female mice, respectively.

particularly pathways involving polyunsaturated fatty acids, in F1 female offspring. In addition, significant alterations were observed in glycerophospholipid metabolism, as well as monoacylglycerophosphocholine and diacylglycerol pathways, which are important for membrane structure and cellular signaling.

Notably, monoacylglycerophosphocholine and diacylglycerol also play key roles in triglyceride turnover and lipid mobilization, highlighting the broader metabolic relevance of these findings. Taken together, the disruption of these interconnected pathways suggests that prenatal paracetamol exposure influences overall lipid homeostasis rather than causing isolated changes in single metabolites. Overall, the pathway analysis supports the idea that prenatal paracetamol exposure may lead to persistent metabolic reprogramming, characterized by alterations in fatty acid metabolism, glycerophospholipid balance, and triglyceride-related pathways in female offspring.

3.5 | Histopathological Evaluation of Reproductive Effects in F1 Female Offspring

To assess general histological parameters, ovarian and uterus tissues were stained with H&E. In addition, Masson's trichrome staining was performed to evaluate stromal connective tissue. Histopathological analysis of ovarian tissues demonstrated a marked decrease in total follicle counts (pool of primary, secondary, and tertiary follicles) and an increase in atretic follicles in the experimental groups compared with controls (Table 3). Numerous atretic follicles were observed at the primordial and primary follicle stages, with primary oocytes showing abnormal morphology and loss of normal cellular integrity. In addition, several secondary and tertiary follicles exhibited granulosa cell degeneration, irregular zona pellucida, and oocyte deformities with poorly defined boundaries. Multi-oocyte follicles (MOF) were observed exclusively in the Cmax/10 group. Mild stromal hyperemia was noted in this group, whereas no significant histological changes were detected in the other dose groups. Moreover, the endometrium, myometrium, and perimetrium layers of the uterus all exhibited normal morphology (Figure 6).

4 | Discussion

The present study aimed to investigate the long-term in vivo effects of prenatal paracetamol exposure on metabolic and reproductive outcomes in F1 female CD1 mice.

Several of the measured systemic metabolic endpoints showed only limited alterations. Body weight did not significantly differ

among groups in 17-week-old F1 female mice, suggesting that prenatal paracetamol exposure did not markedly affect overall body mass under the experimental conditions. Interestingly, food intake was reduced in prenatally exposed Cmax/10 animals. The lack of a corresponding decrease in body weight by reduced food intake may indicate that low doses of paracetamol can alter energy regulation even in the absence of overt changes in body mass.

TABLE 3 | Quantitative analysis of atretic and multi-oocyte follicles (MOFs) across experimental and control groups. Total follicle count: pool of primary, secondary, and tertiary follicles. Data are expressed as mean value in each group. Statistically significant differences relative to the control group are indicated in bold.

	Groups			
Follicle types	**Control**	**Cmax/10**	**Cmax**	**Cmax×10**
Total follicle count	31.52	**8.8*** ($p<0.0001$)**	**12.76*** ($p<0.0001$)**	**24.13* ($p=0.0251$)**
Atretic	2.61	**20.2*** ($p<0.0001$)**	**10.55*** ($p<0.0001$)**	**5.14** ($p=0.0096$)**
MOF	0	**2.4*** ($p<0.0001$)**	0 ($p>0.9999$)	0 ($p>0.9999$)

*$p<0.05$.
**$p<0.01$.
***($p<0.001$).

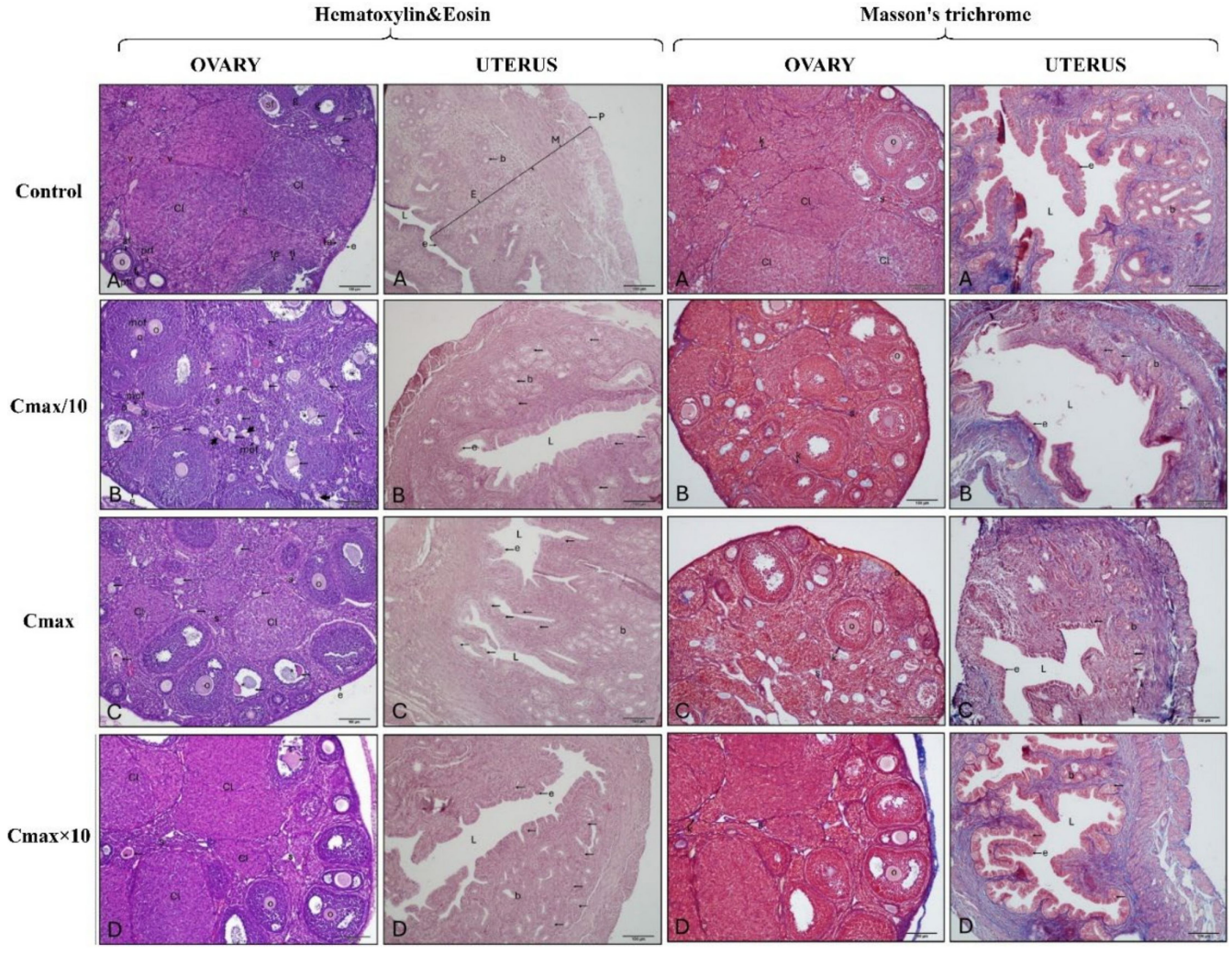

FIGURE 6 | Representative histological images of ovary and uterus from all experimental groups. In ovarian sections: epithelium (e); tunica albuginea (ta); corpus luteum (Cl); oocyte (o); follicular cell (f); granulosa cell (g); theca interna (ti); theca externa (te); atretic follicle (arrow); stromal cell (s); vessel (v); primordial follicle (prf); primary follicle (pf); secondary follicle (sf); multi-oocyte follicle (MOF), hyperemia (thick arrow). In uterine sections: endometrium (E); myometrium (M); perimetrium (P); epithelium (e); lumen (L); uterine glands (B); degeneration (arrow). Magnification ×10.

More pronounced metabolic alterations were observed at the level of adipose tissue distribution and adipogenic signaling. Although total body weight remained largely unchanged, a dose-dependent increase in peritoneal adipose tissue mass was observed. The distribution of adipose tissue is considered a more critical indicator of metabolic health than total fat mass. An increase in visceral white adipose tissue surrounding the abdominal cavity is known to pose a greater metabolic risk than subcutaneous adipose tissue, as it contains abundant macrophages that secrete pro-inflammatory cytokines and is strongly associated with metabolic dysfunction, inflammation, and insulin resistance (Ibrahim 2010). In mice, intra-abdominal visceral fat depots include peritoneal/retroperitoneal and gonadal adipose tissues (Mandarim-de-Lacerda et al. 2021). Expansion of these adipose depots through adipocyte hypertrophy and hyperplasia promotes the release of pro-inflammatory adipokines, contributing to chronic inflammation, insulin resistance, and metabolic dysfunction (Ouchi et al. 2011). Overall, in the present study, the absence of a significant difference in body weight despite reduced food intake in the Cmax/10 group, together with the observed dose-dependent increase in peritoneal adipose tissue in prenatally exposed female mice, suggests that paracetamol may exert metabolic disrupting effects that increase susceptibility to metabolic dysfunction later in life.

Additionally, alterations in glucose homeostasis were detected. GTT revealed elevated glucose concentrations at all measured time points in the Cmax/10 group, accompanied by increased AUC values, indicating impaired glucose tolerance in animals exposed to the lowest dose of paracetamol during prenatal development. In contrast, ITT results did not indicate reduced insulin sensitivity, suggesting that impaired insulin sensitivity may not be the primary driver of the altered glucose response. Instead, these findings may be explained by alterations in endogenous insulin production or modulation of insulin- and glucose-related signaling pathways. Such alterations in glucose homeostasis are among the characteristic features of type 2 diabetes (Vinué and González-Navarro 2015). Interestingly, the persistent increase in water consumption observed across all prenatal paracetamol exposed groups was accompanied by alterations in glucose homeostasis despite unchanged body weight. Although the underlying mechanism was not investigated, these findings may be consistent with subtle disturbances in metabolic homeostasis resulting from prenatal paracetamol exposure. However, the present data do not establish a causal relationship between increased water intake and altered glucose regulation. Because renal function, urine output, and endocrine regulators of fluid balance were not evaluated, the biological basis of the increased water consumption remains unclear. Accordingly, prenatal exposure to subtherapeutic doses of paracetamol may predispose female offspring to an increased risk of glucose intolerance, and within the framework of developmental programming, these early disturbances in glucose regulation may increase susceptibility to metabolic disorders later in life. Further mechanistic and epidemiological studies are required to confirm and better elucidate these observations.

Molecular analyses of peritoneal adipose tissue revealed transcriptional upregulation of several key adipogenic and lipogenic genes, including *Pparγ*, *Lpl*, and *Fasn*. These genes are central regulators of adipocyte differentiation and lipid metabolism (Janesick and Blumberg 2012; Lefterova and Lazar 2009). The observed transcriptional response therefore suggests stimulation of adipogenic and lipogenic pathways following prenatal paracetamol exposure. Although consistent alterations were not detected for all proteins examined, increased LPL protein levels were observed, supporting the transcriptional findings. Subtle changes in gene regulation during critical developmental periods may have lasting physiological consequences, and together with the observed increase in visceral adipose tissue mass, these findings suggest that prenatal paracetamol exposure may influence adipose tissue programming.

The lipidomic alterations observed in this study provide mechanistic insight into the metabolic reprogramming induced by prenatal paracetamol exposure. In particular, the consistent dysregulation of LPC species across all exposure groups is noteworthy. LPCs are well-recognized bioactive lipids involved in inflammatory signaling, membrane remodeling, and metabolic regulation, and elevated LPC levels have been associated with obesity, insulin resistance, and cardiometabolic risk (Barber et al. 2012; Law et al. 2019). Therefore, the increase in LPC species observed in this study may indicate an early shift toward a pro-inflammatory metabolic state. Alterations in PC species further support the disruption of membrane lipid homeostasis. PCs play a central role in maintaining membrane integrity and lipoprotein metabolism, and their imbalance has been linked to metabolic disorders, including hepatic steatosis and insulin resistance (van der Veen et al. 2017). The observed changes in PC species may therefore reflect impaired lipid transport and altered cellular signaling processes. Moreover, the increase in unsaturated fatty acids, including FA 16:1 and long-chain polyunsaturated fatty acids, suggests modulation of lipid synthesis and desaturation pathways. Enhanced activity of lipogenic pathways has been associated with metabolic dysfunction and adipose tissue expansion (Ntambi and Miyazaki 2004). These findings are consistent with the upregulation of adipogenic markers, indicating coordinated transcriptional and metabolic reprogramming. Additionally, alterations in SM further highlight the potential involvement of sphingolipid metabolism. Sphingolipids are critical regulators of apoptosis, inflammation, and insulin signaling, and dysregulation of SM metabolism has been implicated in obesity and insulin resistance through ceramide-mediated pathways (Hannun and Obeid 2018). Thus, the observed changes in SM species may contribute to the altered metabolic phenotype detected in prenatally exposed offspring. At higher exposure levels, the increase in TG species is consistent with enhanced lipid storage and adipose tissue expansion. Elevated circulating and tissue TG levels are a hallmark of metabolic imbalance and are closely associated with increased visceral adiposity (Samuel and Shulman 2016). This aligns with the observed increase in peritoneal fat mass, suggesting that prenatal paracetamol exposure may promote lipid accumulation independently of changes in total body weight. Importantly, lipidomic alterations were also evident at the lowest exposure dose, supporting a non-monotonic dose–response pattern. Such responses are characteristic of endocrine-disrupting chemicals, where low-dose exposures can exert significant biological effects (Vandenberg et al. 2012). Within the DOHaD framework, these findings indicate that even low-level prenatal exposure to paracetamol may induce long-lasting perturbations in lipid metabolism, potentially increasing susceptibility to metabolic disorders later in life.

In addition to its metabolic effects, prenatal paracetamol exposure markedly affected reproductive endpoints in F1 female offspring. Previous studies have shown that endocrine-disrupting compounds can adversely affect ovarian development by interfering with folliculogenesis, oocyte maturation, and overall ovarian function (Cevasco et al. 2008; Gupta et al. 2010; Hannon et al. 2015; W. N. Jefferson et al. 2002; Stoker et al. 2008). Several in vivo studies have also reported that intrauterine paracetamol exposure may reduce follicle numbers and impair fertility-related outcomes in female offspring (Arendrup et al. 2018; de Araújo-Ramos et al. 2026; Holm et al. 2016; Wu et al. 2024). Consistent with these findings, the present study demonstrated a reduction in the total follicle pool, including primary, secondary, and tertiary follicles, across all exposure groups. Because the primordial follicle reserve established during fetal life is a key determinant of reproductive lifespan, such depletion may have important consequences for long-term female fertility.

Histological analysis further revealed several morphological abnormalities in ovarian tissue, including altered primary oocyte morphology, degeneration of granulosa cells, irregular zona pellucida structures, and poorly defined oocyte boundaries. These changes indicate disruption of early folliculogenesis. In addition, consistent with previous in vivo studies (de Araújo-Ramos et al. 2026), increased follicular atresia was observed at the primordial and primary follicular stages. Follicular atresia is a tightly regulated apoptotic process involving granulosa cell apoptosis and disrupted communication between oocytes and surrounding somatic cells. It has been well established that exposure to endocrine disruptors during critical windows of ovarian development increases follicular atresia via oxidative stress, mitochondrial dysfunction, and altered steroidogenic signaling (Hannon and Flaws 2015; Hussein 2005; Matsuda et al. 2012). In particular, early-stage follicles are highly sensitive to environmental and pharmacological exposures due to their limited antioxidant capacity and active cellular remodeling (Hutz et al. 2006; Priya et al. 2021). Increased atresia at these stages therefore suggests depletion of the ovarian reserve, which may ultimately impair fertility in the F1 generation. These ovarian findings should also be interpreted in light of certain methodological limitations. At the end of the experimental period, the estrous stage of all F1 female mice was determined by vaginal cytology immediately before tissue collection, and ovarian and uterine histology were interpreted with consideration of the corresponding estrous stage. However, the animals were not synchronized to the same estrous stage at sacrifice. Therefore, although prenatal paracetamol exposure was associated with increased atretic follicle numbers, the potential influence of cycle-stage differences on follicle counts cannot be completely excluded.

Additional evidence of disrupted ovarian development in F1 female mice was provided by the occurrence of MOFs. MOFs arise from incomplete breakdown of germ cell cysts or defective follicular assembly during the perinatal period. Disruption of ovarian cyst breakdown has been shown to increase MOF incidence (Pepling 2012). Previous studies have shown that endocrine disruptors such as genistein, bisphenol A, and diethylstilbestrol can increase MOF incidence in the ovary by interfering with normal ovarian cyst breakdown (Iguchi et al. 1990; W. Jefferson et al. 2006; Suzuki et al. 2002). In the present study, MOFs were detected exclusively in the Cmax/10 group, suggesting a potential low-dose response to prenatal paracetamol exposure. Such non-monotonic responses are commonly reported for endocrine-modulating compounds (Lagarde et al. 2015). The occurrence of MOFs at the lowest exposure level therefore supports the possibility that paracetamol may exert endocrine-modulating effects during ovarian development.

Overall, the histological findings are consistent with previous literature highlighting the high susceptibility of the developing ovary to intrauterine environmental and pharmacological exposures. Because premature depletion of the follicular reserve is closely associated with impaired reproductive outcomes, further mechanistic investigations focusing on apoptotic pathways, oxidative stress responses, and steroidogenic signaling are warranted to clarify the molecular mechanisms of prenatally paracetamol exposure underlying these effects.

A limitation of the present study is that individual offspring were not prospectively tracked according to their litter of origin after allocation to the different experimental analyses. Consequently, litter-based statistical analyses could not be performed. Because the litter is generally considered the appropriate experimental unit in developmental and reproductive toxicity studies, future investigations should preserve litter identity throughout the experimental workflow and incorporate litter-based statistical approaches to further validate the present findings.

5 | Conclusion

This study demonstrates that intrauterine paracetamol exposure can induce persistent reproductive and metabolic alterations in female offspring. Although systemic metabolic effects were limited, significant changes were observed in adipose tissue distribution, lipid profiling, and the expression of adipogenic and lipogenic regulators. Lipidomic analyses revealed dysregulation of major lipid classes, indicating disturbances in lipid remodeling, signaling, and energy storage pathways. Increased visceral adiposity, elevated triglyceride species, and enhanced adipogenic gene expression suggest metabolic reprogramming that may predispose offspring to metabolic dysfunction later in life, even in the absence of overt changes in body weight. Importantly, these molecular alterations were evident even in the absence of significant changes in overall body weight, highlighting the sensitivity of lipid metabolism as an early indicator of metabolic disturbance. Reproductive effects were more pronounced, with reduced follicle numbers, increased follicular atresia, abnormal follicular morphology, and the presence of MOFs, indicating disruption of folliculogenesis and potential impairment of ovarian reserve. Collectively, these findings identify lipid metabolism and ovarian development as key targets of prenatal paracetamol exposure, support the DOHaD concept that pharmaceutical exposure during critical developmental windows can induce long-term physiological consequences, and underscore the value of integrating lipidomic approaches into developmental toxicology. Further mechanistic studies are warranted to elucidate the molecular pathways linking early-life exposure to adverse metabolic and reproductive outcomes.

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Ethics Statement

Ethical approval for this study was granted by the Local Ethics Committee for Animal Experiments at Ege University (Approval No: 2020-052R2). Furthermore, animal procedures aligned with the ARRIVE guidelines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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