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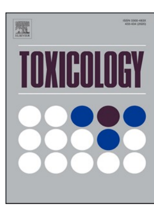

Endocrine and metabolism modulating effects of paracetamol: From *in vitro* signaling to *in vivo* metabolic reprogramming in male mice

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A B S T R A C T

Obesity is a major global health challenge associated with a cluster of comorbidities, including metabolic syndrome and type 2 diabetes, necessitating a deeper understanding of the environmental factors contributing to this epidemic. This study investigated the *in vitro* adipogenic/lipogenic potential of paracetamol and its *in vivo* endocrine and metabolic modulating effects following prenatal exposure. Using the 3T3-L1 preadipocyte model, cells were exposed to paracetamol at physiologically relevant concentrations. Results demonstrated that paracetamol promoted lipid accumulation and upregulated G3PDH activity. Furthermore, low concentrations significantly increased the protein expression of key adipogenic regulators (PPARγ, C/EBPα, LPL, and SREBP1), suggesting interference with transcriptional cascades governing adipogenesis and lipogenesis. To assess *in vivo* effects, pregnant CD1 mice were exposed to paracetamol at three human relevant doses (Cmax/10, Cmax, and Cmax×10). In male F1 offspring, prenatal exposure resulted in increased anogenital distance and a higher incidence of sperm morphological abnormalities, indicating reproductive developmental alterations despite unchanged circulating hormone levels. Metabolically, offspring exhibited dyslipidemia characterized by elevated serum triglycerides and total cholesterol. Although body weight and glucose tolerance remained unaffected, lipidomic profiling of epididymal adipose tissue revealed pronounced remodeling, including the accumulation of neutral lipids and altered membrane phospholipid composition. This was accompanied by the upregulation of the adipogenic genes *Pparγ*, *Lpl*, and *Fasn* in adipose tissue. Collectively, these findings suggest that paracetamol may act as an endocrine modulator and metabolic disruptor when exposed prenatally, inducing latent metabolic dysregulation that may predispose offspring to metabolic syndrome later in life, even in the absence of overt obesity.

1. Introduction

There are approximately 85,000 synthetic chemicals generated as a result of human activities worldwide, and more than 1000 of these have been shown to exert endocrine-disrupting effects (NIH, 2024). Bisphenol A, phthalates, dioxins, polychlorinated biphenyls, and polybrominated diphenyl ethers are among the most extensively studied endocrine disruptors (Bergman et al., 2012). In addition to environmental and industrial chemicals, certain pharmaceuticals are also known to cause endocrine disruption beyond their intended therapeutic actions; the synthetic estrogen diethylstilbestrol (DES) remains the most well-known example (Kabir et al., 2015).

In recent years, considerable attention has focused on the potential role of endocrine disruptors in obesity, particularly during childhood (Diamanti-Kandarakis et al., 2009; La Merrill and Birnbaum, 2011). In the early 2000s, it was hypothesized that certain endocrine disruptors might promote obesity by disrupting these metabolic pathways, and subsequent studies examining exposure during both early and later stages of life have provided evidence supporting this concept (de Cock and van de Bor, 2014).

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Chemicals, including endocrine disruptors, that contribute to obesity in humans and other mammals are collectively referred to as "obesogens". These chemicals can interfere with the normal physiological processes of adipose tissue through multiple mechanisms, thereby promoting excessive fat accumulation (Gluckman et al., 2007). *Adipogenesis*—the differentiation of preadipocytes into mature adipocytes regulated by adipogenic transcription factors—and *lipogenesis*—the storage of triglycerides in mature adipocytes as lipid droplets, are among these crucial physiological processes of adipose tissue, and are the primary targets of obesogens (Kersten, 2001; Sarjeant and Stephens, 2012).

Exposure to obesogens, even at very low doses, during critical windows of fetal development can disrupt metabolic processes and predispose individuals to obesity and related diseases later in life (Braun, 2017). Owing to their lipophilic nature and resistance to metabolic degradation, many obesogens bioaccumulate in adipose tissue, where they may interfere with metabolic regulation. Moreover, these compounds are capable of crossing the placental barrier, exposing the developing fetus and perturbing metabolic pathways during vulnerable stages of development (Elobeid and Allison, 2008). Consequently, it is of great importance to examine the link between prenatal exposure to obesogens and the onset of obesity in later stages of life, as previously shown with DES in a study by Newbold et al., 2007. In the literature, research on obesogens has predominantly focused on industrial chemicals, whereas only a limited number of pharmaceuticals have been investigated for their endocrine-disrupting and obesogenic potential.

Paracetamol was previously classified as Category B in the former U. S. Food and Drug Administration (FDA) pregnancy classification, and it is still generally considered safe for use as an analgesic and antipyretic during pregnancy (Alemany et al., 2021). Recent studies have suggested that maternal use of paracetamol during pregnancy may exert endocrine disrupting effects in the offspring (Lind et al., 2017; Smarr et al., 2017). Epidemiological studies have indicated that fetal exposure to paracetamol may be associated with early onset of puberty in females and reduced anogenital distance in males (Tadokoro-Cuccaro et al., 2022). Moreover, findings over the past decade have highlighted a possible relationship between prenatal paracetamol exposure and an increased incidence of cryptorchidism in male infants (Jensen et al., 2010; Kristensen et al., 2012). Furthermore, several recent reviews have summarized emerging data indicating possible endocrine disrupting effects of paracetamol and have emphasized the need for caution regarding its use during pregnancy (Arendrup et al., 2017; Bauer et al., 2021; Jégou, 2015; Kristensen et al., 2016). Besides, over the past decade, several epidemiological studies have investigated the potential association between prenatal exposure to paracetamol and the risk of childhood obesity (Liew et al., 2019; Murphy et al., 2015; Sorrow et al., 2019). However, no common or conclusive results have been obtained from these studies, and contradictory data have been reported.

Overall, the present study investigated the potential adipogenic and lipogenic mechanisms of paracetamol using 3T3-L1 murine preadipocytes *in vitro*. Subsequently, pregnant CD1 mice were exposed to physiologically relevant concentrations of paracetamol during critical windows of gestation, and the F1 male offspring were evaluated from birth through adulthood to investigate potential endocrine and metabolic modulating effects later in life.

2. Materials and methods

2.1. Materials

3T3-L1 cell line was purchased from the American Type Culture Collection (ATCC-CL-173). Dulbecco's Modified Eagle's Medium (DMEM), Trypsin EDTA 0.25%, Penicillin-Streptomycin (pen-strep), Fetal Bovine Serum (FBS), Bovine Calf Serum (BCS) were purchased from Gibco, Thermo Fisher. 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Phosphate Buffered Saline (PBS), Oil Red O, insulin and paracetamol were obtained from Sigma-Aldrich. Glycerol-3-Phosphate Dehydrogenase (G3PDH) Kit was supplied by Abcam. Formaldehyde and methanol were purchased from Isolab. The kits for adiponectin, FSH, LH, estradiol, testosterone, triglyceride and total cholestrol were supplied by Elabscience. For RT-qPCR analysis, the RNA isolation and cDNA synthesis kits were supplied by BioRad, sybr green mastermix was obtained from abm. Primer and seconder antibodies were purchased from Proteintech.

2.2. Cell culture

3T3-L1 cells (mouse embryo fibroblast cells) were cultured in DMEM supplemented with 10% bovine calf serum (BCS) and 1% penicillin–streptomycin (pen-strep) under humidified conditions at 37 °C with 5% CO_2. Cells were passaged upon reaching approximately 80% confluency. To ensure optimal differentiation capacity, cells were used for experiments up to passage 10 (Kassotis et al., 2021).

2.3. Adipogenic differentiation assay

Adipogenic differentiation assay was performed according to our previous study in 3T3-L1 cells (Entezari et al., 2024). Briefly, cells were incubated with paracetamol at concentrations ranging from 1.23 to 900 μM for a total duration of 8 days, covering the entire differentiation period. The tested concentration range of paracetamol in this study (1.23–900 μM) were selected based on reported maternal and fetal plasma Cmax values in humans (Conings et al., 2019; Rayburn et al., 1986). At the end of adipogenesis assay, differentiated cells were evaluated for morphological changes using microscopy. The three most effective concentrations and relevant to human exposure were selected for subsequent molecular analyses. The three concentrations that induced lipid accumulation were selected for subsequent molecular analyses.

2.4. Lipid accumulation by Oil Red O staining

At the end of the differentiation assay, 3T3-L1 cells were fixed with 4% formalin solution and washed with PBS and 60% isopropyl alcohol, respectively. Cells were incubated with Oil Red O working solution (3 parts 0.5% Oil Red O: 2 parts distilled water) for 30 min in room temperature and thereafter each well were washed distilled water and stained cells were visualized under microscope. Accumulated lipid droplets were quantified by dissolving Oil Red O in 100 μL of 100% isopropanol per well, and measuring the absorbance at 518 nm using a microplate reader (Thermo Scientific VarioScan).

2.5. Glycerol-3-phosphate dehydrogenase (G3PDH) activity and adiponectin secretion

At the end of the differentiation assay, cells were collected and G3PDH activity was measured according to the manufacturer instructions (ab174095). Culture media collected from the wells were used to determine adiponectin levels according to the related kit protocol (E-EL-M0002).

2.6. Gene expression by RT-qPCR

In vitro (3T3-L1 cells) and *in vivo* (adipose tissue) effect of paracetamol on *Pparγ* and its target genes have been investigated by RT-qPCR. Total RNA extraction (Aurum™ Total RNA Mini Kit), cDNA synthesis (iScript™ cDNA Synthesis Kit) and RT-qPCR (BlasTaq™ 2X qPCR MasterMix) analysis were performed according to the manufacturer instructions. Primer sequences for these genes and the RT-qPCR conditions were used as described in our previous study (Entezari et al., 2024).

2.7. Protein expression by Western blot

To assess the effects of paracetamol on the expression of adipogenic and lipogenic proteins, lysates were prepared from 3T3-L1 cells (*in vitro*) (Bozdag et al., 2025) and adipose tissue samples (*in vivo*) (An and Scherer, 2020) as described before. Protein concentrations of lysates were determined by using Bradford reagent. Protein samples were denatured at 95 °C, following electrophoresis, proteins were transferred onto PVDF membranes. The membranes were blocked and incubated overnight at 4 °C with primary antibodies. After washing, membranes were incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence (ECL) substrate on a Fusion X7 imaging system, and band intensities were quantified using ImageJ software.

2.7.1. Animal caring and paracetamol administration

Six-week-old male and female CD1 mice were housed in a 12-hour light/dark cycle with access to food and water ad libitum in the Ege University Laboratory Animal Research and Application Center. The animal experiments followed to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and all procedures were approved by the Ege University Local Ethics Committee for Animal Experiments (Approval No: 2020–052R2). After 7 days of acclimation period in the animal housing facility, seven-week-old male and female mice were housed in the cages by pairing one male with one female for mating. Gestational day (GD) 1 of pregnancy was marked by the presence of a vaginal plug and indicated successful mating. After confirmation of pregnancy, dams were randomly assigned to control and dose groups. Pregnant dams were weighed daily from the onset of pregnancy to ensure precise dosing, and dose calculations were performed by considering the body weight (BW) of dams, according to the formula provided below (Krishnan, 2019). The human equivalent dose was calculated based on a single standard therapeutic dose of 1000 mg for a 70 kg adult, corresponding to a maternal maximum plasma concentration (Cmax) of approximately 100–200 μM (Rayburn et al., 1986). For a 30 g mouse, the corresponding dose (representing the Cmax group) was estimated as 185 mg/kg using allometric scaling formula. Doses for the remaining experimental groups were adjusted accordingly to 18.5 mg/kg (Cmax/10) and 1850 mg/kg (Cmax×10), respectively. To account for physiological weight changes during pregnancy, body weight was monitored daily, and individual doses were adjusted throughout the dosing period from GD 7.5 to GD 16.5.

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

Pregnant dams were administered paracetamol orally by gavage at three different doses (Cmax/10, Cmax, and Cmax×10), while the control group received distilled water. The exposure window from GD 7.5 to GD 16.5 was chosen because it represents a critical period of early to mid-organogenesis in mice, encompassing primordial germ cell migration, genital system formation and differentiation, and the mammary gland development.

Following the completion of the dosing period, pregnant mice were housed individually and monitored until delivery. After birth, the pups were weaned at postnatal day 21 (PND21) and grouped according to the maternal dose groups (Table 1). From postnatal day 21 (PND21) to week 17, food intake and body weight of F1 offspring were measured and recorded weekly. Prior to each analysis, animals displaying visible abnormalities or body weights substantially deviating from the group mean were excluded to avoid potential bias.

Table 1
Number of pups in *in vivo* study.

Groups	Control	Cmax/10	Cmax	Cmax× 10
Female pups (n)	14	11	16	11
Male pups (n)	14	9	17	17

2.8. Assessment of anogenital distance (AGD)

To evaluate the dose-dependent antiandrogenic effects, anogenital distance (AGD) was measured and recorded in 3-week-old F1 offspring. The assessment and normalization of AGD was conducted in accordance with the OECD Test Guideline 443 (OECD, 2011).

2.9. Histological analysis

After euthanasia, testicular tissues were weighed and subsequently fixed in 4% formaldehyde for 24–48 h at room temperature. Following fixation, samples were rinsed in PBS (pH 7.4) overnight to remove residual fixative. The tissues were then dehydrated through a graded ethanol series (80%, 95%, and 100%, 1 h each), cleared in xylene, and infiltrated with paraffin. After embedding, paraffin blocks were allowed to solidify overnight at room temperature. Serial sections were then prepared using a microtome and subjected to histological staining (Hematoxylin and Eosin (H&E), Masson's trichrome) for microscopic evaluation. Testicular sections stained with H&E were examined microscopically for each group. A total of 100 seminiferous tubules per group were evaluated using the Johnsen score system, and the mean testicular Johnsen score was subsequently calculated to assess spermatogenic activity (Johnsen, 1970). Following the euthanasia, sperm samples were collected from the cauda epididymides. The sperm suspensions were used for quantitative and morphological evaluations. Sperm morphology was evaluated using Giemsa staining under light microscopy.

2.10. Measurement of endocrine and metabolic biomarkers

Blood samples were collected from offspring at postnatal weeks 8 and 17, corresponding to the young adult and mature adult stages, respectively. Samples were centrifuged at 1000 × g for 5 min at room temperature to separate the serum fraction. Serum LH (E-EL-M3053), FSH (E-EL-M0511), estradiol (E-EL-0150), testosterone (E-OSEL-M0003) and adiponectin (E-EL-M0002) concentrations were quantified using an ELISA kit, whereas triglyceride (E-BC-K238) and total cholesterol (E-BC-K109-S) levels were determined by enzymatic colorimetric assay kits, following the manufacturer's instructions.

2.11. Glucose and insulin tolerance tests (GTT/ ITT)

At postnatal week 15, F1 offspring were subjected to a GTT following an 18-hour overnight fasting. Baseline blood glucose levels were measured from the tail vein using a glucometer (Accu-Chek®, Roche Diagnostics) prior to glucose administration (time 0). Subsequently, D-glucose was administered intraperitoneally (2 g/kg body weight), and blood glucose concentrations were recorded at 15, 60, and 120 min post-injection. At postnatal week 16, ITT were performed after a 4-hour fasting period to minimize the risk of hypoglycemia. Baseline glucose levels were determined as described above, followed by intraperitoneal injection of human insulin (0.5 IU/kg body weight). Blood glucose measurements were obtained at 15, 30, and 60 min post-injection.

2.12. Lipidomics

Untargeted lipidomic analysis was performed to investigate alterations in lipid metabolism following prenatal paracetamol exposure. Lipidomic profiling was conducted using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC–qTOF–MS). Chromatographic separation was achieved on a reverse-phase column

under optimized gradient conditions. Mass spectrometric detection was performed in both positive and negative electrospray ionization modes to ensure broad coverage of lipid classes. Data were acquired in full-scan mode over a defined mass-to-charge (m/z) range.

Raw data were processed using dedicated software for peak detection, alignment, and normalization. Multivariate statistical analyses, including principal component analysis (PCA) and partial least squares–discriminant analysis (PLS-DA), were applied to evaluate global lipidomic differences between experimental groups. Differentially abundant lipid species were identified using variable importance in projection (VIP) scores > 1.0, combined with fold-change thresholds and statistical significance criteria. Lipid species were annotated using accurate mass measurements and database matching.

2.12.1. Sample preparation

Frozen plasma samples were thawed at room temperature, vortexed, and 200 μL aliquots were subjected to lipid extraction using a methanol:water:chloroform mixture (3:1:3, v/v/v). After vortexing, incubation on ice, and centrifugation (10,000 rpm, 10 min, 4 °C), the lower lipid-containing phase was collected, dried under vacuum, and reconstituted in isopropanol:acetonitrile (7:3, v/v). Quality control (QC) samples were prepared by pooling equal volumes from all samples.

2.12.2. LC-qTOF-MS based lipidomic analysis

Lipidomic analysis was performed using an LC-qTOF-MS system equipped with a C18 column (2.1 × 100 mm, 2.7 μm). Mobile phases consisted of water:acetonitrile (6:4, v/v) and isopropanol:acetonitrile (9:1, v/v), both containing 0.05% formic acid and 10 mM ammonium formate. A 26-min gradient was applied at 0.25 mL/min, with a column temperature of 60 °C and an injection volume of 10 μL. Data were acquired in both positive and negative ionization modes, and QC samples were analyzed in MS/MS mode at multiple collision energies for lipid identification.

Raw data were processed using MS-DIAL (v4.60) for peak detection, alignment, and annotation against public spectral libraries. Data were normalized using total ion chromatogram (TIC), and features with $> 30\%$ RSD in QC samples or $> 50\%$ missing values were excluded. Multivariate analyses (PCA, PLS-DA, OPLS-DA) were performed using SIMCA, and significant lipids were selected based on VIP > 1 and $p < 0.05$, followed by pathway enrichment analysis using LION/web.

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8. The Shapiro–Wilk test was applied to assess data normality. For normally distributed data, one-way ANOVA was conducted, followed by Tukey's or Dunnett's post hoc tests for multiple comparisons. Data not following normal distribution were analyzed via the Kruskal-Wallis test followed by Dunn's multiple comparison test. A p-value of less than 0.05 was considered statistically significant, with statistical significance reported as $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

3. Results

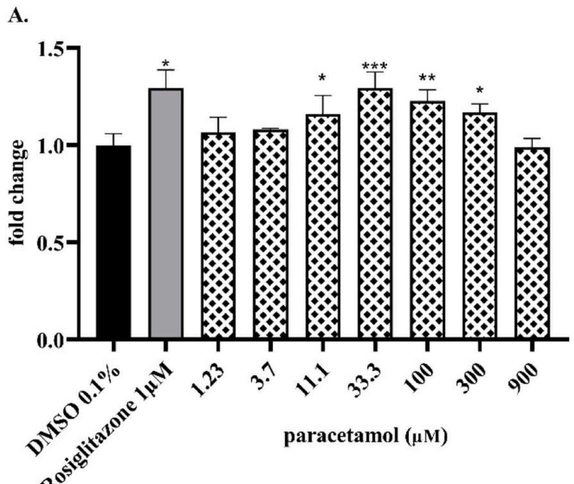
3.1. Evaluation of adipogenic/lipogenic potential of paracetamol in 3T3-L1 cells in vitro

3.1.1. Effects on lipid accumulation

To determine the effect of paracetamol on adipogenesis/ lipogenesis, its effect on lipid accumulation—an end-point biomarker of these pathways—was evaluated across a wide concentration range including Cmax values. The Oil Red O assay was performed, as mentioned in our previous study (Bozdag et al., 2025) by using rosiglitazone, a known PPARγ agonist, as a reference compound. Here, we observed an increase in lipid accumulation (Fig. 1A), and microscopic evaluation revealed that the fibroblastic morphology of the undifferentiated cells was altered to spherical shaped adipocytes by rosiglitazone-treatment (Fig. 1B). These findings show that the adipogenesis assay was successfully performed. Lipid accumulation increased in paracetamol-treated cells at concentrations of 11.1, 33.3, 100, and 300 μM, with inductions of 1.16-, 1.30-, 1.23-, and 1.17-fold, respectively, indicating a slight but significant effect of this pharmaceutical on lipid accumulation (Fig. 1A).

3.1.2. Effects on G3PDH activity and adiponectin secretion

During the differentiation of preadipocytes into mature adipocytes, G3PDH activity elevates due to enhanced lipogenesis, promoting triglyceride accumulation in lipid droplets. Therefore, it serves as a lipogenic biomarker in obesity-related studies (Ojha et al., 2014; Swierczynski et al., 2003). Moreover, adiponectin secretion increases during the adipogenesis pathway, serving as an adipogenic differentiation biomarker (Fu et al., 2005; Trujillo and Scherer, 2005). Both enzyme activity and adiponectin levels significantly increased with rosiglitazone to 11.22 mU/mL and 254.7 pg/mL, respectively,



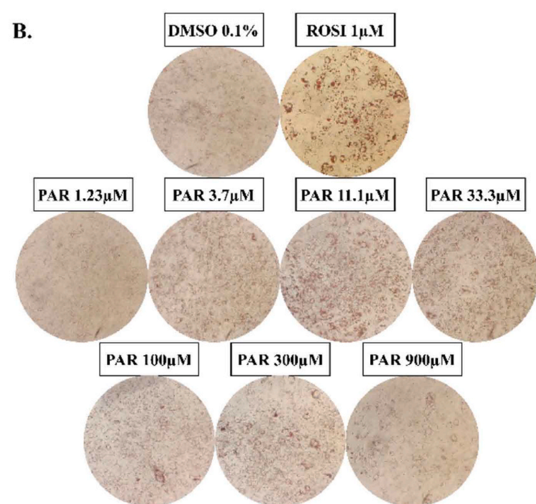


Fig. 1. Effect of paracetamol on lipid accumulation in 3T3-L1 cells assessed with Oil Red O staining. A: fold change in lipid content compared to DMSO 0.1%. B: Oil Red O stained 3T3-L1 cells. PAR: paracetamol; ROSI: rosiglitazone. Bars represent means of three independent experiments. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

compared to DMSO which were measured 5.51 mU/mL and 38.4 pg/mL. Only 100 μM of paracetamol resulted in a modest but significant increase in G3PDH activity compared to DMSO, reaching 7.62 mU/mL (Fig. 2). However, paracetamol did not produce any significant alterations in adiponectin levels within the tested concentration range.

3.1.3. Evaluation of adipogenic and lipogenic gene and protein expressions

To evaluate the effect of paracetamol on adipogenic and lipogenic gene expressions, the mRNA levels of *Pparg* and its target genes, including *Cebpa*, *Fasn*, *Lpl*, and *Srebp1*, were analyzed by RT-qPCR. Expressions of all tested genes significantly increased with rosiglitazone. Among the tested genes, paracetamol increased *Srebp1* mRNA expression at all tested concentrations and the extent of this elevation decreased in a dose-dependent manner. *Srebp1* mRNA expression increased by 1.8 and 1.38 fold at 33.3 and 100 μM, respectively (Fig. 3A). Conversely, paracetamol had no significant effect on *Cepba* and *Lpl* mRNA expressions, while *Pparg* and *Fasn* mRNA expressions were decreased at 33.3 and 300 μM, respectively.

To further investigate the effect of paracetamol on adipogenic and lipogenic factors, protein expression levels of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (CEBPα), fatty acid synthase (FASN), lipoprotein lipase (LPL) and sterol regulatory element-binding protein 1 (SREBP1) were analyzed by western blot. Since no statistically significant effects were found with 300 μM only fetal and maternal Cmax related concentrations (33.3 and 100 μM) were tested. Protein expression levels of all these factors were significantly increased in rosiglitazone treated cells. Although paracetamol induced only *Srebp1* mRNA expression among adipogenic/lipogenic genes, it enhanced the protein expression levels of PPARγ, CEBPα, LPL, and SREBP1 by 1.44-, 1.20-, 1.33-, and 1.14-fold, respectively, at 33.3 μM (Fig. 3B). Furthermore, at 100 μM, paracetamol had no effect on the protein expression of adipogenic/lipogenic factors, except for a slight increase in CEBPα protein expression (1.2-fold increase) (Fig. 3B).

3.2. Evaluation of endocrine modulating potential of paracetamol in vivo

3.2.1. Assessment of anogenital distance

Prenatal exposure to paracetamol resulted in a significant increase in anogenital distance (AGD) in male offspring, specifically in the Cmax/10 (5.7 mm/g) and Cmax (5.5 mm/g) dose groups, compared to control group (4.8 mm/g). Although a similar trend was observed in the Cmax× 10 group, the increase was not statistically significant (Fig. 4).

3.2.2. Effects on the reproductive hormone levels

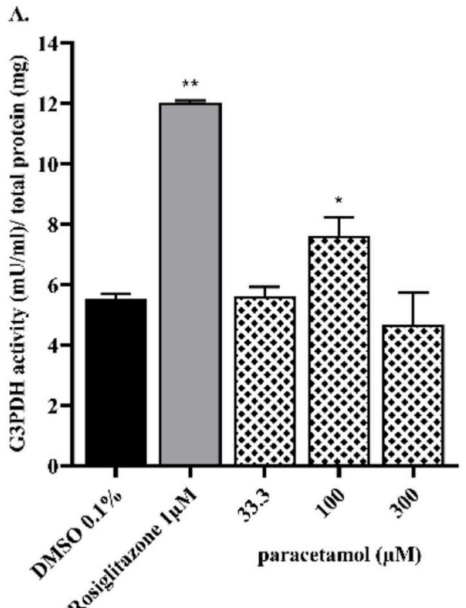
In 8-week-old male offspring, LH levels were elevated in the Cmax/10 group, while FSH levels increased in all dose groups. A statistically significant, dose-dependent decrease in testosterone levels was observed in the Cmax× 10 group, along with a reduction in estradiol levels in the Cmax group. In 17-week-old male offspring, LH levels remained unchanged across all dose groups. By contrast, FSH levels showed a dose-dependent increase, reaching statistical significance in the Cmax× 10 group. Additionally, testosterone concentrations were approximately doubled in the Cmax× 10 group, whereas estradiol levels were significantly elevated only in the Cmax/10 group (Supplementary Figure 1).

3.2.3. Histological analysis

To assess general histological parameters, testicular tissues were stained with H&E. In addition, Masson's trichrome staining was performed to evaluate stromal connective tissue. Evaluation of the seminiferous tubules (t), spermatogenic cells—including spermatogonia (sg), spermatocytes (sp), spermatids (sd), and spermatozoa (sz)—as well as sertoli cells (se) and leydig cells (lc), revealed normal morphology across all male CD1 groups. Similarly, the stromal components of the testis, including interstitial areas (S) and capillary vessels, were also observed to be histologically normal (Fig. 5).

3.2.4. Effects on sperm count and morphology

Following H&E staining of testis samples, an average Johnsen Testicular Score, an indicator of spermatogenesis quality, was calculated based on the evaluation of 100 randomly selected seminiferous tubules per sample, and the score was significantly reduced in both the Cmax and Cmax× 10 groups (Table 2). Comparison of epididymal sperm counts between groups revealed a statistically significant decrease in the Cmax× 10 group compared to the control. Additionally, sperm smears from 9 subjects per group were analyzed, and 50 sperm cells per slide were evaluated for head, neck, and tail morphology. The number of abnormal sperm was recorded and expressed as a percentage for each group. An increase in the percentage of abnormal sperm was observed



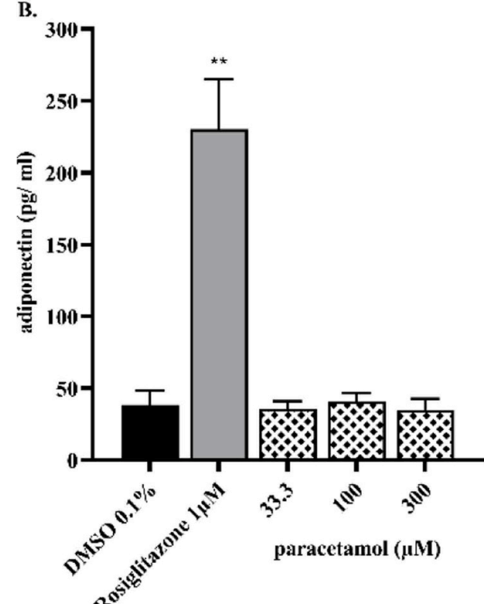


Fig. 2. Effect of paracetamol on G3PDH activity (A) and adiponection levels (B) in 3T3-L1 cells. Bars represent means of three independent experiments. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

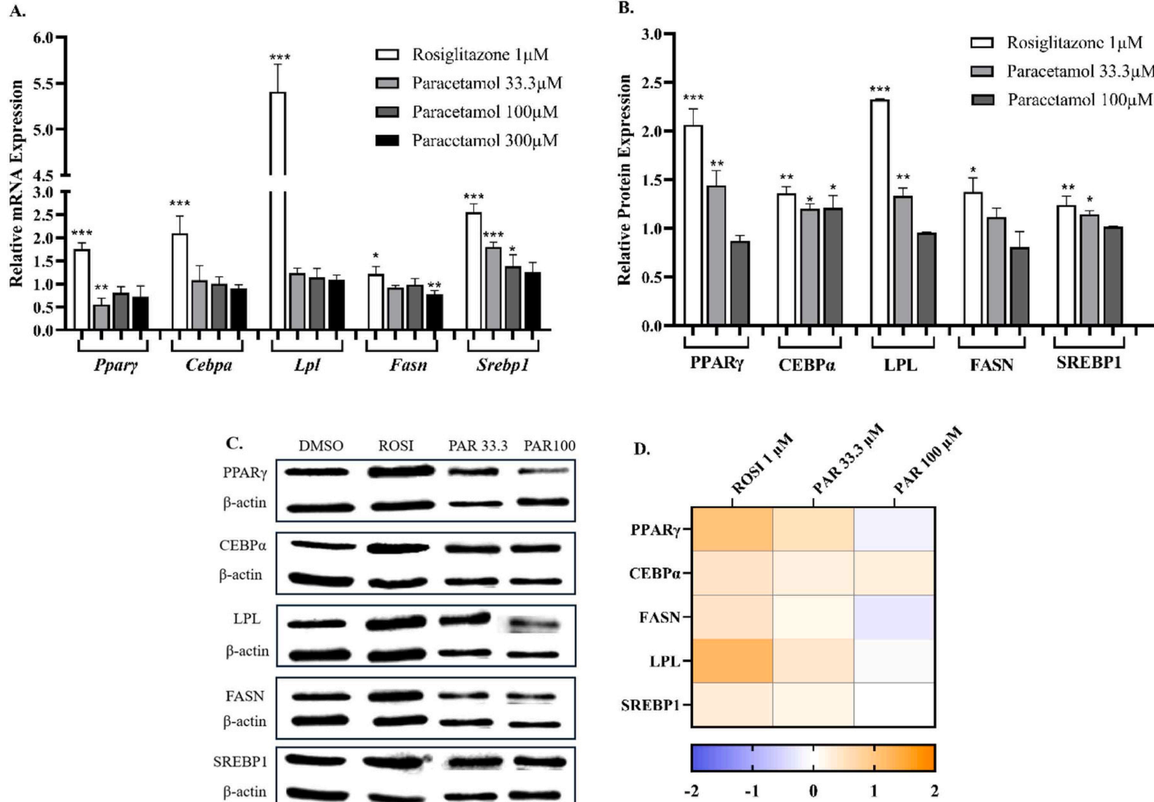


Fig. 3. Effect of paracetamol on adipogenic and lipogenic gene (A) and protein expressions (B) in 3T3-L1 cells. (C) protein bands for selected adipogenic and lipogenic markers. (D) Heatmap illustrating the relative expression profiles of adipogenic and lipogenic proteins. Bars represent means of three independent experiments. ROSI: rosiglitazone; PAR: paracetamol. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to DMSO 0.1%.

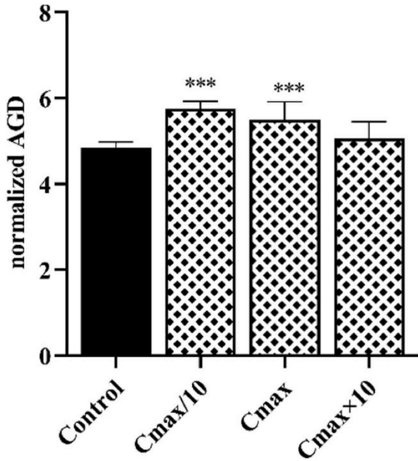


Fig. 4. Anogenital distance (AGD) in male offspring prenatally exposed to paracetamol. Maternal Cmax = 100 μM. Bars represent means of all animals in each group. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group.

across all groups (Table 2).

3.3. Evaluation of the metabolism modulating potential of paracetamol in vivo

3.3.1. Effects on body weight, food and water consumption

Offspring were weaned at postnatal week 3 and they were weighed weekly from postnatal week 4 until week 17. Compared to the control group, no significant differences in body weight were observed across the dose groups (Fig. 6). Additionally, food and water intake were monitored weekly starting from week 5. A reduction in food intake was observed in the Cmax group compared with the other dose groups across all time points. Conversely, water intake was significantly elevated in the Cmax/10 and Cmax groups (Supplementary Figure 2A-D). Furthermore, the organ weights (liver, pancreas and testes) of F1 male mice were evaluated, and no significant differences were found.

3.3.2. Effects on metabolic biomarkers

In 8-week-old male mice, serum adiponectin levels remained unchanged across groups, whereas in 17-week-old males a slight reduction was detected in the Cmax group. Serum triglyceride levels were significantly elevated at both 8 and 17 weeks. At 8 weeks, this elevation was dose-dependent, reaching significance in the Cmax and Cmax× 10 groups, while at 17 weeks, increased triglyceride levels were observed across all dose groups. Additionally, total cholesterol levels were unaffected in 8-week-old males; however, by 17 weeks, a significant increase was evident in all treatment groups compared to controls (Fig. 7).

3.3.3. Glucose and insulin tolerance tests (GTT/ITT)

Blood glucose levels were measured over time following an intraperitoneal injection of 2 g/kg glucose (GTT) and 0.5 IU/kg insulin (ITT). During the GTT, male mice in the Cmax group exhibited lower blood glucose levels compared to controls at 15 and 60 min, returning to control levels by 120 min. Similarly, in the Cmax/10 group, glucose levels were significantly lower relative to controls at 15 min but normalized at subsequent time points.

During the ITT, blood glucose levels declined over time in response to insulin administration. In F1 male mice, blood glucose levels in the Cmax× 10 group were higher relative to controls at 15 min but returned to baseline levels at subsequent time points. Moreover, in Cmax group blood glucose levels was lower than the control after 60 min

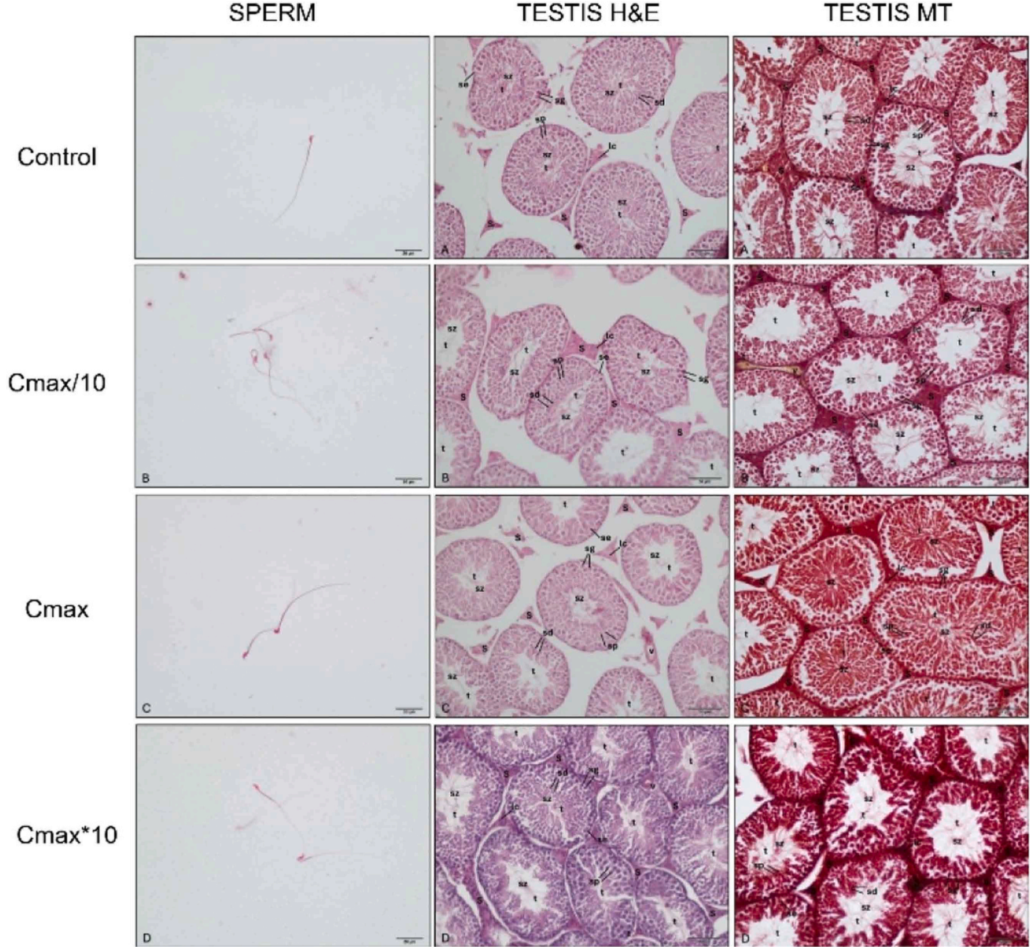


Fig. 5. Representative histological images of sperm and testes from all experimental groups. seminiferous tubules (t), spermatogonia (sg), spermatocytes (sp), spermatids (sd), spermatozoa (sz), sertoli cells (se), stromal areas (S), and leydig cells (lc). Maternal Cmax = 100 μM. Magnifications: smear × 40; testis × 20. Sperm staining: Eosin Y; Testis staining: Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT).

Table 2
Comparison of Johnson Testicular Score, epididymal sperm counts, and sperm anomaly percentages among groups. Maternal Cmax = 100 μM. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group.

Groups	Control	Cmax/10	Cmax	Cmax× 10
Johnson Testicular Score	8.31	8.11	**7.3***** ($p < 0.0001$)	**7.53*** ($p = 0.0108$)
Sperm Count	21×10^6	19×10^6	25×10^6	$\mathbf{14 \times 10^6}$* ($p = 0,0309$)
Abnormal sperm (%)	26%	32%	40%	34%

(Supplementary Figure 3).

3.3.4. Effects on adipogenic/ lipogenic gene and protein expressions in adipose tissue

The expression levels of certain adipogenic/lipogenic genes and proteins in epididymal adipose tissue were investigated by RT-qPCR and western blotting. *Pparγ* mRNA expression was upregulated in a dose-dependent manner, with 1.6-fold and 1.9-fold induction observed in the Cmax and Cmax× 10 groups, respectively. *Lpl* expression was also significantly elevated, showing 3.3-fold and 2.8-fold increases in the Cmax and Cmax× 10 groups. Moreover, *Fasn* expression exhibited 1.6-fold, 2.7-fold and 1.9-fold induction in all dose groups, respectively. In contrast, *Srebp1* expression was reduced in epididymal adipose tissue compared to control (Fig. 8A).

Analysis of adipogenic and lipogenic protein expression revealed reduced levels of LPL and SREBP1 compared with controls. In contrast, FASN expression was increased by 1.35-fold in the Cmax× 10 group, whereas no significant change was observed in PPARγ expression (Fig. 8B-C).

3.3.5. Lipidomics

3.3.5.1. Global lipidomic profile alterations. Untargeted lipidomic analysis revealed clear alterations in the adipose tissue lipid profile following prenatal paracetamol exposure. Multivariate analysis demonstrated a distinct separation between control and paracetamol-exposed groups in PLS-DA score plots, indicating dose-dependent remodelling of the lipidome. Model performance parameters supported the robustness of group discrimination (Fig. 9A).

3.3.5.2. Differential lipid species and lipid classes. Comparative analysis identified significant changes across multiple lipid classes in prenatally paracetamol-exposed offspring. Notably, neutral lipids such as triglycerides (TG), diglycerides (DG), and cholesterol esters (CE) were prominently altered, indicating disrupted lipid storage and cholesterol homeostasis. In addition, changes were observed in membrane-associated phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), suggesting

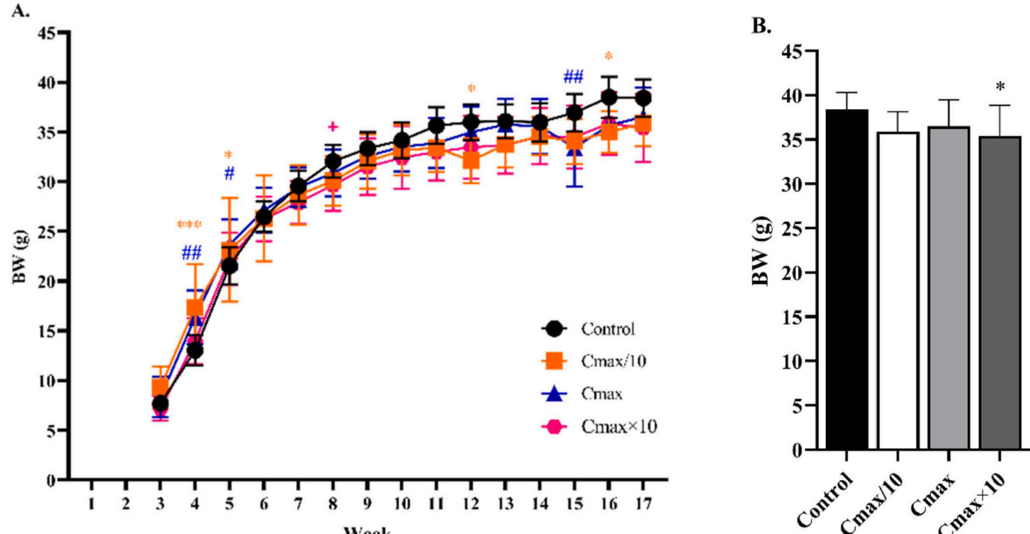


Fig. 6. Effect of prenatal paracetamol exposure on body weight. Weekly body weight (A) and body weight of 17-week-old F1 CD1 male mice. Maternal Cmax = 100 μM. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group.

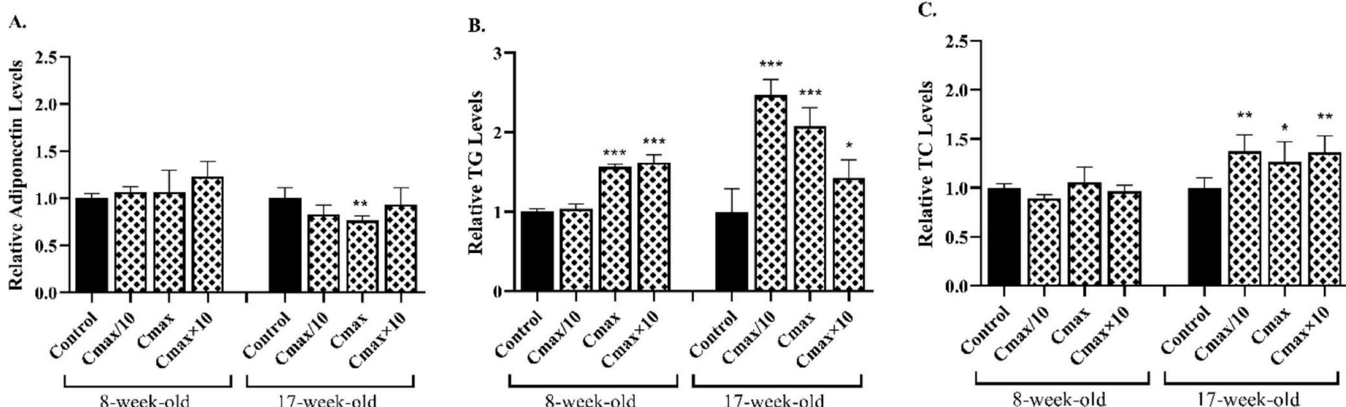


Fig. 7. Effect of prenatal paracetamol exposure on serum levels of adipogenic biomarkers in F1 CD1 males. TG: triglyceride; TC: total cholesterol. Maternal Cmax = 100 μM. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group.

modifications in membrane composition and cellular signalling capacity (Fig. 9 B-D).

PLS-DA score plot and univariate analyses further highlighted dose-related trends in lipid abundance, with several lipid species showing consistent upregulation or downregulation compared to controls. These lipidomic changes occurred in the absence of marked differences in body weight, underscoring a metabolic shift independent of overt obesity (Fig. 9).

In addition, the enrichment of lipid-mediated signalling pathways and lipids with negatively charged headgroups support the presence of membrane structural alterations that may influence cellular signalling and membrane dynamics. Changes in lipid curvature–associated terms further suggest modifications in membrane organization, which can occur during cellular stress responses and inflammatory activation (Fig. 10).

Notably, the concurrent enrichment of lipid storage, lipid droplet, glycerolipid, and triacylglycerol categories implies a metabolic adaptation to increased fatty acid availability. Such responses are often interpreted as protective mechanisms that sequester excess fatty acids to prevent lipotoxicity and maintain cellular energy homeostasis under stress conditions.

Overall, the combined lipidomic signatures point toward inflammation-associated membrane remodelling accompanied by fatty acid mobilization and metabolic reprogramming, highlighting altered lipid turnover as a central feature of the biological response observed in this study.

3.3.5.3. Pathway-level interpretation. Lipid ontology enrichment analysis demonstrated consistent perturbations in fatty acid–related lipid subclasses across all experimental comparisons relative to the control group. The most prominently enriched categories were associated with fatty acids and their conjugates, including long-chain fatty acids (>18 carbons), fatty acids with low degrees of unsaturation (≤2 double bonds), and fatty acids containing 16–18 carbon chains. In addition, significant enrichment of lipid-mediated signalling pathways and lysoglycerophospholipid subclasses, particularly monoacylglycerophosphocholines, suggests substantial remodeling of membrane phospholipids accompanied by activation of lipid signaling processes (Fig. 10).

The enrichment of lipids characterized by negatively charged headgroups and positive intrinsic curvature further supports the presence of alterations in membrane structural properties, which may reflect increased membrane turnover and dynamic remodeling events. Representation of both polyunsaturated and saturated fatty acid categories indicates broad disturbances in fatty acid composition and metabolic regulation. Moreover, the enrichment of lipid storage–related terms,

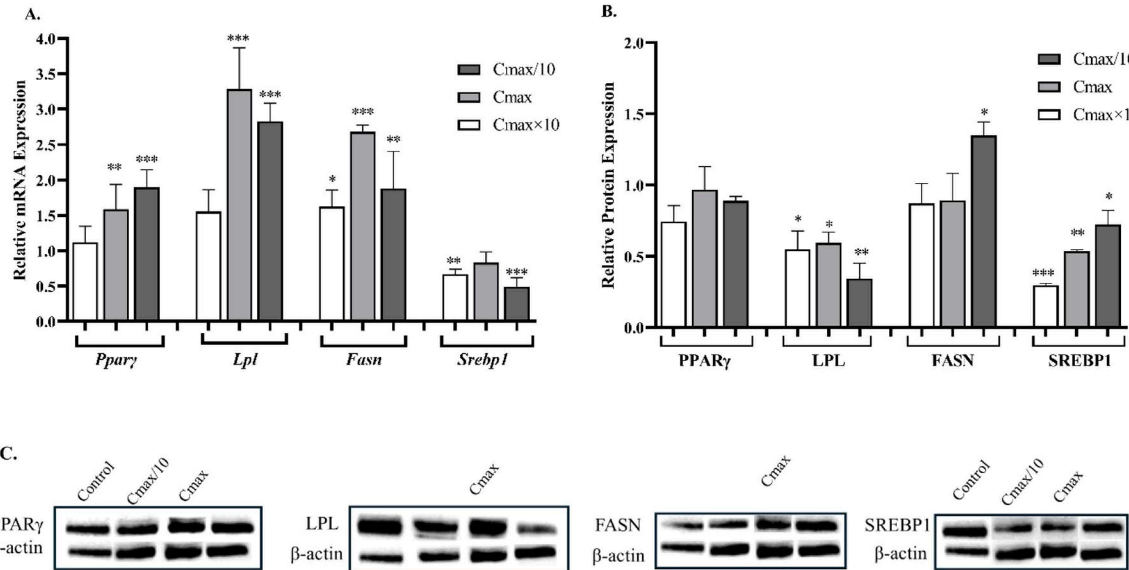


Fig. 8. Effect of prenatal paracetamol exposure on adipogenic/lipogenic gene (A) and protein (B-C) expressions in epididymal adipose tissue, in F1 CD1 males. Maternal Cmax = 100 μM. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group.

including lipid droplets, glycerolipids, and triacylglycerols, points toward metabolic adaptation to altered fatty acid availability and potential shifts in cellular energy handling (Fig. 10).

Notably, the concurrent enrichment of lysoglycerophospholipids and fatty acid subclasses suggests enhanced phospholipid hydrolysis, potentially driven by phospholipase-mediated processes, resulting in the mobilization of free fatty acids and the generation of bioactive lipid mediators. Taken together, these findings indicate that membrane lipid remodeling, fatty acid mobilization, and altered lipid storage dynamics constitute central features of the observed lipidomic alterations, potentially reflecting underlying inflammatory or metabolic stress–related mechanisms.

4. Discussion

The present study aimed to elucidate the adipogenic potential of paracetamol through *in vitro* analyses for the first time, using the murine 3T3-L1 preadipocyte cell line, and to further assess its *in vivo* endocrine and metabolism moldulating effects in CD1 mice following prenatal exposure.

Adipogenesis is a complex, multistep process involving the commitment of mesenchymal stem cells (MSCs) to the preadipocyte lineage (first phase), followed by the differentiation of preadipocytes into lipid containing mature adipocytes (second phase). The 3T3-L1 preadipocyte cell line serves as the gold standard for investigating terminal adipocyte differentiation (Tang and Lane, 2012). Furthermore, the 3T3-L1 model is extensively utilized in adipogenesis research, supported by a substantial body of literature, which facilitates reliable comparison across studies and the use of standardized, validated assays. Given this background, the 3T3-L1 preadipocyte model was used in this study to investigate the *in vitro* adipogenic and lipogenic effects of paracetamol.

In *in vitro* experiments, concentration range of paracetamol was selected based on the reported maternal and fetal maximum plasma concentrations in humans. Following a standard 1 g therapeutic dose of paracetamol, maternal Cmax values have been reported to range between 100–200 μM, while fetal concentrations are typically lower, within 60–80 μM (Conings et al., 2019; Rayburn et al., 1986). In the present study a wide range of paracetamol concentrations (1.23–900 μM) was initially tested to evaluate lipid accumulation. As the effect in lipid accumulation was significant within the Cmax-relevant range (33.3–300 μM), all subsequent experiments were performed within this interval. These concentrations were therefore considered physiologically relevant, allowing the experimental design to mimic potential *in vivo* exposure levels during pregnancy and to better interpret the observed cellular responses in the context of human exposure.

The impact of paracetamol on lipid accumulation was assessed as a terminal endpoint of adipogenic and lipogenic differentiation, using Oil Red O staining—one of the most widely applied methods for visualizing and quantifying intracellular lipid droplets (Kraus et al., 2016)— in differentiated 3T3-L1 adipocytes. Our findings demonstrated that paracetamol promoted lipid accumulation at maternal and fetal Cmax (33.3 and 100 μM), suggesting a potential metabolic disrupting effect within physiologically relevant exposure levels. Moreover, at the 100 μM concentration paracetamol significantly upregulated G3PDH activity, a key enzyme in triglyceride synthesis (Cifuentes et al., 2008; Ojha et al., 2014), suggesting that it may promote lipogenesis through modulating metabolic enzymes associated with lipid biosynthesis.

During adipogenesis, the activation of pro-adipogenic transcription factors (such as C/EBPα, C/EBPβ, and SREBP1) leads to the induction of the master regulator PPARγ (Lefterova and Lazar, 2009). To fully establish adipogenic differentiation, PPARγ acts in harmony with its key downstream effector C/EBPα. These two transcription factors function synergistically, co-binding to over 90% of the same genomic loci to activate the transcription of adipocyte-specific genes such as FASN, LPL, and fatty acid–binding protein 4 (FABP4), which are essential for lipid accumulation and the maintenance of mature adipocyte phenotype (Song et al., 2020). In the present study, *in vitro* analysis of gene and protein expressions revealed that paracetamol increased only *Srebp1* gene expression in 3T3-L1 cells. Notably, at 33.3 μM — lower concentration than the fetal Cmax — paracetamol significantly upregulated PPARγ, C/EBPα, LPL, and SREBP1 protein expressions. The discrepancies between the gene and protein expressions can be attributed to higher protein stability, longer protein halflife, or saturation of mRNA expression (Greenbaum et al., 2003). These results indicate that paracetamol, even at low concentrations, may interfere with the transcriptional cascade, thereby perturbing metabolic pathways in adipose tissue during early development. These findings may provide a potential mechanistic explanation for the previously reported association between paracetamol exposure and childhood obesity (Murphy et al., 2015; Sorrow et al., 2019).

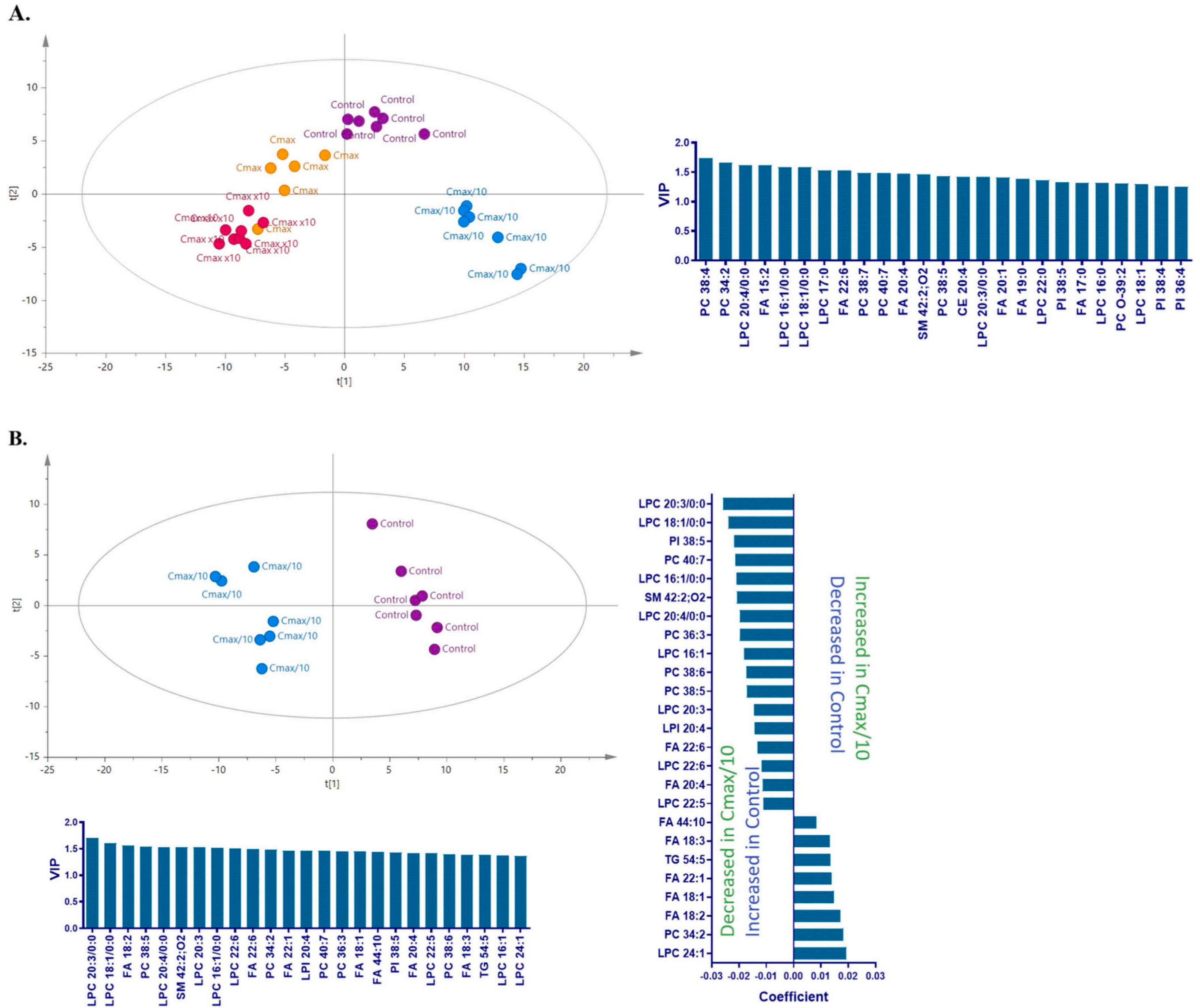


Fig. 9. Multivariate data analysis of lipidomics data from prenatally paracetamol-exposed male mice. A) PLS-DA score plot of all dose groups. (B-D) Comparison of Cmax/10, Cmax, and Cmax× 10 versus control in male mice, respectively.

It is noteworthy that the observed alterations in gene and protein expression and enzymatic activity were characterized by modest but statistically significant shifts. While these magnitudes may appear subtle compared to high-throughput genomic screenings, their biological relevance is underscored by their consistency across multiple regulatory levels—from transcriptional induction to enzymatic activation and, ultimately, to functional phenotypic outcomes such as increased lipid accumulation. In the context of developmental toxicology, such persistent, low-magnitude changes during critical windows of susceptibility are increasingly recognized as drivers of metabolic programming.

To evaluate the *in vivo* endocrine and metabolism modulating potential of paracetamol in male F1 generation, pregnant CD1 mice were exposed to three concentrations of paracetamol (Cmax/10, Cmax, Cmax×10; maternal Cmax = 100 μM). AGD was assessed in male offspring, and significant increase in the Cmax/10 and Cmax groups was observed. This finding contrasts with certain epidemiological studies, where *in utero* paracetamol exposure has been associated with decreased AGD or no measurable effect (Bauer et al., 2021; Fisher et al., 2016; Lind et al., 2017; Navarro-Lafuente et al., 2021). Notably, *in vivo* studies, reporting reductions in AGD, employed paracetamol concentrations at least tenfold higher than the maternal/fetal Cmax levels used in the present study (Kristensen et al., 2011, 2016). It should be emphasized that such supraphysiological concentrations may not accurately reflect relevant human exposures. Taken together, the observed increase in AGD in this study suggests that paracetamol does not exert anti-androgenic effects but may induce endocrine-related alterations at low physiologically relevant concentrations, indicative of a potential low-dose effect on male reproductive development.

In our previous study, paracetamol decreased testosterone levels in H295R cells (Entezari et al., 2023). Consistent with these findings, several *in vivo* studies in rodents have demonstrated that intrauterine exposure to paracetamol decreases fetal testicular testosterone synthesis (Kristensen et al., 2011, 2012). Nevertheless, data from human-based models remain inconclusive. For example, one study utilizing human testicular explants reported no significant alteration in testosterone levels following paracetamol exposure (Mazaud-Guittot et al., 2013), whereas another study employing the same model demonstrated a concentration-dependent biphasic response, with reduced testosterone levels at low and no observable effect at higher concentrations (Albert et al., 2013). Moreover, previous studies have indicated that

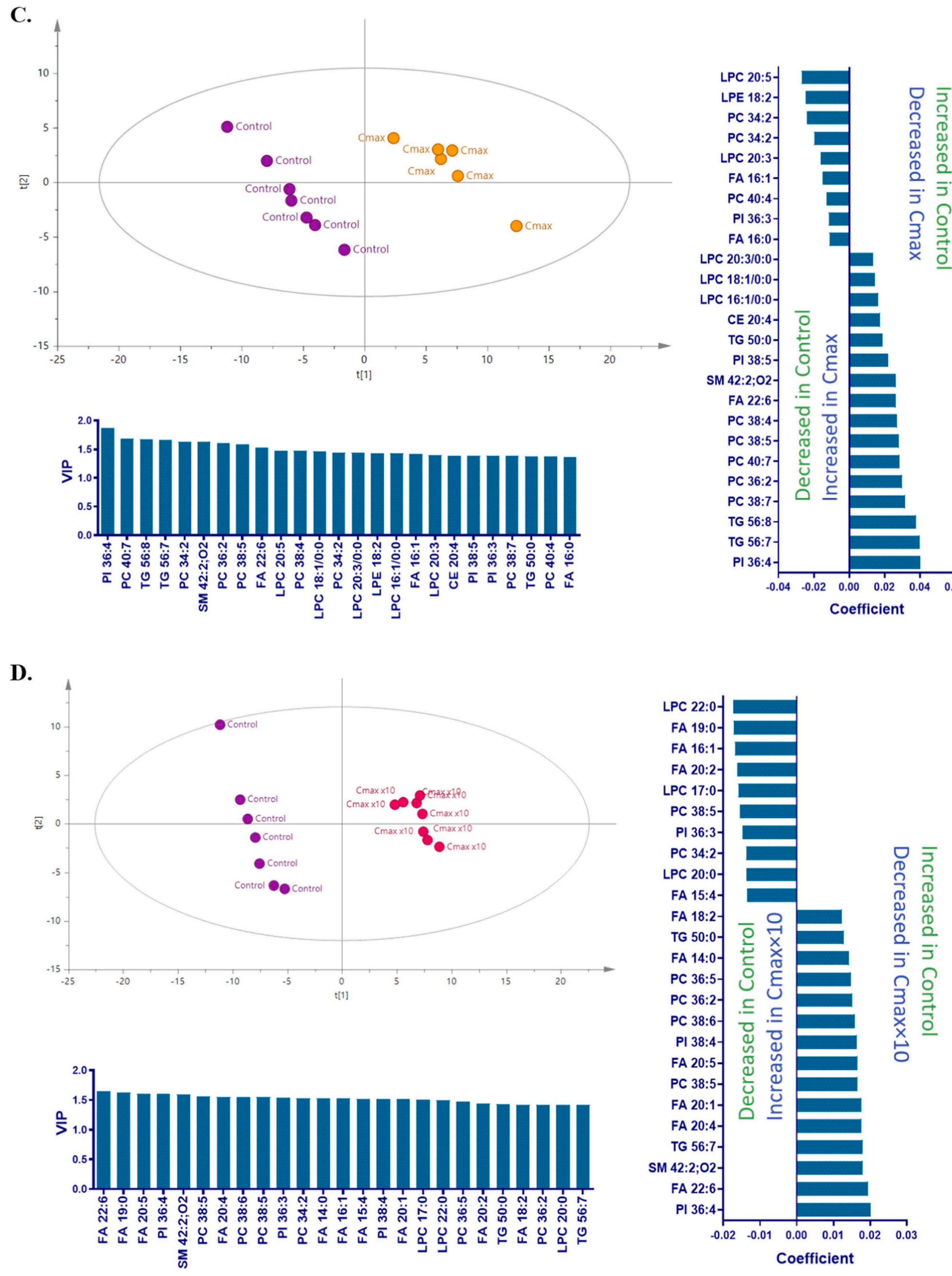
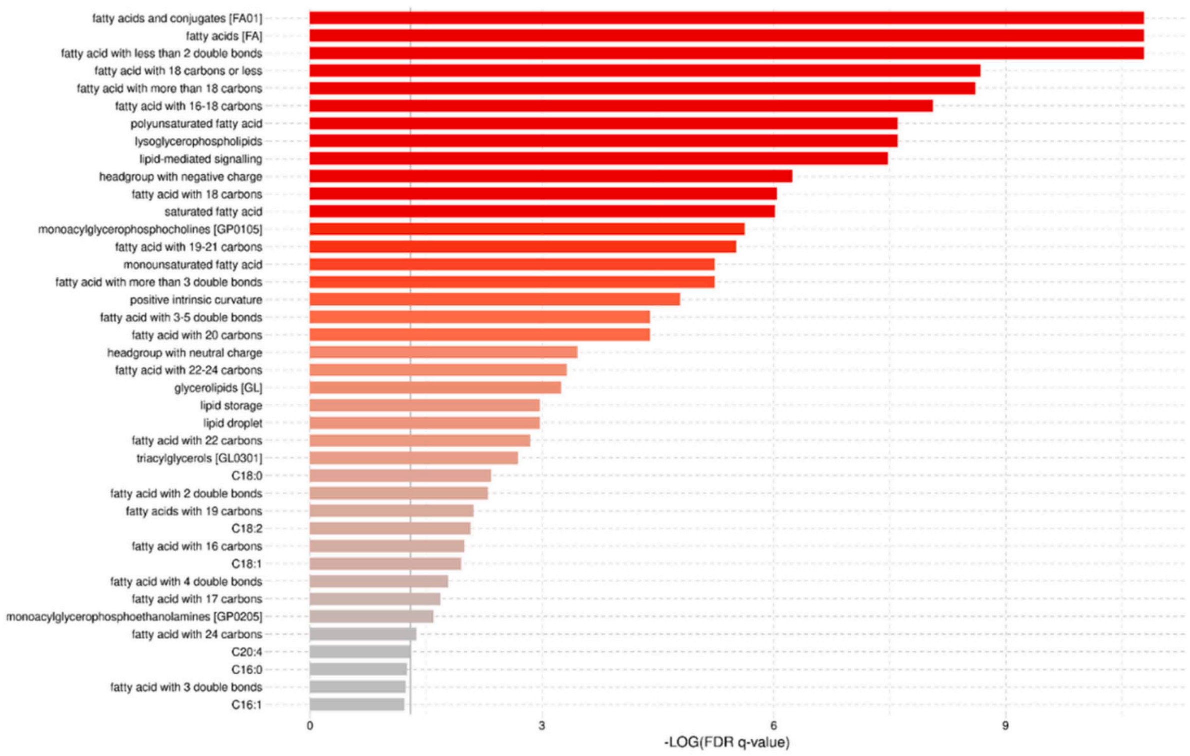


Fig. 9. (*continued*).

paracetamol exposure may impair male reproductive function, as evidenced by decreased sperm counts and increased sperm morphological abnormalities (Smarr et al., 2016, 2017). In the present *in vivo* study, maternal exposure to paracetamol did not produce a statistically



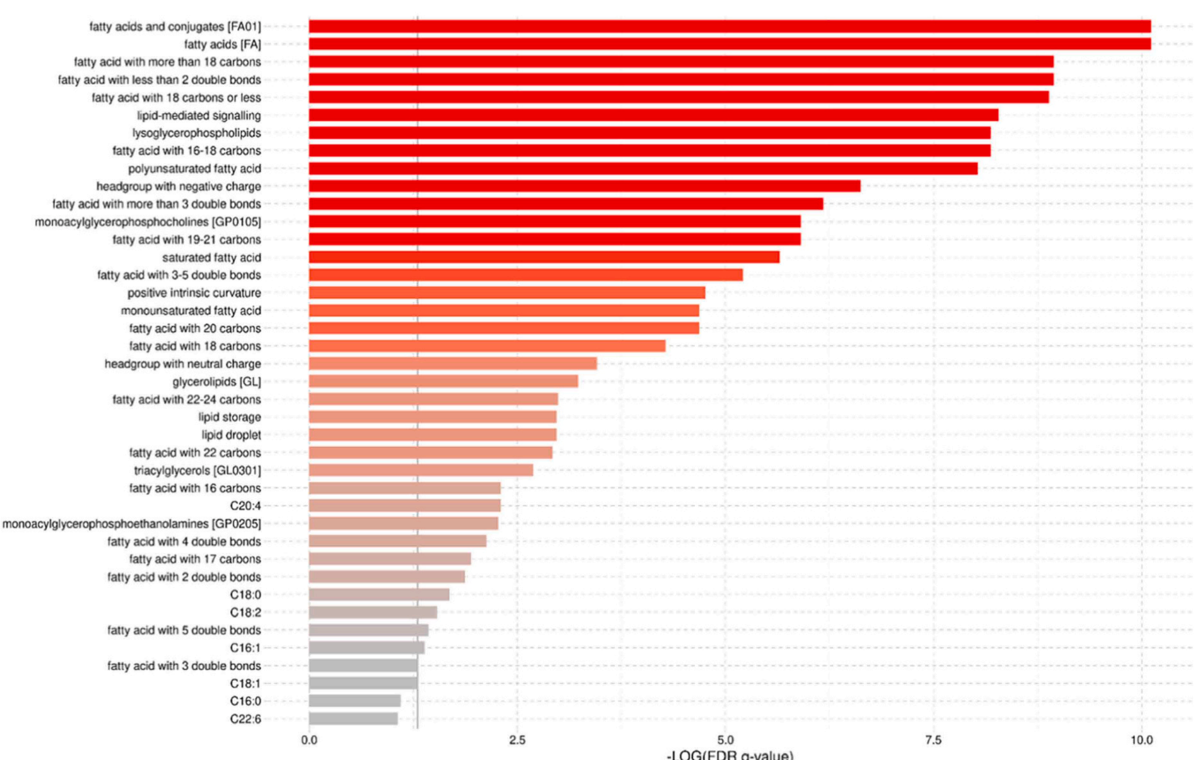


Fig. 10. Pathway enrichment analysis of statistically significant ($p < 0.05$) altered lipid metabolites in paracetamol-treated male mice. (A-C) Pathway analysis results for Cmax/10, Cmax, and Cmax$\times$ 10 groups in male mice, respectively.

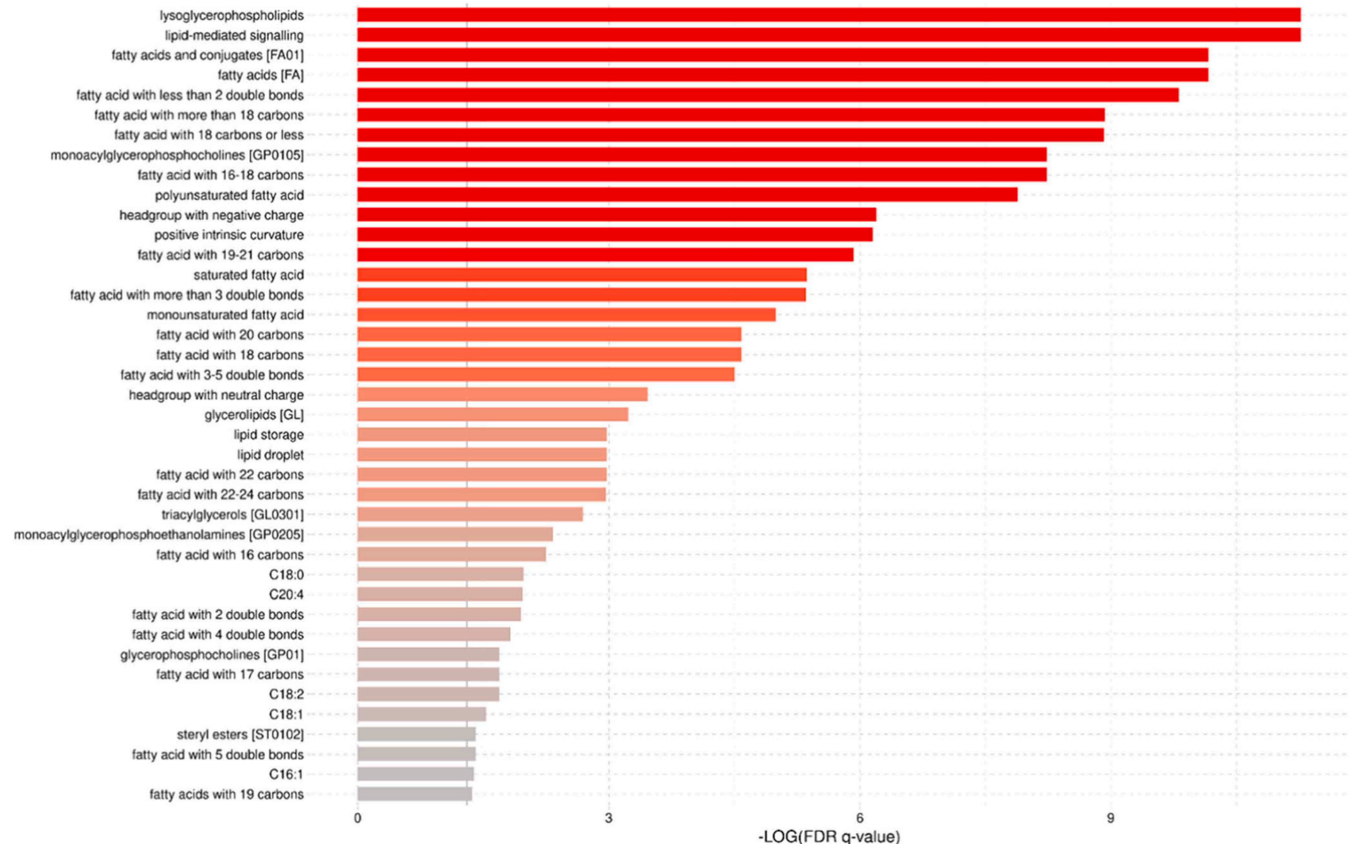


Fig. 10. (*continued*).

significant, dose-dependent alteration in circulating endocrine hormone levels. However, histopathological analysis revealed a reduction in sperm counts in the Cmax× 10 group, along with an increased incidence of sperm morphological abnormalities across all treatment groups, most notably at the Cmax group. Furthermore, reductions in the testicular Johnsen scores in the Cmax group suggest that intrauterine paracetamol exposure may impair spermatogenesis. The absence of pathological alterations in testicular tissue indicates that impairment in spermatogenesis may be functional rather than structural, possibly mediated by cellular or molecular mechanisms such as oxidative stress, mitochondrial dysfunction, or apoptosis induction.

Obesity is often accompanied by dyslipidemia, characterized by elevated triglyceride levels that contribute to the onset of insulin resistance and metabolic syndrome (Klop et al., 2013). Similarly, increased serum total cholesterol is a well-established indicator of cardiovascular disease risk (Singh et al., 2011). In the present study, a marked increase in serum triglyceride and total cholesterol levels was observed in adult male F1 offspring. Notably, this occurred despite the lack of significant changes in body weight within the Cmax group, even though food intake was reduced (Supplementary Figure 2A-B). The lack of significant difference in body weight until week 17 does not rule out the metabolic programming observed *in vitro*. It is well-documented in the DOHaD (Developmental Origins of Health and Disease) framework (Gluckman and Hanson, 2004; Heindel and Blumberg, 2019) that metabolic vulnerabilities often remain latent under standard laboratory diet conditions and only manifest as overt obesity when the organism is challenged with a high-fat diet later in life. Moreover, a slight decrease in adiponectin levels was observed in 17-week-old Cmax group. Given that adiponectin is a key regulator of glucose and lipid homeostasis and enhances insulin sensitivity (Ojha et al., 2014), this reduction—although modest—may indicate the early onset of metabolic alterations. Nevertheles, metabolic tolerance tests (GTT and ITT) revealed no impairment in glucose or insulin responsiveness. All these findings suggest that prenatal exposure to paracetamol may induce long-term alterations in lipid metabolism, potentially predisposing offspring to metabolic dysfunctions such as metabolic syndrome, or cardiovascular disorders later in life.

Analysis of gene and protein expressions revealed discrepancies between mRNA and protein levels, consistent with observations from the *in vitro* studies. Nevertheless, the data indicate that prenatal exposure to paracetamol, at both therapeutic and sub-therapeutic doses, may disrupt metabolic processes during critical developmental windows. Notably, the upregulation of key adipogenic and lipogenic genes, including *Pparγ*, *Lpl*, and *Fasn*, in epididymal adipose tissue suggests stimulation of adipogenesis and lipogenesis pathways in the offspring. Despite the absence of consistent induction at the protein level, this transcriptional response, together with the modest increase in FASN protein levels, may still hold physiological relevance, as subtle shifts in gene regulation during critical developmental windows could influence development later in life. These findings imply that early-life paracetamol exposure can modulate the expression of essential regulatory genes governing lipid metabolism, potentially leading to long-term alterations in adipose tissue function and metabolic homeostasis.

The lipidomic alterations identified in this study provide mechanistic insight into the metabolic consequences of prenatal paracetamol exposure. Despite the absence of significant changes in body weight, the pronounced remodeling of the adipose tissue lipidome indicates the presence of latent metabolic dysregulation, a phenomenon frequently described within the framework of developmental metabolic programming induced by early-life environmental exposures (Heindel et al.,

2017; Janesick & Blumberg, 2012). In particular, the elevation of triglycerides, diglycerides, and cholesterol esters is consistent with the increased circulating triglyceride and total cholesterol levels observed in exposed offspring, suggesting coordinated systemic and tissue-level lipid disturbances. Accumulation of neutral lipids is commonly interpreted as an adaptive response to altered lipid flux and may reflect compensatory lipid storage mechanisms aimed at preventing lipotoxicity (Listenberger et al., 2003; Welte, 2015). Alterations in membrane phospholipids, including phosphatidylcholines, phosphatidylethanolamines, and sphingomyelins, further suggest that prenatal paracetamol exposure may influence adipocyte membrane composition and lipid-mediated signaling pathways. Membrane lipid remodeling is known to play a critical role in adipocyte differentiation, lipid droplet formation, and insulin sensitivity, and disturbances in these processes are closely linked to metabolic dysfunction (van der Veen et al., 2017). Overall, these findings support the notion that paracetamol exposure extends beyond transient changes in gene expression and may instead induce a persistent, maladaptive lipidomic profile in the offspring. Such alterations may represent a latent metabolic vulnerability, potentially lowering the threshold for the development of diet-induced obesity and systemic dyslipidemia later in life. In this context, an important point to consider when interpreting these lipidomic findings is the absence of dietary variation in the experimental design. It is well recognized that dietary composition—particularly total fat intake and fatty acid profile—can substantially influence the lipidome, affecting lipid storage, membrane structure, and lipid-mediated signaling pathways (Quehenberger et al., 2011; Vetrani et al., 2013). In the present study, all animals were maintained on a standard laboratory diet, which allowed us to minimize variability and specifically examine the effects of prenatal paracetamol exposure. However, this controlled setting also limits the direct interpretation of the observed lipidomic alterations in more complex metabolic conditions. Notably, early-life metabolic alterations may remain subtle under standard dietary conditions and become more pronounced only in the presence of additional metabolic challenges, such as high-fat diets (Gluckman and Hanson, 2004; Heindel and Blumberg, 2019). Therefore, the lipidomic changes observed here may reflect an early shift in metabolic regulation rather than overt dysfunction, potentially indicating increased susceptibility to metabolic imbalance under different dietary exposures. Future studies incorporating controlled dietary interventions will be important to further clarify the pathophysiological relevance of these findings.

Importantly, these findings support the concept that early-life exposure to xenobiotics can induce long-lasting molecular changes without immediate phenotypic manifestations such as increased body weight, with metabolic alterations often preceding overt clinical outcomes (Heindel et al., 2017). The lipidomic data complement the observed transcriptional alterations in adipogenic and lipogenic pathways, collectively indicating that paracetamol may act as a metabolic disruptor rather than ma classical obesogen, consistent with emerging concepts in endocrine and metabolic disruption research (Janesick and Blumberg, 2012; La Merrill and Birnbaum, 2011). Overall, the integration of lipidomic profiling strengthens the evidence that prenatal paracetamol exposure induces subtle but persistent reprogramming of lipid metabolism, which may increase susceptibility to metabolic disorders later in life, even in the absence of overt obesity.

Collectively, while paracetamol remains an essential therapeutic option for the management of high fever and severe pain during pregnancy, the findings of this study indicate that its use warrants a more cautious and risk–benefit evaluation. In light of the evidence suggesting latent endocrine and metabolic reprogramming in the offspring, clinical practice should prioritize the use of the lowest effective dose for the shortest possible duration in order to minimize potential long-term developmental risks.

5. Conclusion

The complementary experimental approaches used in this research, were designed to provide a comprehensive understanding of the potential role of prenatally paracetamol exposure in the disruption of reproductive system, lipid metabolism and adipose tissue development. The findings support that paracetamol, at physiologically relevant concentrations, may exert endocrine-modulating and metabolic-disrupting effects via multiple mechanisms. Importantly, lipidomic profiling in male offspring revealed persistent alterations in adipose tissue lipid composition, characterized by remodeling of neutral lipids and membrane phospholipids, suggesting early metabolic reprogramming that may predispose individuals to metabolic dysfunction later in life even in the absence of overt phenotypic changes. Both observational and interventional epidemiological studies are warranted to thoroughly examine the relationship between paracetamol use during pregnancy and long-term outcomes in offspring, including hormonal changes, reproductive impairments, obesity, regulation of body weight and appetite, insulin resistance, and other metabolic dysfunctions.

ARRIVE statement

The animal experiments followed the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and all procedures were approved by the Ege University Local Ethics Committee for Animal Experiments (Approval No: 2020–052R2).

CRediT authorship contribution statement

Gurer-Orhan Hande: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Altug Yavasoglu:** Supervision, Methodology, Investigation. **Deniz Bozdag:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bita Entezari:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Suna Sabuncuoglu:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Aylin Buhur:** Writing – review & editing, Visualization, Methodology, Formal analysis.

Animal ethics statement

All animal experiments described in this study were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The study was carried out in compliance with the EU Directive 2010/63/EU for the protection of animals used for scientific purposes and relevant institutional and national regulations governing the care and use of laboratory animals.

The experimental procedures were reviewed and approved by the Ege University Local Ethics Committee for Animal Experiments (Approval No: 2020–052R2).

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2026.154468.

Data availability

Data will be made available on request.

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