**Title:** Exposure to environmentally persistent free radicals during gestation lowers energy expenditure and impairs skeletal muscle mitochondrial function in adult mice

**Authors:** Erin J. Stephenson1,2,3, Alyse Ragauskas1,2,3, Sridhar Jaligama2,3, JeAnna R. Redd1,2,3, Jyothi Parvathareddy2,3, Matthew J. Peloquin1,2,3 , Jordy Saravia2,3 , Joan C. Han1,2,3 and Stephania A. Cormier1,2,3,, Dave Bridges1,2,3,4

**Author contributions:** Funding (DB, SAC, JCH), Conceptualization (DB, SAC), Experimental design (DB, EJS, SAC), Data acquisition (EJS, JP, AR, MJP), Technical contributions (EJS, AR, MJP, JP, JRR, SJ), Data analysis (EJS, DB, JS), Data interpretation (EJS, DB, SAC, JCH), Drafted manuscript (EJS, DB), Edited manuscript (EJS, DB, SAC, SJ, JCH), Final approval of manuscript (EJS, DB, SAC, SJ, JS, MJP, JP, JRR, AR, JCH)

**Affiliations:**

1Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee, 38163. 2Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee, 38103. 3Children's Foundation Research Institute, Le Bonheur Children's Hospital, Memphis, Tennessee, 38103.

4Corresponding author

**Running head:** Gestational EPFR exposure and energy expenditure

**Address for corresponding author:** email: [dbridge9@uthsc.edu](mailto:dbridge9@uthsc.edu) mail: Department of Physiology, Room 426. 894 Union Avenue, Memphis TN 38163

# Abstract

We have investigated the effects of *in utero* exposure to Environmentally Persistent Free Radicals (EPFR’s) on growth, metabolism, energy utilization and skeletal muscle mitochondrial function in a mouse model of diet-induced obesity. Pregnant dams were treated with laboratory-generated combustion derived particular matter (MCP230). The adult offspring were placed on a high fat diet for 12 weeks, after which we observed a 9.8% increase in their body weight. The increase in body size observed in the MCP230-exposed mice was not associated with increases in food intake, but was associated with a reduction in physical activity and lower energy expenditure when compared to the saline-exposed mice. The reduced energy expenditure in mice indirectly exposed to MCP230 was associated with reductions in skeletal muscle mitochondrial DNA copy number, mRNA levels of electron transport genes and citrate synthase activity. Up-regulation of key genes involved in ameliorating oxidative stress was also observed. These findings suggest that gestational exposure to MCP230 leads to a reduction in energy expenditure, at least in part, through alterations to mitochondrial metabolism in the skeletal muscle.

# Key words

*In utero* exposure, Environmentally Persistent Free Radicals, Whole-body metabolism, Skeletal muscle, Mitochondria

# Introduction

Obesity is a major global health concern and emerging data supports a role for environmental pollutants in the pathogenesis of obesity and its comorbidities (2, 6, 8, 9, 11, 20, 21, 41). Gestational and early-life exposure to combustion-derived particulate matter (PM) has been associated with an increased risk of obesity in humans (8, 11, 16, 20, 21). This association is supported by data obtained from animal studies, where the offspring of pregnant female mice, which have been exposed to diesel exhaust *in utero*, are predisposed to weight gain as adults (5). Furthermore, several studies have linked the exposure to combustion-derived PM to impaired metabolic health in humans (2, 6, 9, 41) and animals (25, 26, 39, 49, 50). Specifically, cross-sectional studies of human subjects who are chronically exposed to combustion derived PM have shown associations with type 2 diabetes and cardiovascular disease (2, 6, 41), whereas murine models of chronic PM exposure indicate that pollutants lead to elevated adipose tissue inflammation and insulin resistance (26, 39, 49). Relatively stable radicals with half-lives of ~21 days exist on the surface of airborne PM (12, 14, 27)  referred to as Environmentally Persistent Free Radicals (EPFR’s).

From a mechanistic stand point, exactly how environmental pollutants result in obesity and other metabolic abnormalities is currently unknown. However, mitochondrial deficiencies and structural abnormalities have been observed in adipose tissue (49, 50), vascular tissue (47) and cardiac muscle (24) following exposure to combustion derived pollutants that should contain EPFR’s. Mitochondria are responsible for oxidative cellular energy production, endogenous reactive oxygen species production and are the primary site of the antioxidant defense system. Thus, reactive oxygen species can impair mitochondrial oxidative capacity can and can correlate with whole body reductions in energy expenditure. The importance of skeletal muscle mitochondrial metabolism for maintaining metabolic health is becoming well recognized (19, 33, 37) with deficits in muscle quality and function, particularly during early development (7), being closely linked to many different metabolic disease states (33). However, the effects of *in utero* exposure to EPFR’s on skeletal muscle mitochondrial quality remains to be determined. In this study, we investigated the effects of *in utero* exposure to EPFR’s on growth, metabolism, energy utilization and skeletal muscle mitochondria in a mouse model of diet-induced obesity. We hypothesized that gestational exposure to EPFR’s reduces energy expenditure and results in mitochondrial impairments in the skeletal muscle.

# Methods and Materials

## MCP230 Preparation and Treatment

EPFR-containing particles (i.e. MCP230) were generated and characterized by our colleagues as previously described (27). Suspensions of MCP230 and cabosil, a non EPFR-containing amorphous silica particle control, (1mg/ml) were prepared in irrigation saline containing 0.02% tween 80 and the resulting particle suspension was monodispersed by probe sonication. Breeder mice (6 wk of age) were mated and pregnant dams were administered 50 µl of MCP230 particle suspension via oropharyngeal aspiration on days 10 and 17 of gestation, as described earlier (46). Control mice received 50 µl saline or cabosil. Mice were anesthetized by inhalant anesthetic isoflurane (5%) and anesthetized mice were placed upright in a holder and physically supported in an upright position. The suspension was instilled just above the vocal cords while holding the tongue with forceps to prevent swallowing.

## Animal Housing and High Fat Diet

C57BL/6NHsd mice were purchased from Harlan (Indianapolis, IN). Mice were maintained in a 12h light/dark cycle room at constant temperature and humidity and allowed unrestricted access to food and water. Offspring were weaned at 4 weeks of age. Male mice were selected for the study and were fed standard rodent chow until 10 weeks of age. At 10 weeks of age, mice were switched from chow to a high fat diet (HFD), consisting of 45% of calories from fat (Research Diets catalog D12451). Mice were maintained on HFD for 12 weeks. One mouse, an MCP230 treated animal, had malocclusion and was removed from all data analyses. The UTHSC Institutional Animal Care and Use Committee approved all mouse procedures.

## Metabolite Assays

Blood was collected in both the fed and 16- hour fasted state. Blood glucose was determined using an AccuCheck glucometer. Serum hormone levels were determined using a Bio-Plex pro mouse diabetes multiplex immunoassay, BioRad (#171-F7001M) following the manufacturer’s instructions using a MAGPIX LMX200 system. HOMA-IR was calculated from 16-hour fasting glucose and insulin values.

## Body Composition and Metabolic Cages

Mice were weighed weekly, at approximately ZT10. Body composition was determined non-invasively using an echo-MRI 100. Food intake during the HFD phase was determined on a per-cage level by weighing the food on a weekly basis. For food intake pre-HFD, this was determined by scaled feeders within the CLAMS system.

VO2, energy expenditure, ambulatory locomotor activity and respiratory exchange ratios were determined in a home-cage style comprehensive laboratory animal monitoring system (Columbus Instruments). Mice were placed in the cages at approximately ZT10 and monitored for 3-4 days. Data from the first 6 h were discarded as this was the amount of time determined to be necessary for the mice to acclimate to their new environment. Oxymax software (Columbus Instruments) calculated the volumes of O2, CO2, the respiratory exchange ratio, the ambulatory x- and y-phase physical activity and the food consumption. Heat production was calculated using the Lusk equation (28) via the Oxymax software:

Heat = (3.815 + 1.232 \* RER) \* VO2

## Tissue Collection and Nucleic Acid Preparation

After the 12 week HFD phase, mice were fasted overnight, anesthetized with ketamine/xylazine (180/10 mg/kg, respectively) delivered IP. Quadriceps muscles were carefully dissected out, cleared of any visible adipose and connective tissue and snap frozen in liquid N2. Nucleic acids were isolated from frozen quadriceps samples via Trizol extraction in a Qiagen Tissue Lyser (30Hz for 5 min). Following careful and complete removal of the RNA-containing aqueous phase and its subsequent column purification (PureLink mRNA kit from Life Technologies). Genomic and mitochondrial DNA extraction buffer (Tris base [1 M], sodium citrate dibasic trihydrate [50 mM], guanidine thiocyanate [4 M]) was added to the tubes containing the remaining Trizol-separated interphase and infranatant. Tubes were shaken vigorously and centrifuged at 12,000 g at room temperature for 30 minutes. The aqueous phase was then collected and the genomic DNA precipitated in isopropanol. Samples were re-spun at 12,000 G at 4°C to pellet the DNA. The DNA pellet was then washed in 70% ethanol, re-spun and, after careful ethanol removal, re-suspended in TE buffer. cDNA was generated from purified RNA using the Applied Biosystems cDNA Synthesis Kit.

## qPCR Analysis of Mitochondrial DNA Copy Number and mRNA Transcripts

## Primers designed for three mitochondrial-encoded gene regions were used to assess mitochondrial DNA (mtDNA) copy number in genomic DNA and primers designed for quantitative RT-PCR were used to assess transcript levels (Table 1). Briefly, genomic DNA or cDNA from each sample extraction was added to the appropriate working qPCR master mix (containing SYBR Green and the relevant primers at a final concentration of 100 nM each). PCR conditions included an activation cycle of 95 ⁰C for 10 min followed by 45 amplification cycles of 15 s at 95 ⁰C, 15 s at 60 ⁰C, and 10 s at 73 ⁰C. Cp values were quantified on a Light Cycler 480. Nucleic acid levels were calculated using the ∆∆Ct method, with data for mtDNA copy number being normalized to values obtained for a nuclear-encoded genomic locus (*Tsc2*) and mRNA levels being normalized to *Rpl13a*, which was determined to be unaffected by MCP230 treatment in comparison to other normalization controls including XX.

## Preparation of protein lysates and western blotting

Skeletal muscle homogenates were prepared from ~30-50 mg of frozen quadriceps in RIPA buffer using stainless steel beads and a Qiagen Tissue Lyser (30Hz for 5 min). Homogenates were centrifuged at 4⁰C for 10 min at 14,000 g, after which the protein concentration of supernatants was determined by Bradford assay. Lysates of equal protein concentration were prepared in 2x Laemmli buffer containing 2-mercaptoethanol and heated at 37⁰C (for mitochondrial proteins) or 95 ⁰C (for PGC-1α) for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for western blotting. After ponceau staining to ensure equal protein loading, membranes were blocked in BSA for 1 hr and incubated overnight in total OXPHOS rodent WB antibody cocktail (Abcam #ab110413), anti-PGC-1α (Sigma #SAB4200209), anti-phospho AMPKT172 (Cell Signaling #2535S), anti-AMPK (Cell Signaling #2793S), anti-phospho S6KT389 (Cell Signaling #9206), anti-S6K (Cell Signaling #2708), anti-phospho AktS473 (Cell Signaling #4060), anti-Akt (Cell Signaling #9272) or anti-β-Actin (Proteintech #60008-1-Ig) at 4⁰C. Blots were visualized after a 1 hr incubation with infrared anti-mouse or anti-rabbit secondary antibody, using a LI-COR Odyssey fluorescent western blotting system. Protein expression was quantified using densitometry (Image Studio Lite, LI-COR) and normalized to Akt, which was unchanged by the treatments.

## Citrate synthase activity

Muscle homogenates were prepared in KCl-EDTA buffer (pH 7.4) from ~10-40 mg of frozen quadriceps. Following 3 freeze-thaw cycles, samples were centrifuged at 4⁰C for 10 min at 1000 g to settle cellular debris. Supernatants were analyzed for citrate synthase activity using a modified method described in (36). Briefly, aliquots of supernatant were added to the appropriate wells of a 96 well microplate containing an assay solution comprised of 72.5 mM Tris, 0.45 mM acetyl CoA and 0.1 mM 5,5’-dithiobis-2-nitrobenzoate (DTNB) at a pH of 8.3. After monitoring the plate for possible background activity, activity reactions were initiated by the addition of 0.5 mM oxaloacetic acid to each well. Changes in absorbance at 405 nm were recorded for each well every 9-11 sec over 3 min at room temp. Citrate synthase activity was calculated using the extinction coefficient for DTNB (which is reduced by the CoA-SH released during the cleavage of acetyl-CoA by citrate synthase).

## Statistics

All raw data, and analysis scripts are available at <http://bridgeslab.github.io/ObesityParticulateTreatment> (38). Statistics and calculations were performed using R version 3.1.1 (30). For longitudinal data, mixed linear models were used and χ2 tests were performed to determine the significance of the MCP230 treatment. Mixed linear models used the R package lme4 (version 1.1-7; (4)). In all cases, normality of the data and models were determined via Shapiro-Wilk Test and equal variance was tested using Levene’s test from the car package (version 2.0-21; (17)). In cases where cabosil and saline treatment were not significantly different, these data were combined and designated as a single control group. For energy expenditure calculations, we performed an ANCOVA analysis with lean body mass and the treatment group as non-interacting covariates and the averaged light or dark VO2 as the responding variable as described in (44). Statistical significance was designated as a p-value <0.05.

# Results

## Gestational exposure to MCP230 leads to increased body size on a high fat diet

To test the whole-body metabolic effects of gestational exposure to EPFR’s, pregnant females were exposed to MCP230 on days 10 and 17 of gestation. As controls, mice were either exposed to cabosil (the non-conjugated particulate without the EPFR group) or saline. After birth, these mice were left with their dams until weaning onto standard rodent chow at 28 days of age. At 10 weeks of age, mice were placed on a HFD consisting of 45% of calories from lard, in order to induce obesity (Figure 1A).

As shown in Figure 1B, at 10 weeks of age, mice that were exposed to MCP230 had a 7.6% higher body weight than the saline-exposed mice and remained heavier, gaining more weight throughout the HFD phase (p=3.5x10-5). After 12 weeks of HFD, the MCP230-exposed mice were 4.5 g heavier than saline-exposed mice (9.8%, p<0.001; Figure 1B). We assessed body composition after 12 weeks of HFD and observed significant elevations in both fat mass (10.1% increase, p=0.011) and lean mass (10.2% increase, p=2.2x10-4) in the MCP230-exposed mice (Figures 1C and D). The relative adiposity of these mice, as determined by the percent fat mass, was not different between groups (Figure 1E).

## MCP230-exposed mice have reduced caloric intake and increased serum concentrations of leptin, ghrelin and GLP-1

To determine how energy balance was affected in MCP230-exposed mice, we examined their food intake longitudinally throughout the study. As shown in Figure 2A, all mice tended to eat less food each week, though this did not reach statistical significance. Cumulatively, the MCP230-exposed mice ate less food throughout the diet (-6.3 +/- 1.8 kcal/week/mouse, χ2=11.6, p=8.0 x 10-4, Figure 2B). Throughout the 12 week HFD treatment, this corresponds to a 20% reduction in total caloric intake. During the metabolic cage experiments the MCP230-exposed mice tended to eat less food per feeding bout, whereas each feeding bout also tended to be shorter in duration; however, neither of these parameters were significantly different (data not shown). There were no differences between groups for the frequency of feeding bouts. Taken together, these data suggest that the increased body mass of MCP230-exposed mice (Figure 1B) was not due to increased caloric intake, as the caloric intake of MCP230-exposed mice was 19.2% less than that of the saline-exposed mice at the end of the experimental period (Figure 2B).

Leptin concentrations were modestly elevated in both fasted and fed state serum from MCP230-exposed mice (main effects for both feeding state, p=0.002, and treatment, p=0.011, by 2-way ANOVA, with post-hoc *t-*test p-values of 0.058 for fasting and p=0.097 for fed leptin concentrations, Figure 2C). Elevations in circulating leptin levels are consistent with the modest increase in fat mass observed in MCP230-exposed mice (Figure 1C). We observed significant serum elevations in both the fasting and fed state for ghrelin (main effects for both feeding state, p=0.001, and treatment, p=6.5 x 10-6, by 2-way ANOVA, with post-hoc *t-*test p-values of 0.024 for fasting and p=0.0002 for fed ghrelin concentrations, Figure 2D), which is consistent with a reduction in food intake (Figure 2A and B) and reduced energy expenditure (Figure 4A-E) observed in the MCP230-exposed mice (10, 42, 43, 48). Similarly, GLP-1 was elevated in serum from MCP230-exposed mice in both the fasting and fed state (main effects for both feeding state, p=0.002, and treatment, p=3.6 x 10-5, by 2-way ANOVA, with post-hoc *t-*test p-values of 0.024 for fasting and p=0.001 for fed GLP-1 concentrations, Figure 2D), which is also consistent with the MCP230-exposed mice eating less (Figure 2A and B) (3, 45). There was a main effect of feeding state for GIP concentrations (p=6.0 x 10-9 by 2-way ANOVA, Figure 2F) and GIP was elevated in serum from MCP230-exposed mice in the fasting state, although these values did not attain statistical significance (p=0.069 for fasted GIP-1 levels by Wilcoxon Rank Sum Test). Although there was a main effect of feeding state for PAI-1 via 2-way ANOVA, neither PAI-1 nor resistin concentrations were different between the two groups at either time point (data not shown).

We next evaluated the extent of obesity related co-morbidities in these mice after 12 wk of high fat feeding. We observed no differences in fasting blood glucose as a result of MCP230 exposure (Figure 3A). As shown in Figure 3B, there was a main effect of feeding state on serum insulin concentrations (p=3.3 x 10-6), however, MCP230 exposure had no effect. Calculation of the HOMA-IR revealed that both the saline and MCP230-exposed groups had similar HOMA-IR score (12.77 ± 1.29 vs. 12.14 ± 0.96 for Saline and MCP230, respectively; p=0.74, Figure 3C). Taken together, these findings suggest that insulin sensitivity of both the saline and MCP230 exposed mice responded similarly to the HFD but that acute *in utero* MCP230 exposure did not exacerbate this effect. Consistent with this, we observed no changes in the levels of fasted Akt phosphorylation in muscle tissue (data not shown). A main effect of both feeding state (p=7.3 x 10-5) and treatment (p=4.0 x 10-3) was observed for serum glucagon concentrations, with MCP230-exposed mice having elevated glucagon concentrations in the fasted and fed state, although fed state levels did not quite attain statistical significance (p=0.009 and p=0.059, respectively, by post-hoc *t*-test analysis; Figure 3D).

## MCP230 Mice Have Reduced Energy Expenditure

Since the MCP230 mice did not appear to be larger due to excessive caloric intake, we next examined their energy utilization. To evaluate energy expenditure, we individually housed 9 week old mice (prior to HFD) in metabolic cages for indirect calorimetry, physical activity monitoring and evaluation of gas exchange rates. As shown in Figures 4A and B, the MCP230 exposed mice had lower oxygen consumption (VO2) in both the light and dark phases (-19.1%, p=0.031 and -16.8%, p=0.019, respectively). This reduction in VO2 translated to a similar reduction in energy expenditure in both the light and dark phase (-18.4%, p=0.032 and -16.4%, p=0.021, respectively; Figures 4C and D). In Figures 4B and 4D, each dot represents the average value for each individual mouse plotted against fat-free mass. Accounting for change in lean mass is necessary due to known associations between this covariate and rates of oxygen consumption (44). To determine whether these decreases in energy expenditure were associated with changes in physical activity we monitored the ambulatory movements of these mice while they were housed in the metabolic cages. As shown in Figure 4E, compared to the control group, we observed 21.4% (p=0.040) and 26.2% (p=0.0099) reductions in physical activity for the MCP230-exposed mice in the dark and light phases, respectively.

We next evaluated energy substrate preference by analyzing the respiratory exchange ratio of the three groups. When this ratio nears 1, that indicates preference for predominately carbohydrate as fuel and as it nears 0.7 it indicates utilization of mainly lipids (18). Although there was no difference in the respiratory exchange ratio between MCP230 and cabosil exposed mice, we did observe a significant elevation (carbohydrate preference) in the saline exposed mice during both the light and dark phases relative to mice exposed to either the vehicle control (cabosil) or MCP230 (the EPFR) (Figure 4F). These data indicate that particle exposure alone (cabosil) altered substrate preference; and therefore exposure to the EPFR did not alter substrate preference.

## Skeletal Muscle from MCP230 Treated Mice Have Reduced mtDNA Copy Number and a Lower Citrate Synthase Activity

Due to the observed reductions in whole-body oxygen consumption and total energy expenditure, we next explored the hypothesis that MCP230 exposed mice have skeletal muscle mitochondrial deficits. To test this, we first determined mtDNA copy number in quadriceps muscle after the 12 week HFD phase. Figure 5A demonstrates that MCP230-exposed mice have a marked reduction in mtDNA copy number relative to the saline-exposed mice, as determined using primers designed for three distinct mtDNA-encoded gene regions. Decreases of 61.2%, 68.0% and 51.9% were observed for the mitochondrial D-loop, *mt-Cytb* and *mt-Nd1*, respectively (p=0.039, p=0.031 and p=0.032, respectively) suggesting that MCP230 exposed mice may have reduced skeletal muscle mitochondrial content. Since citrate synthase activity is better associated with skeletal muscle mitochondrial content than mtDNA copy number (and is also a good indicator of tricarboxylic acid cycle activity (23), we measured citrate synthase activity to further evaluate mitochondrial content and function in the skeletal muscle from MCP230 exposed mice. As shown in Figure 5B, maximal citrate synthase activity was reduced 24.1% in the quadriceps from MCP230 exposed mice (p=0.03). Together, reduced mtDNA copy number and lower citrate synthase activity suggest that mice exposed to MCP230 are likely to have reduced mitochondrial oxidative enzyme content and, as a result, reduced skeletal muscle oxidative capacity, which, along with the reduction in physical activity, would likely contribute to the reduced VO2 seen in these mice. Consistent with this hypothesis, mRNA transcript levels for the mitochondrial- and nuclear-encoded electron transport genes *mt-Nd4* (25.2%), *Sdha* (35.9%), *mt-Cytb* (35.4%) and *mt-Co2* (35.1%) were reduced in the quadriceps from MCP230-exposed mice, although not all of these reductions attained statistical significance (p=0.12, p=0.08, p=0.04 and p=0.10, respectively; see Figure 5C).

To test whether these reductions were due to lowered mitochondrial biogenesis, we evaluated the expression level of several known mitochondrial biogenesis genes. We observed increases in the mRNA of *Ppard* and *Ppargc1b* (Figure 6A and C), with there were no differences in the expression levels of *Ppargc1a*, *Nrf1*, *Nfe2l2* or *Tfam* (Figure 6B, D-F). To determine whether differences in skeletal muscle mitochondrial electron transport enzymes were present at the protein level, we measured the relative expression of several electron transport chain proteins via western blotting (Figure 5D). We did not observe differences in the relative abundance of any of the five proteins measured in skeletal muscle from mice exposed to MPC230 compared to the saline-treated mice, nor did we see changes in PGC-1α protein expression (Figure 5D-E). These finding suggests that the mitochondrial deficits we observe in the skeletal muscle of mice exposed to MCP230 are likely to be secondary to the ability of the myocyte to generate mitochondrial enzymes at the protein level. In other words, reduced mtDNA content and impaired citrate synthase activity are likely a response to some kind of cell stress, rather than transcriptional downregulation of mitochondrial biogenesis *per se*. In support of this notion, we found robust increases in the mRNA for *Ucp2*, *Sod1*, *Sod2*, *Cat*, *Gpx1* and *Gclm*, all enzymes activated in response to oxidative stress (Figure 7). We did not see alterations in any of the other markers of skeletal muscle metabolism and growth (phospho-AktS473, phospho-AMPKT172 or phospho-S6KT389, data not shown).

# Discussion

While epidemiological studies have linked exposure to PM with obesity and its comorbidities, few have attempted to define the molecular mechanisms involved in these responses, especially with acute gestational exposure. In this study, we tested some of the metabolic effects of a limited gestational exposure to a recently realized environmental pollutant that is present in most combustion derived PM – EPFR’s. Each exposure of MCP230 that the mothers received was the equivalent to a human breathing 200 µg/m3 of EPFR, which is similar to what would be inhaled on a typical day in one of the major US cities (34). We noted that pups born from mothers that were acutely exposed to PM grew larger, despite reductions in food intake, and that this was associated with reduced energy expenditure and mitochondrial impairments in skeletal muscle.

One potential explanation for reductions in energy expenditure and skeletal muscle mitochondrial function is the observed reduction in physical activity for MCP230-exposed mice. It is also possible that muscle weakness (due to reduced skeletal muscle oxidative capacity (40, 51) could contribute to the reduced physical activity of MCP230-exposed animals. Both of these hypotheses are consistent with cross-sectional studies showing negative associations between ambient air pollutant exposure and leisure time physical activity (31) and exercise performance (13, 29, 32). Our current data are unable to determine whether reduced mitochondrial function is the primary cause of these reductions in energy expenditure or if this observation is secondary to a reduced propensity for physical activity or some other mechanism. However, our observations of reductions in mtDNA, citrate synthase activity and mRNA transcripts support the hypothesis that gestational exposure to EPFRs can affect skeletal muscle mitochondrial oxidative function, which would contribute to the overall changes we observe in energy expenditure.

The mechanisms by which gestational exposure to EPFRs result in reduced mitochondrial function are not yet clear. Our data are consistent with chronic models of PM2.5 exposure, which show reduced mitochondrial numbers in white adipose tissue (49, 50). Analyses of placental tissues from mothers showed a strong correlation between late-gestational PM10exposure and placental mtDNA content (20). Given the elevated sensitivity of mitochondria to free radicals and oxidative stress, it is reasonable to hypothesize that during development, EPFR-mediated mitochondrial damage may result in chronic decreases in mitochondrial oxidative function, either directly, via reactive oxygen species (ROS), or indirectly, via inflammatory processes. Indeed, the bioenergetics proteins in skeletal muscle are highly susceptible to ROS-induced post translational modifications, changes that are thought to be important for reducing endogenous ROS production and protect against irreversible oxidative damage during periods of cellular stress (22). In line with this concept, Siegel and colleagues (35) have shown that mild oxidative stress *in vivo* impairs skeletal muscle oxidative efficiency and reduces oxidative phosphorylation coupling without altering the expression of key electron transport chain proteins or their respiratory activities *ex vivo*. This suggests that reduced skeletal muscle oxidative capacity in response to oxidative stress is probably not due to down-regulation of the mitochondrial biogenesis pathways or irreversible oxidative damage to bioenergetic proteins. Although we do not present any *ex vivo* functional measurements to support our hypothesis, similar to previous reports on oxidative stress-induced mitochondrial dysfunction (35), we did not observe decreases in upstream regulators of mitochondrial biogenesis (Figure 6) or mitochondrial protein expression (Figure 5D and E) as part of the chronic effects of acute *in utero* MCP230-exposure. We did observe marked increases in the transcripts of key enzymes of the antioxidant defense system (*Sod1*, *Sod2*, *Cat*, *Gpx1*), as well as increased expression of *Ucp2*, an uncoupling protein known to be up-regulated as a means to reduce endogenous ROS production (Figure 7; (1, 15), and increases in both the nuclear receptor *Ppard* and the transcriptional co-regulator *Ppargc1β*, both of which are required for the induction of *Sod1* and *Sod2* (REFS). Based on our current protocol, mice are exposed to EPFRs after inheritance of maternal mitochondria, indicating that this mitochondrial damage occurs *in situ* in the progeny. It must be emphasized that this exposure is indirect, through the mother, as there is no evidence at present that the conjugated EPFR crosses the placenta to exert its effect on the muscle directly. However, we hypothesize that the changes we observe in the skeletal muscle mitochondria of the MCP230-exposed mice are, at least in part, a consequence of ROS-induced post-translational changes and chronic oxidative stress. Future studies with more direct measurements of mitochondrial function and the oxidative stress response will provide more mechanistic insight into this process.

In contrast to previous studies that use chronic pollution models (1, 5, 9, 33, 35), we did not observe any indications that glycemic control was impaired to a greater extent in MCP230-exposed mice compared to the control groups following the HFD in spite of differences in fat mass as there were no differences in fasting glucose, insulin, HOMA-IR score (Figure 3A-C) or Akt phosphorylation in muscle tissue (data not shown). We did not measure insulin sensitivity directly, which limits our ability to make strong conclusions about the effects of acute in utero PM exposure on insulin sensitivity. That said, our data suggests that the effects of acute gestational particulate exposure may not mimic the effects of chronic exposure, and the risk profiles and mechanisms associated with these exposures may differ.

In conclusion, we have investigated the effects of limited, gestational exposure to combustion-derived pollutants in a mouse model of diet-induced obesity. Our findings show that even brief gestational exposure to environmental pollutants such as EPFR’s can result in chronic changes in growth, metabolism and energy balance. These changes are associated with skeletal muscle mitochondrial deficits and reductions in physical activity, which likely contribute to reduced energy expenditure and a predisposition to elevated body weight when exposed to a HFD. While the mechanisms behind these changes remain to be determined, the finding that limited *in utero* exposure to EPFR’s can suppress later life energy metabolism highlights a need for further research in this area.

# Acknowledgements

The authors would like the other members of the Bridges, Cormier, O’Connell and Han labs for helpful discussions and insights. The authors would also like to thank the UTHSC Molecular Resource Center including William Taylor and Felicia Waller for assistance with qRT-PCR.

# Grants

We would like to acknowledge funding from the Memphis Research Consortium, NIH grant R01DK107535, Le Bonheur Grant #650700 (to DB) and NIH grants (R01AI090059, R01ES015050, and P42ES013648) to SAC.

# Disclosures

The authors have no conflicts of interest to disclose. **References**

1. **Allister EM**, **Robson-Doucette CA**, **Prentice KJ**, **Hardy AB**, **Sultan S**, **Gaisano HY**, **Kong D**, **Gilon P**, **Herrera PL**, **Lowell BB**, **Wheeler MB**. UCP2 regulates the glucagon response to fasting and starvation. *Diabetes* 62: 1623–1633, 2013.

2. **Andersen ZJ**, **Raaschou-Nielsen O**, **Ketzel M**, **Jensen SS**, **Hvidberg M**, **Loft S**, **Tjønneland A**, **Overvad K**, **Sørensen M**. Diabetes incidence and long-term exposure to air pollution: a cohort study. *Diabetes Care* 35: 92–8, 2012.

3. **Baggio LL**, **Huang Q**, **Brown TJ**, **Drucker DJ**. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. *Gastroenterology* 127: 546–558, 2004.

4. **Bates D**, **Mächler M**, **Bolker B**, **Walker S**. Fitting Linear Mixed-Effects Models using lme4. *ArXiv* 1406.5823: 1–51, 2014.

5. **Bolton JL**, **Smith SH**, **Huff NC**, **Gilmour MI**, **Foster WM**, **Auten RL**, **Bilbo SD**. Prenatal air pollution exposure induces neuroinflammation and predisposes offspring to weight gain in adulthood in a sex-specific manner. *FASEB J* 26: 4743–4754, 2012.

6. **Brook RD**, **Jerrett M**, **Brook JR**, **Bard RL**, **Finkelstein MM**. The relationship between diabetes mellitus and traffic-related air pollution. *J Occup Environ Med* 50: 32–8, 2008.

7. **Brown LD**. Endocrine regulation of fetal skeletal muscle growth: impact on future metabolic health. *J Endocrinol* 221: R13–29, 2014.

8. **Burke V**, **Gracey MP**, **Milligan RA**, **Thompson C**, **Taggart AC**, **Beilin LJ**. Parental smoking and risk factors for cardiovascular disease in 10- to 12-year-old children. *J Pediatr* 133: 206–213, 1998.

9. **Cakmak S**, **Dales R**, **Leech J**, **Liu L**. The influence of air pollution on cardiovascular and pulmonary function and exercise capacity: Canadian Health Measures Survey (CHMS). *Environ Res* 111: 1309–12, 2011.

10. **Cowley M a.**, **Smith RG**, **Diano S**, **Tschöp MH**, **Pronchuk N**, **Grove KL**, **Strasburger CJ**, **Bidlingmaier M**, **Esterman M**, **Heiman ML**, **Garcia-Segura LM**, **Nillni E a.**, **Mendez P**, **Low MJ**, **Sotonyi P**, **Friedman JM**, **Liu H**, **Pinto S**, **Colmers WF**, **Cone RD**, **Horvath TL**. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37: 649–661, 2003.

11. **Crawley HF**, **While D**. Parental smoking and the nutrient intake and food choice of British teenagers aged 16-17 years. *J Epidemiol Community Heal* 50: 306–312, 1996.

12. **dela Cruz ALN**, **Gehling W**, **Lomnicki S**, **Cook R**, **Dellinger B**. Detection of environmentally persistent free radicals at a superfund wood treating site. *Environ Sci Technol* 45: 6356–65, 2011.

13. **Cutrufello PT**, **Rundell KW**, **Smoliga JM**, **Stylianides GA**. Inhaled whole exhaust and its effect on exercise performance and vascular function. *Inhal Toxicol* 23: 658–67, 2011.

14. **Dellinger B**, **Pryor W a.**, **Cueto R**, **Squadrito GL**, **Hegde V**, **Deutsch W a.** Role of free radicals in the toxicity of airborne fine particulate matter. *Chem Res Toxicol* 14: 1371–1377, 2001.

15. **Echtay KS**, **Roussel D**, **St-Pierre J**, **Jekabsons MB**, **Cadenas S**, **Stuart J a**, **Harper J a**, **Roebuck SJ**, **Morrison A**, **Pickering S**, **Clapham JC**, **Brand MD**. Superoxide activates mitochondrial uncoupling proteins. *Nature* 415: 96–9, 2002.

16. **Fleisch AF**, **Rifas-Shiman SL**, **Koutrakis P**, **Schwartz JD**, **Kloog I**, **Melly S**, **Coull BA**, **Zanobetti A**, **Gillman MW**, **Gold DR**, **Oken E**. Prenatal Exposure to Traffic Pollution. *Epidemiology* 26: 43–50, 2015.

17. **Fox J**, **Weisberg S**. *An {R} Companion to Applied Regression*. Second. Thousand Oaks {CA}: Sage, 2011.

18. **Hill A V.**, **Long CNH**, **Lupton H**. Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen. *Proc R Soc B Biol Sci* 96: 438–475, 1924.

19. **Hoeks J**, **Schrauwen P**. Muscle mitochondria and insulin resistance: a human perspective. *Trends Endocrinol Metab* 23: 444–450, 2012.

20. **Janssen BG**, **Munters E**, **Pieters N**, **Smeets K**, **Cox B**, **Cuypers A**, **Fierens F**, **Penders J**, **Vangronsveld J**, **Gyselaers W**, **Nawrot TS**. Placental mitochondrial DNA content and particulate air pollution during in utero life. *Environ Health Perspect* 120: 1346–52, 2012.

21. **Johnson RK**, **Wang MQ**, **Smith MJ**, **Connolly G**. The association between parental smoking and the diet quality of low-income children. *Pediatrics* 97: 312–317, 1996.

22. **Kramer PA**, **Duan J**, **Qian WJ**, **Marcinek DJ**. The measurement of reversible redox dependent post-translational modifications and their regulation of mitochondrial and skeletal muscle function. *Front. Physiol.* 6: 2015.

23. **Larsen S**, **Nielsen J**, **Hansen CN**, **Nielsen LB**, **Wibrand F**, **Stride N**, **Schroder HD**, **Boushel R**, **Helge JW**, **Dela F**, **Hey-Mogensen M**. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* 590: 3349–60, 2012.

24. **Li R**, **Kou X**, **Geng H**, **Xie J**, **Tian J**, **Cai Z**, **Dong C**. Mitochondrial damage: An important mechanism of ambient PM2.5 exposure-induced acute heart injury in rats. *J Hazard Mater* 287C: 392–401, 2015.

25. **Li XY**, **Gilmour PS**, **Donaldson K**, **MacNee W**. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. *Thorax* 51: 1216–1222, 1996.

26. **Liu C**, **Fonken LK**, **Wang A**, **Maiseyeu A**, **Bai Y**, **Wang T-Y**, **Maurya S**, **Ko Y-A**, **Periasamy M**, **Dvonch T**, **Morishita M**, **Brook RD**, **Harkema J**, **Ying Z**, **Mukherjee B**, **Sun Q**, **Nelson RJ**, **Rajagopalan S**. Central IKKβ inhibition prevents air pollution mediated peripheral inflammation and exaggeration of type II diabetes. *Part Fibre Toxicol* 11: 53, 2014.

27. **Lomnicki S**, **Truong H**, **Vejerano E**, **Dellinger B**. Copper oxide-based model of persistent free radical formation on combustion-derived particulate matter. *Environ Sci Technol* 42: 4982–4988, 2008.

28. **Lusk G**. ANIMAL CALORIMETRY: TWENTY-FOURTH PAPER. ANALYSIS OF THE OXIDATION OF MIXTURES OF CARBOHYDRATE AND FAT. A Correction. *J Biol Chem* 59: 41–42, 1924.

29. **Marr LC**, **Ely MR**. Effect of air pollution on marathon running performance. *Med Sci Sports Exerc* 42: 585–91, 2010.

30. **R Core Team**. R: A Language and Environment for Statistical Computing. .

31. **Roberts JD**, **Voss JD**, **Knight B**. The association of ambient air pollution and physical inactivity in the United States. *PLoS One* 9: e90143, 2014.

32. **Rundell KW**, **Caviston R**. Ultrafine and fine particulate matter inhalation decreases exercise performance in healthy subjects. *J Strength Cond Res* 22: 2–5, 2008.

33. **Russell AP**, **Foletta VC**, **Snow RJ**, **Wadley GD**. Skeletal muscle mitochondria: A major player in exercise, health and disease. *Biochim Biophys Acta - Gen Subj* 1840: 1276–1284, 2014.

34. **Saravia JS**, **You D**, **Thevenot P**, **Lee GI**, **Shrestha B**, **Lomnicki S**, **Cormier SA**. Early-life exposure to combustion-derived particulate matter causes pulmonary immunosuppression. *Mucosal Immunol* 7: 694–704, 2014.

35. **Siegel MP**, **Kruse SE**, **Knowels G**, **Salmon A**, **Beyer R**, **Xie H**, **van Remmen H**, **Smith SR**, **Marcinek DJ**. Reduced coupling of oxidative phosphorylation In Vivo precedes electron transport chain defects due to mild oxidative stress in mice. *PLoS One* 6, 2011.

36. **Srere PA**. Citrate synthase. In: *Methods in enzymology*. 1969, p. 3–11.

37. **Stephenson EJ**, **Hawley JA**. Mitochondrial function in metabolic health: A genetic and environmental tug of war. *Biochim Biophys Acta - Gen Subj* 1840: 1285–1294, 2014.

38. **Stephenson EJ**, **Ragausksas A**, **Jaligama S**, **Redd JR**, **Parvathareddy J**, **Peloquin MJ**, **Saravia JS**, **Han JC**, **Cormier SA**, **Bridges D**. Dataset for Particulate Studies and Obesity. *ZENODO*: 2016.

39. **Sun Q**, **Yue P**, **Deiuliis J a**, **Lumeng CN**, **Kampfrath T**, **Mikolaj MB**, **Cai Y**, **Ostrowski MC**, **Lu B**, **Parthasarathy S**, **Brook RD**, **Moffatt-Bruce SD**, **Chen LC**, **Rajagopalan S**. Ambient air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity. *Circulation* 119: 538–46, 2009.

40. **Taivassalo T**, **Jensen TD**, **Kennaway N**, **DiMauro S**, **Vissing J**, **Haller RG**. The spectrum of exercise tolerance in mitochondrial myopathies: a study of 40 patients. *Brain* 126: 413–23, 2003.

41. **Thiering E**, **Cyrys J**, **Kratzsch J**, **Meisinger C**, **Hoffmann B**, **Berdel D**, **von Berg A**, **Koletzko S**, **Bauer C-P**, **Heinrich J**. Long-term exposure to traffic-related air pollution and insulin resistance in children: results from the GINIplus and LISAplus birth cohorts. *Diabetologia* 56: 1696–704, 2013.

42. **Toshinai K**, **Mondal MS**, **Nakazato M**, **Date Y**, **Murakami N**, **Kojima M**, **Kangawa K**, **Matsukura S**. Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem Biophys Res Commun* 281: 1220–5, 2001.

43. **Tschöp MH**, **Smiley DL**, **Heiman ML**. Ghrelin induces adiposity in rodents. *Nature* 407: 908–13, 2000.

44. **Tschöp MH**, **Speakman JR**, **Arch JRS**, **Auwerx J**, **Brüning JC**, **Chan L**, **Eckel RH**, **Farese R V**, **Galgani JE**, **Hambly C**, **Herman M a**, **Horvath TL**, **Kahn BB**, **Kozma SC**, **Maratos-Flier E**, **Müller TD**, **Münzberg H**, **Pfluger PT**, **Plum L**, **Reitman ML**, **Rahmouni K**, **Shulman GI**, **Thomas G**, **Kahn CR**, **Ravussin E**. A guide to analysis of mouse energy metabolism. *Nat Methods* 9: 57–63, 2011.

45. **Turton MD**, **O’Shea D**, **Gunn I**, **Beak SA**, **Edwards CM**, **Meeran K**, **Choi SJ**, **Taylor GM**, **Heath MM**, **Lambert PD**, **Wilding JP**, **Smith DM**, **Ghatei MA**, **Herbert J**, **Bloom SR**. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 379: 69–72, 1996.

46. **Wang P**, **Thevenot P**, **Saravia JS**, **Ahlert T**, **Cormier SA**. Radical Containing Particles Activate DCs and Enhance Th17 Inflammation in a Mouse Model of Asthma. *Am J Respir Cell Mol Biol* 45: 977, 2011.

47. **Westbrook DG**, **Anderson PG**, **Pinkerton KE**, **Ballinger SW**. Perinatal tobacco smoke exposure increases vascular oxidative stress and mitochondrial damage in non-human primates. *Cardiovasc Toxicol* 10: 216–226, 2010.

48. **Wilson JL**, **Enriori PJ**. Molecular and Cellular Endocrinology A talk between fat tissue , gut , pancreas and brain to control body weight. *Mol. Cell. Endocrinol.* (2015). doi: 10.1016/j.mce.2015.08.022.

49. **Xu X**, **Liu C**, **Xu Z**, **Tzan K**, **Zhong M**, **Wang A**, **Lippmann M**, **Chen L-CC**, **Rajagopalan S**, **Sun Q**. Long-term exposure to ambient fine particulate pollution induces insulin resistance and mitochondrial alteration in adipose tissue. *Toxicol Sci* 124: 88–98, 2011.

50. **Xu Z**, **Xu X**, **Zhong M**, **Hotchkiss IP**, **Lewandowski RP**, **Wagner JG**, **Bramble L a**, **Yang Y**, **Wang A**, **Harkema JR**, **Lippmann M**, **Rajagopalan S**, **Chen L-C**, **Sun Q**. Ambient particulate air pollution induces oxidative stress and alterations of mitochondria and gene expression in brown and white adipose tissues. *Part Fibre Toxicol* 8: 20, 2011.

51. **Zurlo F**, **Larson K**, **Bogardus C**, **Ravussin E**. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest* 86: 1423, 1990.

# Figure Legends

**Figure 1: *In utero* exposure to MCP230 results in increased body size.** (A) Schematic of the experimental design. (B) Body weight throughout the high fat diet phase of the intervention. (C) Absolute body fat, (D) fat-free mass and (E) percent body fat after 12 weeks of high fat diet (ZT12). Data shown is the group mean ± SE. § indicates p<0.05 via mixed linear model, compared by χ2 test (B), whereas \* indicates p<0.05 via a Student’s *t*-test (C-D). The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Figure 2: Gestational exposure to MCP230 causes a reduction in food intake and alters ‘hunger hormone’ concentrations on a high fat diet.** Food intake per mouse was calculated on a (A) weekly and (B) cumulative basis throughout the high fat diet phase of the intervention. MCP230-exposed mice had elevated serum concentrations of (C) leptin, (D) Ghrelin and (E) GLP-1 after access to the high fat diet. (F) Serum GIP tended to be elevated during the fasted state, although this did not attain statistical significance. Fed serum was collected at ZT12. Fasting serum was collected following an overnight fast (~16 hr) at ZT4. Data shown is the group mean ± SE. n=8-14/group. § indicates p<0.05 by mixed linear model, compared by χ2 test (B). † indicates a main effect for feeding state (C-F) and ‡ indicates a main effect for MCP230-exposure by 2-way ANOVA (C-E). \* indicates p<0.05 via a Wilcoxon Rank Sum Test (D-E). The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Figure 3: Gestational exposure to MCP230 causes an increase in serum glucagon but does not differentially alter glucose or insulin concentrations following exposure to a high fat diet.** (A)Fasting blood glucose, (B) serum insulin, (C) HOMA-IR and (D) serum glucagon concentrations were determined after a 16h fast at ~ZT4. Fed serum was collected at ZT12 and analyzed for insulin (B) and glucagon (D). Data shown is the group mean ± SE. n=8-14/group. † indicates a main effect for feeding state (B and D) and ‡ indicates a main effect for MCP230-exposure by 2-way ANOVA (D). \* indicates p<0.05 via a Wilcoxon Rank Sum Test (D). The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Figure 4: *In utero* exposure to MCP230 reduces energy expenditure and lowers physical activity.** (A) O2 consumption rates (VO2) and (B) VO2 analysis, normalized to fat-free mass during both the light and dark phase. Each dot represents the average O2 consumption of each mouse. (C) Time course of energy expenditure and (D) energy expenditure normalized to fat-free mass during both the light and dark phase. Each dot represents the average energy expenditure of each mouse. (E) Quantification of ambulatory movement during the light and dark phases. (F) Respiratory exchange ratio of each group. Saline and cabosil groups were not combined for this analysis as there was a significant reduction in the respiratory exchange ratio for both the cabosil- and MCP230- exposed groups. Data shown is either the individual (B, D) or group mean (A, C, E and F) ± SE (E and F). n=18, 6 or 14 for MCP230, saline and cabosil groups, respectively. § indicates p<0.05 by ANCOVA (B). \*indicates p<0.05 by Student’s *t*-test (E), or Wilcoxon-Rank Sum Test (F). The saline-exposed mice are depicted in black, the cabosil-exposed mice are depicted in white and the MCP230-exposed mice are depicted in grey. Where the saline- and cabosil- exposed groups are combined, these mice are depicted in black and white stripes.

**Figure 5: Exposure to MCP230 *in utero* results in skeletal muscle mitochondrial abnormalities following high fat diet consumption as adults**. (A) mtDNA copy number, (B) citrate synthase activity and (C) mRNA levels of oxidative phosphorylation genes were reduced in the quadriceps muscles of mice that were indirectly exposed to MCP230 *in utero* and subjected to 12 wk of high fat diet as adults. Quadriceps PGC-1α and select electron transport chain protein expression was unchanged in the MCP230-exposed mice (D, representative blots and E, relative quantification). Data shown is the group mean ± SE. \*indicates p<0.05 via Student’s *t*-test. n=7-12/group. The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Figure 6: Indirect exposure to MCP230 *in utero* is not associated with reductions in the mRNA of upstream regulators of mitochondrial biogenesis** (A) *Ppard* and (C) *Ppargc1b* mRNA was elevated in the MCP230-exposed mice, whereas (B) *Ppargc1a*, (D) *Nrf1*, (E) *Nfe2l2* and (F) *Tfam* mRNA were not different*.*  Data shown is the group mean ± SE. \*indicates p<0.05 via Student’s *t*-test (A) or Wilcoxon-Rank Sum Test (C). n=7-12/group. The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Figure 7: The antioxidant defense system is upregulated in the quadriceps of MCP230-exposed mice.** Data shown is the group mean ± SE. \*indicates p<0.05 via Student’s *t*-test (*Cat*), Welch’s *t*-test (*Sod1*, *Sod2*, *Gpx1*) or Wilcoxon-Rank Sum Test (*Ucp2*, *Gclm*). n=7-12/group. The saline-exposed mice are depicted in black and the MCP230-exposed mice are depicted in grey.

**Tables**

**Table 1.** Mitochondrial DNA copy number and relative gene expression were determined using the following primer sequences. *Tsc2* and *Rpl13a* were used for normalization of genomic DNA and mRNA respectively.

|  |  |  |
| --- | --- | --- |
| **Region/gene** | **Forward primer** | **Reverse primer** |
| **d-Loop** | GGC CCA TTA AAC TTG GGG GT | TTC TTC ACC GTA GGT GCG TC |
| ***mt-Nd1*** | CGT CCC CAT TCT AAT CGC CA | ATG GCG TCT GCA AAT GGT TG |
| ***mt-Cytb*** | CTT CAT GTC GGA CGA GGC TT | CCT CAT GGA AGG ACG TAG CC |
| ***mt-Nd4*** | TAA TCG CAC ATG GCC TCA CA | GCT GTG GAT CCG TTC GTA GT |
| ***Sdha*** | TCT TCG CTG GTG TGG ATG TC | CTT CAG CAC CTG TCC CTT GT |
| ***mt-Co2*** | AAC CGA GTC GTT CTG CCA AT | CTA GGG AGG GGA CTG CTC AT |
| ***Ppard*** | ACA TGG AAT GTC GGG TGT GC | CGG AAG AAG CCC TTG CAC C |
| ***Ppargc1a*** | TGA TGT GAA TGA CTT GGA TAC AGA CA | GCT CAT TGT TGT ACT GGT TGG ATA TG |
| ***Ppargc1b*** | TTG TAG AGT GCC AGG TGC TG | GTG TAT CTG GGC CAA CGG AA |
| ***Nrf1*** | AGA AAC GGA AAC GGC CTC AT | GGC TCT GAG TTT CCG AAG CA |
| ***Nfe2l2*** | TGG ACT TGG AGT TGC CAC C | TCT TGC CTC CAA AGG ATG TCA |
| ***Tfam*** | TCG CAT CCC CTC GTC TAT CA | AGT TTT GCA TCT GGG TGT TTA GC |
| ***Ucp2*** | TGC GGT CCG GAC ACA ATA G | GCC TCC AAG GTC AAG CTT CT |
| ***Ucp3*** | ACA AAG GAT TTG TGC CCT CC | TCA AAA CGG AGA TTC CCG CA |
| ***Sod1*** | GGA ACC ATC CAC TTC GAG CA | CCC ATG CTG GCC TTC AGT TA |
| ***Sod2*** | TTC TGG ACA AAC CTG AGC CC | GTC ACG CTT GAT AGC CTC CA |
| ***Cat*** | CAC TGA CGA GAT GGC ACA CT | TGT GGA GAA TCG AAC GGC AA |
| ***Gpx1*** | TTC GGA CAC CAG GAG AAT GG | TAA AGA GCG GGT GAG CCT TC |
| ***Gclm*** | TGG AGT TCC CAA ATC AGC CC | CAA CTC CAA GGA CGG AGC AT |
| ***Tsc2*** | AAG AAG CCT CTT CTG CTA CC | CAG CTC CGA CCA TGA AGT G |
| ***Rpl13a*** | GGA GTC CGT TGG TCT TGA GG | GGC CAA GAT GCA CTA TCG GA |