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**VANDER SILVA ALVES**

# INSULTOS AMBIENTAIS E NUTRICIONAIS PROGRAMAM PARA A SÍNDROME METABÓLICA NA VIDA ADULTA

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de "Doutor em Ciências Biológicas".

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# BIOGRAFIA

**Vander Silva Alves** nasceu na cidade de Araruna no PR, no dia 24 de Janeiro de 1987. É licenciado em Ciências Biológicas pela Faculdade Integrado de Campo Mourão (ano de 2008), Mestre em Ciências Biológicas (PBC/UEM) (ano de 2012) e atualmente realiza o Doutorado no Programa de Pós-graduação em Ciências Biológicas (PBC/UEM). Possui experiência em Biologia Celular e Fisiologia.

# APRESENTAÇÃO

Esta tese é composta de dois artigos científicos. O primeiro é intitulado "**Particulate matter from air associated to obesity enhances vulnerability to metabolic impairment and oxidative stress markers**". O trabalho demostra que a administração via gavagem de material particulado ($PM_{<10}$), durante a lactação é um agente programador para o indivíduo, possuindo capacidade de exacerbar as consequências provocadas pela hipernutrição no início da vida e subsequente exposição a uma dieta hiperlipídica na vida adulta. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, o artigo foi redigido de acordo com as normas da revista científica *Environmetal Science and Pollution Research* com atual fator de impacto 2,8. O segundo é intitulado "**High fat diet during adolescence in male rats is able to programs blood pressure increase and lead to others metabolic dysfunctions at adulthood**". O trabalho demostra que a exposição à dieta hiperlipídica durante a adolescência programa para o desenvolvimento da síndrome metabólica na vida adulta, evidenciando dessa forma que a adolescência, bem como a gestação e lactação, é também uma janela de programação. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, o artigo foi redigido de acordo com as normas da revista científica *Plos one* com atual fator de impacto 3,234.

## RESUMO GERAL

INTRODUÇÃO - A obesidade e a síndrome metabólica (MetS) são enormes desafios para países desenvolvidos e em desenvolvimento. A alta prevalência dessas doenças tem sido apontada como consequência de mudanças na composição da dieta, aumento da ingestão alimentar, baixos níveis de atividade física, alterações na microbiota intestinal e, mais recentemente, exposição à poluição do ar. Evidências mostram que a exposição a estressores ambientais durante períodos cruciais do desenvolvimento, como gestação, lactação e adolescência, pode levar ao desenvolvimento de distúrbios metabólicos na vida adulta. Essa ideia é conhecida como origens desenvolvimentistas da saúde e doença (DOHaD). Assim, evidências crescentes indicam que a exposição a estressores ambientais durante essas janelas vulneráveis contribuem para a patogênese não transmissível, como obesidade, doença coronariana, diabetes mellitus tipo 2 (DMT2) e alguns tipos de câncer. Além disso, tem sido demonstrado que o excesso de peso, induzido pela hipernutrição precoce durante a vida pós-natal, é um fator crucial para o desenvolvimento da obesidade e disfunções cardiometabólicas. Neste contexto, os animais de ninhada reduzida (NR) têm sido amplamente estudados como um modelo importante para o desenvolvimento da programação metabólica e consequente desenvolvimento de MetS na prole. Os animais NR caracterizam-se pela presença de hiperfagia, resistência à leptina, resistência à insulina (RI) e aumento do estresse oxidativo. Outro modelo consistente para estudar a obesidade e a MetS pode ser a exposição à dieta rica em gordura (HFD). Existe uma relação entre a exposição à HFD durante os períodos de gestação e lactação e as alterações na pressão arterial na vida adulta. Também foi sugerido que a adolescência é uma fase suscetível de programação para a MetS. Por outro lado, um número crescente de evidências tem mostrado que o material particulado (PM) do ar desempenha um papel crucial no desenvolvimento de disfunções metabólicas e doenças crônicas, como a obesidade e o DMT2. Recentemente, dados do nosso grupo mostraram que a exposição materna à PM durante a vida perinatal foi capaz de levar a uma programação metabólica na prole na vida adulta. Embora os efeitos da PM sejam muito discutidos atualmente, as consequências de sua exposição por via oral durante a vida perinatal em modelos animais de obesidade são limitadas.

**OBJETIVOS - (a):** Primeiro: verificar se a exposição materna de $PM_{<10}$ durante a gestação e a lactação poderia exacerbar tanto as disfunções de curto como de longo prazo em prole de NR que ingeriram HFD na idade adulta. **(b)**: Segundo: veriricar se a

exposição a HFD durante a adolescência pode levar a disfunções cardiometabólicas na vida adulta.

**MÉTODOS - (a):** Ratas prenhas foram alojadas em gaiolas individuais e randomizadas em dois grupos no dia 7 da gestação (GD). O primeiro grupo recebeu o veículo óleo de milho (OIL-Mothers) e o segundo recebeu por gavagem PM <10 (PM -Mothers) na dose de 50 µg de PM <10/rata. Durante todo o tratamento as mães receberam água *ad libitum* e alimentaram-se de dieta padrão para roedores (Nuvital®, Curitiba, Brasil). No dia pós-natal (PND) 1, todas as ninhadas foram ajustadas para 9 filhotes por mãe (com equilíbrio entre macho e fêmea). Para induzir a supernutrição precoce, no PND 3, os filhotes foram ajustados para 3 machos por mãe. Os filhotes das mães tratadas com óleo de milho foram denominados SL-OIL, enquanto aqueles das mães tratadas com $PM_{<10}$ foram anotados como SL-PM. Avaliamos um lote da prole ao desmame, no PND 21 (n = 7 / grupo). Outro lote de ratos foi avaliado no PND 90 (n = 7/grupo). Também utilizamos outro lote de descendentes no PND 90 para avaliar o efeito combinado de HFD em ratos que foram expostos ao MP no início da vida (n = 7/grupo). Para isso, o SL-OIL e o SL-PM receberam por 30 dias (do PND 90 até o PND 120) HDF; Banha de 35%; 5,817 kcal/g). Avaliaram-se a composição do leite, homeostase da glicose (ipITT e ivGTT), perfil lipídico, um marcador de inflamação e alguns parâmetros de estresse oxidativo. **(b):** Ratos Wistar adolescentes (30 a 60 dias) foram expostos a dieta hiperlipídica (HFD, 35% de gordura). Os animais de controle tiveram acesso a ração comercial normal (NFD, 4,5% de gordura). A pressão arterial, frequência cardíaca e pressão de pulso foram verificadas em ratos com 120 dias de idade.

**RESULTADOS - (a):** A exposição de $PM_{<10,}$ coletada em área urbana de alta densidade de tráfego, durante a gestação e lactação levou a exacerbação das consequências a curto e longo prazo na superalimentação neonatal (induzida por ninhadas reduzidas) e subsequente ingestão de HFD na idade adulta. Neste estudo, mães tratadas com $PM_{<10}$ durante a gestação e lactação apresentaram exacerbação das disfunções metabólicas causadas pela exposição à hipernutrição pós-natal precoce na prole, tanto no desmame quanto no final da vida. Além disso, $PM_{<10}$ durante a vida perinatal foi capaz de agravar o fenótipo obeso na idade adulta após um segundo desafio com ingestão de HFD. **(b):** adolescentes com dieta hiperlipídica apresentaram alterações na ingestão calórica durante o período da dieta, bem como no ganho de peso, perfil lipídico e parâmetros cardiovasculares na vida adulta. Em conjunto, estes achados sugerem que a HFD durante

a adolescência, tem consequências a longo prazo na saúde e, desta forma, programa para o desenvolvimento da MetS mais tarde na vida.

**CONCLUSÃO - (a):** Exposição materna a $PM_{<10}$ durante a gestação e lactação, foi capaz de exacerbar disfunções no metabolismo em modelos animais de obesidade, aumentando a intolerância à glicose e dislipidemia mais tarde. Entretanto, após um desafio nutricional com HFD na idade adulta, as disfunções metabólicas se tornam mais evidentes, demonstrando que a exposição ao MP no início da vida piora quando associada a insultos nutricionais, aumentando o estresse oxidativo, obesidade e levando à dislipidemia na idade adulta. **(b):** exposição à dieta hiperlipídica durante a adolescência levou a níveis mais elevados de pressão arterial sistólica e pressão arterial média na vida adulta. Além disso, a redução exacerbada da pressão arterial em resposta à injeção de hexametônio observada em animais com HFD sugere que o aumento da pressão arterial pela HFD durante a adolescência pode ser devido a uma maior atividade do sistema nervoso simpático.

## GENERAL ABSTRACT

**INTRODUCTION** – Obesity is an enormous challenge for developed and developing countries. This great prevalence have been pointed as consequence of changes in diet composition, increases in food intake, low levels of physical activity, changes in gut microbiome and more recently air pollution exposure. Several evidences have been shown that exposure to environmental stressors during crucial periods of developmental such as gestation, lactation and adolescence could lead to health or development of metabolic disorders in adulthood. This idea is known as developmental origins of health and disease (DOHaD) concept. Thus, increasing evidence has indicated that exposure to environmental endocrine and immune disruptors during these vulnerable windows contribute to the noncommunicable pathogenesis, such as obesity, coronary heart disease, type 2 diabetes mellitus (T2DM), and some types of cancer. It has been shown that overweight inducing by early overnutrition during postnatal life is a crucial factor for development of obesity and cardiometabolic dysfunctions, in this context, the small litter (SL) animals have been widely studied as an important model for development of metabolic programming and metabolic syndrome (MetS) in the offspring. SL animals are characterized by present hyperphagia, leptin resistance, insulin resistance (IR) and increase of oxidative stress. Other consistent model to study obesity and MetS may be exposure to high-fat diet (HFD). There is a negative relationship between exposure to HFD during the gestation and lactation periods and changes in blood pressure in adult life. It has also been suggested that adolescence is a susceptible phase for programming to MetS. On the other hand, a growing number of evidences have been shown that PM from air plays a crucial role in the development of metabolic dysfunctions and chronic disease such as obesity and T2DM. Recently data of our group shown that maternal PM exposure during perinatal life was able to lead a metabolic programing in the offspring at adulthood. Although the effects of PM are much discussed nowadays, the consequences of its exposure by the oral route during perinatal life in obesity animal models are limited. **AIMS: (a) -** First: we hypothesized that maternal PM<10 exposure during pregnancy and lactation could exacerbate both short- and long-term dysfunctions on small litter offspring who fed HFD at adulthood. **(b)** – The second: our hypothesis argues that HFD during adolescence can lead to cardiometabolic dysfunctions at adult life.

**METHODS - (a) :** Pregnant rats were housed in individual cages and randomized into two groups on the gestation day (GD) 7. The first group received the vehicle corn oil

(OIL-Mothers) and second received by gavage $PM_{<10}$ (PM-Mothers) at a dose of 50 µg of $PM_{<10,}$ collected from a high-density-traffic urban area, during gestation and lactation. During all treatment mothers received water *ad libitum* and fed a balanced chow diet (Nuvital®, Curitiba, Brazil). On the post-natal day (PND) 1, all the litters were adjusted to 9 pups per dam (with balance between male and female). To induce early overnutrition, on the PND 3, the pups were adjusted to 3 male per dam. Offspring from the mothers treated with corn oil were denominated as SL-OIL, whereas those from the mothers treated with $PM_{<10}$ were noted as SL-PM. We evaluated a batch of the offspring at weaning, on the PND 21 (n=7/group). Another batch of rats was evaluated at PND 90 (n=7/group). We also used another batch of offspring at PND90 to evaluate the effect between HFD in rats that were exposure to PM in early life (n=7/group). For this, the SL-OIL and SL-PM received for 30 days (from PND 90 until PND 120) HDF; 35% lard; 5.817 kcal/g). It was evaluated the milk composition, glucose homeostasis (ipITT and ivGTT), lipid profile, one inflammation marker and some oxidative stress parameters. **(b):** Adolescent Wistar rats (30 to 60 day-old) were exposed to a HFD (35% of fat). Control animals had access to normal commercial chow (NFD, 4.5% of fat). Blood pressure, heart rate and pulse pressure were verified in 120-day-old rats. Student t-test was used to compare groups.

**RESULTS - (a)**: $PM_{<10}$ exposure during pregnancy and lactation may exacerbate in offspring the short- and long-term consequences on neonatal overfeeding (induced by small litter) and subsequent ingestion of HFD at adulthood. In this study, mothers treated with $PM_{<10}$ during critical periods of life (gestation and lactation) presented exacerbation of the metabolic dysfunctions caused by expose to early postnatal overnutrition in male offspring, both at early and later in life. Furthermore, $PM_{<10}$ during perinatal life was able to aggravate the obese phenotype at adulthood after a second challenge with HFD ingestion. **(b):** HFD adolescents showed changes in calorie intake during the diet period, as well in the weight gain, lipid profile and cardiovascular parameters at adulthood. Taken together, these finding suggest that HFD during adolescence, have long term consequences on health and this way programs the development of cardiometabolic syndrome later in life.

**CONCLUSION – (a):** Maternal exposure to PM<10 was able to exacerbate dysfunctions on metabolism in obese animal model offspring, increasing glucose intolerance and dyslipidemia later in life. However, after a nutritional challenge with HFD at adulthood, metabolic dysfunctions become more evident, demonstrating that PM exposure in early

life worsens when combined with nutritional insults, increasing oxidative stress, obesity and leading to dyslipidemia at adulthood. **(b):** HFD exposition during adolescence programs to higher levels of systolic blood pressure and mean blood pressure later in life. In addition, the exacerbated blood pressure reduction in response to hexamethonium injection observed in HFD animals suggest that the increased blood pressure programed by HFD during adolescence may depends on sympathetic nervous system, which is an important predictor for cardiovascular death.

# Particulate matter from air associated to obesity enhances vulnerability to metabolic impairment and oxidative stress markers in male rat offspring

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Air pollution • Particulate matter • Perinatal life • Metabolic programming • Insulin resistance • Oxidative stress

## Abstract

**Background/Aims:** Several evidences have been shown that exposure to environmental stressors during crucial periods of development such as gestation, lactation and adolescence could lead to disease at adulthood. Furthermore, some reports have been shown that particulate matter ($PM_{<10}$) exposure combined with high fat diet (HFD) was able to exacerbate obesity potentiating chronic inflammation and insulin resistance in mice. We hypothesized that $PM_{<10}$ exposure during pregnancy and lactation could exacerbate both short- and long-term dysfunctions on neonatal overfeeding rats. **Methods:** Samples of $PM_{<10}$ from an urban area of Cotonou, Benin, were suspended in corn oil and administered by gavage (50 μg $PM_{<10}$/day) in pregnant and lactating rats (PM-Mothers group). The offspring were analyzed in three different ages: post-natal day (PND) 21, 90 and 120. **Results:** Our results showed that PM exposure lead to a several consequences in the offspring at weaning, where SL-PM at PND 21 showed an increase in body weight (bw) ($p < 0.001$), retroperitoneal ($p < 0.05$) and mesenteric ($p < 0.001$) fat pad stores and fasting glucose ($p < 0.05$). At PND 90 no statistical difference was found on the bw, fat pad stores, fasting glucose and fasting insulin. However, SL-PM showed an increased in total cholesterol (TC) ($p < 0.01$) and triglycerides (TG) ($p < 0.05$). Furthermore, during intravenous glucose tolerance test (ivGTT), an increase of glucose plasma levels ($p < 0.01$) was observed at SL-PM in the AUC, followed by a blood insulin level increment in the first 5 minutes at the test ($p < 0.05$). After HFD challenge, at PND 120 the SL-PM showed higher weight gain ($p < 0.01$), increase of the retroperitoneal ($p < 0.05$) and mesenteric ($p < 0.05$) fat pad stores and higher insulin resistance (IR) compared to the SL-OIL. In addition, a significative reduction of the levels of glutathione ($p < 0.05$), glutathione transferase ($p < 0.05$) and superoxide dismutase ($p < 0.005$), was observed in the SL-PM compared to SL-OIL. **Conclusion:** Our data demonstrated that $PM_{<10}$ exposure during pregnancy and lactation was able to exacerbate in the offspring metabolic disfunctions caused by exposure to early postnatal overnutrition, both at weaning and later in life, mainly after a second challenge with HFD ingestion. Thus, we suggest that places with poor air quality can lead to exacerbation of preexisting metabolic dysfunctions.

## 1. Introduction

Obesity is an important challenge for developed and developing countries, since promote a great impact on public health, and in this way, interfering on social and economic aspects from individuals and communities (Fruhbeck et al. 2013, Visscher et al. 2017). Reaching pandemic status, it has been estimated the quantity of overweight or obese among adults is 40% (Sung et al. 2019) and, this increased prevalence have been pointed as consequence of changes in diet composition, increases in food intake (Astrup &Brand-Miller 2012, Swinburn et al. 2009), low levels of physical activity (Hill &Peters 2013), changes in gut microbiome (Tilg &Kaser 2011) and more recently ongoing air pollution exposure (Sun et al. 2009).

Several evidences have been shown that exposure to environmental factors and endocrine and immune disruptors during crucial periods of developmental such as gestation, lactation and adolescence could contribute to development of noncommunicable pathogenesis, such as obesity, coronary heart disease, type 2 diabetes mellitus (T2DM), and some types of cancer at adulthood (de Oliveira et al. 2013, Grandjean et al. 2015). This idea is known as developmental origins of health and disease (DOHaD) concept (de Oliveira et al. 2013, Grandjean et al. 2015, Reynolds et al. 2015, Vickers 2014).

It has been shown that overweight induced by early overnutrition during postnatal life is a crucial factor for development of obesity and cardiometabolic dysfunctions, in this context, the small litter (SL) animals have been widely studied as an important model for development of metabolic programming and metabolic syndrome (MetS) in the offspring (Schmidt et al. 2001, Stefanidis &Spencer 2012). Other consistent model to study obesity and MetS may be exposure to high-fat diet (HFD). This diet is known by to lead an increase fat pad deposition and gain in body weight, both in humans and experimental (Halima et al. 2018).

Particulate matter (PM) from air plays a crucial role in the development of these dysfunctions (Brook et al. 2017, Rajagopalan &Brook 2012). The mechanisms by which the PM leads to chronic disease may be related to tissue or systemic inflammation and oxidative stress (Brook et al. 2017, Laing et al. 2010, Rajagopalan &Brook 2012, Rao et

al. 2015). Indeed, some reports have shown that PM exposure combined with HFD was able to exacerbate obesity by potentiate chronic inflammation and insulin resistance in mice, evidencing a relationship between HFD and PM (Sun et al. 2005, Sun et al. 2009).

Recently, data from our group showed that maternal PM exposure during pregnancy and lactation was able on leadin metabolic programing in the rat offspring at adulthood (Miranda et al. 2018). Furthermore, it has been defended that organism with preexisting disease, such as MetS are more sensitive to PM exposure, than which may exacerbate their symptoms besides increase their risk for hospitalization and chronic disease (Clementi et al. 2019). Although the effects of PM are much discussed nowadays, the consequences of its exposure by the oral route during perinatal life associated to obesity at adulthood are limited.

Thus, we hypothesized that maternal PM <10 exposure during pregnancy and lactation in rats lead to metabolic programming in the offspring and it could exacerbate both short- and long-term dysfunctions, such as increased body fat deposition, glycemia, insulinemia and lipid profile on SL offspring who fed HFD at adulthood.

## 2. Material and Methods

*Animals and experimental groups*

The experiments and results were reported in accordance with the ARRIVE guidelines for experiments involving animals (Kilkenny et al. 2010). All experimental protocols were approved by the Ethics Committee in Animal Research (CEUA nº 5681231014) and conducted at the Laboratory of Secretion Cell Biology of the State University of Maringá.

Adult male and female Wistar rats, from the Central Animal House at the State University of Maringá, Paraná, Brazil, were housed in the Animal House of the Department of Biotechnology, Genetics and Cellular Biology in polypropylene cages (45 cm/30 cm/15 cm) under light controlled conditions with a 12h light-dark cycle (07:00 a.m. to 07:00 p.m.) and a temperature of 22.0 ± 2 °C. After, the animals were mated at a proportion of three females to each male. We daily verified changes in the oestrous cycle by vaginal smears and pregnancy was confirmed by the presence of sperm. Pregnant rats were housed in individual cages and randomized into two groups on the gestation day (GD) 7. By gavage, both, the first group received the vehicle corn oil (OIL-Mothers) and second received $PM_{<10}$ (PM-Mothers) at a dose of 50 µg of $PM_{<10,}$ once a day, during gestation and lactation. During all treatment mothers received water *ad libitum* and were fed with a balanced chow diet (Nuvital®, Curitiba, Brazil).

We used 7 dams/group to obtain the litters and for this study, we choose randomly one offspring/group/litter. On the post-natal day (PND) 1, all the litters were adjusted to 9 pups per dam (with balance between male and female). To induce early overnutrition, on the PND 3, the pups were adjusted to 3 male per dam. Offspring from mothers treated with corn oil were denominated as SL-OIL, whereas those from mothers treated with $PM_{<10}$ were designated as SL-PM (Figure 1).

We evaluated a group of offspring at weaning, on the PND 21 (n=7/group). Another group of rats was evaluated at PND 90 (n=7/group). We also used another batch of offspring at PND90 to evaluate the effect between HFD in rats that were exposure to PM in early life (n=7/group). For this, the SL-OIL and SL-PM received for 30 days (from PND 90 until PND 120) HDF (35% lard; 5.817 kcal/g) produced basing on Barella et al. (2012) (Figure 1).

*Particulate matter sampling*

Particulate matter <10 µm ($PM_{<10}$) samples were collected in an urban area of Cotonou (Benin) and characterized for physicochemical composition at Dunkerque (France) as previously described Cachon et al. The PM is a mix of compounds that includes inorganic and organic components, such as Al, Fe, Mg, Mn, Na, Pb, Zn and carbonaceous materials; volatile organic compounds such benzene, toluene, ethylbenzene and o-xylene; polycyclic aromatic hydrocarbons, mainly fluoranthene, pyrene, chrysene, benzo [a] pyrene; paraffins such as tricosane, tetracosane, heptacosane, tritriacontane; and fatty acids such as hexadecanoic acid, octadecanoic acid, oleic acid and tetradecanoic acid (Cachon et al. 2014).

*$PM_{<10}$ reconstitution*

The $PM_{<10}$ stock solution was prepared by reconstitution with 3 mL of corn oil and was sonicated for 30 minutes (Sonic Dismembrator Model 100, Fischer Scientific, Waltham, MA, USA). The gavage solutions were individually prepared using a stock solution (Miranda et al. 2018).

*Dose solution administration*

The treatment started at the gestation day (GD) 7 and the female rats who received 50 µg of $PM_{<10}$ solution daily corresponding to 200 µg $PM_{<10}$/kg of body weight (bw) were called PM-Mothers. All rats weighed approximately 250g of bw, so we administered a constant quantity of 250 µL of reconstituted $PM_{<10}$ per day at a dose of 50 µg of PM<10. This dose was constant, mimicking the daily exposure. The calculation for dose administration was performed on a baseline basis on the low dose of diesel oral exposure as described previously by Danielsen et al. (2008). The control group, called OIL-Mothers, received a corn oil solution at a constant volume of 1 mL/kg of bw. These solutions were administered by gavage from GD 7 to PND 21 (from 8:00 am to 9:00 a.m.).

*Milk collection*

Milk samples were collected at PND 21 before 2 hours pups had been weaned from their dams. Dams were anaesthetized with thiopental (0.2 mL, ip), and so to induce milk secretion, a synthetic oxytocin, Oxiton (5 U.I./mL, União Química S/A, São Paulo, Brazil), was injected (0.5 mL ip). Milk was collected in a sterile Pasteur pipette by

manually massaging the nipple as described previously (DePeters &Hovey 2009). Milk samples (1 mL/dam) were stored at -80º C for subsequent analysis.

*Intraperitoneal insulin tolerance test (ipITT)*

After 6-hour fast the offspring at 120 days old (n = 6), was submitted to ipITT. After that, they received an injection of insulin (1 U/kg of body weight), and blood samples were collected, as previously reported (de Oliveira et al. 2011). Blood samples were obtained from the tail vein at 0, 15, 45 and 60 min after injection. Glucose levels were measured using the Accu-Chek Aviva system (Roche Diagnostics). The constant for the insulin tolerance test (Kitt) was calculated using formula Kitt (%/min) = 0.693/t1/2, where t 1/2 was calculated from the slope of the plasma glucose concentration during ipITT (Bonora et al. 1989).

*Intravenous glucose tolerance test (ivGTT)*

At PND 90 and PND 120 the offspring (n=6 for each group and age) underwent surgery consisting of silicone cannula implantation into the right jugular vein as previously described (de Oliveira et al. 2011). After 12-h fasting, a glucose load (1 g/kg bw) was injected into the cannula. Blood samples (400 µl) were collected immediately prior to glucose injection (0 min) and then at 5, 15, 30 and 45 min, and the obtained plasma samples were stored at -20 °C for further analysis. To maintain blood volume, a corresponding volume of saline (0.9%) was infused through the cannula.

*Biochemical analysis*

The blood glucose concentration was determined using the glucose oxidase method (Trinder 1969) with a commercial kit (Gold Analisa®, Belo Horizonte, MG, Brazil). The triglyceride (TG), total cholesterol (TC) and high-density lipoprotein (HDL) levels were measured by colorimetric commercial kits (Gold Analisa®, Belo Horizonte, MG, Brazil). Low-density lipoprotein (LDL) and very low-density lipoproteins (VLDL) levels were found using the Friedewald formula (Friedewald et al. 1972).

The insulin levels of plasma were determined using a radioimmunoassay (Scott et al. 1981) with a gamma counter (Wizard$^2$ Automatic Gamma Counter, TM-2470, PerkinElmer®, Shelton, CT, USA). For the radioimmunoassay, human insulin was used as a standard, and detection was performed using an anti-rat insulin antibody (Sigma-Aldrich®,

St. Louis, MO, USA) and $^{125}$I-labelled recombinant human insulin (PerkinElmer®, Shelton, CT, USA). The intra- and inter-assay coefficients of variation were 12.2% and 9.8%, respectively, for insulin. The detection limit for the insulin levels was 1.033 pmol/l. Homeostasis model assessment: insulin resistance (HOMA-IR) and β-cell function (HOMA-β), were determined by formulas as previously described (Matthews et al. 1985).

*Fat pad stores assessment*

The animals were euthanized by decapitation method and fat pad stores was removed and weighed, to measure fat tissue accumulation.

*Sample collection to oxidative stress and inflammation profile*

The liver of the animals was collected, weighed, washed in Sodium Phosphate Buffer (PBS 0.1 M, pH 7.4) and stored in a freezer at -80 °C until the date of the biochemical tests (n=6 each group and age). A portion of the liver was homogenized 200 mM potassium phosphate buffer (pH 6.5), in a five-fold dilution and uses to determine all parameters (Borges et al. 2018).

*Lipid hydroperoxide (LOOH) levels and Reduced Glutathione (GSH) levels*

The GSH levels in the liver were determined according to the adapted method of Sedlak and Lindsay (Borges et al. 2018, Sedlak &Lindsay 1968), which 25 µL of homogenate from the sample plus 975 µL of 5,5′-dithiobis-2-nitrobenzoic acid were read at 412 nm. Individual values were interpolated based on a GSH standard curve and are expressed as µg of GSH/g of tissue. The other part of the homogenate was centrifuged for 20 min at 9000 × *g*, and a part of supernatant was used to measured lipid hydroperoxide (LOOH). LOOH concentrations were determined using an extinction coefficient of 4.3 mmolar 1/cm, and the results are expressed as mmol/mg of tissue. Readings were performed at 560 nm using a spectrophotometer as previously describe (Jiang et al. 1991).

*Catalase enzyme activity (CAT)*

Determination of CAT activity in the liver was performed using the method of Aebi (1984) with adaptations (Aebi 1984). In a 96-well plate containing 200 µl of a 20

mM solution (5 mM Tris/EDTA Buffer, pH 8.0, 30% hydrogen peroxide and distilled water) were added 10 µl of the diluted sample, or in the case of white, 10 µl of distilled water. The absorbance was then measured at 240 nm.

*Superoxide dismutase (SOD) and Glutathione S- transferase (GST) enzyme activity*

The enzymatic assay for SOD is based on the ability of SOD to inhibit the autooxidation of pyrogallol (Marklund &Marklund 1974). Readings were done at 405 nm using a spectrophotometer. The results are expressed as U of SOD/mg of protein. The enzymatic activity of GST was determined according to the method of Warholm et al. (Warholm et al. 1985). The tests were performed at 340 nm using a spectrophotometer, with an extinction coefficient of 9.6 mmolar 1/cm. The results are expressed as µmol/min/mg of protein.

*Myeloperoxidase activity (MPO)*

The precipitate from liver homogenate centrifugation was resuspended in 80 mM potassium phosphate buffer that contained 0.5% hexadecyltrimethylammonium. The samples were homogenized and centrifuged for 20 min at 11,000 × g at 4 °C. The reaction was done in a 96-well plate using tetramethylbenzidine. Enzymatic activity of myeloperoxidase (MPO) was read at 620 nm using a spectrophotometer. The results are expressed as units of optical density (OD)/min/mg of protein (Borges et al. 2018).

*Statistical analyses*

The data are presented as the mean ± SEM and were subjected to Student's t test. A value of $p < 0.05$ was considered statistically significant using GraphPad Prism version 6.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

*Impact of PM exposure in maternal health and milk composition*

As shown in Table 1, any changes were found in maternal weight during pregnancy and lactation and there was no difference in maternal fat pads. PM-Mothers had higher fasting glucose and lower fasting insulin (14.5%, $p < 0.01$; 50%, $p < 0.01$, respectively). In the plasma profile, we observed that PM-Mothers increased LDL (22% $p < 0.05$), while decreased HDL, HOMA-IR and HOMA-β levels (32.8%, $p < 0.0001$; 40.3%, $p < 0.05$; 68%, $p < 0.0001$, respectively). On the other hand, any difference was observed in the TC, VLDL and TG levels.

In breast milk, PM mothers displayed higher insulin and TG levels compared to SL-OIL (167%, $p < 0.0001$; 22.1%, $p < 0.05$), as showed in Table 2.

*Effects of PM exposure in offspring biometric and biochemical parameters at PND 21*

SL-PM group did not present changes in birth weight (Fig. 2a), however, they were heavier (Fig. 2b) than SL-OIL ($p < 0.05$) at weaning. Retroperitoneal (Fig. 2c) and mesenteric (Fig. 2e) fat pads were increased by 47% and 18% ($p< 0.05$; $p < 0.01$), respectively in SL-PM offspring in PND 21.

As shown in the Table 3, at PND 21, SL-PM presented increased fasting glucose (+11%, $p <0.05$), when compared with SL-OIL. In contrast, fasting insulin level was decreased in these animals (-55%, $p <0.01$). In SL-PM group HOMA-IR and HOMA-β were decreased (-45%, $p < 0.01$; -49%, $p < 0.001$, respectively).

SL-PM group, at PND21, displayed decreased HDL (-14.5%, $p < 0.005$) and increased VLDL and TG levels (+44% and +48%, $p < 0.001$, respectively). No statistical difference was observed in TC and LDL levels (Table 3).

*Effects of PM dams exposure in pregnant and lactating on the biometric and biochemical parameters in the offspring at PND 90*

As shown in the Table 3, at PND 90 no statistical difference was found on the bw, as well as in the fat pad stores between SL-PM and SL-OIL groups. The same was observed in fasting glucose and fasting insulin. On the other hand, at PND 90 the animals presented an increased at TC and TG (49%, $p < 0.01$; 55%, $p < 0.05$, respectively) in SL-PM group compared to the SL-OIL group.

Glucose intolerance was detected in the SL-PM offspring at PND 90 (Fig. 3a). These animals showed an increase of glucose plasma levels during intravenous glucose tolerance test (ivGTT) with an increase of 10.5% ($p < 0.001$) in the AUC compared to the SL-OIL offspring (Fig. 3b). Although, the SL-PM group, at PND 90 (Fig. 3c) showed a blood insulin level increment in the first 5 minutes on the ivGTT compared with the SL-OIL rats ($p < 0.05$), the AUC remained unchanged between the groups (Fig. 3d).

*Effect of HFD on the body weight, food intake and glucose-insulin homeostasis in SL-PM rat offspring at PND 120.*

Although no difference was observed in food intake from PND 90 to PND 120 (Fig. 4a and 4b), in the evolution of the bw, SL-PM showed a higher weight gain from PND 90 until PND 120 (Fig. 4c), represented by the AUC (Fig. 4d), the PM group was 37.1% higher than in the SL-OIL group ($p < 0.01$) (Fig. 4c and 4d).

In addition, as observed in the Table 4, after a HFD ingestion, from PND 90 to PND 120, the SL-PM group exhibited a 12.3% and 25.2% increase in the retroperitoneal and mesenteric fat pad stores compared to the OIL offspring ($p < 0.05$). Despite no statistical difference was observed in plasmas levels of glucose and insulin between the animals at PND 120, the PM group presented an increase in the TC of 25.6% ($p < 0.001$), but no statistical difference was found on TG levels between the groups (Table 4).

During the ivGTT, no difference was observed between the groups at PND 120 (Fig. 5a and 5b). However, the SL-PM group, showed a blood insulin level increment (40%, $p < 0.05$) in the first 5 minutes on the test compared with the SL-OIL, the AUC remained unchanged ($p > 0.05$; Fig. 5c and 5d). During the insulin sensitivity assessment, detected by ipITT (Fig.5e), the glucose uptake in the SL-PM group was expressively decreased compared to the OIL offspring ($p < 0.01$; Fig. 5f), and an increase was observed with HOMA-IR and HOMA-β index (Table 4).

*Effects of PM exposure in pregnant and lactating dams on oxidative stress status in liver after HFD exposure of their offspring at PND 120*

In the assessment of the oxidative status of the liver from offspring at PND 120 no difference was found in the levels of CAT between the groups. The same happened with the levels of LOOH and MPO of the liver. On the other hand, the PM group showed a significative reduction of the levels of GSH, GST and SOD ($p < 0.05$), compared to SL-OIL offspring (Fig. 6).

## 4. Discussion

To the best of our knowledge, this is the first report to describe whether ambient $PM_{<10}$ exposure during pregnancy and lactation may exacerbate in offspring the short- and long-term consequences on neonatal overfeeding induced by SL and subsequent ingestion of HFD at adulthood. In this study, male offspring rats from mothers treated with $PM_{<10}$ during perinatal life (gestation and lactation) presented exacerbation of the metabolic dysfunctions caused by exposure to early postnatal overnutrition, both at early and later in life. Furthermore, $PM_{<10}$ during perinatal life was able to aggravate the obese phenotype at adulthood after a second challenge with HFD ingestion.

Ambient air pollution has been implicated with adverse pregnancy outcomes such as mortality in children and adults (Buka et al. 2006). In the present investigation, $PM_{<10}$ exposure during gestation and lactation did not alter bw from dams. On the other hand, an increase of fasting glucose, LDL, and decrease in fasting insulin, HDL, HOMA-IR and HOMA-β was found. Similar bw and HDL results were found in a previous study conducted in our laboratory, but lower TC and TG levels was observed (Miranda et al. 2018). These discrepancies may be attributed to variations in the experimental models and procedures.

Our data also showed that PM-Mothers presented an increase in insulin and TG levels in breast milk. The breast milk is considered crucial for the adequate development of the offspring. It includes several important substances, such as macronutrients (proteins, carbohydrates and fats), steroids, growth factors and hormones, such as leptin and insulin (Franco et al. 2012, Miranda et al. 2017, Ribeiro et al. 2017). Similar results was observed in breast milk from pregnant rabbits who were exposed to diluted and filtered diesel engine (DE) exhaust fumes from the $3^{rd}$ to the $27^{th}$ days post-conception (total exposure of 20 days out of a 31-day pregnancy) (Hue-Beauvais et al. 2019). Despite had not being possible to measure the difference in milk intake among the litters, we suggest that changes in the breast milk, from PM-Mothers, such as high levels of fat and insulin in its composition lead to exacerbation of the complications on SL-PM at PND 21 and later life.

Epidemiological and experimental studies have consistently associating PM exposure in perinatal life to metabolic diseases such as T2DM and obesity in adult life (Haberzettl et al. 2016, Rao et al. 2015), since the PM maternal exposure can promote

low birth weight, increased adiposity, insulin resistance, and impairs glucose tolerance in adult offspring (Chen et al. 2017b). In addition, small litters are able to promote overnutrition by reduction in milk competition and increasing caloric intake (Ribeiro et al. 2017), as result, the SL animals present increased levels of circulating leptin and insulin resistance (Bei et al. 2015, Hou et al. 2011), companied with overweight or obesity phenotype throughout life (Wu et al. 2016).

In the present study the animals did not present difference at the birth weight. Similarly results also were found in mice from mothers treated with diesel exhaust $PM_{2.5}$ (DEP), from 7-weeks pre-conception until the weaning (Chen et al. 2017a). Nevertheless, at PND21 the SL PM showed an increase of fasting glucose and higher bw and fat deposition, as retroperitoneal and mesenteric fat pads (Table 3). Increased body weight, impaired whole-body glucose tolerance and decreased expression of insulin signaling genes in adipose tissue has also been found in mice exposure to $PM_{<2.5}$ initiated at conception until 5 weeks of age, (Woodward et al. 2019).

The maternal treatment of $PM_{<10}$ during pregnancy and lactation was not able to exacerbate insulin resistance rat offspring at PND 21, since less levels of fasting insulin as well decrease of HOMA-IR and HOMA-β index was observed. Taken together these data suggest that PM combined to SL model may lead to impaired insulin secretion in the β-cell, resulting in an increase of fasting glucose observed, in this sense, we could defend there was worsened the disease phenotype previously stablished.

Unlike PND 21, at PND 90 no statistical difference was found on the bw, body weight evolution (bwe), as well as in the fat pad stores, fasting glucose and fasting insulin, between SL-PM and SL-OIL (Table 3). On the other hand, at ivGTT, the SL-PM offspring showed a significantly increase of glucose plasma levels in the AUC, suggesting a glucose intolerance, same it had not observed hyperinsulinemia during the test. Curiously, an increment of insulin level was detecting in the first 5 minutes of the test compared to the SL-OIL offspring. Consistent with our findings, a pediatric cohort study, childhood elevated exposure to nitrogen dioxide ($NO_2$) and $PM_{2.5}$ between the ages of 10 and 18, presented significant effects on insulin homeostasis, insulin sensitivity, and β-cell function. Although, they defend that PM exposure initially lead to an increase in insulin secretion, including higher fasting insulin levels and higher acute insulin response to glucose. The long-term PM exposure have been associated to β-cell fatigue and

consequently decrease in insulin secretion (Alderete et al. 2017). Thus, we assumed here that PM exposure was able to programs offspring, lead to a worsening in their disease phenotype as early as later in life.

In the current study, after a second challenge with HFD, from PND 90 to PND 120, we did not observed difference at plasmas levels of glucose and insulin on the ivGTT between the groups, however, ipITT data clearly indicated insulin resistance in PM group at PND 120 compared to the OIL offspring, corroborating with HOMA-IR and HOMA-β index. Similarly results was reported by Liu and colleagues in male offspring of Swiss Webster, with overnutrition until PDN 21 and after feeding with HFD between PDN 21-150, showed an disruption in the ability to regulate the food intake, increasing the sensitivity to HFD and increased the fat pad stores and adiposity (Liu et al. 2013). HFD is known by inducing increase bw, fat pad stores, fasting glucose, insulin resistance and total cholesterol, generally independent of animal age (Bock et al. 2016, Ibanez et al. 2017).

Although it was not observed difference in food intake from PND 90 to PND 120 between groups, SL-PM presented increased the body weight gain as well the retroperitoneal and mesenteric fat pad stores, after HFD. Curiously, mice that fed HFD by 16 weeks, presented a decrease on hyperglycemia after to high-intensity exercise, however, when they were exposure to $PM_{<2.5}$ that reduction was not observed (Kostrycki et al. 2019), suggesting a combined effect of PM and HFD that lead to an increase of adiposity in SL-PM group.

Recent results in this field have been shown that chronic PM exposure combined with HFD cause a synergistic effect impairing oxidative homeostasis, changes of lipid accumulation, oxidative stress, inflammation and hepatic steatosis in mice (Ding et al. 2019). In our study the assessment of the oxidative status of the liver from offspring at PND 120, demonstrated a significative reduction of the levels of SOD and GST. And lower levels of GSH were found. These enzymes are both oxidative stress markers and play an important role to protect against the cellular damage, while SOD is able to eliminate superoxide radicals converting, GST may acting detoxifying xenobiotic compounds and lipidic hydroperoxides, in addition, decrease of GSH represents lower cell capacity to eliminate reactive species and peroxides (Borges et al. 2018). This way,

559 a decrease on SOD activity may be suggest a lower capacity to eliminate reactive oxygen
560 species due to damage to cell machinery.

561 In previous studies with squamous cell carcinoma of the nasal septum human, the
562 $PM_{2.5}$ was able to increase inflammatory response and decrease the activities of SOD,
563 catalase and glutathione peroxidase (Hong et al. 2016). In another finding, $PM_{2.5}$
564 exposure, for 48 hours, in HBE and BEAS-2B cells was able to increase reactive oxygen
565 species (ROS) levels and IL-6 secretion in this cell culture. Notably, IL-6 is known as
566 important mediator and a strong indicator of the inflammatory response (Yuan et al.
567 2019). In addition, six-week-old C57BL/6 male mice that were exposed to concentrated
568 ambient $PM_{2.5}$ by 60 days, presented an increase in endoplasmic reticulum stress and
569 inflammatory status in the lung and liver (Laing et al. 2010). However, when the
570 C57BL/6 male mice were fed HFD and after exposure to concentrated ambient $PM_{2.5}$, it
571 was observed markedly worsened insulin and glucose homeostasis, besides increase in
572 inflammation (Sun et al. 2009).

573 Indeed, the liver is considered the main organ and a critical place of lipid
574 metabolism (Xu et al. 2019), so, although our data did not presented difference in lipid
575 peroxidation and Myeloperoxidase, we observed that SL-PM group presented alteration
576 of the lipid profile at all ages evaluated. Curiously, mice that were exposure to different
577 concentrations of $PM_{2.5}$ for 6 months, showed that genes related to lipid accumulation
578 who was resulting with increases in liver TG, TC and VLDL levels, followed by IR,
579 glucose intolerance and abnormal blood pressure. In this sense is clearly there is a strong
580 association among an oxidative stress, inflammation and insulin resistance (Giacco
581 &Brownlee 2010, Xu et al. 2019) and as we could observed, several studies shown that
582 PM exposure might lead to oxidative stress, inflammation and consequently development
583 of the insulin resistance (Park 2017, Thiering et al. 2013).

584 In fact, the animals evaluated in this work were early overfeed and submitted to
585 the HFD in adult life, being these consistent models for studies of metabolic dysfunctions.
586 Thus, our major aim in the current study was to test the hypothesis that maternal
587 exposition to $PM_{<10}$ during gestation and lactation could exacerbate dysfunctions already
588 found on offspring metabolism, as short- and long-term consequence. Although, this
589 study has some limitations about a better explanation upon the mechanism behind
590 malprogramming consequences from $PM_{<10}$, further experiments with this model are

essential to verify the mechanisms by which PM programming exacerbate the dysfunctions on insulin and lipid metabolism in mothers and offspring and oxidative stress in the offspring at adulthood. Nevertheless, our results present very important evidences to hypothesis confirmation that environmental pollutants exposure during perinatal period combined with early overnutrition and HFD at adulthood worsened metabolic dysfunction in the descendants.

## 5. Conclusion

In summary, we demonstrate that maternal exposure to PM<10, collected from a high-density-traffic urban area, during gestation and lactation, was able to exacerbate dysfunctions on metabolism in obese animal model offspring, increasing glucose intolerance and dyslipidemia later in life. Furthermore, after a nutritional challenge with HFD at adulthood, metabolic dysfunctions become more evident, demonstrating that PM exposure in early life worsens when combined with nutritional insults, increasing oxidative stress, obesity and leading to dyslipidemia and insulin resistance at adulthood. While previous animal studies have yielded similar results, our findings are the first to show the effect of programming of $PM_{<10}$ on recognized obesity models, such as early overnutrition combined with HFD at adulthood.

## Declaration of interest

The authors declare no conflict of interest associated with this manuscript.


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889 **Table 1. Effects of PM on biometric and biochemical parameters of mothers at weaning**

| Parameters | OIL-Mothers | PM-Mothers |
|---|---|---|
| AUC bw gestation | 5985 ± 209.9 | 5787 ± 177.4 |
| AUC bw lactation | 6184 ± 194.4 | 6121 ± 151.3 |
| Retroperitoneal fat pad (g/100 g bw) | 1.41 ± 0.12 | 1.01 ± 0.14 |
| Ovarian fat pad (g/100 g bw) | 0.84 ± 0.15 | 0.70 ± 0.14 |
| Uterine fat pad (g/100 g bw) | 1.39 ± 0.16 | 1.02 ± 0.15 |
| Mesenteric fat pad (g/100 g bw) | 0.72 ± 0.07 | 0.69 ± 0.08 |
| Fasting glucose (mg/dL) | 97.4 ± 3.22 | 111.6 ± 1.12** |
| Fasting insulin (ng/dL) | 0.122 ± 0.01 | 0.061 ± 0.01** |
| HOMA-IR | 0.65 ± 0.08 | 0.39 ± 0.06* |
| HOMA-β | 29.96 ± 2.81 | 9.73 ± 2.16**** |
| Total cholesterol (mg/dL) | 93.49 ± 4.37 | 82.91 ± 4.2 |
| HDL cholesterol (mg/dL) | 51.7 ± 3.01 | 34.7 ± 1.03**** |
| VLDL cholesterol (mg/dL) | 10.42 ± 0.23 | 10.7 ± 0.81 |
| LDL cholesterol (mg/dL) | 32.52 ± 1.36 | 39.89 ± 2.18* |
| Triglycerides (mg/dL) | 53.9 ± 2.06 | 57.3 ± 3.79 |

890 Data are expressed as the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001
891 by Student's *t* test (n = 7 rats/group).

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**Table 2. Effects of PM on milk biochemical parameters at weaning.**

| Parameters | OIL-Mothers | PM-Mothers |
|---|---|---|
| Milk glucose (mg/dL) | 190.1 ± 11.81 | 200.1 ± 16.58 |
| Milk insulin (ng/mL) | 0.768 ± 0.09 | 2.054 ± 0.114**** |
| Milk triglycerides (mg/dL) | 931.7 ± 51.25 | 1138.0 ± 70.77* |
| Milk total cholesterol (mg/dL) | 85.3 ± 8.11 | 73.18 ± 6.97 |

Data are expressed as the mean ± SEM; *$p < 0.05$, ****$p < 0.0001$ by Student's t test ($n = 7$ rats/group).

**Table 3. Effects of maternal PM treatment on the biometric and biochemical parameters in offspring at PND 21 and PND 90**

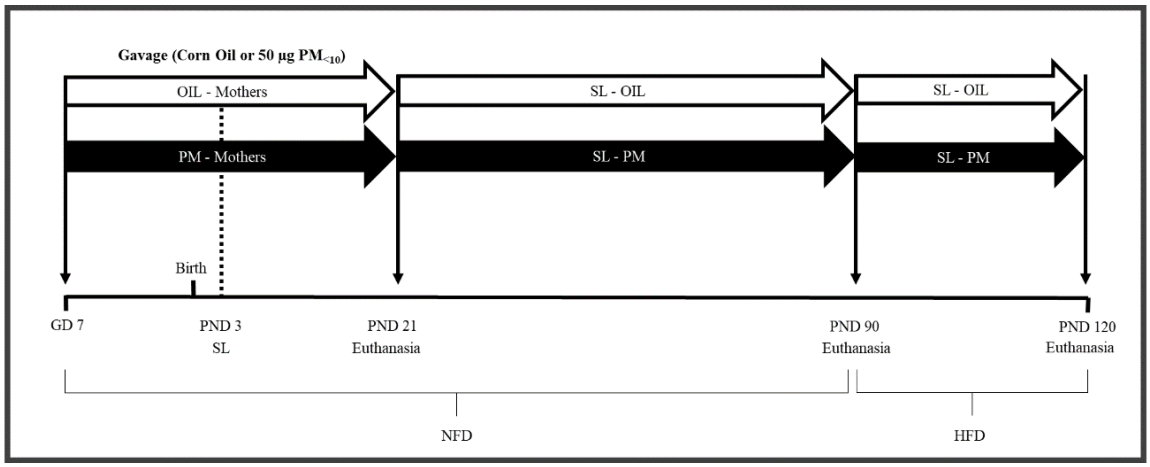
| Parameters | SL-OIL | SL-PM |
|---|---|---|
| **PND 21** | | |
| Fasting glucose (mg/dL) | 103.2 ± 3.89 | 114.8 ± 3.46* |
| Fasting insulin (ng/dL) | 0.1978 ± 0.03 | 0.0873 ± 0.03** |
| HOMA-IR | 1.15 ± 0.09 | 0.63 ± 0.14** |
| HOMA-β | 34.44 ± 2.69 | 17.35 ± 3.58*** |
| Total cholesterol (mg/dL) | 69.92 ± 3.07 | 68.62 ± 3.75 |
| HDL cholesterol (mg/dL) | 25.15 ± 0.89 | 21.5 ± 0.92** |
| VLDL cholesterol (mg/dL) | 7.8 ± 0.31 | 11.23 ± 0.87*** |
| LDL cholesterol (mg/dL) | 40.22 ± 1.79 | 41.43 ± 2.83 |
| Triglycerides (mg/dL) | 39.0 ± 1.54 | 56.17 ± 4.36*** |
| **PND 90** | | |
| Fasting glucose (mg/dL) | 78.8 ± 1.7 | 84.1 ± 2.0 |
| Fasting insulin (ng/dL) | 0.329 ± 0.03 | 0.327 ± 0.02 |
| HOMA-IR | 1.406 ± 0.09 | 1.427 ± 0.05 |
| HOMA-β | 216.7 ± 2.69 | 137.2 ± 3.58* |
| Body Weight (g) | 371.8 ± 5.76 | 389.9 ± 11.51 |
| Retroperitoneal fat pad (g/100 g bw) | 1.70 ± 0.06 | 1.54 ± 0.08 |
| Mesenteric fat pad (g/100 g bw) | 0.829 ± 0.04 | 0.723 ± 0.05 |
| Total cholesterol (mg/dL) | 61.06 ± 5.76 | 91.06 ± 5.65** |
| Triglycerides (mg/dL) | 53.58 ± 2.22 | 83 ± 13.37* |

Data are expressed as mean ± SEM; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Student's t test (n = 6/group from 6 different liters).

**Table 4. Effects of maternal PM treatment on the biometric and biochemical parameters in offspring at PND 120 under the HFD treatment**

| Parameters | SL-OIL | SL-PM |
|---|---|---|
| Body Weight (g) | 484.5 ± 9.80 | 466.5 ± 7.48 |
| Retroperitoneal fat pad (g/100 g bw) | 2.92 ± 0.05 | 3.28 ± 0.14* |
| Mesenteric fat pad (g/100 g bw) | 1.19 ± 0.08 | 1.49 ± 0.08* |
| Fasting glucose (mg/dL) | 75.9 ± 6.42 | 75.2 ± 2.26 |
| Fasting insulin (ng/dL) | 0.438 ± 0.07 | 0.658 ± 0.08 |
| Total cholesterol (mg/dL) | 78.84 ± 2.17 | 99.06 ± 4.57*** |
| Triglycerides (mg/dL) | 61.0 ± 3.8 | 58.3 ± 4.1 |
| HOMA-IR | 1.791 ± 0.35 | 2.985 ± 0.32* |
| HOMA-β | 210.3 ± 30.9 | 501.8 ± 110.8* |

Data are expressed as the mean ± SEM; *p < 0.05, **p < 0.01 and ***p < 0.001 by Student's t test (n = 6/group from 6 different liters).

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955 **Figure 1. Experimental design:** OIL - Mothers, mothers who received corn oil; PM-Mother,
956 mothers who received $PM_{<10}$ solution; SL - OIL, offspring from OIL - Mothers; SL – PM,
957 offspring from PM - Mothers; PND, post-natal day; NFD, normal fat diet; HFD, high fat diet.

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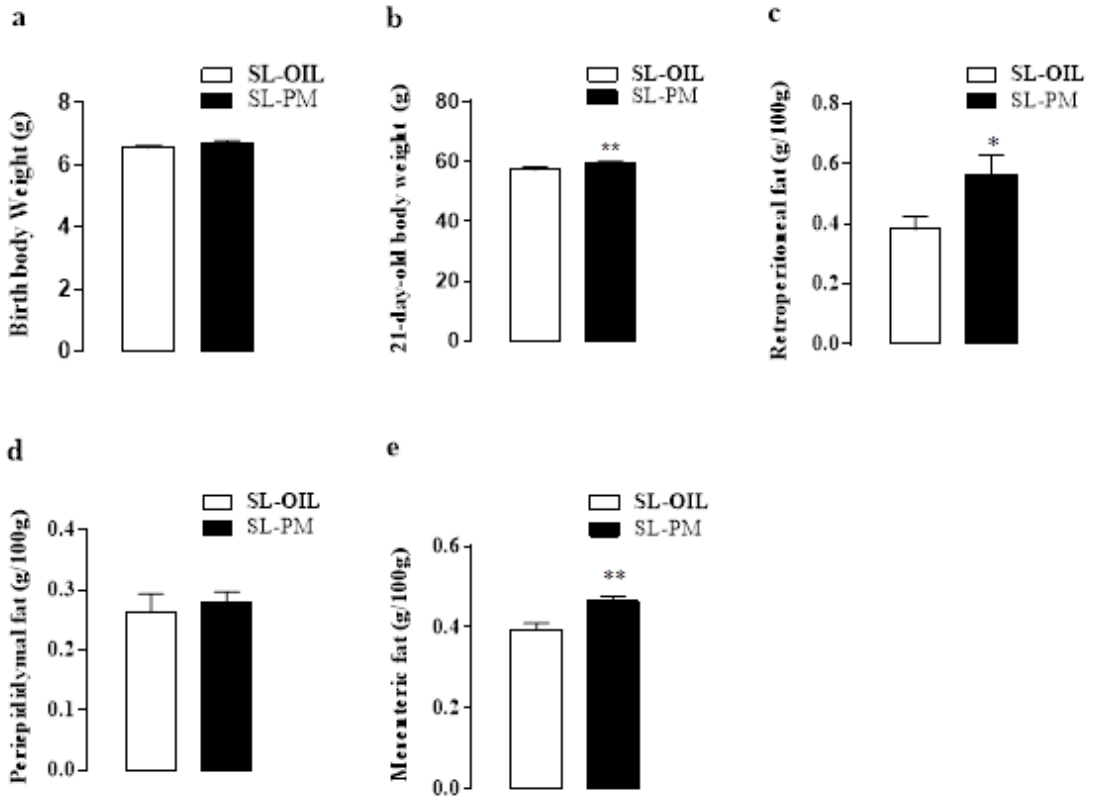


**Figure 2. Effect of $PM_{<10}$ during gestation and lactation on birth weight, body weight and tissue fat accumulation in the offspring at PND 21.** Birth weight in OIL and PM offspring (a) (n = 21/group from 7 litters), body weight at PND 21 (b) (n=21/group from 7 litters), retroperitoneal (c) (n = 6-9/group from 6 different liters), periepididymal (d) (n = 6-9//group from 6 different liters) and mesenteric fat pads (e) (n = 6-9//group from 6 different liters). Data are expressed as the mean ± SEM; *p < 0.05, **p < 0.01 by Student's t test.

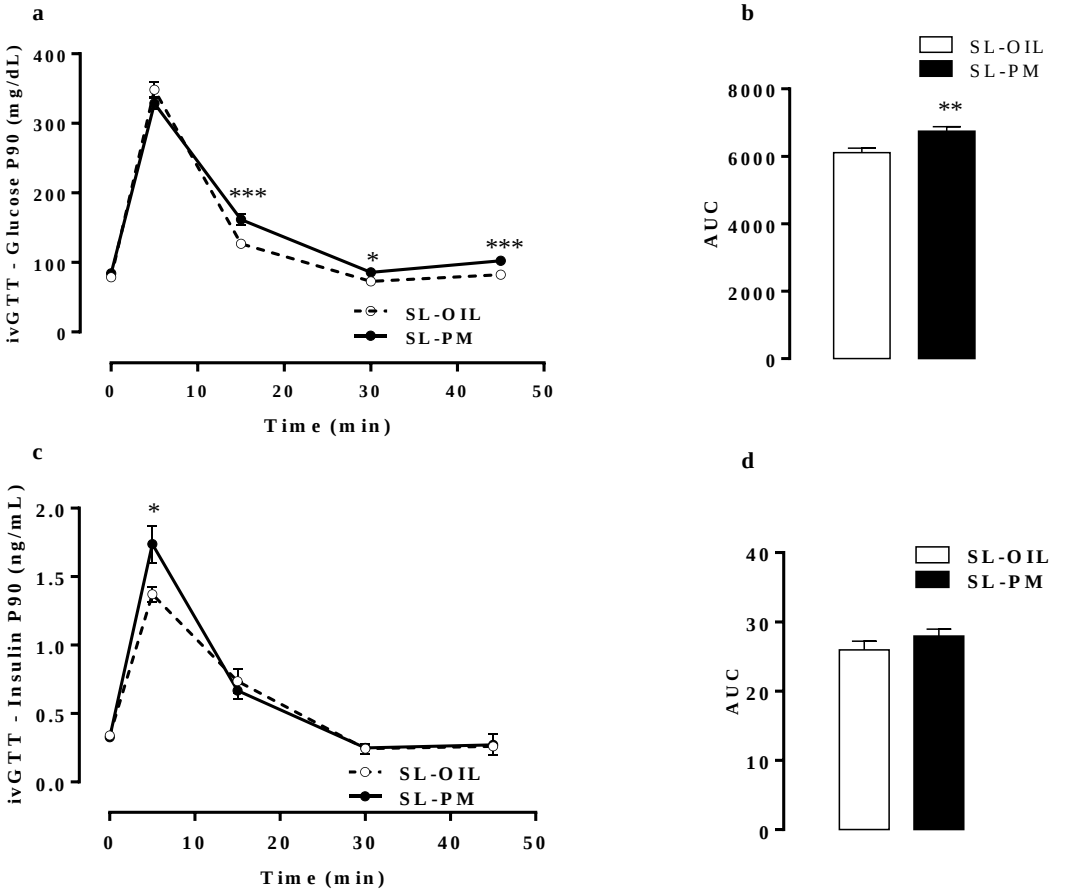


**Figure 3. Effect of $PM_{<10}$ on plasma glucose and insulin levels during ivGTT in PND 90 offspring.** Glucose (a) and insulin (b) levels in ivGTT. Data are expressed as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 by Student's *t* test (n = 6/group from 6 different liters).

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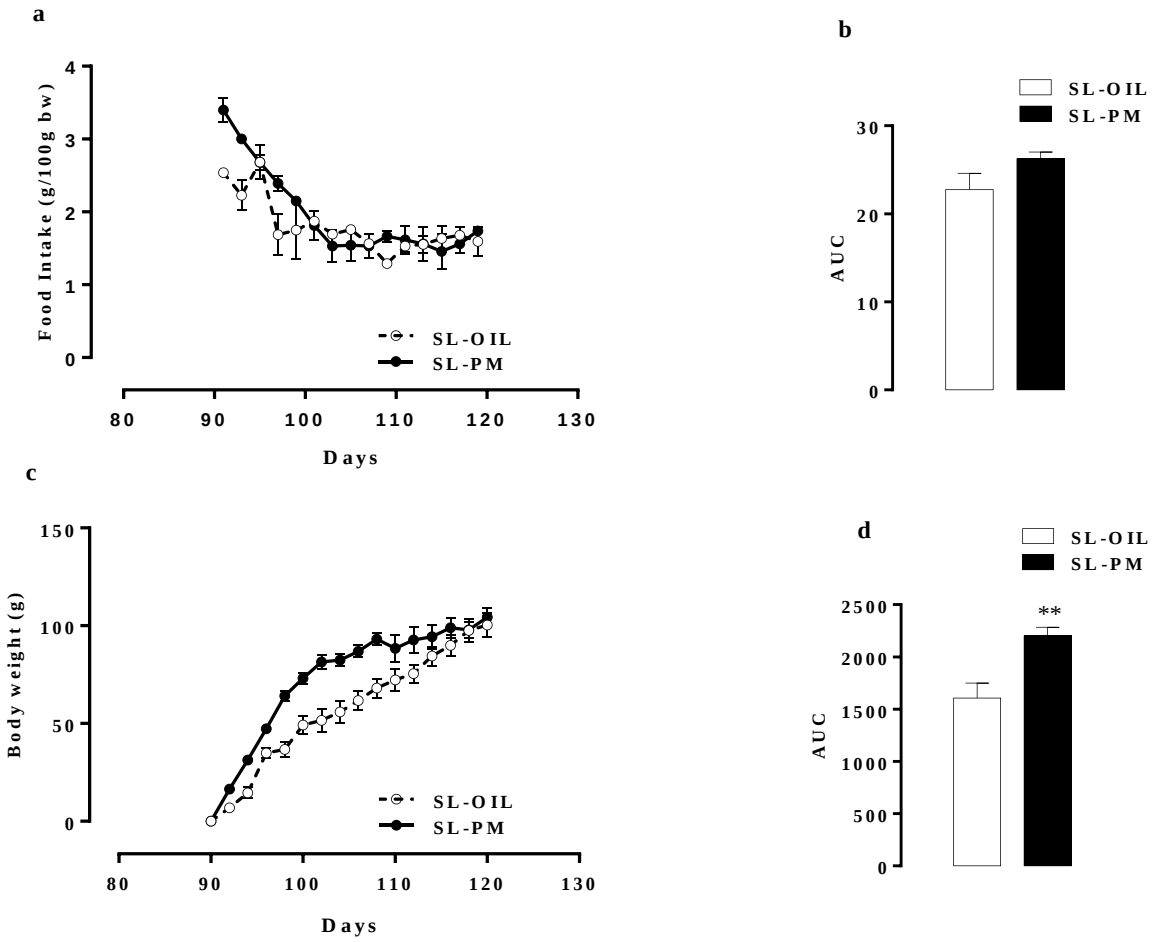


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1007 **Figure 4. Effect of $PM_{<10}$ during gestation and lactation on body weight evolution and food**
1008 **intake of the offspring from PND 90 until PND 120.** Body weight (a), AUC of body weight
1009 (b), Food intake (c) and AUC of food intake (d). Data are expressed as mean ± SEM; $p < 0.05$ by
1010 Student's *t* test (n = 6/group from 6 different liters).

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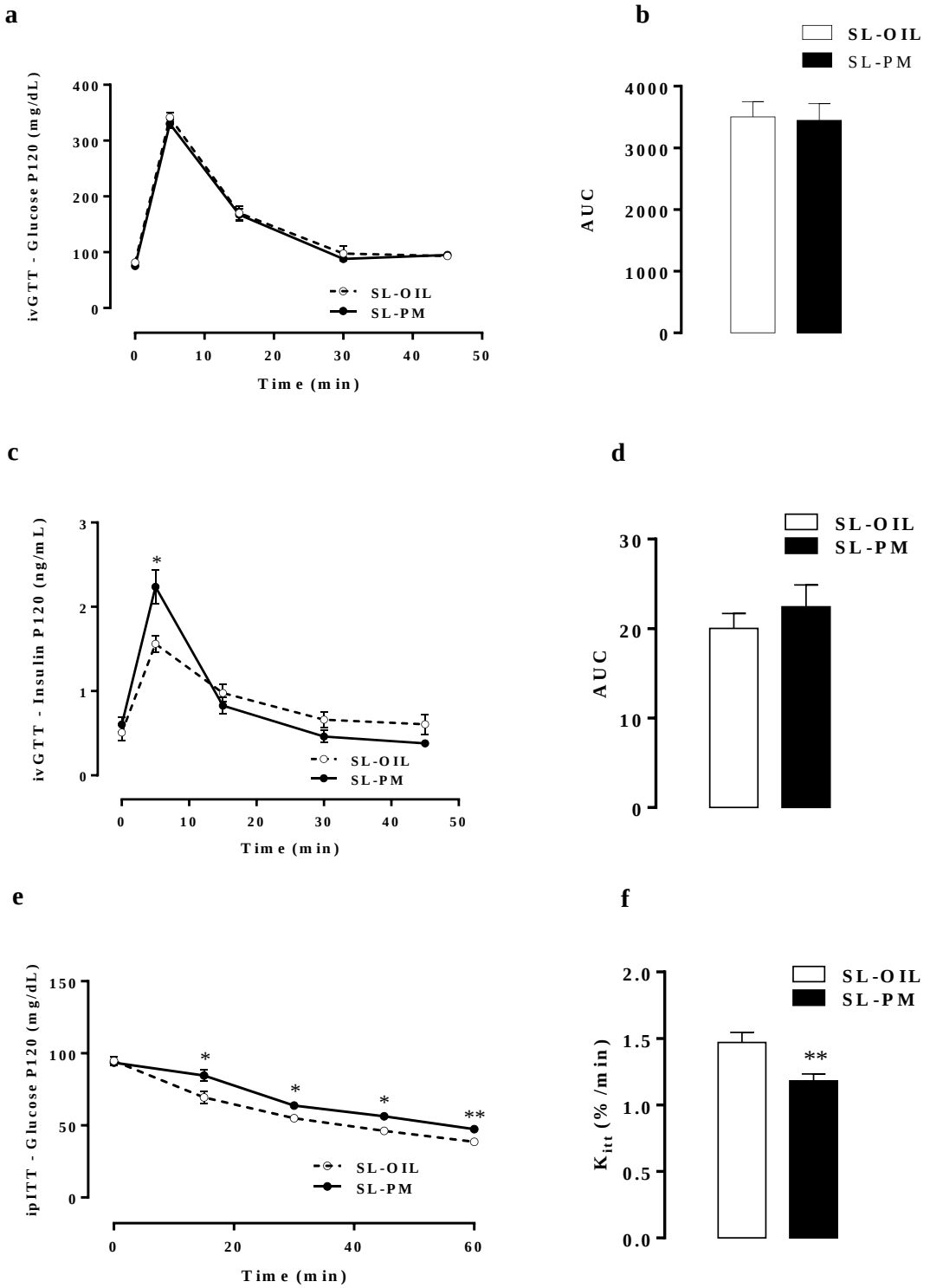


**Figure 5. Effects of $PM_{<10}$ on glucose and insulin plasma during ivGTT and glucose plasma in ipITT in PND 120 offspring.** Glucose (a) and insulin (b) levels in ivGTT .Glucose level (c) in ipITT. Data are expressed as mean ± SEM; *p < 0.05, **p < 0.005 by Student's *t* test (n = 6/group from 6 different liters).

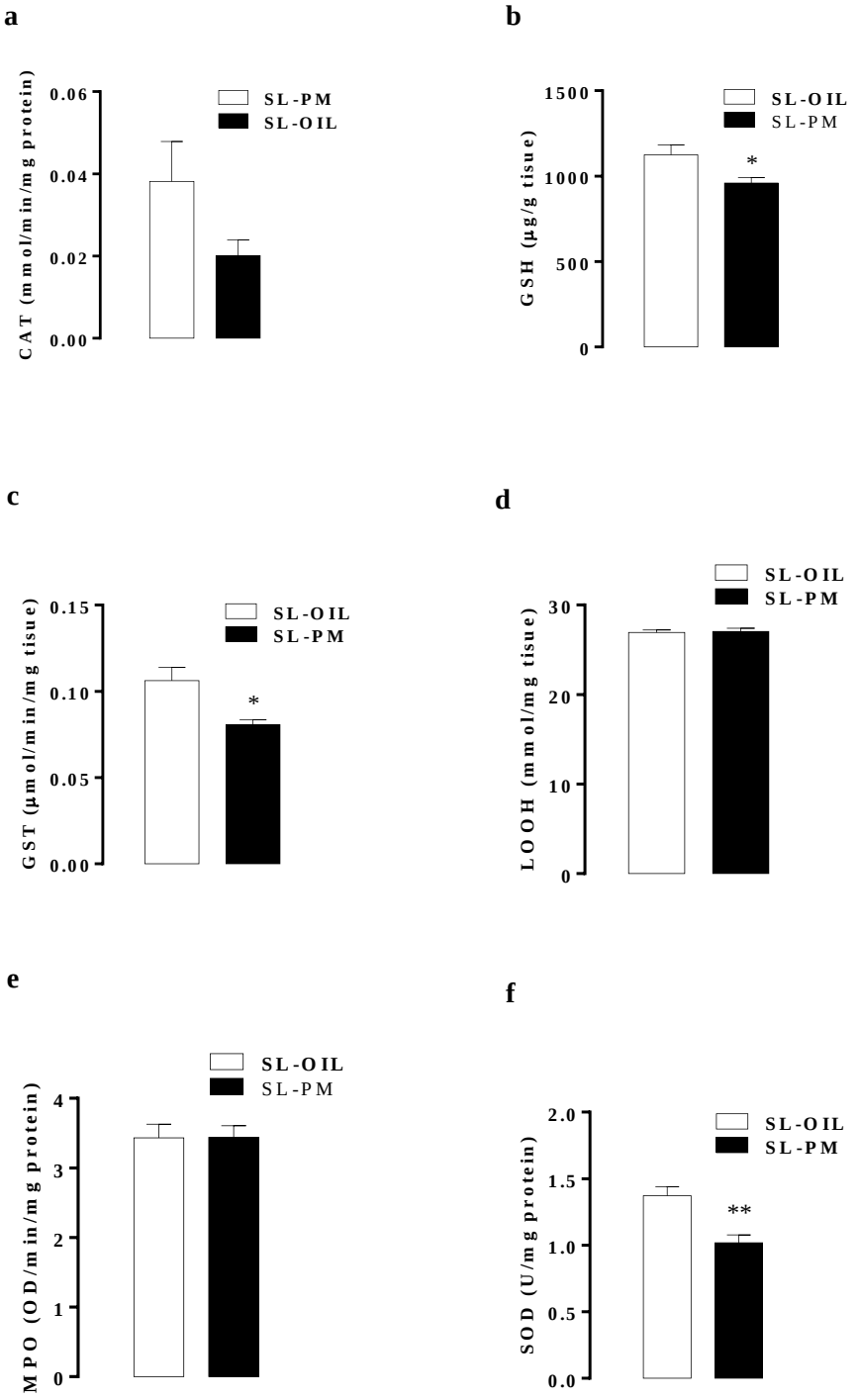


**Figure 6. Effect of $PM_{<10}$ on oxidative stress and myeloperoxidase in PND 120 offspring**. Catalase (a), GSH: glutathione (b), GST: glutathione transferase (c) LOOH: lipid hydroperoxide (d), MPO: myeloperoxidase (e) and SOD: Superoxide Dismutase (f). Data are expressed as mean ± SEM; *p < 0.05, **p < 0.005 by Student's *t* test (n = 6/group from 6 different liters).

# High fat diet during adolescence in male rats programs cardiometabolic dysfunctions in adulthood

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**Background/aims:** There is a negative relationship between exposure to high fat diet (HFD) in early life and changes in blood pressure in adulthood. It has also been suggested that adolescence is a susceptible phase for programming to metabolic syndrome (MetS). Thus, our hypothesis argues that HFD during adolescence can lead to cardiometabolic dysfunctions at adulthood. **Methods:** Adolescent Wistar rats (30 to 60 day-old) were exposed to a HFD (35% of fat). Control animals had access to normal commercial chow (NFD, 4.5% of fat). Glycemia, lip profile, food intake, body weight, fat pad stores, blood pressure, heart rate and pulse pressure were verified in 120-day-old rats. Student t-test was used to compare data between groups. **Results:** At basal test, HFD animals showed increased systolic blood pressure (SBP) and mean blood pleasure (MBP) compared with control animals (SBP: 125.4±1.8 mmHg vs. 117 ± 1.9 mmHg, respectively, $p< 0.05$; MBP: 95.78 ± 1.8 vs. 88.85 ± 1.6 mmHg, respectively, $p< 0.05$ ). Diastolic blood pressure (DBP) was similar between groups, as well as pulse pressure and heart rate. Furthermore, was observed an increase in body weight gain, as well fat pad stores, hypertriglyceridemia and glycemia in the intravenous glucose tolerance test (ivGTT). Blood pressure decrease in response to hexamethonium injection (30mg/kg of body weight) was greater in HFD animals compared with control animals (ΔSBP -43.5 ± 3.1 vs. -33.8 ± 3.1 mmHg; ΔMBP -37.9 ±3.3 vs. -27.8 ± 1.9 mmHg; ΔDBP -33.2 ±3.5 vs. -23.7 ± 1.1 mmHg respectively, $p< 0.05$). No difference between groups was observed in pulse pressure (HFD: -10.2 ± 2.1 and NFD -9.4 ± 2.2 mmHg, $p=0.4$) and in heart rate response (HFD: -5.9 ± 20.2 and NFD -3.3 ± 6.9 bpm, $p=0.2$) in response to hexamethonium. **Conclusions:** HDF during adolescence was able to program rats to development MetS, leading to higher levels of adiposity, hypertriglyceridemia, SBP and MBP later in life. In addition, the exacerbated blood pressure decrease in response to hexamethonium, points to higher sympathetic activity in HFD rats than in control ones, taken together these data suggest that pubertal period is an important programming window.

**Keywords:** Adolescence, metabolic disease, high-fat diet.

## 1. Introduction

Metabolic syndrome (MetS) has been main cause of morbidity and mortality. In addition, presenting a close association with obesity, type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD), the prevalence of MetS and obesity together have reaching an epidemic status in worldwide [1, 2]. It has been estimated that number of overweight or obese among adult people is about 40% [3]. In this context, the high prevalence of MetS may be due the changes in the lifestyle in the last decades, including lower levels of physical activity, increased in adiposity and socioeconomic status [4]. Furthermore, other factors as smoking, difference in genetic inheritance, cases of T2DM on family and education seems to have a strong association with MetS and its other diseases associated [5]. Although there is no a single definition to MetS, it may be defined as a cluster of three or more of the following risk factors together: obesity, glucose intolerance, hypertension and dyslipidemia, specifically hypertriglyceridemia [1, 6].

Among the several risk factors associated to MetS and obesity, the changes in diet composition seems to have a key role to development of obesity and consequent MetS. In this sense, diet with high fat content (HFD) are often related to increase of body weight (bw) by increase in fat pad stores, both in humans and experimental [7]. Furthermore, this diet lead to high level of fasting glucose, total cholesterol and insulin resistance (IR), generally independent of exposure age [8, 9]..

Several studies have shown that gestation and lactation are critical periods of body development, thus, it has been suggested that exposure to environmental disruptors, during early life, could lead to programming of the offspring to cardiometabolic disease later in life [10-13]. This exposure may lead to noncommunicable disease, such as obesity, coronary heart disease, T2DM, and some types of cancer at adulthood [10, 11]. Corroborating this findings, previous study of our group have show that offspring from mothers exposure to HFD during gestation and lactation presented increase in bw and adiposity at weaning, in addition these pups showed hyperglycemia, hyperleptinemia and elevated blood pressure at adulthood [14, 15].

Recent studies have shown that peri-pubertal period also is critical period for body development as well of the sexual maturation and development of central nervous system (CNS) [9, 11, 16]. In addition, HFD was able to induce changes in both

reproductive system and metabolism, and consequently led to metabolic dysfunctions at adulthood [9]. Nevertheless little is known about the effect of HFD during adolescence and its role on cardiometabolic syndrome later in life. Thus, we hypothesized that consumption of HFD during peri-pubertal period is able to programing changes and induce to cardiometabolic syndrome at adulthood.

## 2. Materials and Methods

### 2.1. Experimental model and diet

Twenty-five day-old male Wistar rats were supplied by the Central Animal House at the State University of Maringá, Paraná and kept in the Animal House of Department of Biotecnology, Genetics and Cellular Biology in light controlled conditions with a 12-h light-dark cycle (07:00 a.m. to 07:00 p.m.) and temperature of 22.0±2 °C. After five days of adaptation, the animals were randomly distributed in five per cage. At 30-days-old a group of animals were fed with high fat diet (HFD; hypercaloric diet with 35% lard/ 5.817 kcal/g) [17] for thirty days (HFD group). After that, the rats were switched to standard diet containing an adequate amount of protein (20.5% protein, Nuvital®, Curitiba/PR, 3.801 kcal/g) (NFD group). Controls animals were fed with standard diet during all protocol. Water and food were provided *ad libitum* in both groups. Experimental assays were performed with 15 animals per group and the protocol was approved by the Ethics Committee of the State University of Maringá (protocol number 1527130815).

### 2.2. Food intake, body weight, evolution and fat tissue accumulation (biometric and biochemical parameters)

Between 30 and 120 days-old, the body weight and food intake were assessed, once and three times a week, respectively. The average of food intake was calculated per rat per day and expressed relative to 100g of body weight. At 120-days of age, a group of animals were euthanized by decapitation method and fat pad stores was removed and weighed, to measure fat tissue accumulation. Plasma was used for quantify, fasting glucose, total cholesterol and triglycerides by enzymatic method using a specific colorimetric commercial kit (Gold Analisa®, Belo Horizonte, Minas Gerais, Brazil).

### 2.3. Intravenous glucose tolerance test (ivGTT)

At PND 120 the offspring (n=6 for each group and age) underwent surgery consisting of silicone cannula implantation into the right jugular vein as previously described (de Oliveira et al. 2011). After 12-h fasting, a glucose load (1 g/kg bw) was injected into the cannula. Blood samples (400 µl) were collected immediately prior to glucose injection (0 min) and then at 5, 15, 30 and 45 min, and the obtained plasma samples were stored at -20

°C for further analysis. To maintain blood volume, a corresponding volume of saline (0.9%) was infused through the cannula.

### 2.3. Surgery for arterial catheter implantation

At 120 days of life, another group of the rats were submitted to intramuscular anesthesia (Ketamine-xylazine; 3 and 0.6 mg/100g of body weight, respectively) and a P10 catheter (P10 cannula-Micro-Renathane linked to a P50 cannula-ClearTygon) that was filled with 0.1 ml of heparinized saline (500 units/mL) was installed into the femoral artery and advanced (4 cm) until the tip entered the abdominal aorta. During surgery a Doxycycline dose (2 mg/kg of body weight, intra-arterial) was administered and the next two days after surgery analgesic metamizole was provided (30mg/kg). After surgery, animals were housed in individual cages. To eliminate any clot, 0.1 ml of heparinized saline (500 units/mL) was injected through the cannula [18] and blood pressure recordings were performed four days later [19].

### 2.4. Protocol and experimental procedures for blood pressure assessment

After 1 hour of adaptation the experiments were performed in 3 animals from NFD and HFD groups running in parallel. It was wait thirty minutes in adaptation to allow blood pressure stabilization and a baseline recording during 30 minutes, when animals were relaxed and quiet or sleeping. All protocol were performed during the inactive period of animals, between 1 and 4 p.m. [20]. Following, a dose of Hexamethonium (30 mg/kg) was injected intraperitoneally and blood pressure recording for subsequent 30 minutes.

### 2.5. Measurement of blood pressure and heart rate

The arterial cannula was connected to a fluid-filled blood pressure transducer (MLT0699, AD Instruments, Dunedin, New Zealand), which was linked to a signal amplifier (Insight, Ribeirão Preto/SP Brazil). Thus, continuous recordings of arterial pressure were sampled at 1000Hz using an analog-to-digital converter board (CODAS, 1-kHz sampling frequency, Dataq Instruments, Inc, Akron, OH) with freely moving rats in their home cage. To measure systolic, diastolic and calculated mean arterial pressure (SBP, DBP and MBP, respectively) and pulse interval (PI; a surrogate for the R-R interval) values. Analyses were made on a beat-to-beat basis [21] over 20 min and during Hexamethonium test.

## 2.6. Statistical analyses

Statistical analyses and graph design were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA), being the data expressed as mean ± SEM. Students t-test was used and p value $< 0.05$ was considered as statistical significant differences between groups.

## 3. Results

### 3.1 Food intake, body weight evolution and fat tissue accumulation

HFD adolescent rats showed a decreased food intake during the diet period treatment compared to control animals, however no difference was observed after this period between groups (Fig. 1A, $p < 0.0001$). In addition, the content of calories intake was higher in the HFD group only during the diet period and no difference was verified in calories intake after that (Fig. 1B, $p < 0.005$). Furthermore, an increase in the weight gain was observed during and after HFD treatment (23.9%, $p < 0.01$; 14.7%, $p < 0.01$, respectively; Fig. 1C). Besides, an increase of bw, the retroperitoneal, periepididymal and mesenteric fat pad stores was observed, compared to controls (14.7%, $p < 0.01$; 54.7%, $p < 0.01$; 36.8%, $p < 0.01$ and 56.2%, $p < 0.01$, respectively; Fig. 2).

### 3.2 Lipid profile and glucose homeostasis

Although the total cholesterol did not show significant difference between the groups ($p = 0.0602$), triglyceride levels were elevated in HFD animals (59.5%, $p < 0.001$; Fig. 3). As indicated in the Figure 4, an increase of plasma glucose levels was observed in HFD animals at the intravenous glucose tolerance test (ivGTT) in the AUC compared to controls (13.5%, $p < 0.05$%), this increase of glucose plasma levels was observed mainly at the five minutes after glucose injection in HFD group (40.6%, $p < 0.01$).

### 3.3 Cardiovascular measurements

Cardiovascular analyses during resting period did not show difference in pulse interval, heart rate and pulse pressure, animals exposed to HFD during adolescence presented an increase in SBP and MBP in adulthood (9.3%, $p < 0.05$ and 7.1%, $p < 0.05$, respectively). Representative signals of blood pressure recordings of controls and HFD animals are shown in Fig 5.

### 3.4 Cardiovascular response to hexamethonium

After hexamethonium exposure, an expressive decrease in the DBP, SBP and MBP (35%, $p < 0.05$; 19%, $p < 0.01$ and 30.3%, $p < 0.05$, respectively) was observed in HFD group compared to control group. However, differences on heart rate were not statistically significant.

## 4. Discussion

In this study HFD adolescent rats showed changes in calorie intake during the diet period treatment, as well in the weight gain, lipid profile and cardiovascular parameters at adulthood. Taken together, these finding suggest that chronic HFD exposure during adolescence, have long term consequences in health and programs the development of cardiometabolic syndrome later in life. Gestation, lactation and adolescence are particularly crucial periods for brain structures development. Thus, several of studies have shown that the exposure to environmental stressors in each one of these periods could be responsible to development of metabolic diseases at adulthood, pointing to the developmental origins of health and disease (DOHaD) concept [9-13].

Male rats fed with HFD by thirty days, during adolescence, presented changes on cardiometabolic parameters at 120 days of life signaling to increased risk of cardiovascular disease and MetS. These founds agree with previous reports [9]. It has been discussed that deleterious effect of insults during adolescence are both related to behavior and metabolic changes at adulthood, pointing to the sensibility of central nervous system (CNS) during adolescence due it maturation [16, 22]. In this sense, the expressively increase of weight gain, mainly after the HFD period, may be attributed to less energy expenditure observed in HFD animals, since the HFD exposure is related to decrease of mobility [22, 23]. Indeed HFD exposure has been related to damage on arcuate nucleus from hypothalamus [24], and, as it has already been widely known, that food intake, weight gain and metabolic parameters are regulates by orexigenic (anabolic) and anorexigenic (catabolic) neuropeptides produced by hypothalamic regions, that are related and integrated with satiety signals like glucose, insulin and leptin [25]. However, this study does not discuss that into metabolic programming context. On the other hand, studies about maternal programming by HFD exposure have shown that mice offspring presents increased changes in food intake as well as increased body weight at adulthood [26, 27]. Furthermore, our group showed that HFD during perinatal life led to hyperleptinemia and leptin resistance in the offspring at weaning, and these effects are strongly associated with higher body weight and adiposity at adulthood [14].

Notably, a previous study of our group discussed that HFD exposure at peripubertal phase led to more drastic consequences than HFD exposure at adult life. Thus, it has been suggest that adolescence as well gestation and lactation periods are

susceptible programming window, where some regions that are responsible by food intake and body weight regulation, e. g. hypothalamus, might be seriously affected and consequently promote changes in metabolism [17]. In this context, organization problems in these hypothalamic nucleus, (e.g. paraventricular nucleus, which are involved in the blood pressure regulation), may leads to long-term cardiovascular disease [15, 28].

Our data shows that HFD in adolescence leads rats to present an increase in SBP and MBP at adulthood, corroborating with studies of metabolic programming by HFD maternal exposure [15, 29]. Studies outside metabolic programming context, have widely discussed that cardiovascular function is controlled by diverse mechanisms, mainly sympathetic and parasympathetic activity that innervate the blood vessels and heart. After hexamethonium exposure, an expressive decrease in the DBP, SBP and MBP was observed in HFD group compared to control group. Thus, the increase in BP showed by HFD animals in later life could be due to a programming effect of HFD during pubertal period that lead to sympathetic nervous system (SNS) disfunction. In addition, the higher sensitivity to ganglionic blockade of hexamethonium chloride, and consequent expressive decrease in BP, strongly demonstrate that increased BP observed here support this idea. Some reports have shown that male Wistar rats shown higher MBP as well as renal sympathetic nerve activity in HFD rats compare to control [30]. In addition, rabbits fed HFD, for only four weeks, also shown higher BP combined with increase in renal sympathetic nerve activity [31]. Although both studies above are outside metabolic programming concept, taken together, these data suggest that HFD exposure has a major impact on development of CNS during adolescence, like as other previous period of life [26, 27, 32].

Besides the impairment of HFD exposure on control of BP, in this study HFD exposure during adolescence leads to dyslipidemia with increase in triglyceride levels at adulthood. Although no difference was observed in total cholesterol between de groups, similarly was found in other programming window [29, 33]. Triglyceride (TGL) is considered as an important biomarker of cardiovascular risk and this increase due to HFD exposure is well discussed in a lot of studies [34, 35]. In addition, no difference was observed in fasting glucose, HFD exposure is often associated to glucose intolerance [9, 17]. Accordingly, in our study HFD treatment in adolescent rats showed to be able on increased body weight gain associated to glucose intolerance at adulthood, corroborating with previous studies [17, 23, 36]. Taken together, our data appear to reveal that dietary

insult during adolescence might lead to programming to increase risk factors of cardiometabolic syndrome at adulthood and emphasized peripubertal period as a crucial window of development, as well as on gestation and lactation [9, 11, 37].

## 5. Conclusion

In summary, we demonstrate that HFD consumption during adolescence is able to program cardiometabolic dysfunctions in male rats, and in this sense is strongly associated to cardiovascular risk, leading rats to dyslipidemia, mainly hypertriglyceridemia, obesity, glucose intolerance and hypertension at adulthood. The present study confirms the susceptibility of adolescence as a critical window of development, pointing to the need of intervention and control of access to HFD in this life period to prevent cardiometabolic disease.

## Declaration of interest

The authors declare no conflict of interest associated with this manuscript.


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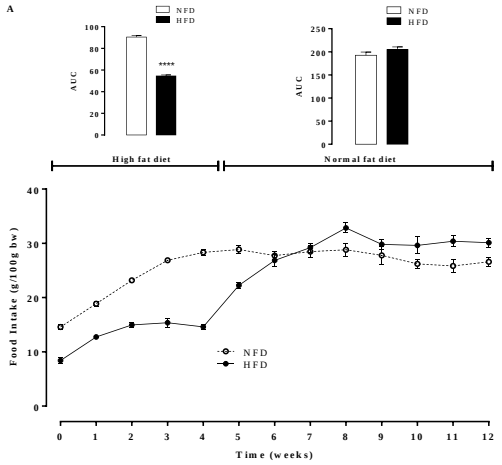


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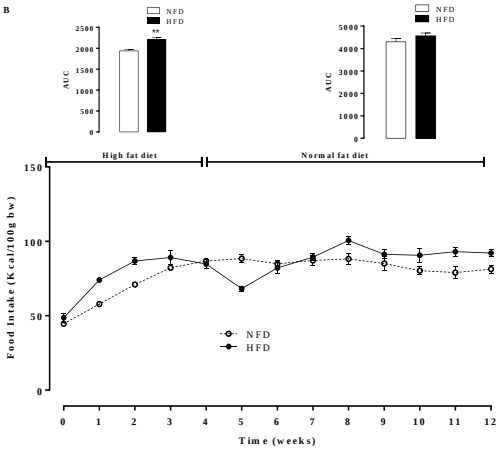


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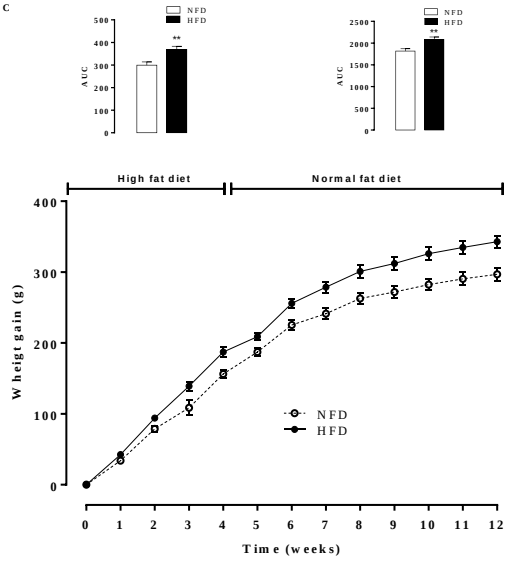


1534

1535 **Fig 1:** Food intake relative to 100g of body weight and its total area-under-the-curve (AUC) (A),
1536 Food intake relative to kcal100g of body weight and its AUC (B), body weight gain and its AUC
1537 (C) from 30 to 120 days of life from animals exposed to normal diet (NFD, white) and high-fat-
1538 diet (HFD, black). n = 20 animals; values are mean ± SEM; ****$p<0.0001$, **$p<0.01$ indicate
1539 statistical significance by Student's t-test.

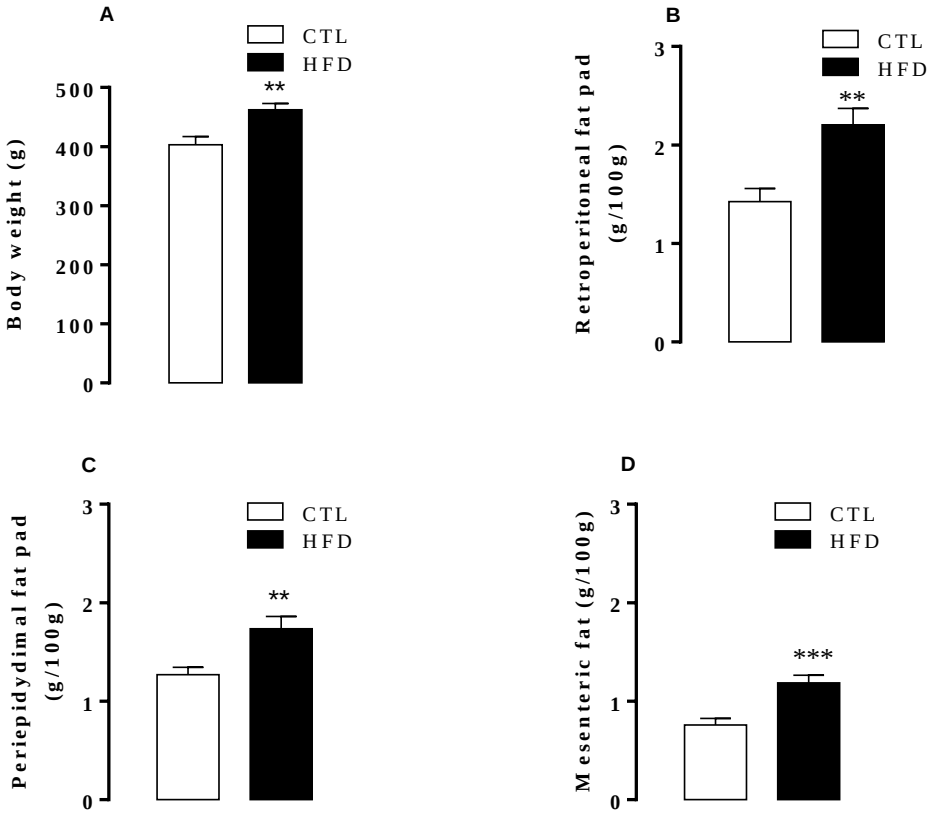


**Fig 2:** Body weight (A), retroperitoneal (B), periepididymal (C), and mesenteric fat stores (D) from 120–days-old rats exposed to normal fat diet (NFD, white) and high-fat-diet (HFD, black) at adolescence. n = 10 animals; values are mean ± SEM; ***p<0.001, **p<0.01 indicate statistical significance by Student's t-test.

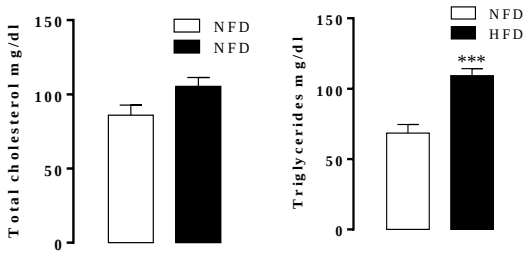


**Fig 3:** Total cholesterol (A) and triglycerides (B), from 120-days-old rats exposed to normal fat diet (NFD, white) and high-fat-diet (HFD, black). n = 6 animals for total cholesterol and n = 6-8 for triglycerides; values are mean ± SEM; ***p<0.001 indicate statistical significance by Student's t-test.

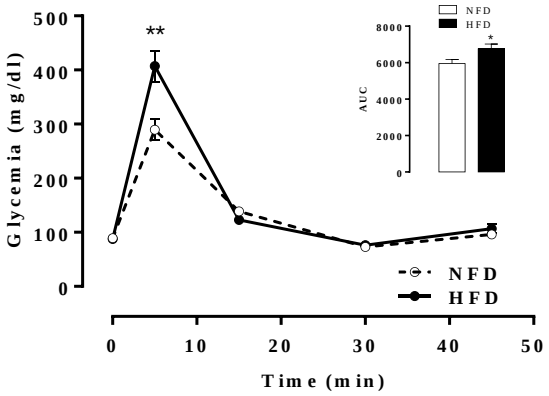


**Fig 4:** Glucose tolerance test and its total area-under-the-curve (AUC) from 120-days-old rats exposed to normal fat diet (NFD, white) and high-fat-diet (HFD, black). n = 6-9 animals for test; values are mean ± SEM; **p<0.001, *p<0.05 indicate statistical significance by Student's t-test.

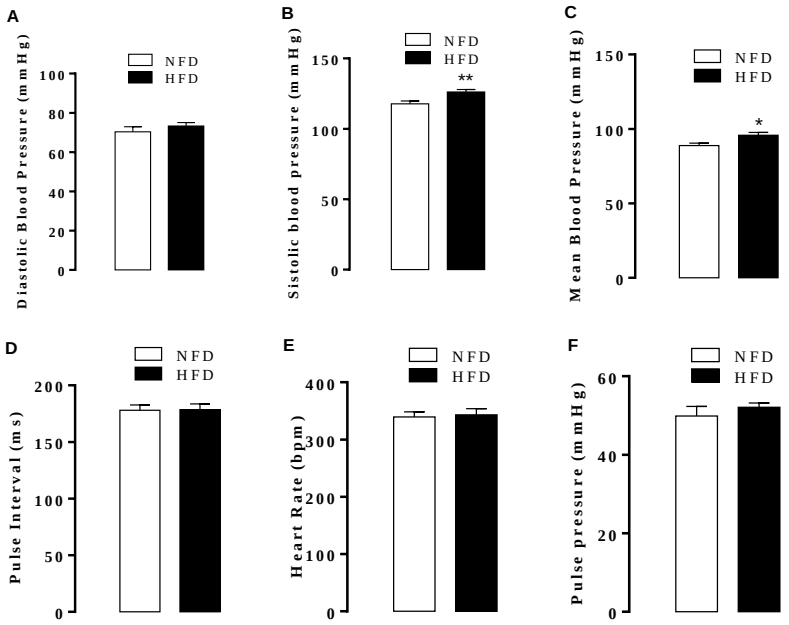


**Fig 5:** Diastolic blood pressure (DBP; A), systolic blood pressure (SBP; B), mean blood pressure (MBP; C), pulse interval (PI; D), heart rate (HR; E) and pulse pressure (PP; F) from 120–days-old rats exposed to normal fat diet (NFD, white; n = 11) and high-fat-diet (HFD, black; n = 13). Values are mean ± SEM; **p<0.001 and *p<0.05 indicate statistical significance by Student's t-test.

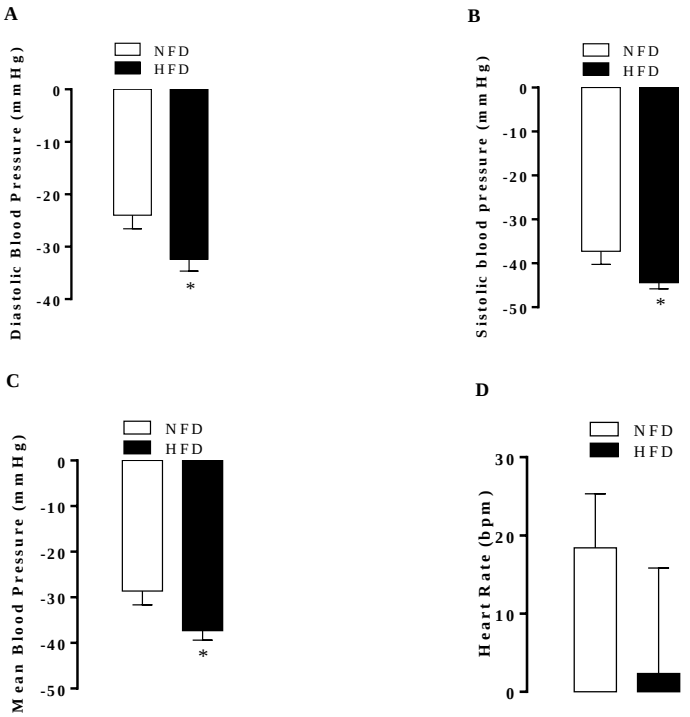


**Fig 6:** Depressant response to hexamethonium in diastolic blood pressure (DBP; A), systolic blood pressure (SBP; B), mean blood pressure (MBP; C), and heart rate (HR; D) in 120–days-old rats exposed to normal fat diet (NFD, white; n = 11) and high-fat-diet (HFD, black; n = 13). Values are mean ± SEM; *$p<0.05$ indicate statistical significance by Student's t-test.