UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE CIÊNCIAS MÉDICAS

OLIVIA PIZETTA ZORDÃO

MATERNAL EXPOSURE TO AIR POLLUTION PROGRAMS ENERGY BALANCE AND THE GUT MICROBIOTA IN A TIMING AND GENDER-SPECIFIC MANNER

EXPOSIÇÃO MATERNA À POLUIÇÃO DO AR PROGRAMA O BALANÇO ENERGÉTICO E A MICROBIOTA EM UM PERÍODO E GÊNERO ESPECÍFICO DA PROLE

CAMPINAS

2021

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Thesis presented to the Faculty of Medical Sciences of the State University of Campinas as part of the requirements for obtaining the title of doctor in Sciences, at Clinical Medicine area.

Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de doutora em Ciências, na área de Clínica Médica.

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ESTE TRABALHO CORRESPONDE À VERSÃO
FINAL DA TESE DEFENDIDA PELA
ALUNA OLIVIA PIZETTA ZORDÃO, E ORIENTADA PELA
PROFA. DRA. PATRÍCIA DE OLIVEIRA PRADA.

CAMPINAS

2021

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Médicas
Maristella Soares dos Santos - CRB 8/8402

Z769e

Zordão, Olivia Pizetta, 1990-
Exposição materna à poluição do ar programa o balanço energético e a microbiota em um período e gênero específico da prole / Olivia Pizetta Zordão. – Campinas, SP : [s.n.], 2021.

Orientador: Patrícia de Oliveira Prada.
Coorientador: Mariana Matera Veras.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Poluição atmosférica. 2. Gestação. 3. Lactação. 4. Crianças adultas. 5. Obesidade. I. Prada, Patrícia de Oliveira, 1971-. II. Veras, Mariana Matera. III. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Maternal exposure to air pollution programs energy balance and the gut microbiota in a timing and gender-especific manner
Palavras-chave em inglês:
Air pollution
Pregnancy
Lactation
Adult children
Obesity
Área de concentração: Clínica Médica
Titulação: Doutora em Ciências
Banca examinadora:
Mariana Matera Veras [Coorientador]
Soraia Katia Pereira Costa
Patricia Cristina Lisboa da Silva
Adriana Souza Torsoni
Leandro Pereira de Moura
Data de defesa: 27-05-2021
Programa de Pós-Graduação: Clínica Médica

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-0596-1954
- Currículo Lattes do autor: http://lattes.cnpq.br/9767380162724176

COMISSÃO EXAMINADORA DA DEFESA DE MESTRADO/DOUTORADO

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Data de Defesa: 27/05/2021

DEDICATÓRIA

Aos meus pais, Arquelau e Luzia, por sempre me apoiarem, incentarem e darem o suporte necessário em todas as situações, por mais desafiadoras que fossem.

Ao meu irmão Luís Henrique, que sempre me ouviu e deu forças para continuar, mesma na distância.

Ao Marcelo, por todo amor, parceria, paciência e pelo companheirismo de toda essa trajetória**.**

Ao meu avô pelo suporte, orações e torcida para que tudo desse certo.

A república Hellmann's que me incentiva, admira e esteve ao meu lado nos piores e melhores momentos desde sempre.

A mim mesma, que me superei, me desafiei e venci essa etapa em tempos difíceis para a ciência.

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

A Profa. Dra. Patrícia de Oliveira Prada, pela orientação, oportunidades, ensinamentos e dedicação durante todas as etapas. Obrigada pela paciência e confiança dedicada a mim e também por fazer parte de uma fase tão importante na minha vida.

A Profa. Dra. Mariana Matera Veras pela coorientação, possibilidades, ensinamentos e disponibilidade ao longo de todo o processo.

A Gisele, Paula e Laís, que dedicaram tempo no início do trabalho para que eu aprendesse novas técnicas e experimentos. Agradeço à amizade, ao companheirismo e a paciência.

A Sónia, Duda e Clara por estarem sempre presentes, dividirem alegrias, felicidades e angústias durante da trajetória e por se tornarem mais do que colegas de trabalho.

Aos colegas do laboratório Tamires, Vitor, Raquel, Clílton, Bruna, Raquel, Angie e as duas Vanessas pelo companheirismo.

A Dioze, Heloísa, ao Guilherme e Andrey pelas contribuições durante as análises.

Ao Prof. Dr. Mario por ceder um local para os experimentos e análises, além das contribuições acadêmico científicas ao longo dos 5 anos.

A Faculdade de Ciências Médicas, pela oportunidade de realização deste trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001

A todos funcionários da Secretaria da Pós-Graduação e da Biblioteca da FCM-UNICAMP.

E a todos que direta ou indiretamente fizeram parte deste sonho.

RESUMO

Objetivo do estudo foi investigar se a exposição ao material particulado ($MP_{2,5}$) altera o metabolismo energético em filhotes e se a microbiota pode contribuir com o fenótipo. Camundongos C57BL/6J não expostas foram acasaladas e a exposição ao $MP_{2,5}$ ou ao ar filtrado (AF) aconteceu apenas na gestação ($MP_{2,5}$/AF) ou na lactação (AF/$MP_{2,5}$). Nós estudamos os filhotes dos dois gêneros. Observamos que a exposição ao $MP_{2,5}$ durante a gestação aumentou peso desde o nascimento até a idade adulta , ingestão alimentar, níveis de leptina e prejudicou a tolerância a glicose nos filhotes machos. Nos filhotes fêmeas, a exposição ao $MP_{2,5}$ aumentou o peso na idade adulta, ingestão alimentar, níveis de insulina sem alteração na tolerância a glicose e na sensibilidade a insulina. Surpreendentemente, a exposição ao $MP_{2,5}$ durante a lactação aumentou o peso nos filhotes machos no desmame mas não na idade adulta. Esse resultado foi associado com a redução no consumo de oxigênio nos filhotes machos. Nas fêmeas da prole, a exposição durante a lactação ao $MP_{2,5}$ reduziu os níveis de glicose no sangue em jejum e melhorou a sensibilidade a insulina. A expressão gênica de NPY e AGRP foram aumentadas nos machos da prole do grupo AF/$MP_{2,5}$ e nas fêmeas foi observado um aumento no NPY no grupo $MP_{2,5}$/AF. Para determinar se a exposição materna ao $MP_{2,5}$ pode afetar a microbiota intestinal da prole, nós analisamos a alfa e a beta diversidade. A exposição durante a gestação foi associada com uma redução na alfa diversidade da microbiota. Nós observamos uma dissimilaridade entre os três grupos, demonstrando que a exposição ao $MP_{2,5}$ modificou a composição da microbiota. Comparado ao grupo que foi exposto apenas ao ar filtrado, o gênero *Akkermansia* foi aumentado nos filhotes machos e fêmeas do grupo $MP_{2,5}$/AF; *Oscillibacter* foi reduzido em filhotes machos do grupo $MP_{2,5}$/AF; *Allistipes* estava aumentado nas fêmeas do grupo AF/$MP_{2,5}$. Em conclusão, nossos dados sugerem que a exposição materna ao $MP_{2,5}$ durante a gestação afetou negativamente o metabolismo energético de machos; durante a lactação, nós observamos uma pequena melhora na sensibilidade a insulina nas fêmeas mas não nos machos. Nós podemos também concluir que a exposição materna a poluição provoca efeitos negativos na microbiota intestinal da prole.

Palavras-chave: poluição atmosférica, gestação, lactação, prole, obesidade

ABSTRACT

We aimed to determine the most critical period to maternal exposure to particulate matter ($PM_{2.5}$) to impair offspring energy metabolism and whether the gut microbiota could contribute to the phenotype. Unexposed female C57BL/6J mice were mated, and the exposure to $PM_{2.5}$ or filtered air (FA) occurred only in pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). We studied the offspring from both genders. We observed that pregnancy exposure to $PM_{2.5}$ increased body mass from birth to adulthood, food intake, leptin levels, and impaired glucose tolerance in male offspring. In female offspring, pregnancy exposure to $PM_{2.5}$ increased adulthood body mass, food intake, insulin levels without altering glucose tolerance and insulin sensitivity. Surprisingly, lactation exposure to $PM_{2.5}$ increased male body mass at weaning but not in adulthood. This result was associated with increased O_2 consumption in male offspring. In female offspring, lactation exposure to $PM_{2.5}$ decreased fasting blood glucose levels and improved insulin sensitivity. NPY and AGRP expression levels were increased in male offspring from the group FA/$PM_{2.5}$, and in females, we observed an increase in NPY of the $PM_{2.5}$/FA group. To determine if maternal exposure to $PM_{2.5}$ could affect the offspring's gut microbiota, we analyzed alpha and beta -diversity, the Linear Discriminant Analysis (LDA), and Effect Size (LEfSe). Pregnancy exposure was associated with decreased alpha diversity in the gut microbiota. We observed a spatial separation among the three groups, demonstrating that exposure to $PM_{2.5}$ changed the microbiota composition, as indicated by principal coordinate analysis (PCoA) of Bray-Curtis distances among samples of each group. Compared with FA/FA, the genus *Akkermansia* was increased in the male and female offspring of the $PM_{2.5}$/FA group; *Oscillibacter* was reduced in the male offspring of $PM_{2.5}$/FA; *Allistipes* was increased in the FA/$PM_{2.5}$ of the female offspring. In conclusion, our data suggest that maternal exposure to $PM_{2.5}$ in pregnancy negatively affects the energy metabolism of males; during lactation, we observed a slight improvement in insulin sensitivity in female offspring and not in male offspring. Also we can conclude that maternal air pollution exposure provokes negative effects on the offspring gut microbiota.

Keywords: air pollution, pregnancy, lactation, offspring, obesity.

SUMÁRIO

INTRODUÇÃO

A prevalência de indivíduos obesos no mundo vem aumentando de forma significativa e alcançou 650 milhões de adultos obesos em 2016 (1). No Brasil, o excesso de peso cresceu 26,3% em dez anos; nos adultos a prevalência de obesidade é de 18,1% entre os homens e 19,6% entre as mulheres (2). A obesidade é caracterizada pela acumulação em excesso de lipídios no tecido adiposo e outros órgãos, sendo considerada uma doença crônica e evidenciada sendo fator de risco de doenças cardiometabólicas (1,3–11). Diante desse quadro alarmante, é urgente que se entenda as causas da obesidade para que se possa empregar medidas preventivas e/ou terapêuticas adequadas.

Condições adversas que podem ocorrer no ambiente perinatal têm sido implicadas na etiopatogenia de algumas doenças crônicas, incluindo obesidade, diabetes mellitus tipo 2 (DM2) e doenças cardiovasculares (DCV) (12–14). Estudos clínicos, epidemiológicos e experimentais sugerem que o ambiente ao qual estamos expostos durante os períodos iniciais do nosso desenvolvimento pode ter um impacto permanente na estrutura e metabolismo de tecidos, afetando a saúde a longo prazo (12,15). A exposição perinatal a alguns fatores ambientais como desnutrição ou obesidade, tabagismo e estresse maternos pode ter um impacto negativo na saúde da prole (16,17). Essas observações deram origem ao conceito de “programação metabólica”, a qual sugere que a exposição ao longo do período perinatal a fatores ambientais adversos pode desencadear uma resposta de adaptação molecular, celular e bioquímica do organismo de forma permanente. Portanto, mesmo que cesse o estímulo inicial, as adaptações poderão ainda ser observadas (12).

Uma série de estudos revela que um aporte insuficiente de nutrientes durante a gestação pode causar danos no desenvolvimento fetal e baixo peso ao nascer. Essas alterações tanto no desenvolvimento fetal quanto o reduzido peso ao nascer foram associadas com o aparecimento de DM2 e DCV na vida adulta (18,19). A “hipótese do fenótipo econômico” foi proposta por Hales e Barker, 1992 pela demonstrando a relação entre o baixo peso ao nascer e um maior risco de intolerância à glicose, DM2 e doenças cardiovasculares na vida adulta de humanos. Essa hipótese sugere que um ambiente intrauterino

escasso de nutrientes pode induzir uma programação no feto voltada a “economizar” nutrientes, mesmo que na vida adulta haja abundância dos mesmos. De fato, no modelo experimental, no qual roedores fêmeas são submetidos à restrição proteica durante a gestação, observa-se que a prole nasce com baixo peso e tem grande chance de desenvolver obesidade e maior preferência por alimentos ricos em gordura na vida adulta (20,21). Em conjunto, esses estudos sugerem que o peso ao nascer pode ser um importante preditor de doenças futuras e a programação metabólica, induzida por déficit de nutrientes durante a gestação, predispõe ao desenvolvimento de doenças crônicas cardiometabólicas na vida adulta (22,23).

O desenvolvimento de neurocircuitos hipotalâmicos consiste de duas principais fases. Primeiro, determina-se o número de células neuronais, por meio da neurogênese, migração neuronal e diferenciação. Na segunda fase, ocorre a formação dos neurocircuitos funcionais por meio do estabelecimento de projeções neuronais e conexões sinápticas. Ressalta-se que em humanos, as duas fases descritas acima ocorrem no útero. No entanto, em roedores, a primeira fase ocorre no útero, mas a segunda fase, onde ocorre a formação das projeções neuronais, incluindo as do hipotálamo, só será finalizada na terceira semana de vida (24–27). Logo, como a maioria dos estudos é realizada em roedores, os dois ambientes perinatais, gestação e lactação, precisam ser considerados na investigação do *imprinting* metabólico (28). O ambiente nutricional durante a lactação em roedores tem impactos duradouros sobre o peso corpóreo e a susceptibilidade ao ganho de peso induzido por diferentes tipos de dieta (29,30). Recentemente, foi demonstrado que a dieta materna com alto teor de gordura saturada durante a lactação e não gestação, predispôs a prole à obesidade, hiperinsulinemia e intolerância à glicose em camundongos. Esses desfechos foram associados a má formação de projeções dos neurônios POMC e AgRP localizados no núcleo arqueado do hipotálamo ao núcleo paraventricular do hipotálamo. A interrupção do sinal da insulina, em neurônios POMC da prole, preveniu as malformações das projeções neuronais, assim como na inervação parassimpática pancreática, restaurando a secreção de insulina estimulada pela glicose (31). Esse estudo reforça a ideia de que o período da lactação é fundamental para que a prole tenha um desenvolvimento neuronal satisfatório que impacta no metabolismo

da glicose, na regulação do consumo alimentar e no gasto energético na vida adulta.

A poluição atmosférica vem ganhando destaque como um importante fator ambiental envolvido na etiopatogenia de várias doenças crônicas, dentre essas, a DM2 (32–34). Dos componentes da poluição atmosférica, o material particulado (MP) parece ter um papel importante no desencadeamento de doenças (35). O MP pode conter partículas tanto sólidas quanto líquidas que ficam suspensas na atmosfera, variando de diâmetro entre menores ou iguais a 2,5 μm chegando até 30 μm. O $MP_{2,5}$, em geral, tem como sua principal fonte de emissão os automóveis e é composto por carbono, óxidos de enxofre e nitrogênio, amônia, metais, íons de hidrogênio e lipopolissacárides (LPS) (36). Essas partículas finas podem interferir diretamente as trocas gasosas que ocorrem no sistema respiratório. O MP de diâmetro maior que 2,5 μm, em geral, é composto de silício, titânio, alumínio, ferro, sódio e cloro provenientes de queimadas, sendo o MP_{10}, o mais estudado no desenvolvimento de doenças (37). Uma metanálise recente comparou o efeito da exposição materna ao MP2,5 com a exposição ao MP10 sobre o peso ao nascimento da prole. Como resultado, foi demonstrado que a exposição materna ao $MP_{2,5}$, durante a gestação, induziu um peso ao nascimento menor do que as gestantes expostas ao MP_{10} (38), sugerindo, nesse contexto, um efeito mais deletério da exposição ao $MP_{2,5}$.

A possível ligação entre os efeitos da poluição atmosférica e a susceptibilidade à doença metabólica crônica pode ocorrer por meio de múltiplos mecanismos. A ativação do sistema imune inato e do sistema imune adaptativo, resposta inflamatória e estresse oxidativo são alguns dos mecanismos descritos na fisiopatologia de doenças crônicas desencadeadas pela poluição atmosférica (33). Evidências dos últimos anos têm sugerido uma relação de causa e efeito entre a poluição atmosférica e a susceptibilidade ao desenvolvimento tanto de obesidade quanto de DM2 (34). Essa relação de causa e efeito envolve a ativação do sistema imune, inflamação e o estresse oxidativo que são mecanismos comuns da etiopatogenia da obesidade, DM2 e doenças cardiovasculares. Nesse sentido, pode-se aventar que a maior exposição à poluição atmosférica que ocorre atualmente pode contribuir significativamente para o aumento da prevalência de tais doenças (33,39). De

fato, nosso grupo investigou o efeito da exposição aguda e crônica ao $MP_{2,5}$ sobre o peso corpóreo, ingestão e gasto energético em camundongos C57BL/6J machos adultos mantidos em dieta padrão e observou que essa exposição leva à resistência a leptina, hiperfagia e redução do gasto energético.

Além do efeito direto da exposição à poluição atmosférica na saúde, fêmeas quando expostas durante a gestação produzem ninhadas com baixo peso ao nascimento (40–42), sugerindo que a exposição à poluição nesse momento da vida compromete o desenvolvimento intrauterino do feto. Duas metanálises realizadas recentemente, em estudos com humanos, concluíram que há uma nítida associação entre a exposição ao MP no período gestacional e o baixo peso ao nascer, e que o período mais crítico de exposição ao $MP_{2,5}$ durante à gestação parece ser o terceiro trimestre (38,43). Outro estudo realizado com 2001 gestantes demonstrou que as mulheres expostas ao dióxido de nitrogênio e ao $MP_{2,5}$ durante a gestação elevou as concentrações de IL-33 (interleukin-33) e TSLP (thymic stromal lymphopoietin) no cordão umbilical da prole, sugerindo que a poluição pode modificar o desenvolvimento do sistema imunológico do feto, ativando vias pró-inflamatórias (44). Em roedores, foi demonstrado que a exposição ao $MP_{2,5}$ antes e durante a gestação elevou as concentrações de IL-4 (interleukin-4) no soro e na placenta, sugerindo que a poluição pode induzir uma reação inflamatória placentária que pode ter contribuído para o baixo peso ao nascimento que foi encontrado (45). De fato, como os fetos e as crianças recém-nascidas têm imaturidade no seu sistema imunológico, acabam sendo mais vulneráveis aos efeitos deletérios da exposição à poluição atmosférica nesse período da vida (46). Outro estudo investigou 250 mulheres no Irã e encontrou uma correlação significativamente positiva entre exposição ambiental ao monóxido de carbono (CO) e ozônio (O_3) e os marcadores de disfunção endotelial no cordão umbilical, incluindo VCAM-1 (vascular adhesion molecule) e ICAM-1 (intercellular adhesion molecule). Nesse mesmo estudo, a exposição ao MP10 e SO_2 (dióxido sulfúrico) foram significativamente associados com o aumento das concentrações de Endotelina-1 no feto, sendo esta um marcador de desenvolvimento precoce de aterosclerose (47). Veras et al, 2008 (46) demonstrou que a segunda geração de mães, expostas à poluição atmosférica durante a gestação, gerou ninhadas

com peso significativamente menor em comparação à prole de mães expostas ao ar filtrado, sugerindo um comprometimento no desenvolvimento fetal (46).

Em conjunto, os estudos são contundentes a respeito de que a exposição intrauterina à poluição atmosférica interfere nos processos de desenvolvimento fetal ligados ao controle do metabolismo energético tanto em humanos quanto em roedores. Entretanto, ainda não está claro qual seria o momento (gestação ou lactação) mais significativo de alteração do metabolismo energético da prole de mães expostas à poluição. Nesse sentido, esse estudo teve como objetivo investigar qual seria o momento mais crítico (gestação ou lactação) de exposição à poluição atmosférica para alterar o metabolismo energético da prole, considerando o peso corpóreo, ingestão alimentar, gasto energético, sensibilidade à insulina e metabolismo da glicose em ambos os gêneros.

OBJETIVOS

Objetivo geral

Investigar qual seria o momento mais crítico (gestação ou lactação) de exposição à poluição atmosférica capaz de alterar o metabolismo energético da prole, considerando o peso corpóreo, ingestão alimentar, gasto energético, sensibilidade à insulina e metabolismo da glicose em ambos os gêneros.

Objetivos específicos

1. Investigar os efeitos da exposição ao material particulado com diâmetro igual ou menor que 2,5 μm ($MP_{2,5}$) durante a gestação ou lactação sobre o peso corpóreo, ingestão alimentar, gasto energético e expressão de neuropeptídios (AgRP, NPY, POMC e TRH) e marcadores inflamatórios (TLR4 e TNFa) no hipotálamo da prole adulta de ambos os gêneros.
2. Investigar os efeitos da exposição ao $MP_{2,5}$ durante a gestação ou lactação sobre a glicemia e concentração de insulina sérica de jejum, tolerância à glicose e sensibilidade à insulina da prole adulta de ambos os gêneros.
3. Investigar os efeitos da exposição ao material particulado com diâmetro igual ou menor que 2,5 μm ($MP_{2,5}$) durante a gestação ou lactação sobre a microbiota intestinal da prole adulta

METODOLOGIA

Animais

Todos os animais do presente estudo camundongos da linhagem C57BL/6J fornecidos pelo Biotério Central da Unicamp. Protocolo CEUA/UNICAMP nº 4627-1. Os camundongos foram acondicionados em novo ambiente mantido a 25°C, com ciclos escuro - claro fixos (12/12 horas), recebendo água potável e dieta padrão *ad libitum* por 1-2 semanas para adaptação ao novo local. Com oito semanas de idade, as fêmeas foram acasaladas com camundongos machos, sendo estes da mesma idade. Para o acasalamento, três fêmeas e um macho permaneceram por 3 dias em gaiolas de plástico. No primeiro dia de gestação, as mães foram divididas aleatoriamente em três grupos experimentais: 1) mães expostas à poluição durante a gestação e ao ar filtrado durante a lactação ($MP_{2,5}$/AF); 2) mães expostas ao ar filtrado durante a gestação e a poluição durante a lactação (AF/$MP_{2,5}$); 3) mães expostas ao ar filtrado durante a gestação e lactação (AF/AF). As proles nunca foram expostas, considerando então que receberam somente ar filtrado desde o nascimento até a eutanásia. Após o desmame (21 dias) a prole de cada grupo experimental, foi acondicionada em gaiolas plásticas, recebendo somente dieta padrão para camundongos. Na idade adulta as proles foram analisadas quanto aos seguintes parâmetros: peso corpóreo, ingestão alimentar, consumo de oxigênio (O_2), produção de dióxido de carbono (CO_2), quociente respiratório (RER), concentrações de glicose no sangue e concentrações séricas de insulina em jejum, tolerância à glicose (GTT), à insulina (ITT) que serão descritos a seguir. A coleta de sangue, tecidos e a posteriori o estudo da expressão gênica de neuropeptídios e marcadores inflamatórios no hipotálamo, foram feitos após eutanásia dos animais.

Exposição ao material particulado 2,5 ($MP_{2,5}$) e Ar filtrado (AF)

O equipamento utilizado para expor os animais ao $MP_{2,5}$/ar filtrado foi o Concentrador de Partículas Finas Ambientais - CPA, instalado na Universidade de São Paulo (USP). Este equipamento foi criado e desenvolvido pelos cientistas da *Harvard School of Public Health*, a fim de concentrar em até 30x o

MP, equivalente a um dia inteiro de exposição a tal poluente em uma cidade com altos níveis de poluição (600 μg/m^3) (48,49). Os grupos experimentais foram expostos ao $MP_{2,5}$ ou ar filtrado durante a gestação ou lactação e após o desmame levados à Faculdade de Ciências Médicas da Universidade Estadual de Campinas (FCM-UNICAMP), para as análises descritas nessa seção. É importante ressaltar que as mães eram expostas por um tempo médio de 1 hora por dia.

Ingestão alimentar e peso corporal

Com 13 semanas foi acompanhada a ingestão alimentar da prole; os animais foram acondicionados individualmente durante uma semana e a ração pesada a cada 24 horas. O peso corporal de todos os animais foi mensurado ao nascer, ao desmame, na idade adulta (8 semanas de vida) e na eutanásia.

Mensuração do gasto energético

Com 19 semanas de idade foi determinado o volume de oxigênio (VO_2) e de dióxido de carbono (VCO_2), além da razão de troca respiratória (RER) por meio de calorimetria indireta (Oxymax Deluxe System; Columbus Instruments, Columbus, OH) de 48h. Antes das medidas serem executadas, os animais foram adaptados por um período de 24 horas.

Teste de Tolerância à Insulina (ITT)

O teste de tolerância à insulina (ITT) foi realizado nos animais com 13 semanas de idade, após jejum de 6 horas para avaliar a sensibilidade periférica à insulina no animal intacto. Foi administrada insulina regular (1,5 U/kg, intraperitoneal) e o sangue coletado pela veia caudal no tempo zero após 5, 10, 15, 20, 25 e 30 minutos da injeção. Ao final do experimento foram administradas 250μl de solução de glicose intraperitoneal, em uma concentração de 25%.

Teste de Tolerância à Glicose (GTT)

A prole adulta (13 semanas de idade) após jejum de 12 h, foi submetida ao teste de tolerância à glicose. Este teste consiste na injeção intraperitoneal

de glicose a 25% (dose= 1 g/kg de peso corporal dos animais) e então a glicemia obtida no sangue pela veia caudal no tempo zero e após 15, 30, 45, 60 e 120 minutos da injeção.

Extração de tecido para análise de expressão gênica por qPCR

Com aproximadamente 25 semanas de idade, após *overdose* de quetamina (240 mg/kg) e xilazina (30 mg/kg) por injeção intraperitoneal, os camundongos foram eutanasiados por decaptação. Então o hipotálamo e o tecido adiposo marrom foram rapidamente congelados em nitrogênio e em seguida armazenado a uma temperatura de -80°C até a análise de RNA. Assim como previamente descrito (Quaresma 2015, 2016). Para avaliar os níveis relativos de expressão gênica foram utilizados os seguintes insumos: RNeasy Mini Kit da Qiagen (cat#74106; Qiagen Inc, CA, USA), Nanodrop 2000, High Capacity cDNA Reverse Transcription Kit (cat#4368814, Applied Biosystem, CA, USA), TaqMan e Quant Studio 6 Flex Real-Time PCR System (#4485694) e Data Assist™ software Applied Biosystems, CA, USA). Os *primers* usados foram: Npy, Mm003048253_m1; Pomc, Mm00435874_m1; Agrp, Mm00475829_g1; Ucp1, Mm01244861_m1; Tnf, Mm00443258_m1; Tlr4, Mm00445273_m1; Hprt, Mm01545399_m1; Trh, Mm_009426.3.

Dosagens hormonais – ELISA

Após jejum de 12h os animais receberam uma overdose de uma mistura de cloridrato de cetamina (300 mg / kg) e cloridrato de xilazina (30 mg / kg) por meio de injeção intraperitoneal (IP). Após a confirmação do efeito da anestesia, os camundongos foram sacrificados. O sangue foi coletado, centrifugado e o soro armazenado a -80°C até a análise hormonal. Determinamos os níveis séricos de insulina e leptina (# EZRMI-13K, # EZML-82K, Millipore; Billerica, MA, EUA, respectivamente) por kits comerciais de ELISA.

Análise da Microbiota

As amostras de fezes foram coletadas, congeladas em nitrogênio líquido e armazenadas a -80°C até serem analisadas. O DNA genônico foi extraído usando o QIAamp DNA Stool Mini Kit (Qiagen, Alemanha). Para cada amostra,

a V3–V4 hiper-variável da região 16S rRNA do gene da bactéria foi amplificado, seguido do sequenciamento metagenômico Illumina 16S (Illumina Technical Note 15044223). A composição taxonômica das comunidades bacterianas foram obtidas analisando as regiões V3 e V4 do 16S rRNA gene usando a plataforma Illumina® MiSeq. As construções das bibliotecas de sequenciamento de DNA foram realizadas de acordo com as instruções (Illumina, San Diego, CA, EUA) e seguidos os mesmos protocolos descritos por by Caporaso et al. (2012) (Yatsunenko et al., 2012). Usando as 300 bp leituras pareadas e os reagents MiSeq v3, os finais de cada leitura foram sobrepostos para gerar leituras de alta qualidade das regiões V3 e V4. Mais de 100 mil leituras são geradas por amostra, comumente reconhecidas como suficientes para pesquisas metagenômicas. As sequências fastq foram analisadas usando o Illumina 16S Metagenomics software, que classifica taxonomicamente as regiões v3/v4 do gene 16S rRNA usando o banco de dados DADA2 (Illumina, 2014; Alishum, 2019).

Análise estatística de resultados

Os valores estão expressos como média ± erro padrão da média (SEM). Os dados foram avaliados por análise de variância One ou Two-way ANOVA seguido por teste de significância (Bonferroni test) ou test T não pareado (two tailed) dependendo das comparações realizadas entre os grupos experimentais. Erro α menor do que 5% foi aceito para rejeitar a hipótese de nulidade. Além disso, realizamos análise de abundância pareada de dados usando o software IBM SPSS® 20.0 (Wilcoxon Signed Ranks Test) para a análise da microbiota intestinal. A análise estatística da diversidade alfa e beta foi realizada por meio do software EZbioCloud (Yoon et al., 2017). Os gráficos foram gerados pelo GraphPad Prism 7.0. Software (San Diego, CA, EUA). $P < 0,05$ foi considerado estatisticamente significativo.

RESULTADOS

Maternal exposure to air pollution programs energy balance and the gut microbiota in a timing and gender-specific manner

Maternal exposure to $PM_{2.5}$ programs metabolism and microbiota

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Keywords: $PM_{2.5}$, metabolic programming, air pollution, obesity

Abstract

We aimed to determine the most critical period to maternal exposure to particulate matter ($PM_{2.5}$) to impair offspring energy metabolism and whether the gut microbiota could contribute to the phenotype. Unexposed female C57BL/6J mice were mated, and the exposure to $PM_{2.5}$ or filtered air (FA) occurred only in pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). We studied the offspring from both genders. We observed that pregnancy exposure to $PM_{2.5}$ increased body mass from birth to adulthood, food intake, leptin levels, and impaired glucose tolerance in male offspring. In female offspring, pregnancy exposure to $PM_{2.5}$ increased adulthood body mass, food intake, insulin levels without altering glucose tolerance and insulin sensitivity. Surprisingly, lactation exposure to $PM_{2.5}$ increased male body mass at weaning but not in adulthood. This result was associated with increased O_2 consumption in male offspring. In female offspring, lactation exposure to $PM_{2.5}$ decreased fasting blood glucose levels and improved insulin sensitivity. NPY and AGRP expression levels were increased in male offspring from the group FA/$PM_{2.5}$, and in females, we observed an increase in NPY of the $PM_{2.5}$/FA group. To determine if maternal exposure to $PM_{2.5}$ could affect the offspring's gut microbiota, we analyzed alpha and beta -diversity, the Linear Discriminant Analysis (LDA), and Effect Size (LEfSe). Pregnancy exposure was associated with decreased alpha diversity in the gut microbiota. We observed a spatial separation among the three groups, demonstrating that exposure to $PM_{2.5}$ changed the microbiota composition, as indicated by principal coordinate analysis (PCoA) of Bray-Curtis distances among samples of each group. Compared with FA/FA, the genus *Akkermansia* was increased in the male and female offspring of the $PM_{2.5}$/FA group; *Oscillibacter* was reduced in the male offspring of $PM_{2.5}$/FA; *Allistipes* was increased in the FA/$PM_{2.5}$ of the female offspring. In conclusion, our data suggest that maternal exposure to $PM_{2.5}$ in pregnancy negatively affects the energy metabolism of males; during lactation, we observed a slight improvement in insulin sensitivity in female offspring and not in male offspring. Also we can conclude that maternal air pollution exposure provokes negative effects on the offspring gut microbiota.

Introduction

Obesity reaches a very high prevalence worldwide, and it is associated with several chronic metabolic diseases, including obesity and type 2 diabetes (T2DM) (Srinivasan and Patel, 2008; Symonds *et al*., 2009; Fernandez-twinn and Ozanne, 2010; Ozanne, 2014; Vogt *et al*., 2014; Chang *et al*., 2019). The etiology of obesity implicates multiple factors involving environmental changes, genetic and epigenetic modification (Conway and Rene, 2004; Kaushik and Anderson, 2016).

Clinical, epidemiological, and experimental studies consistently demonstrate that abnormal maternal caloric intake or environment disturbance during the perinatal period favor the development of cardiometabolic diseases in the offspring (Waterland and Garza, 1999; Smith and Ryckman, 2015). The mechanism by which altered environment during the perinatal period induces obesity is linked, at least in part, to impairment in neurogenesis, synaptogenesis, and myelination of several brain structures linked to the control of energy metabolism (Bouret, Draper and Simerly, 2004; Plagemann, 2006; Sullivan and Grove, 2009; Vogt *et al*., 2014; Lippert *et al*., 2020).

The hypothalamus has been considered a pacemaker integrating peripheral signals with neural circuitry to keep energy homeostasis through the control of food intake and energy expenditure (EE) (Timper and Brüning, 2017; Seong *et al*., 2019). The arcuate nucleus of the hypothalamus (ARC) displays several neurons, which those expressing pro-opiomelanocortin (POMC) or the Agouti-related peptide (AgRP) co-localized with neuropeptide Y (NPY) are the most recognized and studied (Cowley *et al*., 1999; Timper and Brüning, 2017). Fasting activates the orexigenic AgRP and NPY neurons inducing food intake (Barsh, Farooqi and Rahilly, 2000). In contrast, feeding activates POMC neurons, which project to second-order neurons located in the paraventricular nucleus of the hypothalamus (PVH), increasing other mediators such as thyrotropin-releasing hormone (TRH), which suppress feeding while increasing energy expenditure (Cowley *et al*., 1999; Fekete *et al*., 2000; Kim *et al*., 2000). A failure during POMC and AgRP neurocircuitry development could predispose the offspring to obesity and its comorbidities through life (Steculorum and Vogt, 2013; De Paula Simino *et al*., 2017). Changes in the maternal diet composition, such as an increased high-saturated fat diet, cause obesity, glucose intolerance (Vogt *et al.*, 2014) and elicits preference for sucrose (Lippert *et al*., 2020).

Not only maternal nutrients are able to change the offspring's metabolic phenotype. Maternal smoking, even at a low-intensity frequency during pregnancy, is associated with reduced fetal growth and an increased risk of preterm birth (Abraham *et al*., 2017; Liu *et al*., 2020). In a recent study, Peixoto *et al*. (2021) demonstrated that maternal tobacco consumption during lactation affected dopaminergic circuitry, increasing the risk of hyperphagia through adulthood in the female offspring of rats.

Exposure to some fractions of air pollution known as particulate matter with a diameter ≤ of 2.5 μm ($PM_{2.5}$) is associated with obesity and T2DM (Brook *et al*., 2008; Liu *et al*., 2013; Weinmayr *et al*., 2015). The ability of $PM_{2.5}$ to induce metabolic diseases is based, mainly, on inflammatory responses induced by its chemical composition, which can also include lipopolysaccharides (LPS) (Krewski and Rainham, 2007; Block and Calderón-garcidueñas, 2010; Rajagopalan and Brook, 2012; Franklin, Brook and Iii, 2015; Yoda, Tamura and Shima, 2017). LPS is a potent agonist of TLR4, and it is linked to the etiopathology of obesity and T2DM (Conde *et al*., 2014; Yoda, Tamura and Shima, 2017). Recently we showed that short-term exposure (five days) to $PM_{2.5}$ was sufficient to increase the expression of inflammatory markers as Tlr4, Ikbke, TNF alpha in the hypothalamus causing leptin resistance and obesity at long-term of $PM_{2.5}$-exposure (Campolim *et al*., 2020).

Due to lower diameter, $PM_{2.5}$ can spread into tissues through lungs and olfactory epithelium (Block and Caldero, 2009; Milojevic *et al*., 2014; Perera, 2017; Landrigan *et al*., 2018). A portion of $PM_{2.5}$ can also be transported to the oropharynx and ingested, reaching the gastrointestinal tract where altered gut permeability and local microbiota (Semmler-Behnke *et al*., 2007; Beamish, Osornio-Vargas and Wine, 2011; Mutlu *et al*., 2019). Changes in the gut morphophysiology and microbiota facilitate inflammatory responses and might be related to causing metabolic diseases such as obesity and T2DM (Patterson *et al*., 2016; Saad, Santos and Prada, 2016; Fan and Pedersen, 2021).

Evidence suggested that perinatal exposure to $PM_{2.5}$ might alter the energy metabolism of the offspring (Lee *et al*., 2011; Guxens *et al*., 2014; Fleisch *et al*., 2015, 2016, 2017; Hsu *et al*., 2015; Lakshmanan *et al*., 2015; Chen *et al*., 2017; Chen, Liang, *et al*., 2017b; Xu *et al*., 2019). However, due to different approaches and protocols of $PM_{2.5}$ exposures, it is challenging to compare these studies and conclude. For instance, some studies employed intratracheal instillation of liquid diesel exhaust $PM_{2.5}$ and saline as control (Liu *et al*., 2016; Chen, Liang, *et al*., 2017a). In contrast, other studies employ the exposures via concentrated ambient chambers using $PM_{2.5}$ or filtered air

captured from the atmosphere (Veras *et al*., 2008; Chen *et al*., 2017; Xu *et al*., 2019). Besides, the timing points elected to maternal exposures are considerably distinctive between studies, including preconception exposures in some of them (Chen *et al*., 2017; Chen, Liang, *et al*., 2017b; Xu *et al*., 2019), which lead to maternal metabolic and inflammatory responses before conception. Considering that the time point of maternal $PM_{2.5}$ exposure is crucial to determine the impact in the metabolic outcomes, specifically on the regulation of feeding and energy expenditure, and to tease apart the effect of preconception maternal exposure to $PM_{2.5}$ in the present study, we exposed mothers to $PM_{2.5}$ only during pregnancy or lactation. We had a control group, which received filtered air at the same periods.

Therefore, we aimed to investigate male and female offspring under chow diet whether different time points of maternal exposure to $PM_{2.5}$ (pregnancy or lactation) alter: 1) body mass, food intake, and energy expenditure; 2) glucose and insulin metabolism; 3) hypothalamic neuropeptides and pro-inflammatory markers involved in the regulation of energy balance; 4) the gut microbiota diversity and composition.

Materials and Methods

Ethical committee approval

All experiments, animal handling, and breeding were performed following the National Institute of Health guidelines for experimental animals' use and the approval of the Care of Animals and Ethical Committee for Animal Research of the State University of Campinas (CEUA Protocol 4627-1).

Animals

Eight-week-old male and female C57BL/6J mice were provided by the multidisciplinary center for biological research from the State University of Campinas, SP, Brazil (CEUA Protocol 4627-1). Mice stayed in the animal facility with constant light/dark cycle (12 h/12 h), room temperature (22°C), humidity, and a high-efficiency particulate air filter (HEPA) receiving a standard rodent chow (3.39 kcal/g; Nuvilab CR-1, Nuvital Quimtia, Brazil) and water *ad libitum*.

After one week of acclimation, we set up breeding cages with two females and one male. Usually, three days were sufficient to initiate the pregnancy of at least one female. On the first day of pregnancy, females were randomly divided into three experimental groups:

1. Mothers exposed to $PM_{2.5}$ during pregnancy and FA during lactation ($PM_{2.5}$/FA)
2. Mothers exposed to FA during pregnancy and $PM_{2.5}$ during lactation (FA/$PM_{2.5}$)
3. Mothers exposed to FA during pregnancy and lactation (FA/FA)

The offspring only received FA from birth to euthanasia. At birth, animals were weighed, and the number of puppies per mother was normalized (n=6-8). At weaning (21 days of age), female and male offspring were separated in plastic cages, receiving only a standard diet and water *ad libitum*. Food intake and body weight were measured weekly. The offspring were not exposed to $PM_{2.5}$. Almost all data were obtained from adulthood mice.

Exposure to $PM_{2.5}$ or Filtered Air (FA)

The exposures were performed using the equipment ambient particle concentrator located at the University of Sao Paulo (USP) in São Paulo, Brazil. We followed the same protocol used in several previous studies (Andrade *et al*., 2012;

Yoshizaki *et al*., 2016; Barros *et al*., 2019; Campolim *et al*., 2020). In summary, the equipment separates fine particles according to aerodynamic sizes, concentrates them from ambient air, and has two chambers, one filled with $PM_{2.5}$ and another filled with FA. Female mice were placed according to the groups. We adjusted the dose of $PM_{2.5}$ as described previously (Campolim *et al*., 2020) following the EPA's current methodology (https://www.epa.gov/node/81739/view) equivalent to 600 μg/m^3 in 24 hours. $PM_{2.5}$ composition has been characterized along the course of the experiments consisting of black carbons, polycyclic aromatic hydrocarbon, and metal trace elements such as Na, Al, Si, P, S, K, Ca, Ti, V, Fe, Ni, Cu, Zn, Pb (Mauad *et al*., 2008; Andrade *et al*., 2012; Miranda *et al*., 2012; Yoshizaki *et al*., 2016; Barros *et al*., 2019). Female mice returned immediately to their home cages after each exposure.

Energy Expenditure

After acclimation, we placed random fed male or female offspring in CLAMS (Oxymax Deluxe System; Columbus Instruments, Columbus, OH, USA) to measure oxygen (O_2) consumption, carbon dioxide (CO_2), and respiratory exchange ratio (RER).

Glucose tolerance test (GTT)

Overnight fasted mice received an intraperitoneal (IP) injection of glucose (1 g/kg). Blood samples were collected from the tail, and blood glucose was measured by glucometer immediately before IP injection and after 15, 30, 45, 60, 90, and 120 minutes.

Insulin tolerance test (ITT)

Six hours fasted mice (8 a.m. to 2 p.m.) received an intraperitoneal (IP) injection of insulin (1 UI/kg in males) (0.8 UI/kg in females). Blood samples were collected from the tail, and blood glucose was measured by glucometer immediately before IP injection and after 5, 10, 15, 20, 25, and 30 minutes.

Hormones Measurements

Overnight fasted mice received an overdose of a mixture of ketamine hydrochloride (300 mg/kg) and xylazine hydrochloride (30 mg/kg) via intraperitoneal (IP) injection. After the confirmation of the effect of anesthesia, mice were euthanized. Blood was collected, centrifuged and the serum was stored at -80°C until hormone

analysis. We determined serum insulin and leptin (#EZRMI-13K, #EZML-82K, Millipore; Billerica, MA, USA, respectively) levels by commercial ELISA kits.

Tissue collection for mRNA and gene expression by qPCR analysis.

Mice were euthanized by decapitation, after an overdose of ketamine hydrochloride (300 mg/kg) and xylazine hydrochloride (30 mg/kg) via IP injection. BAT and the hypothalamus were quickly frozen in N2 and stored at -80°C until RNA analysis. As previously described (Quaresma *et al*., 2015, 2016), RNeasy Mini Kit from Qiagen (cat#74106; Qiagen Inc, CA, USA), Nanodrop 2000, High Capacity cDNA Reverse Transcription Kit (cat#4368814, Applied Biosystems, CA, USA), TaqMan and QuantStudio 6 Flex Real-Time PCR System (#4485694) and Data Assist™ software Applied Biosystems, CA, USA) were used to get the relative expression levels of the genes. Used primers were: Npy, Mm003048253_m1; Agrp, Mm00475829_g1; Pomc, Mm00435874_m1; Trh, Mm_009426.3; Ucp1, Mm01244861_m1; Tnf, Mm00443258_m1; Tlr4, Mm00445273_m1; Hprt, Mm01545399_m1.

Microbiota analysis

Fecal samples were collected, frozen in liquid nitrogen, and stored at –80°C until use. The genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). For each sample, the V3–V4 hyper-variable region of the bacterial 16S rRNA gene was amplified, followed by the Illumina 16S Metagenomic Sequencing Library Preparation guide (Illumina Technical Note 15044223, no date). The taxonomic composition of the bacterial communities was obtained by analyzing the V3 E V4 region of the 16S rRNA gene using the Illumina® MiSeq platform. The constructions of the DNA sequencing libraries were performed according to the manufacturer's instructions (Illumina, San Diego, CA, USA) and followed the same flow described by Caporaso *et al*. (2012) (Yatsunenko *et al*., 2012). Using 300 bp paired readings and MiSeq v3 reagents, the ends of each reading are overlaid to generate complete high-quality readings from the V3 and V4 regions. More than 100,000 readings are generated per sample, commonly recognized as sufficient for metagenomic research. The fastq sequences were analyzed using the Illumina 16S Metagenomics software, which performs the taxonomic classification of the v3 / v4 region of the 16S rRNA gene using the DADA2 database (Illumina, 2014; Alishum, 2019).

Statistical analysis

All statistical analysis and number of variables were stated in the Figure Legends. For metabolic studies, we expressed data as mean ± standard error of the mean (SEM). We applied the Unpaired t-test two-tailed or One or Two Way ANOVA with Bonferroni post hoc test Bonferroni as statistical analysis using GraphPad Prism 7.0. Software (San Diego, CA, USA). Besides, we performed data paired abundance analysis using the IBM SPSS® 20.0 software (Wilcoxon Signed Ranks Test) for the gut microbiota analysis. The statistical analysis of alpha and beta diversity was performed using the EZbioCloud software (Yoon *et al*., 2017). The graphs were generated by GraphPad Prism 7.0. Software (San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

Maternal exposure to $PM_{2.5}$ increases the birth and weaned weight of male but not female offspring.

Maternal exposure to $PM_{2.5}$ during pregnancy was sufficient to increase the birth weight (g) of the offspring (mean ± SEM: FA/FA: 1.24 g ± 0.03, n=6; $PM_{2.5}$/FA: 1.39 g ± 0.04, n=17; Unpaired t-test two-tailed: p=0.0472). At this time point, we did not separate male and female offspring. Upon weaning, we separated males and females and included one more group of study in which the maternal exposure to $PM_{2.5}$ occurred only during lactation (referred to as FA/$PM_{2.5}$).

Maternal exposure to $PM_{2.5}$ reprograms the energy metabolism of adult male offspring

At weaning, we observed an increased body weight in male offspring of mothers exposed to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$) compared to the control group (FA/FA) (Fig. 1 A). Adult male offspring at eight weeks old from mothers exposed to $PM_{2.5}$/FA displayed a significant increase in body mass than male offspring from both groups, FA/FA- and FA/$PM_{2.5}$-exposures (Fig. 1 B). To get insight into the mechanism by which we observed increased body weight, we measured food intake during seven days and observed an increased food intake at the fifth day of measure for the group $PM_{2.5}$/FA compared to the group FA/FA. This difference persisted during the sixth and seventh days of measurement. On the sixth and seventh day, male offspring from the $PM_{2.5}$/FA group also ate more than group FA/$PM_{2.5}$ (Fig. 1 C). The AUC of food intake suggested an increased food intake in the group $PM_{2.5}$/FA compared to the FA/FA group (Fig. 1 D). Interestingly, O_2 consumption risen only in the group from mothers exposed to FA/$PM_{2.5}$ than the FA/FA group while there was no change in the group $PM_{2.5}$/FA compared to FA/FA (Fig. 1 E). CO_2 production was similar among groups (Fig. 1 F). $PM_{2.5}$/FA and FA/$PM_{2.5}$ displayed decreased RER compared to the group from mothers exposed to FA/FA (Fig. 1 G). We observed higher UCP1 expression in the $PM_{2.5}$/FA and FA/$PM_{2.5}$groups compared to FA/FA and lower UCP1 expression in the FA/$PM_{2.5}$than $PM_{2.5}$/FA in the brown adipose tissue (BAT) of male offspring (Fig. 1 H).. We also evaluated fasting serum leptin levels and observed a higher value in $PM_{2.5}$/FA when compared to FA/$PM_{2.5}$ and FA/FA (Fig. 1 I). To assess the impact of maternal exposure to $PM_{2.5}$ on glucose metabolism and insulin resistance,

we measured fasting blood glucose and serum insulin levels and performed GTT and ITT. Fasting blood insulin and glucose levels were similar among the three groups (Fig. 1 J and K). No changes in insulin sensitivity was observed during ITT (Fig. L). In contrast, we observed glucose intolerance in male offspring from the group $PM_{2.5}$/FA compared to FA/FA. Blood glucose obtained at 15, 30, and 45 minutes after glucose injection was elevated in $PM_{2.5}$/FA compared to FA/FA. Surprisingly, the group FA/$PM_{2.5}$ kept significantly lower blood glucose from 15 minutes up to 120 minutes compared to the $PM_{2.5}$/FA; however, only at 30 minutes, the FA/$PM_{2.5}$ group had significantly lower blood glucose values than the FA/FA control group (Fig. 1 M). The AUC revealed a significant increase of AUC for the $PM_{2.5}$/FA compared to the FA/$PM_{2.5}$ group (Fig. 1 N).

Maternal $PM_{2.5}$ exposure reprograms the energy metabolism of adult female offspring.

Body mass at weaning was similar among the three groups of female offspring (Fig. 2 A). In contrast, we observed increased body mass of female offspring at eight weeks old from mothers exposed to $PM_{2.5}$/FA compared to the other two groups (Fig. 2 B). Female offspring from the $PM_{2.5}$/FA group demonstrated increased food intake compared to the other two groups, particularly in the beginning of feeding determination. This result did not persist along the seven days of measurements. We also observed increased food intake in the FA/$PM_{2.5}$ compared to FA/FA on the second day of evaluation (Fig. 2 C). The AUC of FI suggested an increased food intake for the groups $PM_{2.5}$/FA and FA/$PM_{2.5}$ compared to the FA/FA group (Fig. 2 D). O_2 consumption risen only in the group FA/$PM_{2.5}$ compared to the FA/FA group, while there was no change in O_2 consumption in the group $PM_{2.5}$/FA (Fig. 2 E). CO_2 production was similar among groups (Fig. 2 F). Regardless of the same CO_2 production for the three groups, decreased RER in the group FA/$PM_{2.5}$ was seen compared to the group FA/FA (Fig. 2 G). Surprisingly, we observed a reduction in UCP1 in BAT in the group FA/$PM_{2.5}$ compared to the two other groups (Fig. 2 H). No differences were observed in fasting serum leptin levels among groups (Fig. 2 I). To evaluate the impact of different timing points of maternal exposure to $PM_{2.5}$ on glucose metabolism and insulin resistance of the offspring, we measured fasting serum insulin and blood glucose levels and performed GTT and ITT. Fasting serum insulin levels were increased in group FA/$PM_{2.5}$ compared to FA/FA (Fig. 2 J). Unexpected, there was a slight decrease

in fasting blood glucose levels and a mild increase in insulin sensitivity (ITT) in the group FA/$PM_{2.5}$ compared to FA/FA group (Fig. 2 K) and the $PM_{2.5}$/FA and FA/FA groups (Fig. 2 L). During the ITT, we observed a decrease in blood glucose in the group FA/$PM_{2.5}$ after 5 minutes of insulin injection, which persisted up to 10 minutes. The AUC of ITT did not detect blood glucose drop in the FA/$PM_{2.5}$ group observed from 5 to 10 minutes (Fig. 2 M). Although we observed lower blood glucose in the basal time point during the GTT in the group FA/$PM_{2.5}$, this group displayed higher blood glucose at 15 and 30 minutes of GTT, suggesting a mild glucose intolerance despite lower levels of basal glycemia (Fig. 2 M). The AUC of GTT did not detect the slight fluctuations in blood glucose in the FA/$PM_{2.5}$ group, as there was no statistical difference in the AUC between the three groups (Fig. 2 N).

Maternal exposure to $PM_{2.5}$ alters offspring's hypothalamic neuropeptides involved in the regulation of energy balance.

We observed an increase in the % of NPY gene expression in the hypothalamus of male offspring from mothers exposed to $PM_{2.5}$ during lactation (FA/$PM_{2.5}$) compared to the FA/FA and $PM_{2.5}$/FA group. An increase in the % of AgRP gene expression was observed in the hypothalamus of FA/$PM_{2.5}$ compared to the $PM_{2.5}$/FA group. We did not find differences in the % of POMC and TRH gene expression in the hypothalamus among the three groups of male offspring (Fig. 3 A). Regarding the female offspring, the % of NPY gene expression showed an increase only in the hypothalamus of female offspring from mothers exposed to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) compared to the FA/$PM_{2.5}$ group. We did not find differences in the % of other neuropeptides (AgRP, POMC, and TRH) gene expression in the hypothalamus among three groups of female offspring (Fig. 3 B).

Maternal exposure to $PM_{2.5}$ does not alter TLR4 and TNFα expression in the offspring's hypothalami.

We did not observe significant differences in the % of TLR4 and TNFα gene expression in the hypothalamus of male or female offspring (Fig. 3 C and D).

Maternal exposure to $PM_{2.5}$ reprograms the gut microbiota of adult male offspring.

To investigate whether air pollution affects male offspring's gut microbiota, we first analyzed the alpha-diversity (α-diversity), estimated by Simpson and Shannon indexes. The α-diversity was reduced in the group $PM_{2.5}$/FA compared to FA/FA, using both indexes (Simpson: (p=0.0089); Shannon: (p=0.0185) (Fig. 4 A and B). No differences in the α-diversity were observed between the FA/FA and FA/$PM_{2.5}$ by Shannon index. However, the Simpson index suggested a reduced (p=0.0110) α-diversity in the group $PM_{2.5}$/FA than the FA/$PM_{2.5}$ group (Fig. 4 B).

The Linear Discriminant Analysis (LDA) and Effect Size (LEfSe) were used to characterize the differences between biological conditions. When compared the FA/FA with the $PM_{2.5}$/FA group, the *Emergencia*, *Clostridium*, *Frisingicoccus*, *Eubacterium*, *Oscillibacter*, and *Alloprevotella* genera were increased in FA/FA group. In contrast, the *Akkermansia* genera were raised in the $PM_{2.5}$/FA group (Fig. 4 C). On the other hand, when comparing the FA/FA and FA/$PM_{2.5}$ groups, only the genus *Clostridiales* was increased in the FA/$PM_{2.5}$ group (Fig. 4 D). When compared the $PM_{2.5}$/FA and FA/$PM_{2.5}$ groups, the genera *Akkermansia* was raised in the $PM_{2.5}$/FA group, while the *Oscillibacter* was increased in FA/$PM_{2.5}$ group (Fig. 4 E).

The Bray–Curtis dissimilarity showed that FA/FA displayed a spatial separation with $PM_{2.5}$/FA but not FA/$PM_{2.5}$ (PERMANOVA: p=0.007 and p=0.46, respectively). $PM_{2.5}$/FA showed a spatial separation with FA/$PM_{2.5}$ (PERMANOVA: p=0.004) (Fig. 5 A-C).

Maternal exposure to $PM_{2.5}$ reprograms the gut microbiota of adult female offspring.

The α-diversity was reduced in the group $PM_{2.5}$/FA compared to FA/FA, using both indexes Simpson (p=0.0025) and Shannon (p=0.0199) (Fig. 6 A and B). No differences in the α-diversity were observed between FA/FA and FA/$PM_{2.5}$ using the Shannon index. However, the Simpson index suggested a reduced (p=0.0025) α-diversity in the group $PM_{2.5}$/FA than the FA/$PM_{2.5}$ group (Fig. 6 A and B).

The LDA and LEfSe revealed that the group $PM_{2.5}$/FA displayed increased *Verrucomicrobia*, *Olsenella*, *Akkermansia* and *Alloprevotella* compared to the FA/FA group (Fig. 6 C). In contrast, *Muribaculum*, *Alistipes*, *Clostridium*, and *Turibacter* genera were increased in FA/$PM_{2.5}$ group, while *Odoribacter* was increased in the FA/FA group (Fig. 6 D). When compared $PM_{2.5}$/FA and FA/$PM_{2.5}$ groups, the genera *Akkermansia*, *Eubacterium*, and *Odoribacter* were increased in the FA/$PM_{2.5}$ group.

Turibacter, *Ruminococcus*, *Muribaculum*, *Clostridium*, and *Alistipes* genera were increased in the $PM_{2.5}$/FA group (Fig. 6 E).

Regarding the Bray–Curtis dissimilarity, we observed a spatial separation between the FA/FA group with the other two groups (PERMANOVA: p=0.001 and p=0.030, respectively). $PM_{2.5}$/FA showed a spatial separation with FA/$PM_{2.5}$ (PERMANOVA: p=0.002) (Fig. 7 A-C).

Discussion

The present study demonstrated that maternal exposure to air pollution affects the offspring's energy metabolism, and this effect is dependent on the timing point of exposure and the offspring's gender. We also observed that maternal exposure to $PM_{2.5}$ reduces the gut microbiota diversity and changes the composition independently of the offspring's gender. Our data support the hypothesis that exposure to air pollution during pregnancy is more harmful to metabolism than exposure during lactation. Male offspring had a more pronounced unfavorable metabolic phenotype than female offspring.

According to the Developmental Origins of Health and Disease (DOHaD)'s postulation, birth weight is a valuable parameter to determine intrauterine growth (Barker, 2007; Mandy and Nyirenda, 2018). Our study demonstrated that maternal exposure to $PM_{2.5}$ during pregnancy increased male offspring body mass at birth, which persisted during weaning up to adulthood. This result was unexpected because some studies that exposed mothers to $PM_{2.5}$ during pregnancy consistently found lower or unchanged birth weight for their offspring (Chen *et al*., 2017; Chen, Liang, *et al*., 2017b; Xu *et al*., 2019). Even though Chen *et al*. (2017) and Xu *et al*. (2019) observed low birth weight in male offspring from mothers exposed to $PM_{2.5}$ prenatal, these mice evolved with increased body mass in adulthood (Chen *et al*., 2017; Xu *et al*., 2019). It is essential to mention that the most harmful period detected in all these studies was the preconception maternal exposure but pregnancy (Xu *et al*., 2019). The difference between our results might be due to different time points of maternal exposure to $PM_{2.5}$. All three studies (Chen *et al*., 2017; Chen, Liang, *et al*., 2017b; Xu *et al*., 2019) started maternal exposure seven weeks before pregnancy and ended with the offspring's weaning. Xu *et al*. (2019) also exposed mothers to $PM_{2.5}$ during pregnancy and lactation, but no result came out. Notably, inconsistency among studies may rely on the route of administration of the $PM_{2.5}$ and the source of $PM_{2.5}$. Some studies employed the intratracheal liquid instillation (Chen, Liang, *et al*., 2017b). Other studies (Chen *et al*., 2017; Xu *et al*., 2019) and our study employed concentrated $PM_{2.5}$ into specialized chambers and different sources of pollutants. Together, these data suggest that the timing, route, and composition of pollutants are essential for building the offspring's phenotype.

We observed a high body mass in males offspring from mothers exposed during pregnancy and lactation. Nonetheless, only the group $PM_{2.5}$/FA kept this high body mass at adulthood. Male offspring from mothers exposed during lactation displayed similar body mass as FA/FA in adulthood. This discrepant difference may be due explained by differences in feeding and energy expenditure outcomes seen in $PM_{2.5}$/FA and FA/$PM_{2.5}$ groups. On the one hand, male $PM_{2.5}$/FA displayed high food intake, which accounts for increased body mass. On the other hand, male FA/$PM_{2.5}$ showed high O_2 consumption and low RER, despite no changes, which may be account for decreased body mass compared to the $PM_{2.5}$/FA group. These data suggest that maternal exposure to $PM_{2.5}$ during pregnancy-induced sustained weight gain from birth until adulthood in male offspring. Maternal exposure to $PM_{2.5}$ during lactation somehow protected the offspring from gaining as much weight as males from the $PM_{2.5}$/FA group. The energy homeostasis adjustment may be linked to energy expenditure and not feeding regulation.

Nutrition in early stages of growth, like breastfeeding, and the beneficial effects of this on obesity diabetes are widely studied and strongly support the concept of metabolic programming (Schack-Nielsen and Michaelsen, 2006; Saad, Santos and Prada, 2016; Badillo-Suárez, Rodríguez-Cruz and Nieves-Morales, 2017). Vieira Borba, Sharif and Shoenfeld (2018) reviewed the protector factor of breastfeeding in human health, pointing to the importance of lactation reducing the risk of type 2 diabetes and obesity in the long term and its absence being a significant risk factor. Considering these discussed studies above and our work, we hypothesize that breast milk protected the offspring from maternal exposure to $PM_{2.5}$ during lactation.

The development of hypothalamic neurocircuits consists of neurogenesis, migration, differentiation neural, and the establishment of projections and synaptic connection (Plagemann, 2006; Bouret, 2009). In humans, these steps occur in the uterus. However, in rodents, neuronal projections happen after birth until the third week of life, equivalent to the lactation period (Koutcherov, Mai and Paxinos, 2003; Bouret, Draper and Simerly, 2004; Grayson *et al*., 2006; Bouret, 2009). Studies in rodents showed that a high saturated fat diet during lactation rather than pregnancy predisposed the offspring to obesity, hyperinsulinemia, and glucose intolerance (Vogt and Bru, 2013). The mentioned outcomes were associated with impaired POMC and AgRP neuron projections from the arcuate nucleus to the paraventricular nucleus of the hypothalamus (Vogt and Bru, 2013). More recently, Lippert *et al*. (2020) demonstrated that maternal

exposure to HFD during lactation revealed sexually dimorphic expression of dopamine phenotypes, males showing hyperlocomotion, and females high preference for sucrose (Lippert *et al*., 2020). Another study exhibited that maternal HFD exposure during pregnancy and lactation predispose the offspring to develop insulin resistance and obesity by activating inflammatory pathways (Costa *et al*., 2020). In contrast, maternal undernutrition causes epigenetic alterations in the hypothalamus leading to postnatal hyperphagia, increasing the risk of the offspring developing metabolic diseases (Block and El-osta, 2017). In our study, the female offspring from the FA/$PM_{2.5}$ group showed signals of metabolic improvement, at least in part, due to a decrease in fasting glycemia and a slight improvement in insulin sensitivity. Male offspring from the FA/$PM_{2.5}$ group, despite displaying elevated body weight at weaning, at eight weeks of age, the body weight was similar to the FA/FA group. In contrast, we observed an improvement in EE and no impairment in insulin sensitivity nor glucose tolerance compared to the FA/FA group. Taken together, data on maternal exposure to HFD and $PM_{2.5}$ suggest that maternal exposure to high-fat feeding during lactation might be more harmful to the energy metabolism than what we observed with $PM_{2.5}$-exposure.

Several studies found metabolic unchanged in female but male offspring whose mothers were exposed to $PM_{2.5}$ during the perinatal period, suggesting that this gender somehow has milder effects (Chen *et al*., 2017; Xu *et al*., 2019). Our data indicated that female offspring from maternal exposure to FA/$PM_{2.5}$ had a slight negative impact on metabolism compared to males. Adulthood female offspring from the $PM_{2.5}$/FA group displayed increased body mass and food intake, but the O_2 consumption was unexpectedly high, potentially maintaining energy homeostasis as a compensatory mechanism. Adulthood female offspring from the $PM_{2.5}$/FA group demonstrated higher fasting insulin levels; however, they displayed equivalent insulin sensitivity and glucose tolerance as the group FA/FA. Unpredictable, the FA/$PM_{2.5}$ group showed lower fasting blood glucose compared to FA/FA group. In addition, we observed a slight improvement in insulin sensitivity in this group of females. That was not the case for male offspring. Male offspring from the $PM_{2.5}$/FA group did not display elevated fasting insulin or blood glucose levels nor insulin resistance measured by ITT. However, this group had pronounced glucose intolerance measured by GTT.

Evidence demonstrated that exposure to air pollution affects gastrointestinal health (Kim *et al*., 2014; Mutlu *et al*., 2019); however, the results are conflicting. Kim *et al*. (2014) showed that exposure to air pollution is involved with reduced biodiversity

in the gut microbiota. In contrast, Mutlu *et al*., (2019) suggested that $PM_{2.5}$ exposure leads to increased gut microbiota alpha diversity. Also, several studies (Liu *et al*., 2019; Tanya *et al*., 2019; Bailey *et al*., 2020) highlighted the influence of air pollution exposure in the development of obesity and T2DM through gut microbiota. Our study observed that maternal $PM_{2.5}$ exposure displayed altered offspring gut microbiota composition, characterized by changes in bacterial diversity and different amounts of some taxon. Similar to other studies (Kish *et al*., 2013; Wang *et al*., 2018; Bailey *et al*., 2020), our results showed that male and female offspring $PM_{2.5}$/FA group displayed decreased alpha diversity (Shannon or Simpson index) compared to the FA/FA and to the FA/$PM_{2.5}$ groups.

In contrast, Liu *et al*. (2020) described an increase in alpha diversity associated with maternal $PM_{2.5}$ exposure during the gestational period. In the same study, despite an increase in alpha diversity, beta diversity analysis revealed an apparent dissociation between the control and $PM_{2.5}$ exposed group (Liu *et al*., 2020). In our study, we also observed an evident dissociation among the three groups. The abundance of some taxon was not similar among the three groups. Female $PM_{2.5}$/FA offspring showed lower proportions of *Bacteroidetes* phylum and higher proportions of *Verrucomicrobia* when compared to female FA/FA or FA/$PM_{2.5}$. Similar to female, male $PM_{2.5}$/FA offspring showed higher proportions of *Verrucomicrobia* phylum but lower proportions of *Firmicutes* phylum when compared to male FA/FA or FA/$PM_{2.5}$. Unexpectedly, in our study, larger amounts of *Akkermansia* (*Verrucomicrobia* phylum) were observed in the male and female offspring from the $PM_{2.5}$/FA group compared to FA/FA group. *Akkermansia* has been associated with improved glucose homeostasis and weight loss (Saad, Santos and Prada, 2016; Cani and de Vos, 2017; Debédat, Clément and Aron-Wisnewsky, 2019; Liu *et al*., 2020). As we observed, FA/$PM_{2.5}$ displayed a larger amount of *Akkermansia* in microbiota diversity than the $PM_{2.5}$/FA group, which might justify an improvement of insulin sensitivity in female offspring whose mothers were exposed to air pollution during lactation.

Some previous studies of our group (Saad, Santos and Prada, 2016; Bagarolli *et al*., 2017) highlighted that genetic and environmental factors provoke alterations in the microbiota, mainly in the obese state, and partially concluded that it is also essential to mention the microbiota composition can variate within an individual.

Serino *et al*. (2012) described that increased *Allistipes* genus could be a protective factor to type 2 diabetes in an HFD scenario. A study of our group (Bagarolli

et al., 2017) demonstrated that probiotic administration in DIO animals could increase these genera. Unexpectedly, in our study, we observed increased amounts of *Allistipes* in females FA/$PM_{2.5}$ compared to FA/FA and $PM_{2.5}$/FA compared to FA/$PM_{2.5}$. This result could also be explained the less harmful effect of maternal exposure to $PM_{2.5}$ in this group.

Thingholm *et al*. (2020) observed a significant association of some individual microbial genera with obesity, pointing to the decrease of *Akkermansia*, *Oscillibacter,* and *Allistipes*. There were similarities in our results with this mentioned study. We also observed increased amounts of *Oscillibacter* at males FA/FA and FA/$PM_{2.5}$ compared to $PM_{2.5}$/FA. Still, our data were slightly different from Thingholm *et al*. (2020) when analyzed *Akkermansia* and *Allistipes*, already discussed above.

However, some studies demonstrate that excessive mucin degradation can favor the access of pathogens to the mucosa (Lindén, Florin and McGuckin, 2008; Ganesh *et al*., 2012). Polluting particles can affect the protective characteristics of the mucus in the intestinal tract, changing the genetic expression, permeability, and composition of the microbiota, impairing the health of the host (Gillois *et al*., 2018). Although the offspring have not been subject to pollution, maternal exposure during pregnancy can result in placental epigenetic modification and fetus reprogramming (Maghbooli *et al*., 2018). This reprogramming can modulate the expression of genes such as NPY or Leptin that act in regulating food intake and consequently modifying the microbiota.

Changes found in the microbiota of the offspring FA/$PM_{2.5}$ exposure may have occurred due to contamination in breast milk by particulate pollutants (Iszatt *et al*., 2019).

Our data demonstrate that maternal exposure to ambient $PM_{2.5}$ programs offspring diseases, calling particular attention to protecting women from exposure to particulate air pollution.

Acknowledgments

We thank Heloísa Assalin, Guilherme Zweig and Raquel Patrícia Ataíde Lima for technical assistance, Bruna Oliveira Matos for assisting with blood glucose measurements. We thank Dioze Guadagnini for the hormones measurements. This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo): 2017/18498-6 (POP), 2017/11518-1 (CMC), 2017/19703-2 (RPAL), São Paulo, Brasil. CNPq INCT (Instituto Nacional de Ciência e Tecnologia de Obesidade e Diabetes), Brasil: 573856/2008-7 (POP), and a grant from the National Institutes of Health (R01DK106076 to YBK).

Author contributions

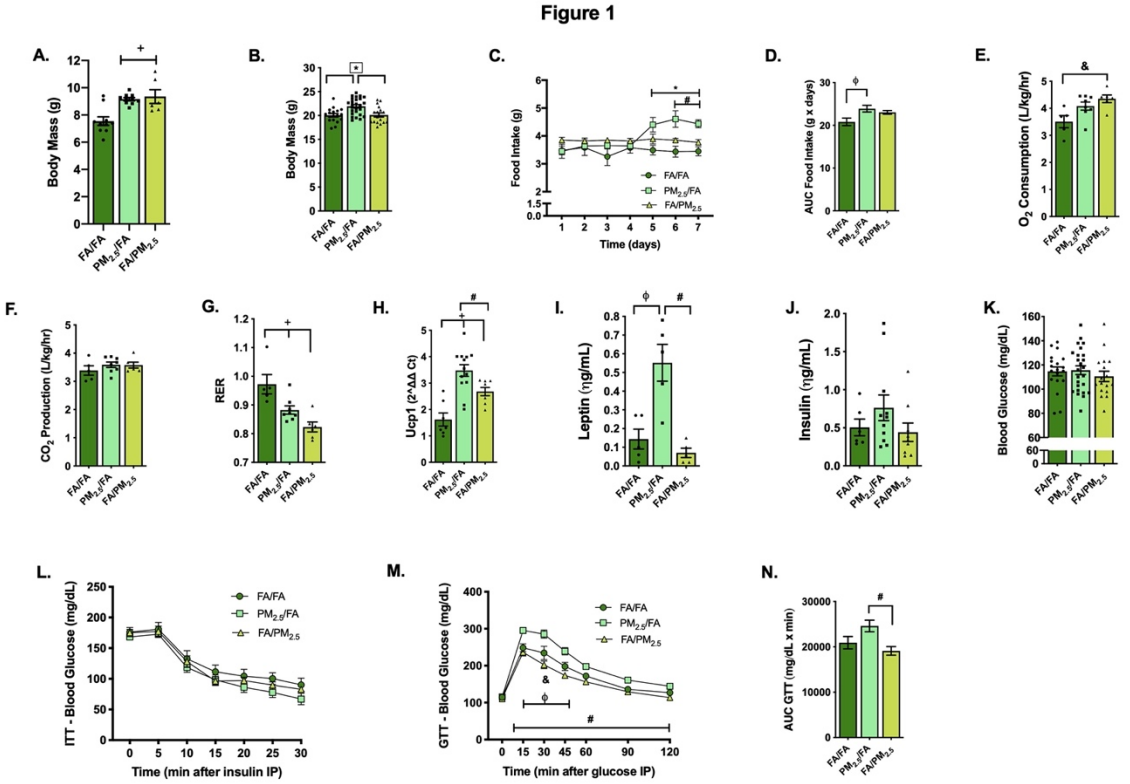
P.O.P., M.M.V., A.S., and P.H.N.S. conceived the study, designed experiments, and interpreted the results. O.P.Z., C.M.C., V.Y.Y., G.C., C.K.O.F., A.S., S.N., M.M.V., and P.O.P. performed all experiments. P.O.P. wrote the manuscript. O.P.Z., C.M.C., Y.B.K., A.S., and M.M.V. reviewed and edited the manuscript.

Additional information

The authors declare no competing interests.

FIGURES

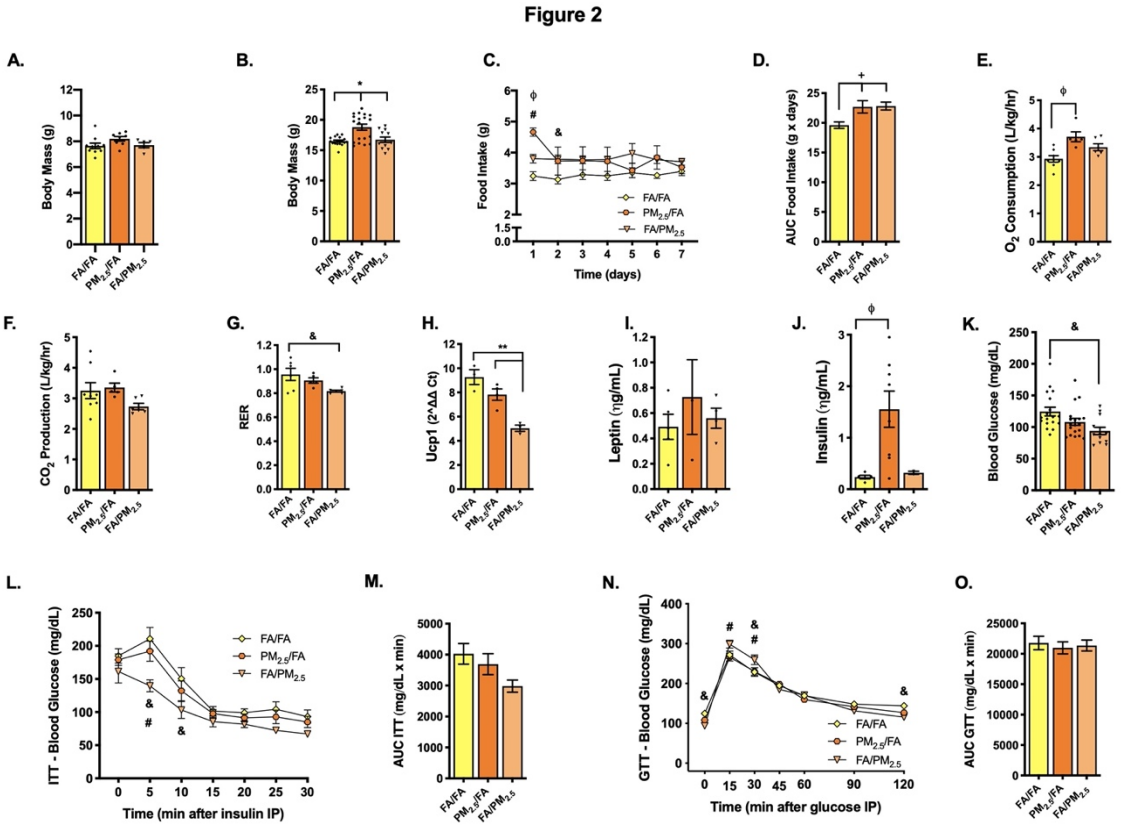
Figure 1. Maternal exposure to $PM_{2.5}$ alters the energy metabolism of male offspring



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. **A**: Body mass (g) at weaning, n=6-10. **B**: Body mass (g), n=17-26. **C:** Food intake (g), n=5 for each group. **D**: Area under the curve of food intake (g x days), n=5 for each group. **E**: oxigen (O_2) consumption (L/kg/hr), n=5-8. **F**: carbon dioxide (CO_2) production (L/kg/hr), n=5-8. **G**: Respiratory exchange ratio (RER), n=5-8. **H**: Uncoupling Protein 1(UCP1) gene expression in the brown adipose tissue, n=8-13. **I:** Fasting serum leptin level (ηg/mL), n=5 for each group. **J:** Fasting serum insulin levels (ηg/mL), n=6-12. **K:** Fasting blood glucose (mg/dL), n=17-25. **L**: Insulin tolerance test (ITT), n=14-25. **M**: Glucose tolerance test (GTT), n=17-25. **N**: Area under the curve of GTT (mg/dL x minutes), n=17-25. All mice were 8 -13 weeks of age, except for A, mice were 3 weeks old (at weaning), and E, F and G, mice were 19 weeks of age. Data were expressed as the mean ± SEM. One-way ANOVA followed by the Bonferroni post hoc test was used for the statistical analysis of Panels A, B, D, E, F, G, H, I, J, K and N and two-way ANOVA

followed by the Bonferroni post hoc test was used for the statistical analysis of Panels C, L and M. **A:** $^{+}$P<0.05 ($PM_{2.5}$/FA and FA/$PM_{2.5}$ *vs* FA/FA). **B:** *P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$ and FA/FA). **C:** *P<0.05 ($PM_{2.5}$/FA *vs* FA/FA) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$). **D:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA). **E:** $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA). **G:** $^{+}$P<0.05 (FA/FA *vs* $PM_{2.5}$/FA and FA/$PM_{2.5}$). **H:** $^{+}$P<0.05 (FA/FA *vs* $PM_{2.5}$/FA and FA/$PM_{2.5}$) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$). **I:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$). **M:** $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$), ϕP<0.05 ($PM_{2.5}$/FA *vs* FA/FA) and $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA). **N:** $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$).

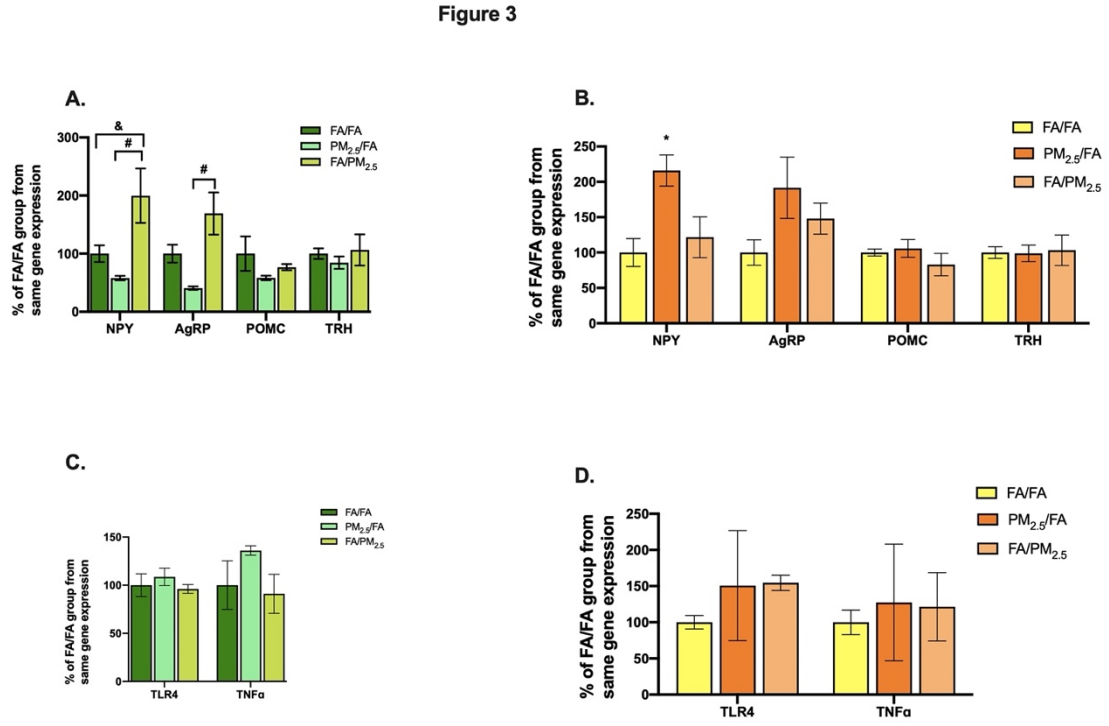
Figure 2. Maternal exposure to $PM_{2.5}$ alters the energy metabolism of female offspring



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. **A**: Body mass (g) at weaning, n=5-10. **B**: Body mass (g), n=12-19. **C:** Food intake (g), n=5 for each group. **D**: Area under the curve of food intake (g x days), n=5 for each group. **E**: oxigen (O_2) consumption (L/kg/hr), n=5-8. **F**: carbon dioxide (CO_2) production (L/kg/hr), n=5-

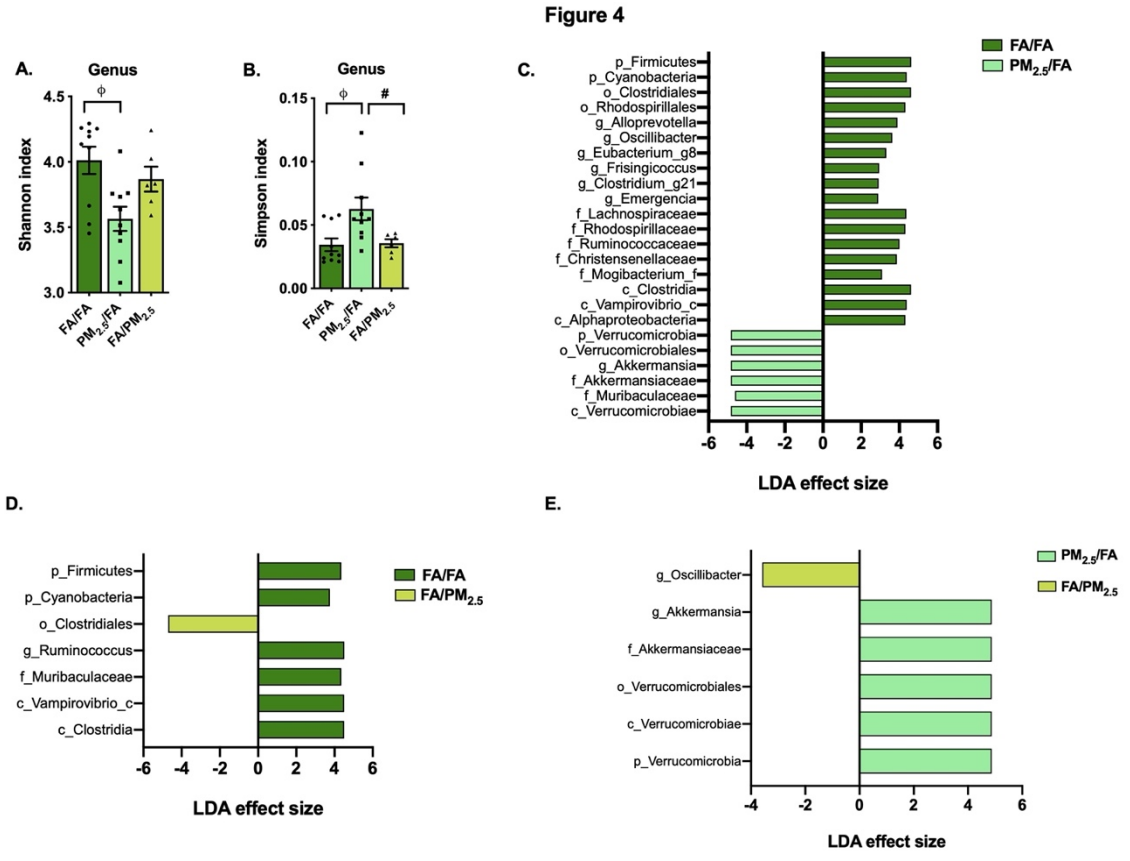
8. **G**: Respiratory exchange ratio (RER), n=5-8. **H**: Uncoupling Protein 1(UCP1) gene expression, n=3-5. **I:** Fasting serum leptin level (ηg/mL), n=4-6. **J:** Fasting serum insulin levels (ηg/mL), n=3-8. **K:** Fasting blood glucose (mg/dL), n=13-19. **L**: Insulin tolerance test (ITT), n=10-19. **M:** Area under the curve of ITT (mg/dL x minutes), n=10-19. **N**: Glucose tolerance test (GTT), n=13-19. **O**: Area under the curve of GTT (mg/dL x minutes), n=13-19. All male mice were 8 -13 weeks of age, except for A mice were 3 weeks old (at weaning) and E, F and G mice were 19 weeks of age. Data were presented as the mean ± SEM. One-way ANOVA followed by the Bonferroni post hoc test was used for the statistical analysis of Panels A, B, D, E, F, G, H, I, J, K and N and two-way ANOVA followed by the Bonferroni post hoc test was used for the statistical analysis of Panels C, L and M. **B:** *P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$ and FA/FA). **C:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA), $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$) and $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA). **D:** $^{+}$P<0.05 ($PM_{2.5}$/FA and FA/$PM_{2.5}$ *vs* FA/FA). **E:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA). **G:** $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA). **H:** **P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA and $PM_{2.5}$/FA). **J:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA) **K:** $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA). **L:** $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$). **O:** $^{\&}$P<0.05 (FA/$PM_{2.5}$ *vs* FA/FA) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$).

Figure 3. Maternal exposure to $PM_{2.5}$ alters hypothalamic neuropeptides expression involved in energy balance mainly in male offspring



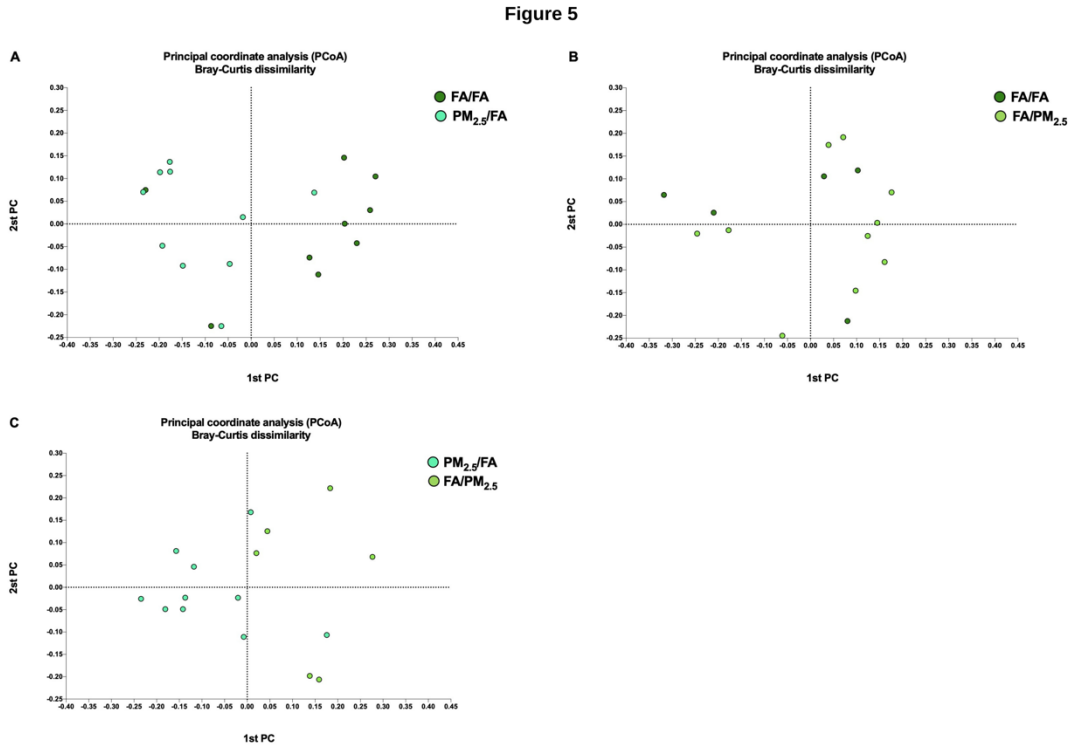
Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. **A**: Male Npy (neuropeptide Y), Agrp (Agouti-related protein), Pomc (Pro-opiomelanocortin) and Trh (Thyrotropin-releasing hormone) expression in the hypothalamus n=3-6. **B:** Female Npy (neuropeptide Y), Agrp (Agouti-related protein), Pomc (Pro-opiomelanocortin) and Trh (Thyrotropin-releasing hormone) expression in the hypothalamus n=3-6. **C:** Male Tlr4 (Toll-like receptor 4) and Tnfα (Tumor necrosis factor alpha) expression in the hypothalamus n=3-6. **D:** Female Tlr4 (Toll-like receptor 4) and Tnfα (Tumor necrosis factor alpha) expression in the hypothalamus n=3-6. Data was expressed as % of FA/FA group for each gene expression. All of the mice studied were 25-30 weeks of age. Data were presented as the mean ± SEM. One-way ANOVA followed by the Bonferroni post hoc test was used for the statistical analysis. **A:** $^{\&}P<0.05$ (FA/$PM_{2.5}$ *vs* FA/FA) and $^{\#}P<0.05$ ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$). **B:** *P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$ and FA/FA).

Figure 4. Maternal $PM_{2.5}$ exposure alters male offspring gut microbiota alpha diversity and composition.



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. **A:** Alpha diversity, estimated by Shannon index (Shannon: FA/FA *vs* $PM_{2.5}$/FA p=0.0089) and **B.** Alpha diversity, estimated by Simpson index (Simpson: FA/FA *vs* $PM_{2.5}$/FA p=0.0185 or $PM_{2.5}$/FA vs FA/$PM_{2.5}$ p=0.0110). Linear discriminant analysis (LDA) effect size indicated differences in phyla and genera between groups FA/FA and $PM_{2.5}$/FA **(C)**, FA/FA and FA/$PM_{2.5}$ **(D)** and **(E)** $PM_{2.5}$/FA and FA/$PM_{2.5}$. For A and B panels data are presented as means ± SEM from n≥5 per group and for C, D and E the taxa with LDA score >2 and significance of a <0.05 were determined by Wilcoxon signed-rank test. **A:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA). **B:** $^{\phi}$P<0.05 ($PM_{2.5}$/FA *vs* FA/FA) and $^{\#}$P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$).

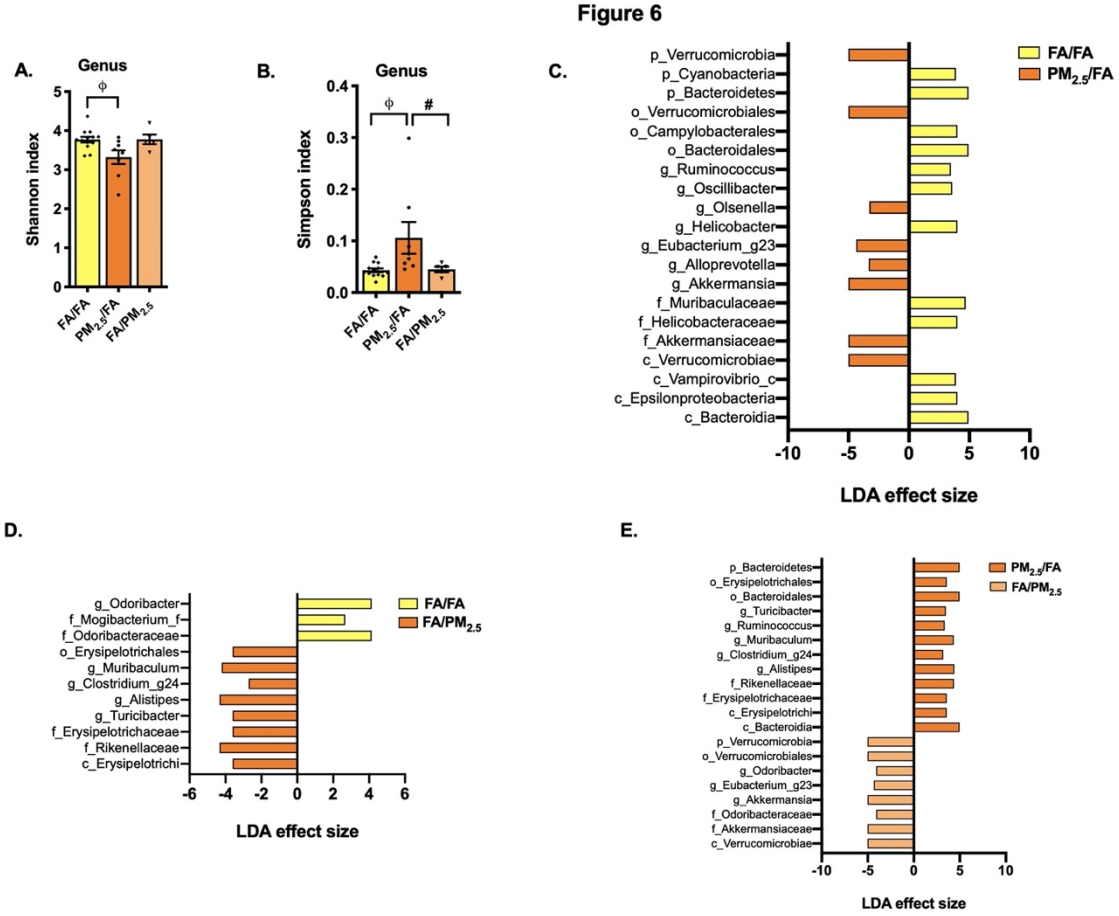
Figure 5. Male Offspring principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity.



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. Ordination (PCoA) generated by using the Bray–Curtis dissimilarity metric sampled. Samples are colored according to group. **A.** Bray-Curtis PCoA ordination. Results revealed that $PM_{2.5}$/FA samples clustered separately from FA/FA sample (p-value= 0.007 n^{o} of

permutations 999). **B.** Bray-Curtis PCoA ordination. The results did not reveal that FA/$PM_{2.5}$ samples clustered separately from FA/FA sample (p-value=0.46 nº of permutations 999). **C.** Bray-Curtis PCoA ordination. Results revealed that $PM_{2.5}$/FA samples clustered separately from FA/$PM_{2.5}$ sample (p-value= 0.004 nº of permutations 999).

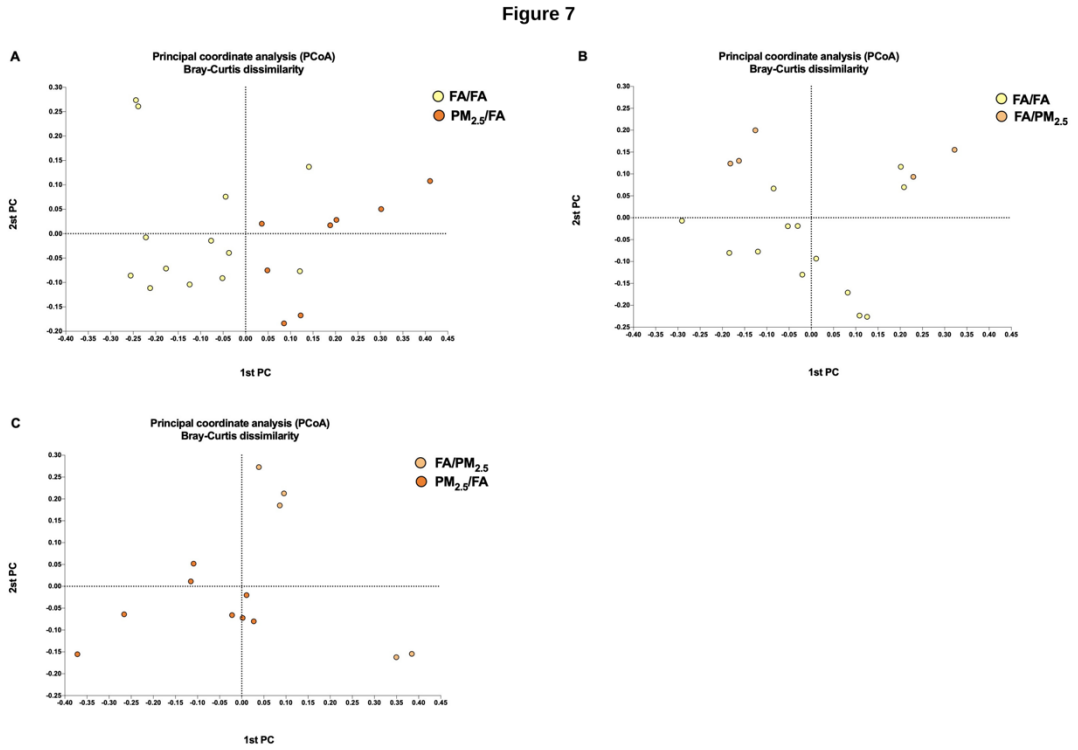
Figure 6. Maternal $PM_{2.5}$ exposure alters female offspring gut microbiota alpha diversity and composition.



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. **A:** Alpha diversity, estimated by Shannon indice (Shannon: FA/FA *vs* $PM_{2.5}$/FA p=0.0089) and **B.** Alpha diversity, estimated by Simpson indice (Simpson: FA/FA *vs* $PM_{2.5}$/FA p=0.0185 or $PM_{2.5}$/FA vs FA/$PM_{2.5}$ p=0.0110). Linear discriminant analysis (LDA) effect size indicated differences in phyla and genera between groups FA/FA and $PM_{2.5}$/FA **(C)**, FA/FA and FA/$PM_{2.5}$ **(D)** and **(E)** $PM_{2.5}$/FA and FA/$PM_{2.5}$. For A and B panels data are presented as means ± SEM from n≥5 per group and for C, D and E the taxa with LDA

score >2 and significance of a <0.05 were determined by Wilcoxon signed-rank test. **A:** ϕP<0.05 ($PM_{2.5}$/FA *vs* FA/FA). **B:** ϕP<0.05 ($PM_{2.5}$/FA *vs* FA/FA) and #P<0.05 ($PM_{2.5}$/FA *vs* FA/$PM_{2.5}$).

Figure 7. Female principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity.



Maternal exposure to $PM_{2.5}$ during pregnancy ($PM_{2.5}$/FA) or lactation (FA/$PM_{2.5}$). Filtered air during pregnancy and lactation (FA/FA) as control group. Ordination (PCoA) generated by using the Bray–Curtis dissimilarity metric sampled. Samples are colored according to group. **A.** Bray-Curtis PCoA ordination. Results revealed that $PM_{2.5}$/FA samples clustered separately from FA/FA sample (p-value= 0.001 nº of permutations 999). **B.** Bray-Curtis PCoA ordination. Results revealed that FA/$PM_{2.5}$ samples clustered separately from FA/FA sample (p-value= 0.030 nº of permutations 999). **C.** Bray-Curtis PCoA ordination. Results revealed that $PM_{2.5}$/FA samples clustered separately from FA/$PM_{2.5}$ sample (p-value= 0.002 nº of permutations 999).

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CONCLUSÃO

Em conclusão os resultados sugerem que a exposição à poluição atmosférica por meio da programação metabólica se mostrou capaz de modificar o metabolismo energético e microbiota intestinal da prole, com desfechos observados ao nascer, ao desmame e na vida adulta, afetando principalmente os filhotes machos. Tais achados evidenciam a necessidade de políticas públicas voltadas à saúde não somente de gestantes mas também de lactantes, principalmente em regiões com maior exposição à poluição atmosférica.

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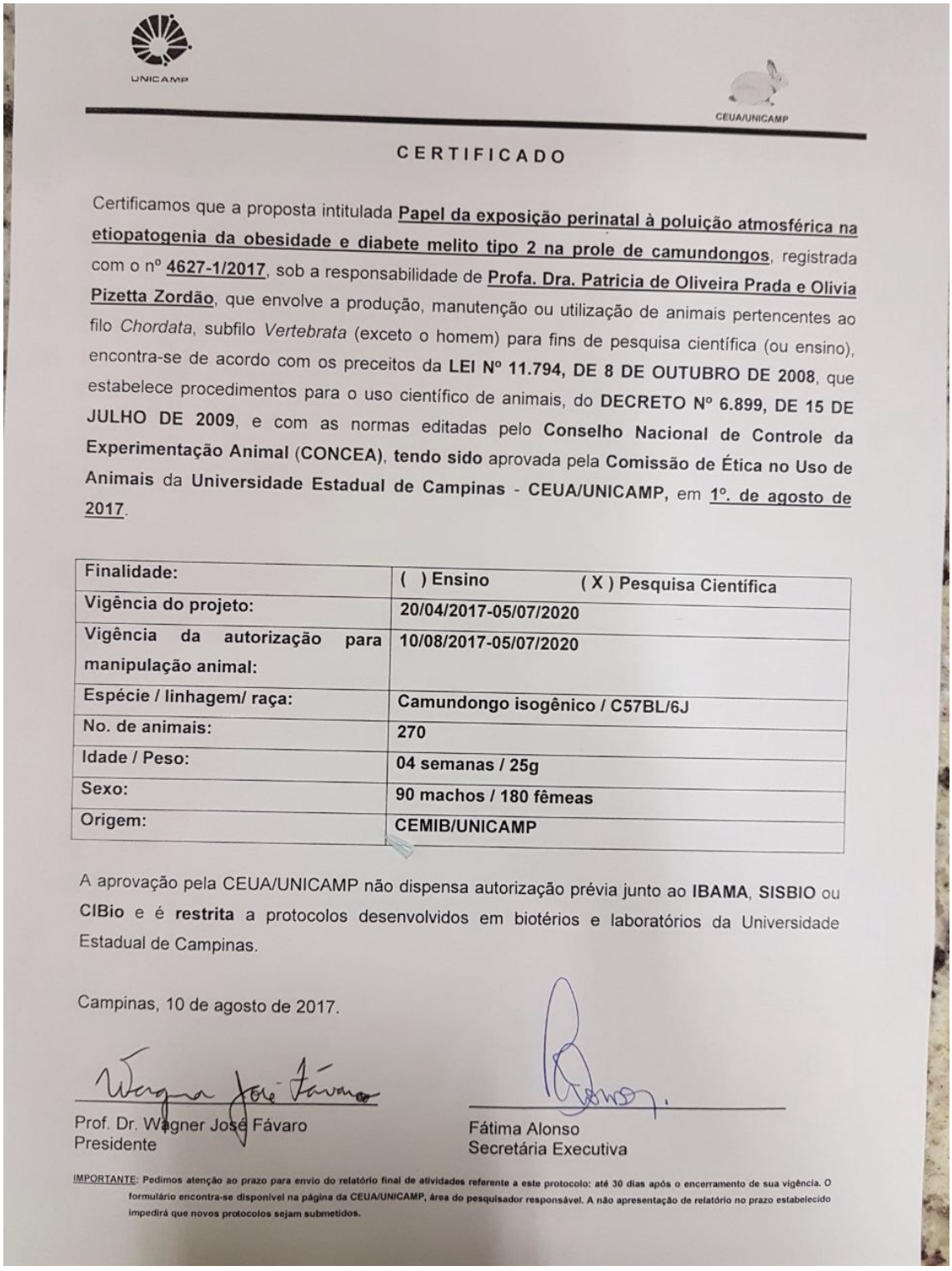
ANEXOS



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CERTIFICADO

Certificamos que a proposta intitulada **Papel da exposição perinatal à poluição atmosférica na etiopatogenia da obesidade e diabete melito tipo 2 na prole de camundongos**, registrada com o nº **4627-1/2017**, sob a responsabilidade de **Profa. Dra. Patricia de Oliveira Prada e Olivia Pizetta Zordão**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, **tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP**, em **1º. de agosto de 2017**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**20/04/2017-05/07/2020**
Vigência da autorização para manipulação animal:	**10/08/2017-05/07/2020**
Espécie / linhagem/ raça:	**Camundongo isogênico / C57BL/6J**
No. de animais:	**270**
Idade / Peso:	**04 semanas / 25g**
Sexo:	**90 machos / 180 fêmeas**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 10 de agosto de 2017.

Prof. Dr. Wagner José Fávaro
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.