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Instituto de Biologia Roberto Alcântara Gomes

Iala Milene Bertasso

Avaliação do metabolismo hepático em ratos Wistar adultos de ambos os sexos em modelos de programação no período da lactação: papel da exposição materna à nicotina, do desmame precoce e da restrição proteica materna

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Orientadora: Prof.ª Dra. Patrícia Cristina Lisbôa da Silva

Coorientador: Prof. Dr. Egberto Gaspar de Moura

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Iala Milene Bertasso

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DEDICATÓRIA

Aos meus pais, aos quais sempre dedicarei toda e qualquer conquista.

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RESUMO

BERTASSO, Iala Milene. **Avaliação do metabolismo hepático em ratos Wistar adultos de ambos os sexos em modelos de programação no período da lactação**: papel da exposição materna à nicotina, do desmame precoce e da restrição proteica materna. 2021. 131f. Tese (Doutorado em Biociências) - Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2021.

Estudos experimentais mostram que a restrição proteica, o desmame precoce e a exposição materna à nicotina durante a lactação são fatores que promovem alterações endócrino-metabólicas na prole adulta do sexo masculino, predispondo-os à prejuízos da função hepática. Em nosso grupo de pesquisa, observamos que ratos Wistar induzidos ao sobrepeso e obesidade na vida adulta, pelos modelos experimentais de desmame precoce e exposição materna à nicotina, apresentam comprometimento da função hepática, com a presença de microesteatose e alterações do balanço redox. Por outro lado, filhotes programados para o baixo peso por meio da dieta materna restrita em proteínas apresentam massa e arquitetura hepática inalteradas. Devido às alterações metabólicas anteriormente descritas, é possível que as vias de manutenção da homeostase glicêmica e lipídica no fígado estejam funcionando de modo alterado para suprir as necessidades do organismo. Assim, na tentativa de investigar as vias moleculares envolvidas nos fenótipos hepáticos caracterizados nestes três modelos de programação exclusivamente causados por *imprinting* na lactação, nesta Tese analisamos as principais enzimas e fatores transcricionais relacionados as vias do metabolismo glicêmico e lipídico do fígado (gliconeogênese, lipogênese, β-oxidação e montagem de VLDL), além da histologia e resposta deste órgão a administração intraperitoneal de substrato gliconeogênico. A prole fêmea adulta foi estudada pela primeira vez pelo nosso grupo, tendo em vista a possibilidade de respostas distintas associadas ao gênero. Modelo de programação pela exposição à nicotina: Dois dias após o parto, foram implantadas minibombas nas ratas lactantes, formando os grupos Nicotina (6 mg/kg/dia por 14 dias) e Controle (solução salina por 14 dias). Os machos apresentaram alterações no metabolismo de triglicerídeos, sendo com acúmulo preferencialmente intra-hepático e independente de sobrepeso e resistência à insulina. De modo oposto, o acúmulo de lipídios nas fêmeas ocorreu preferencialmente no plasma e a arquitetura do hepatócito foi preservada, com alterações principalmente no metabolismo de colesterol. Modelo de programação pelo desmame precoce: As ratas lactantes foram separadas em: 1) grupo DP não farmacológico: envolvidas com uma bandagem adesiva nos 3 últimos dias de lactação; 2) DP farmacológico: receberam bromocriptina (1mg/kg PC/dia) nos 3 últimos dias da lactação; e 3) Controle: cujos filhotes foram desmamados aos 21 dias. Apenas os machos do DP não farmacológico apresentaram alterações na citoarquitetura do fígado associadas às diferenças na expressão proteica da Ácido Graxo Sintase (FAS) e Estearoil-CoA Desaturase 1 (SCD-1). As fêmeas, independente do tipo de desmame, não desenvolveram esteatose hepática, embora tenham maior conteúdo da enzima relacionada a exportação de triglicerídeos hepáticos, o que pode influenciar no melhor perfil lipídico observado. Modelo de programação pela dieta hipoprotéica: Durante a lactação, as ratas lactantes receberam dieta padrão (23% de proteína) ou dieta hipoproteica (8% de proteínas). A dieta hipoproteica materna na lactação não programa o acúmulo de lipídios hepáticos na prole adulta, embora várias vias de reparo da homeostase tenham sido alteradas em machos e fêmeas, possivelmente comprometendo a manutenção da função hepática.

Palavras-chave: Lactação. Desmame precoce. Nicotina. Restrição protéica. Programação metabólica. Esteatose hepática.

ABSTRACT

BERTASSO, Iala Milene. **Evaluation of liver metabolism in adult Wistar rats of both sexes in programming models during lactation**: role of maternal exposure to nicotine, early weaning and maternal protein restriction**.** 2021. 131p. Tese (Doutorado em Biociências) - Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2021.

Experimental studies show that protein restriction, early weaning and maternal exposure to nicotine during lactation are factors that promote endocrine-metabolic alterations in adult male offspring, increasing the risk for liver dysfunction. In our research group, we observed that Wistar rats programmed to overweight and obesity in adulthood, by experimental models of early weaning and maternal exposure to nicotine, have impaired liver function, with the presence of microvesicular steatosis and redox imbalance. On the other hand, offspring programmed for low weight by maternal protein restriction have unchanged liver mass and architecture. However, due to metabolic changes previously described, it is possible that the maintenance pathways of glycemic and lipid homeostasis in the liver are functioning in an altered way in order to supply the body's needs. Thus, to investigate the molecular pathways involved in liver phenotypes in these three programming models exclusively caused by imprinting during lactation, in this Thesis we analyze the main enzymes and transcription factors related to the pathways of liver glycemic and lipid metabolism (gluconeogenesis, lipogenesis, β-oxidation and VLDL assembly), histology as well as the liver response to intraperitoneal administration of gluconeogenic substrate. The adult female offspring was studied for the first time by our group, considering the possibility of distinct responses associated with gender. Programming model by nicotine exposure: Two days after birth, minipumps were implanted in lactating rats, which were divided into Nicotine group (6 mg/kg/day for 14 days) and Control group (saline solution for 14 days). Males showed alterations in triglyceride metabolism, preferentially with intrahepatic accumulation, independently of overweight and insulin resistance. Conversely, the accumulation of lipids in females occurred preferentially in the plasma and the hepatocyte architecture was preserved, with alterations mainly in the cholesterol metabolism. Programming model by early weaning: Lactating rats were divided into: 1) non-pharmacological EW group: wrapped with an adhesive bandage in the last 3 days of lactation; 2) Pharmacological EW: received bromocriptine (1mg/kg BW/day) in the last 3 days of lactation; and 3) Control: whose pups were weaned at 21 days. Only males from the non-pharmacological EW showed alterations in the liver cytoarchitecture associated with differences in the Fatty Acid Sintase (FAS) and Stearoil-CoA Desaturase-1 (SCD-1) protein expression. Females, regardless of the type of weaning, did not develop liver steatosis, although they have a higher content of the enzyme related to the export of hepatic triglycerides, which may influence the best lipid profile observed. Programming model by low-protein diet: Throughout lactation period, lactating dams received a standard diet (23% protein) or a low-protein diet (8% protein). Maternal protein restriction during lactation does not program the accumulation of hepatic lipids in the adult offspring, although several repair pathways for homeostasis have been altered in males and females, possibly compromising the maintenance of liver function.

Keywords: Lactation. Early weaning. Nicotine. Protein restriction. Metabolic programming. Hepatic steatosis.

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LISTA DE ABREVIATURAS E SIGLAS

H_2O_2	Peróxido de hidrogênio
α-MSH	Hormônio estimulante de alfa-melanócitos
α2-AR	Receptor adrenérgico do tipo alfa 2
β3-AR	Receptor adrenérgico beta 3
4HNE	4-hidroxinonenal
11βHSD1	11β-hidroxiesteróide desidrogenase tipo 1
ACC-1	Acetil-CoA carboxilase-1
AG	Ácido graxo
AGCL	Ácido graxo de cadeia longa
AGL	Ácido graxo livre
AKT	Proteína quinase B
AKTp	Proteína quinase B fosforilada
AMP	Monofosfato de adenosina
ATF6	*Activating transcription factor 6*
ATP	Trifosfato de adenosina
AMPK	Proteína quinase ativada por monofosfato de adenosina
AMPKp	Proteína quinase ativada por monofosfato de adenosina fosforilada
ApoB	Apolipoproteína B
AR	Receptor de androgênio
BRO	Bromocriptina
CART	Fator de transcrição cocaína-anfetamina dependente
C/EBP-β	Proteína de ligação ao intensificador beta
CHOP	*C/EBP-Homologous Protein*
ChREBP	Proteína de ligação do elemento responsivo à carboidrato
COL	Colesterol
CPT-1α	Carnitina palmitoil-transferase-1alfa
CPT-2	Carnitina palmitoil-transferase-2
D1	Desiodase tipo 1
D2	Desiodase tipo 2
DAG	Diacilglicerol

DGAT	Diacilglicerol aciltransferase
DHGNA	Doença hepática gordurosa não alcoólica
DM2	Diabetes *mellitus* tipo 2
DNA	Ácido desoxirribonucleico
DOHaD	Origens desenvolvimentistas da saúde e da doença
DP	Desmame precoce
ECA	Estatuto da Criança e do Adolescente
eIF2α	Fator de iniciação da tradução eucariótica 2α
ER-α	Receptor de estrogênio-alfa
ERAD	*Endoplasmic Reticulum Associated Degradation*
FADH	Dinucleotídeo de flavina e adenina
FAS	Ácido graxo sintase
FXR	*Farnesoid X receptor*
G3P	Gliceraldeído-3-fosfato
G6Pase-α	Glicose-6-fosfatase-alfa
GCK	Glicoquinase
GFAP	Proteína ácida fibrilar glial
GLUT2	Transportador de glicose tipo 2
GLUT4	Transportador de glicose tipo 4
GPx	Glutaniona peroxidase
GR-α	Receptor de glicocorticoide alfa
HDL	Lipoproteína de alta densidade
IBA-1	Molécula adaptadora de ligação a cálcio ionizada 1
IL-6	Interleucina-6
IL-10	Interleucina-10
IRβ	Subunidade beta do receptor de insulina
IR	Receptor de insulina
IRE1	*Inositol-requiring enzyme* 1
IRS1	Substrato do receptor de insulina 1
LDL	Lipoproteína de baixa densidade
LIR	Domínio de ligação à LC3
LPA	Ácido lisofosfatídico
LPK	Piruvato quinase hepática

LXR	Receptor X do fígado
MCP-1	Proteína-1 quimioatrativa de monócitos
MDA	Malondialdeído
miRNA	MicroRNA
MTTP	Proteína de transferência de triglicerídeos microssomal
nAChRs	Receptores nicotínicos colinérgicos
NADPH	Fosfato de dinucleotídeo de nicotinamida e adenina
NIC	Nicotina
NIS	Co-trasnportador sódio-iodeto
NPY	Neuropeptídeo Y
ObB	Receptor de leptina
OGTT	Teste oral de tolerância à glicose
OMS	Organização Mundial da Saúde
PA	Ácido fosfatídico
pACC	Acetil-CoA carboxilase fosforilada
PC	Peso corporal
PDX1	Fator de transcrição homeobox pancreático e duodenal 1
PEPCK-1	Fosfoenolpiruvato-carboxiquinase-1
PERK	*PKR-like ER kinase*
PGC1α	Co-ativador-1 do receptor ativado por proliferador do peroxissoma alfa
PN	Pós-natal
POMC	Pró-opiomelanocortina
PPAR-α	Receptor ativado por proliferadores de peroxissoma-alfa
PPAR-γ	Receptor ativado por proliferadores de peroxissoma-gama
RE	Retículo endoplasmático
RNA	Ácido ribonucléico
RNAm	RNA mensageiro
ROS	Espécies reativas de oxigênio
RP	Restrição proteica
SCD-1	Estearoil-CoA desaturase-1
SIRT-1	Sirtuina-1
SNS	Sistema nervoso simpático
SOCS3	Supressor da sinalização de citocinas 3

SOD	Superóxido desmutase
SREBP-1c	Proteína de ligação do elemento regulador de esterol-1c
SUB	Tecido adiposo branco subcutâneo
TAB	Tecido adiposo branco
TAM	Tecido adiposo marrom
T3	Triiodotironina
T4	Tiroxina
TCA	Ciclo do ácido tricarboxílico
TG	Triglicerídeo
TH	Tirosina hidroxilase
TNF-α	Fator de necrose tumoral-alfa
TPO	Tireoperoxidase
TRβ	Receptor de hormônio tireoidiano beta
TRH	Hormônio liberador de tireotrofina
TSH	Tireotrofina
TSHr	Receptor de tireotrofina
UBA	Domínio de ligação com proteínas ubiquitinadas
UCP-1	Proteína desacopladora do tipo 1
UPR	*Unfolded Protein Response*
VDR	Receptor de vitamina D
VISC	Tecido adiposo branco visceral
VLDL	Lipoproteína de muito baixa densidade
XBP1	*X Box Protein 1*

LISTA DE SÍMBOLOS

%	Porcentagem
α	Alfa
β	Beta
γ	Gama
µg	Micrograma
mg	Miligrama
g	Grama
kg	Kilograma
dL	Decilitro
mL	Mililitro
l	Litro
rpm	Rotações por minuto
Min	Minuto
H	Hora
±	Mais ou menos
ºc	Graus celsius
<	Maior
>	Menor
n	Número
vs	Versus

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INTRODUÇÃO

Origens Desenvolvimentistas da Saúde e da Doença (DOHaD)

Atualmente quase dois bilhões de adultos no mundo estão acima do peso, e mais da metade são obesos, enquanto quase um terço das crianças apresentam baixo crescimento e desenvolvimento (HOFFMAN et al., 2021). Esse fenômeno onde o excesso de peso coexiste com baixo peso e deficiências nutricionais, vem sendo chamado de "duplo fardo da desnutrição" (POPKIN; CORVALAN; GRUMMER-STRAWN, 2020), uma vez que pesquisas têm demonstrado que ambos os fenótipos podem ter causas similares (VICTORA et al., 2021). A hipótese mais aceita é a de que insultos em momentos críticos do desenvolvimento poderiam programar o indivíduo para o desenvolvimento de doenças crônicas na vida adulta, conhecida por Origens Desenvolvimentistas da Saúde e da Doença (em inglês: *Developmental Origins of Health and Disease* – DOHaD).

O conceito DOHaD aborda a inter-relação entre fatores maternos, ambientais, de crescimento e desenvolvimento fetal/infantil atuantes no início da vida e a predisposição do indivíduo ao desenvolvimento de doenças em longo prazo (ALMEIDA et al., 2019). Os períodos da concepção à gravidez, lactação à adolescência constituem as principais janelas de tempo susceptíveis a eventos capazes de moldar o metabolismo de um indivíduo ao longo da vida, por mecanismos ainda não totalmente compreendidos, mas ligados a modulação epigenética da expressão de diversos genes (SUZUKI, 2017). Os estudos nesta área iniciaram-se com as observações de Ravelli e colaboradores (1976), retomadas por Barker e Osmond (1986), mostrando a associação epidemiológica entre o baixo peso ao nascer e maior risco de doenças cardiovasculares na vida adulta. Levando em consideração o estado nutricional e sua forte relação com o desenvolvimento, Hales e Barker propuseram a teoria do “fenótipo econômico” sugerindo que o desenvolvimento do feto é sensível ao ambiente nutricional (HALES; BARKER, 1992). Essa programação fetal tem inicialmente caráter adaptativo e protetor, de modo que em situações de ambiente gestacional adverso, o feto pode responder com alterações em seu metabolismo e estrutura, as quais irão possibilitar sua sobrevivência (ROGERS, 2019).

As alterações derivadas dessas condições são denominadas modificações epigenéticas (CHRISTOFOROU et al., 2020). Esse conceito foi introduzido por Waddington em 1939, e

mais tarde, foi relacionado a alterações na expressão gênica hereditária, nesse caso não há alterações na sequência do DNA, mantendo-se a identidade celular e integridade do genoma (WADDINGTON, 1939; ZHANG; LU; CHANG, 2020), mas que podem ser parcialmente transmitidas aos descendentes (BAROUKI et al., 2018; SCHAEFER; NADEAU, 2015). Os três mecanismos epigenéticos envolvidos no controle da transcrição gênica são metilação do DNA, acetilação/desacetilação das histonas e regulação por microRNAs, pequenas moléculas de RNA não codificantes de proteínas (GOYAL; LIMESAND; GOYAL, 2019). Quanto ao ultimo mecanismo, cabe destacar que mais de 2.000 miRNAs são expressos no corpo humano regulando pelo menos 1/3 de todos os genes (MISHRA; TANDON; BYRAREDDY, 2020; FRIEDLÄNDER et al., 2014). Portanto, o metabolismo de um indivíduo apresenta grande capacidade de se adequar a diversos fatores ambientais, gerando ampla variabilidade genética e capacidade de sobrevivência (SCHEINER, 1993).

Todavia, em longo prazo, a programação metabólica, hormonal e/ou morfológica pode aumentar a predisposição ao desenvolvimento de doenças que podem repercutir na vida adulta, em especial quando o individuo é exposto a um ambiente adverso (BARKER, 2007). Estudos têm mostrado que, embora os insultos durante a vida fetal programem um fenótipo "econômico" para promover adaptações de curto prazo para a sobrevivência, um segundo insulto ocorrendo após o nascimento poderia amplificar a programação precoce contribuindo assim para o desenvolvimento ou gravidade das disfunções metabólicas (GLUCKMAN et al. 2008, PADMANABHAN; CARDOSO; PUTTABYATAPPA, 2016).

Atualmente sabe-se que o mesmo insulto pode programar diferentes fenótipos na vida adulta de acordo com a janela de programação. Por exemplo, em ratos, a restrição de proteínas durante a infância e adolescência programa a prole para obesidade e disfunções cardiometabólicas na vida adulta, enquanto que a mesma restrição apenas durante a lactação programa a prole para um fenótipo magro (AGNOUX et al., 2014; MALTA et al., 2016; PASSOS; RAMOS; MOURA, 2000). Sendo assim, apesar do número crescente de estudos, os mecanismos de programação e seus impactos nas diferentes janelas não são totalmente conhecidos.

É de senso comum a importância da lactação para o desenvolvimento do indivíduo, tendo em vista que qualquer alteração na composição do leite materno, o nosso primeiro alimento após o nascimento, pode comprometer seriamente a saúde do recém-nascido, afetando células e órgãos periféricos (PICÓ et al., 2021). Em roedores, protocolos de adoção cruzada têm mostrado que o leite materno de mães obesas programa para obesidade na ninhada de mães

magras, em oposição, o leite materno de mães magras pode prevenir a obesidade na ninhada programada pela influência materna obesa (AGNOUX et al., 2014).

Além da condição nutricional e metabólica materna, sabe-se que a exposição à poluentes ambientais e drogas durante o desenvolvimento, que são capazes de interagir com receptores endócrinos (disruptores ou interferentes endócrinos), pode promover alterações morfo-funcionais na prole em longo prazo (ASHLEY-MARTIN et al., 2015). Dentre os diversos disruptores endócrinos ambientais, a exposição dos descendentes ao tabagismo materno assume carater preocupante, uma vez que estes podem ser expostos tanto à fumaça do cigarro (fumante passivo) como à nicotina transferida pelo leite materno (NEWBOLD, 2010). Estudos epidemiológicos tem reportado que fumar durante a gestação e/ou lactação pode ser um fator de risco para o desenvolvimento de obesidade e hipertensão em crianças e adolescentes (VON KRIES et al., 2002; WIDEROE et al., 2003).

Repercussões do tabagismo materno sobre o metabolismo dos descendentes

A fumaça do tabaco contém aproximadamente 7.000 compostos químicos, dentre os quais, 70 são comprovadamente cancerígenos (WHO, 2019). Os custos globais associados ao consumo de tabaco somam 1,4 trilhões de dólares e mais de 7 milhões de vidas por ano (WHO, 2019), somente no Brasil, 443 pessoas morrem a cada dia (INCA, 2020). A nicotina é o principal alcalóide do tabaco e atua como agonista dos receptores nicotínicos colinérgicos (nAChRs), sendo responsável pela dependência ao cigarro (WATKINS et al., 2000; TALHOUT et al., 2011). A nicotina também é um importante contaminante ambiental capaz de se propagar para o solo e atmosfera (SELMAR et al., 2018), tendo sido encontrado inclusive em garrafas de água mineral (ALONSO et al., 2012) e no cabelo de grávidas (LI et al., 2017).

Apesar das evidências sobre os efeitos nocivos do fumo no indivíduo em idade adulta ou em estágios iniciais do desenvolvimento, 9-20% das mães continuam fumando durante a gestação e, o retorno ao tabagismo ocorre em 50–80% dos casos dentro de seis meses após o parto (HAMMOUD et al., 2005; EINARSON; RIORDAN, 2009; WARREN et al., 2014; MEERNIK; GOLDSTEIN, 2015). O aleitamento associado ao tabagismo aumenta a absorção de nicotina pelo bebê em comparação a exposição exclusiva pela fumaça, uma vez que a meia-vida da nicotina é maior no leite do que no soro; além de reduzir o seu tempo de eliminação, que é 3-4 vezes mais lento em bebês do que em adultos (LUCK; NAU, 1985; DAHLSTRÖM

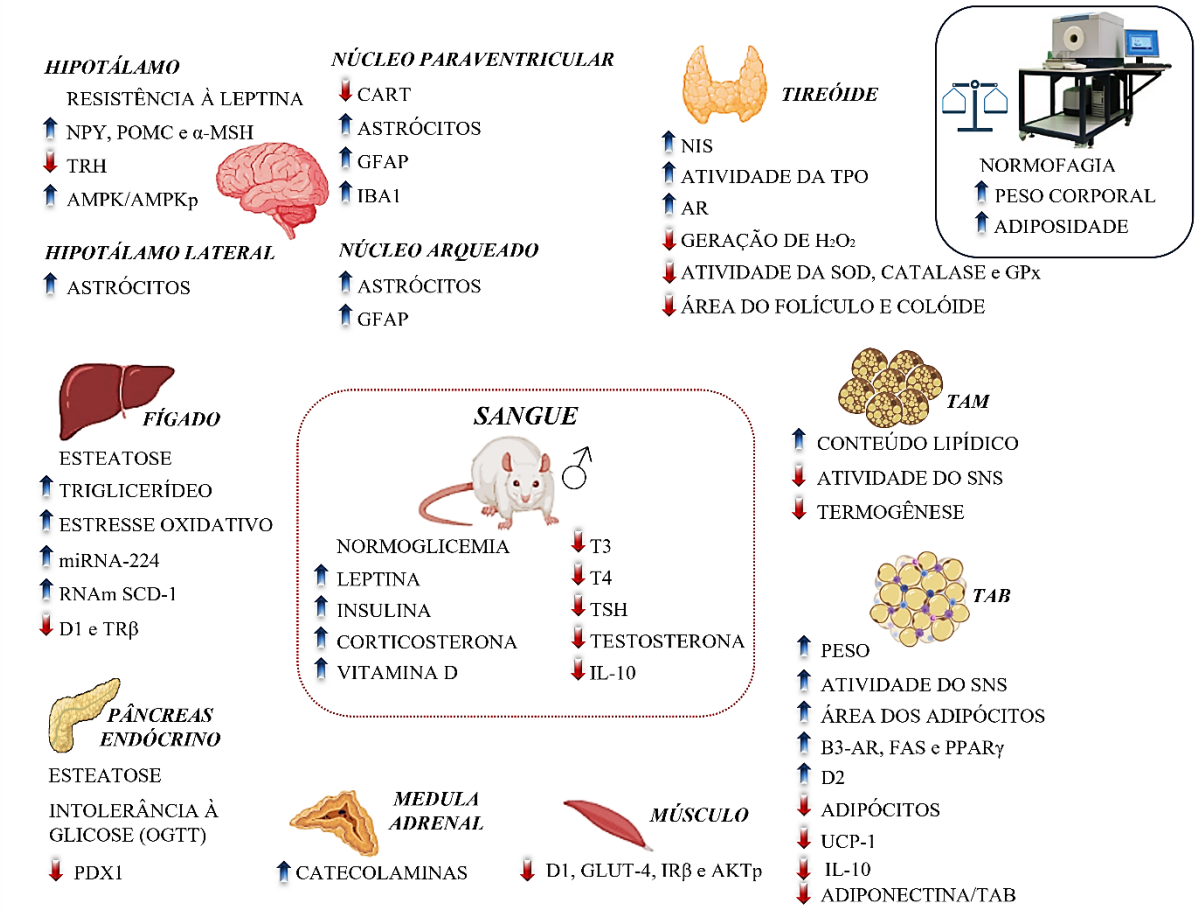
et al., 1990; SCHULTE-HOBEIN et al., 1992; DEMPSEY et al., 2000, BENOWITZ; HUKKANEN; JACOB, 2009). É importante ressaltar que o uso prolongado de terapia de reposição de nicotina e cigarros eletrônicos também são associados ao aumento dos níveis de nicotina no leite materno (WICKSTRÖM, 2007).

A exposição à nicotina por meio do tabagismo materno está associada à inúmeras consequências fetais e após o nascimento, incluindo parto prematuro, baixo peso ao nascer, síndrome da morte súbita infantil, distúrbios do desenvolvimento neurológico, transtorno de déficit de atenção e hiperatividade, inibição do desenvolvimento/função pulmonar e, propensão ao desenvolvimento de diabetes (BANDERALI et al., 2015; KNOPIK et al., 2016; SUZUKI et al., 2016; HUANG et al., 2018; MARCEAU et al., 2018; BUCK et al., 2019; HE et al., 2020).

Neste contexto, nosso laboratório desenvolveu um modelo experimental no qual ratas lactantes são expostas à nicotina do 2º ao 16º dia de lactação, em uma concentração similar à uma fumante pesada, através da implantação de minibombas osmóticas (OLIVEIRA et al., 2009). Verificamos que ao final do período de exposição, o soro e leite das ratas lactantes continham quantidade considerável de cotinina, evidenciando a exposição indireta da prole à nicotina (OLIVEIRA et al., 2010). Aos 180 dias de idade (figura 1), a prole macho apresenta elevado peso corporal e adiposidade central, hiperleptinemia, normoglicemia sustentada por hiperinsulinemia característica do quadro de resistência insulínica (OLIVEIRA et al., 2009; OLIVEIRA et al., 2010; SANTOS-SILVA et al., 2010).

Durante os últimos dois anos, nos dedicamos a estudar o fenótipo das fêmeas neste modelo experimental. Observamos redução da termogênse (PEIXOTO et al., 2020), estresse oxidativo na tireóide (MIRANDA; de MOURA; LISBOA, 2020) e hiperglicemia (RODRIGUES et al., 2021) e comprometimento da homeostase glicêmica e sinalização periférica à insulina (PIETROBON et al., 2021) além de alterações na expressão de miRNAs relacionados à regulação do metabolismo hepático nesta prole (PEIXOTO et al., 2021). As fêmeas programadas pela nicotina apresentam alguns parâmetros diferentes dos machos (figura 2).

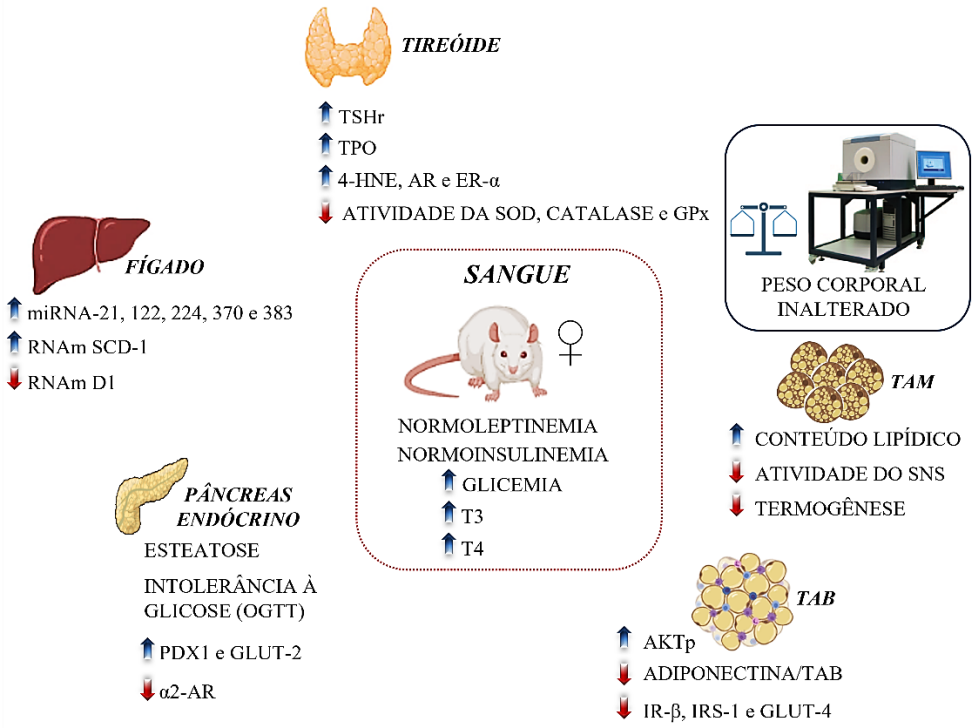
Figura 1 - Principais características da prole macho adulta oriunda de mães expostas à nicotina na lactação



Legenda: **α-MSH:** Hormônio estimulante de alfa-melanócitos; **β3-AR:** Receptor adrenérgico beta 3; **AKTp:** Proteína quinase B fosforilada; **AMPK:** Proteína quinase ativada por AMP; **AMPKp:** Proteína quinase ativada por AMP fosforilada; **AR:** Receptor de androgênio; **CART:** Fator de transcrição cocaína-anfetamina dependente; **D1:** Desiodase tipo 1; **D2:** Desiodase tipo 2; **FAS:** Ácido graxo sintase; **GFAP:** Proteína ácida fibrilar glial; **GLUT4:** Transportador de glicose do tipo 4; **GPx:** Glutationa peroxidase; **IBA-1:** Molécula adaptadora de ligação a cálcio ionizada 1; **IL-10:** Interleucina 10; **IR:** Receptor de insulina; **miR:** micro RNA; **NIS:** Co-trasnportador sódio-iodeto; **NPY:** Neuropeptídeo Y; **OGTT:** Teste oral de tolerância à glicose; **PDX1:** Fator de transcrição homeobox pancreático e duodenal 1; **POMC:** Pró-opiomelanocortina; **PPARγ:** Receptores ativados por proliferador de peroxissoma gama; **RNAm:** RNA mensageiro; **SCD1:** Estearoil-CoA desaturase-1; **SNS:** Sistema nervoso simpático; **SOD:** Superóxido dismutase; **T3:** Triiodotironina; **T4:** Tiroxina; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TPO:** Tireoperoxidase; **TRβ:** Receptor de hormônio tireoidiano beta; **TRH:** Hormônio liberador de tireotrofina; **TSH:** Tireotrofina; **UCP-1:** Proteína desacopladora 1; **H_2O_2:** Peróxido de hidrogênio.

Fonte: Disponíveis em: Oliveira et al., (2009, 2010); Younes-Rapozo et al., (2013, 2015); Lisboa et al., (2015); Conceição et al., (2015); Peixoto et al., (2020, 2021); Miranda et al., (2020); Pietrobon et al., (2021) e Rodrigues et al., (2021).

Figura 2 - Principais características da prole fêmea adulta oriunda de mães expostas à nicotina na lactação



Legenda: **4HNE:** 4-hidroxinonenal; **α2-AR:** Receptor adrenérgico do tipo α2; **AKTp:** Proteína quinase B fosforilada; **AR:** Receptor de androgênio; **D1:** Desiodase tipo 1; **ER-α:** Receptor de estrogênio alfa; **GLUT2:** Transportador de glicose do tipo 2; **GLUT4:** Transportador de glicose do tipo 4; **GPx:** Glutationa peroxidase; **IRβ:** Subunidade beta do receptor de insulina; **IRS1:** Substrato do receptor de insulina 1; **miR:** micro RNA; **OGTT:** Teste oral de tolerância à glicose; **PDX1:** Fator de transcrição homeobox pancreático e duodenal 1; **RNAm:** RNA mensageiro; **SCD1:** Estearoil-CoA desaturase-1; **SNS:** Sistema nervoso simpático; **SOD:** Superóxido dismutase; **T3:** Triiodotironina; **T4:** Tiroxina; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TPO:** Tireoperoxidase; **TSHr:** Receptor de tireotrofina.

Fonte: Disponíveis em: Peixoto et al., (2020, 2021); Miranda et al., (2020); Pietrobon et al., (2021) e Rodrigues et al., (2021).

Efeitos da restrição ao leite materno ao final da lactação

Interessantemente, impactos metabólicos muito parecidos são observados em descendentes adultos provenientes de duas condições opostas no início da vida. Por exemplo, tanto a oferta excessiva como a escassez de nutrientes durante a gestação podem causar sobrepeso, acúmulo de gordura abdominal, alterações na homeostase glicêmica e lipídica e maior incidência de comorbidades associadas, como diabetes mellitus tipo 2 (DM2) e doenças cardiovasculares na vida adulta (ABENHAIM et al., 2007; SEGOVIA et al., 2014).

A desnutrição infantil pode ser resultante da privação do aleitamento materno, impedindo que a criança receba os nutrientes necessários para seu crescimento e

desenvolvimento. O aleitamento exclusivo é definido pela Organização Mundial da Saúde (OMS) como o consumo exclusivo de leite materno, durante os seis primeiros meses de vida, sem consumo de qualquer outro tipo de alimento, suco ou até mesmo água (WHO, 2001). Assim, a introdução de qualquer tipo de alimento na dieta de uma criança antes dos 6 meses de vida é considerado desmame precoce (CANDEIAS et al., 1983). Apesar dos inúmeros benefícios relacionados ao aleitamento exclusivo durante os seis primeiros meses de vida, este ocorre em apenas 45,7% da população brasileira (MS, 2020). Essa "epidemia de desmame precoce" está associada com a redução da performance intelectual, assim como alterações no perfil lipídico, infecções intestinais e respiratórias, alergias, desenvolvimento cardiovascular, DM2, sobrepeso e obesidade ao longo da vida (HORTA et al., 2007; EIDELMAN, 2012; YIN et al., 2012; WHO, 2013; HORTA et al., 2015; LEWANDOWSKI et al., 2016; VICTORA et al., 2016; HORTA et al., 2018).

Neste contexto, nosso laboratório desenvolveu dois modelos animais de desmame precoce, com o objetivo principal de estudar os mecanismos envolvidos na interrupção do aleitamento materno antes do recomendado pela OMS. Nos dois modelos, os filhotes não ficam separados da mãe e o que os diferencia é a forma de cessar a oferta de leite nos três últimos dias de lactação. Ressaltamos que um dia da vida do rato equivale a cerca de 9 dias da vida humana (QUINN, 2005). Assim, o desmame precoce de 3 dias equivalem a aproximadamente um mês de vida do bebê humano, ou seja, o nosso modelo experimental simula a amamentação exclusiva por apenas cinco meses e não seis, como o recomendado pela OMS.

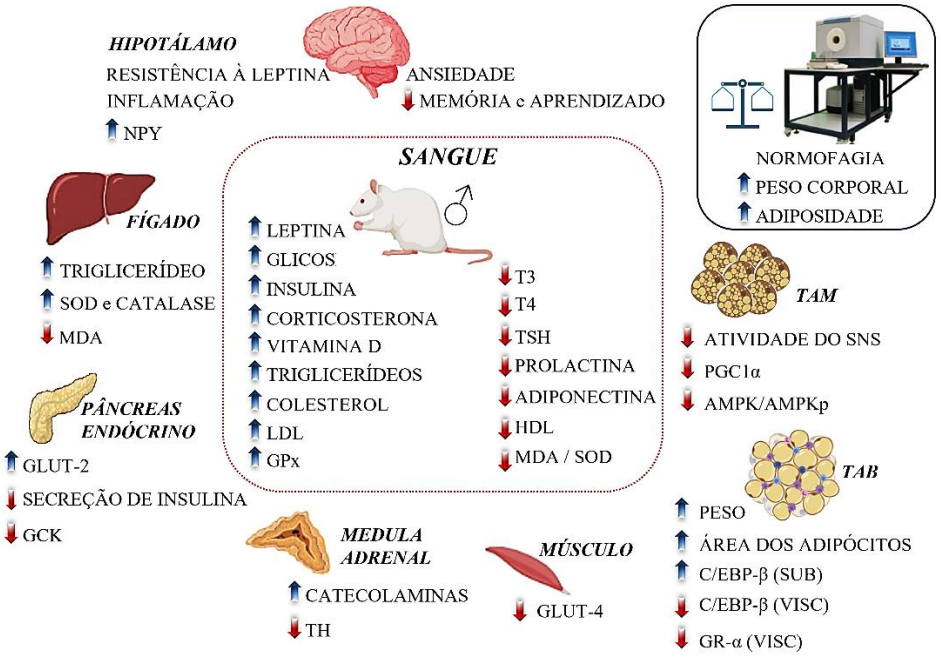
O primeiro modelo, está relacionado ao tratamento materno com bromocriptina, agonista dopaminérgico tipo 2 (inibidor de prolactina), que induz hipoprolactinemia com consequente redução da produção de leite (BONOMO et al., 2005). Neste modelo farmacológico de indução do desmame precoce pelo uso de bromocriptina (modelo BRO), as ratas lactantes recebem 1 mg de bromocriptina administrada intraperitonealmente em 2 doses/dia durante os três últimos dias prévios ao desmame padrão em roedores (BONOMO et al., 2005). Observamos que aos 21 dias de vida (idade padrão para o desmame de ratos), os filhotes machos são hiperleptinêmicos e apresentam menor peso corporal e adiposidade (BONOMO et al., 2005). Quando adultos, esses animais possuem sobrepeso acompanhado de hiperleptinemia, dislipidemia, além de resistência à insulina caracterizada pela hiperglicemia, normoinsulinemia e hipoadiponectinemia (MOURA et al., 2009; PEIXOTO-SILVA et al., 2014; figura 3).

O segundo modelo de desmame precoce é não-farmacológico (modelo DP), sendo realizado pelo uso de uma barreira física. As ratas lactantes são enfaixadas com uma atadura

para impedir o acesso dos filhotes aos mamilos nos últimos três dias de lactação, impedindo a sucção pelos filhotes (LIMA et al., 2011). Aos 21 dias de vida, os filhotes machos possuem baixo peso e gordura, são hipoleptinêmicos, hipoglicêmicos e hipoinsulinêmicos (LIMA et al., 2011). Na vida adulta, a prole macho desenvolve sobrepeso, apresentando aumento da adiposidade visceral, hiperfagia, hiperleptinemia, hipertrigliceridemia, hiperglicemia, normoinsulinemia e hipoadiponectinemia, o que pode estar relacionado ao desenvolvimento de resistência à insulina (LIMA et al., 2011; LIMA et al., 2013; LIMA et al., 2014; NOBRE et al., 2016; figura 5).

A partir de 2020, publicamos o fenótipo das fêmeas programadas pelo desmame precoce. Fêmeas BRO adultas são normofágicas e normoleptinêmicas, apresentam maior ganho de peso corporal, acúmulo de gordura visceral e massa adiposa associada a hipertrofia dos adipócitos retroperitoneais, além de redução de estradiol sérico (PIETROBON; BERTASSO et al., 2020). Fêmeas BRO também apresentam hipercorticosteronemia (MIRANDA et al., 2020), redução da atividade do sistema nervoso simpático no tecido adiposo marrom (TAM), relacionado a redução da expressão de marcadores de termogênese neste tecido, além de redução do conteúdo de catecolaminas na adrenal (PEIXOTO et al., 2020b) e de secreção de insulina (PIETROBON et al., 2020). As fêmeas DP são hiperfágicas, hiperleptinêmicas, apresentam acúmulo de gordura visceral associada a hipertrofia dos adipócitos retroperitoneais (PIETROBON; BERTASSO et al., 2020), além de redução da secreção de insulina (PIETROBON et al., 2020) e do conteúdo de catecolaminas na adrenal, e da expressão de marcadores de termogênese no TAM (PEIXOTO et al., 2020b). As principais características da prole fêmea destes dois modelos de programação metabólica pelo desmame precoce estão dispostas nas figuras 4 e 6, respectivamente.

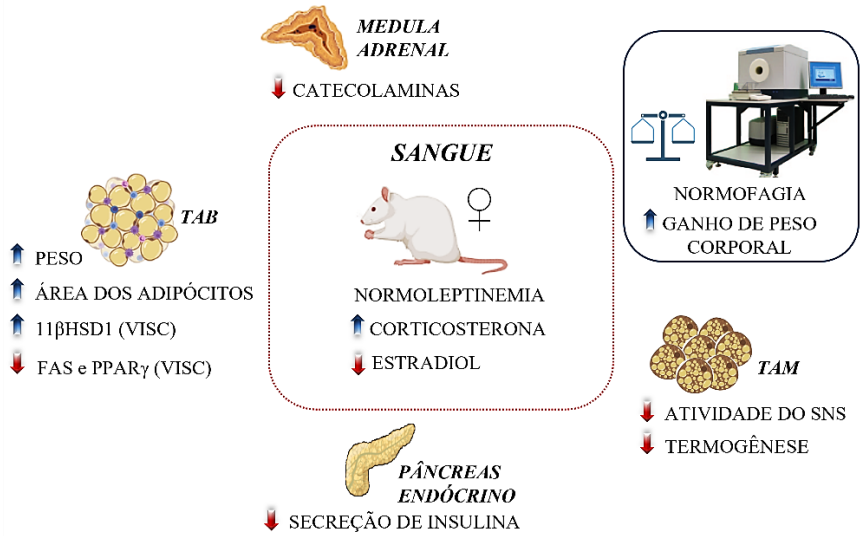
Figura 3 - Principais características dos machos adultos que foram desmamados precocemente pela administração materna de bromocriptina (BRO)



Legenda: **AMPK:** Proteína quinase ativada por AMP; **AMPKp:** Proteína quinase ativada por AMP fosforilada; **C/EBP-β:** Proteína de ligação ao intensificador beta; **GCK:** Glicoquinase; **GLUT2:** Transportador de glicose do tipo 2; **GPx:** Glutationa peroxidase; **GR-α:** Receptor de glicocorticóide alfa; **HDL:** Lipoproteína de alta densidade; **LDL:** Lipoproteína de baixa densidade; **MDA:** Malondialdeído; **NPY:** Neuropeptídeo Y; **PGC1α:** Co-ativador-1 do receptor ativado por proliferador do peroxissoma alfa; **SOD:** Superóxido dismutase; **SNS:** Sistema nervoso simpático; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TH:** Tirosina hidroxilase; **TSH:** Tireotrofina; **T3:** Triiodotironina; **T4:** Tiroxina.

Fonte: Disponíveis em: Bonomo et al., (2007, 2008); Moura et al., (2009); Passos et al., (2011); Albuquerque-Maia et al., (2014); Fraga et al., (2014); Peixoto-silva et al., (2014); Younes-Rapozo et al., (2015)b; Pietrobon e Bertasso et al., (2020); Miranda et al., (2020); Peixoto et al., (2020) e Pietrobon et al., (2020).

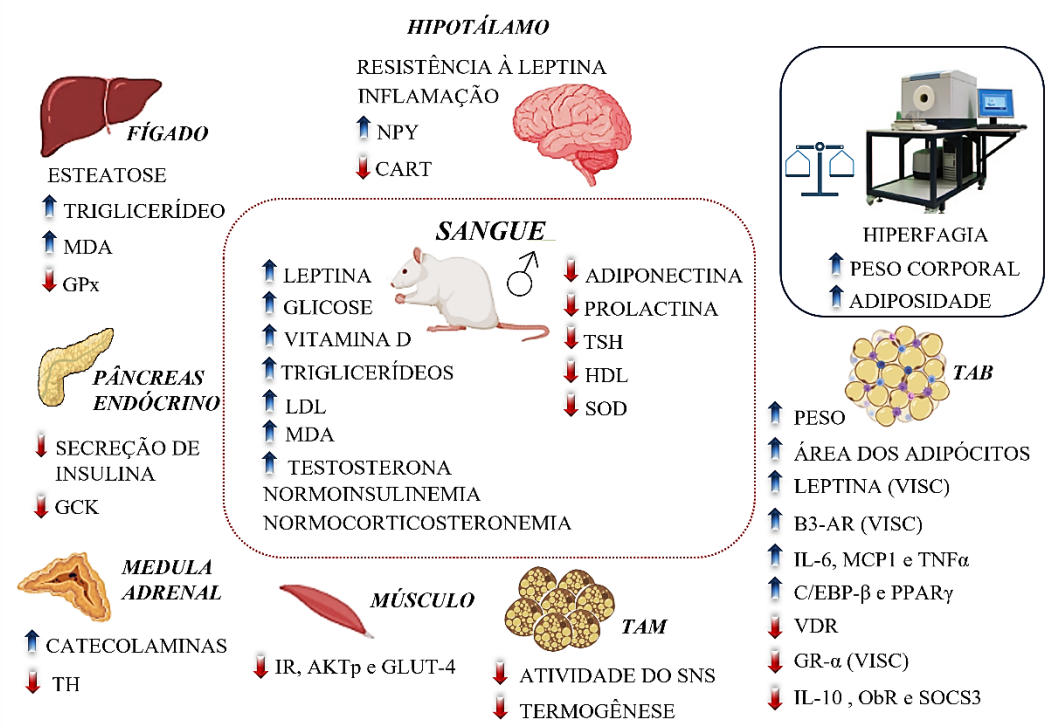
Figura 4 - Principais características das fêmeas adultas que foram desmamados precocemente pela administração materna de bromocriptina (BRO)



Legenda: **FAS:** Ácido graxo sintase; **PPARγ:** Receptores ativados por proliferador de peroxissoma gama; **SNS:** Sistema nervoso simpático; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **VISC:** TAB visceral; **11βHSD1:** 11β-hidroxiesteróide desidrogenase tipo 1.

Fonte: Disponíveis em: Pietrobon e Bertasso et al., (2020); Miranda et al., (2020); Peixoto et al., (2020) e Pietrobon et al., (2020).

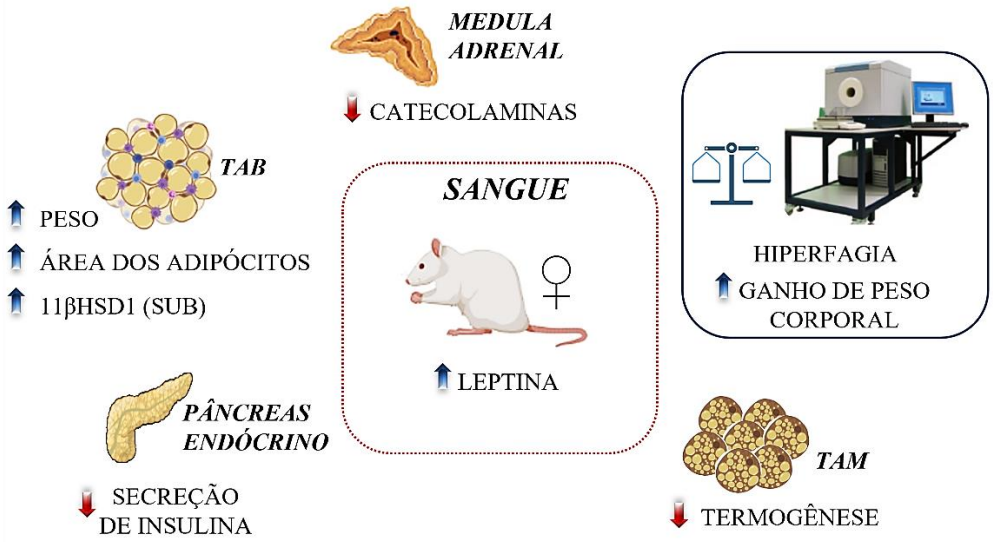
Figura 5 - Principais características dos machos adultos que foram desmamados precocemente no modelo não farmacológico (DP)



Legenda: **β3-AR:** Receptor adrenérgico beta 3; **CART:** Fator de transcrição cocaína-anfetamina dependente; **C/EBP-β:** Proteína de ligação ao intensificador beta; **GCK:** Glicoquinase; **GPx:** Glutationa peroxidase; **GRα:** Receptor de glicocorticóide alfa; **HDL:** Lipoproteína de alta densidade; **IL-6:** Interleucina 6; **IL-10:** Interleucina 10; **LDL:** Lipoproteína de baixa densidade; **MCP-1:** Proteína-1 quimioatrativa de monócitos; **MDA:** Malondialdeído; **NPY:** Neuropeptídeo Y; **ObR:** Receptor de leptina; **PPARγ:** Receptores ativados por proliferador de peroxissoma; **SNS:** Sistema nervoso simpático; **SOD:** Superóxido dismutase; **SOCS3:** Supressor da sinalização de citocinas 3; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TH:** Tirosina hidroxilase; **TNF-α:** Fator de necrose tumoral α; **TSH:** Tireotrofina**; VDR:** Receptor de vitamina D; **VISC:** TAB visceral.

Fonte: Disponíveis em: Lima et al., (2011, 2013); Younes-Rapozo et al., (2012); Franco et al., (2013); Albuquerque-Maia et al., (2014); Nobre et al., (2016); Pietrobon e Bertasso et al., (2020); Miranda et al., (2020); Peixoto et al., (2020) e Pietrobon et al., (2020).

Figura 6 - Principais características das fêmeas adultas que foram desmamados precocemente no modelo não farmacológico (DP)



Legenda: **11βHSD1:** 11β-hidroxiesteróide desidrogenase tipo 1; **SUB:** TAB subcutâneo; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom.
Fonte: Pietrobon e Bertasso et al., (2020); Miranda et al., (2020); Peixoto et al., (2020) e Pietrobon et al., (2020).

Efeitos da dieta materna restrita em proteínas sobre o metabolismo dos descendentes

O direito à alimentação adequada e saudável é garantido pela Constituição Federal e reafirmado pelo Estatuto da Criança e do Adolescente (ECA, Lei nº 8.069, de 13 de julho de 1990; BRASIL, 2019). Na história brasileira, a trajetória intelectual de Josué de Castro se confunde com os marcos norteadores da Política de Segurança Alimentar. Sua trilogia de livros: Geografia da Fome (1946), Geopolítica da Fome (1951) e o Livro Negro da Fome (1960) trouxe essa temática para a agenda política do país (SILVA; NUNES, 2017). Entretanto, o direito básico a alimentação ainda não é garantido, plenamente. O atual aumento da insegurança alimentar está associado a práticas de alimentação infantil subótima, relacionadas ao fornecimento insuficiente de leite por mulheres em estado de insegurança alimentar e agravado pela pandemia de COVID-19 (GAIN, 2020; ROBERTON et al., 2020; UNICEF, OMS, PMA e FAO, 2020; WHO, 2020; ROSEBOOM et al., 2021). Por exemplo, no Ceará, um dos estados mais pobres do Brasil, durante a pandemia houve aumento de 15,5% de mães sob insegurança alimentar (julho a agosto de 2020), principalmente relacionado à redução do emprego formal (ROCHA et al., 2021). Neste contexto, em populações de baixa renda, a substituição de

proteínas por carboidratos é uma prática antiga e comum; adicionar carboidratos à mamadeira, na tentativa de fortalecê-la, reduz o teor de proteínas do leite (TEODÓSIO et al., 1990).

O baixo peso materno e as deficiências de micronutrientes específicos durante a gestação podem resultar em atraso no crescimento intrauterino, baixo peso ao nascer, distúrbios metabólicos, dislipidemias e risco de arterosclerose (RAVELLI, et al., 1998; SZOSTAK-WEGIEREK, 2014). Nesse contexto, diferentes modelos animais experimentais empregam a restrição de determinados nutrientes, oxigênio e hormônios na tentativa de se compreender os efeitos deletérios da programação metabólica sobre o desenvolvimento dos descendentes (SEDAGHAT; ZAHEDIASL; GHASEMI, 2015).

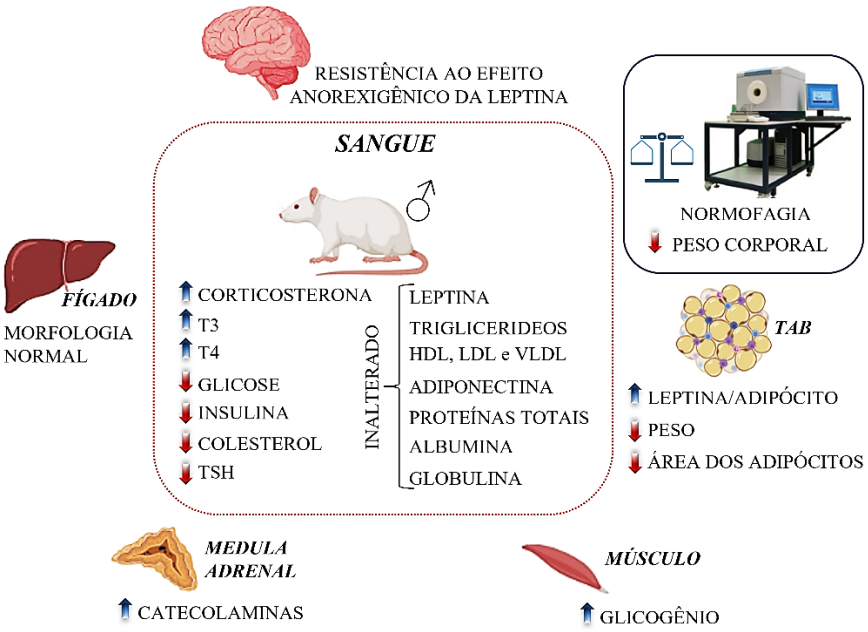
Entre as diversas formas de desnutrição estudadas ao longo dos anos, a restrição protéica (RP) tem atenção especial devido ao fato das proteínas serem um dos nutrientes mais importantes, contendo aminoácidos essenciais para a síntese de proteínas estruturais, enzimas, neuropeptídios e neurotransmissores (VALADARES et al., 2010). A gestação é a etapa do desenvolvimento mais estudada na literatura, no entanto, como o desenvolvimento de alguns órgãos não está completo ao nascimento na maioria dos mamíferos, o período de aleitamento também constitui um período importante para a maturação de muitos sistemas fisiológicos, em aspecto histológico e funcional (PLAGEMANN et al., 1992). Assim, nos propusemos estudar o impacto da oferta de uma dieta RP exclusivamente durante a lactação de ratos Wistar sobre o metabolismo de sua prole.

O nosso laboratório estuda os efeitos da RP na mãe lactante e na prole até a idade adulta, dia 180 pós-natal (PN180). O modelo utilizado é de restrição através da dieta, composta por ração contendo 8% de proteína em relação à ração padrão (23%) (PASSOS; RAMOS; MOURA, 2000). As ratas lactantes apresentam hipofagia, redução do peso corporal e da produção de leite (PASSOS; RAMOS; MOURA, 2000), hiperleptinemia e hipoprolactinemia (LISBOA et al., 2006) no desmame da prole.

Durante a lactação, os filhotes machos apresentam redução de peso corporal e, mesmo após o desmame, quando passam a receber dieta padrão, o peso corporal reduzido é mantido (PASSOS; RAMOS; MOURA, 2000). O conteúdo de proteínas totais, albumina e globulina é reduzido no soro dos filhotes ao desmame (FAGUNDES et al., 2007). Esses animais também apresentam aumento de adiponectina sérica e glicogênio hepático (FAGUNDES et al., 2009) e hiperleptinemia, o que já era esperado uma vez que as mães RP nesta fase também apresentam hiperleptinemia, sugerindo que este hormônio pode ser transferido para a prole através do leite (LISBOA et al., 2006). Quando adulta, a prole macho torna-se normoleptinêmica e normofágica. A administração aguda e sistêmica de leptina neste modelo não promoveu

redução da ingestão alimentar, sugerindo um quadro de resistência hipotalâmica a este hormônio (PASSOS et al., 2004). Além disso, foi observada hipoglicemia, hipoinsulinemia e redução do colesterol sérico nestes animais (FAGUNDES et al., 2007; FAGUNDES et al., 2009). As principais características deste modelo estão descritas na figura 7.

Figura 7 - Principais características dos animais adultos do sexo masculino cujas mães se alimentaram de dieta RP durante a lactação



Legenda: **HDL:** Lipoproteína de alta densidade; **LDL:** Lipoproteína de baixa densidade; **TAB:** Tecido adiposo branco; **TSH:** Tireotrofina; **T3:** Triiodotironina; **T4:** Tiroxina; **VLDL:** Lipoproteína de muito baixa densidade.

Fonte: Passos et al., (2000, 2004); Teixeira et al., (2002); Dutra et al., (2003); Vicente et al., (2004); Moura et al., (2007); Fagundes et al., (2007, 2009); Lisboa et al., (2008, 2012); Corrêa et al., (2011).

Metabolismo hepático e doença hepática gordurosa não alcoólica (DHGNA)

Em estado pós-prandial, os metabólitos provenientes da digestão são transportados até o fígado, por meio do sistema porta hepático. A glicose é transportada por um processo de difusão facilitada para o interior do hepatócito, principalmente pelo transportador de glicose tipo 2 (GLUT2), que não é sensível à insulina e possui elevado Km para a glicose, operando, portanto, abaixo dos níveis de saturação mesmo sob altas concentrações de glicose. No citoplasma do hepatócito, a glicose é então fosforilada à glicose-6-fosfato por ação da enzima glicoquinase (GCK), que se diferencia das demais hexoquinases por possuir alto Km para

glicose e não ser inibida pelo seu produto. Essa característica torna a GCK adequada para atuar em concentrações relativamente altas de glicose existentes na célula hepática, mas também para direcionar o fluxo dos carbonos da glicose para a via glicolítica e para a síntese de glicogênio. A GCK é sensível à insulina, acelerando a fosforilação da glicose. Esta ação associada a ativação da glicogênio sintetase e a inibição simultânea da glicogênio fosforilase, estimula a síntese e armazenamento de glicogênio (RUI, 2014).

Quando a capacidade de armazenamento de glicogênio hepático é saturada, o excesso de glicose é redirecionado para síntese de ácidos graxos (AG), processo denominado de lipogênese *de novo* hepática (RUI, 2014). Neste momento, a glicose entra no fluxo glicolítico gerando gliceraldeído-3-fosfato (G3P), fosfoenolpiruvato e piruvato, por meio da enzima piruvato quinase (YAMASHITA et al., 2001). A oxidação do piruvato produzido gera acetil-CoA, o qual é conduzido à mitocôndria para se integrar ao ciclo do ácido tricarboxílico (TCA). A elevada concentração de ATP e fosfato de dinucleotídeo de nicotinamida e adenina (NADPH), decorrente do estado alimentado, inibe a progressão do citrato no TCA, induzindo ao acúmulo mitocondrial deste metabólito. Ocorre então o transporte de citrato ao citoplasma do hepatócito, sendo convertido à acetil-CoA (YAMASHITA et al., 2001). A enzima acetil-CoA carboxilase (ACC) catalisa a transformação de acetil-CoA à malonil-CoA, o qual é transformado em ácido palmítico por adições repetidas de grupos acetil pelo complexo enzimático ácido graxo sintase (FAS). O ácido palmítico pode ser desaturado pela enzima estearoil-CoA desaturase-1 (SCD-1), formando AG monoinsaturados. A enzima glicerol-3-fosfato aciltransferase catalisa a esterificação do G3P com o AG monoinsaturado recém-sintetizado, gerando ácido lisofosfatídico (LPA). O LPA é substrato para a enzima acilglicerol fosfato aciltransferase catalisar a formação de ácido fosfatídico (PA). O PA é processado à diacilglicerol (DAG) pela enzima lipina, seguido pela formação de triglicerídeos (TG) por ação da enzima acil-CoA:diacilglicerol aciltransferase (DGAT) (BECHMANN et al., 2012; KAWANO; COHEN, 2013).

Tanto os TG hepáticos provenientes da lipogênese *de novo* quanto os oriundos da dieta são transportados à periferia pelas lipoproteínas de densidade muito baixa (VLDL). A síntese desta lipoproteína tem início no retículo endoplasmático do hepatócito através da proteína de transferência de triglicerídeos microssomal (MTTP). Essa proteína realiza a transferência de fosfolipídeos, TG e colesterol (COL) para a apolipoproteína B (apoB) dando origem a molécula de pré-VLDL. Posteriormente, essa molécula se une a MTTP, formando a VLDL madura, a qual é secretada pelo fígado (MCGARRY; FOSTER, 1980; TOMKIN; OWENS, 2015).

No estado de jejum, o fígado atua no controle da homeostase glicêmica sintetizando glicose a partir de aminoácidos, lactato e glicerol. Este processo denominado gliconeogênese, consiste na reversão da via glicolítica. A etapa desta via catalisada pela piruvato quinase é contornada, produzindo fosfoenol-piruvato a partir de piruvato, pela ação da enzima piruvato carboxilase e fosfoenolpiruvato-carboxiquinase (PEPCK) (HATTING et al., 2018). A etapa catalisada pela fosfofrutoquinase é revertida pela frutose-1,6-difosfatase, que culminam na formação de glicose-6-fosfato, que pode ser direcionada para a síntese de glicogênio ou glicose, por ação da enzima glicose-6-fosfatase (G6Pase) (JITRAPAKDEE et al., 2012). A insulina exerce importante papel regulatório sobre a síntese hepática de glicose, justamente por inibir gliconeogênese e glicogenólise (ROPELLE et al., 2009).

No jejum, os TG armazenados no tecido adiposo necessitam ser mobilizados para fornecimento energético. O TG dos adipócitos é hidrolisado, liberando ácidos graxos livres (AGL) e glicerol. No sangue, os AGL são transportados pela albumina até os tecidos, entre eles o fígado (TANG et al., 2017). No hepatócito, os ácidos graxos de cadeia longa (AGCL) são oxidados via β-oxidação para manutenção das quantidades de ATP necessária, o que pode ocorrer na mitocôndria ou no peroxissomo (MURTHY; PANDE, 1994; LIMA et al., 2005). Na β-oxidação mitocondrial, os AGL sofrem esterificação com a coenzima A na membrana mitocondrial externa, formando tio-ésteres Acil-CoA graxos. Estes interagem com a enzima carnitina palmitoil transferase 1α (CPT-1α), localizada na membrana externa da mitocôndria e responsável por sua conversão em ésteres graxos do tipo Acil-carnitina, que são então conduzidos através da membrana interna até a matriz mitocondrial, onde o grupo Acil-CoA graxo interage com a carnitina palmitoil transferase 2 (CPT-2), formando novamente acilCoA (KERNER; HOPPEL, 2000). Em seguida, o produto da CPT-2 passa por ciclos de β-oxidação, onde NADH e FADH também são formados, os quais transferem seus elétrons para a cadeia respiratória, que culminam com a geração de ATP (PESSAYRE et al., 2001).

Durante o jejum e em condições que simulam o estado de baixa energia, as funções dos PGC-1α (Co-ativador-1 do receptor ativado por proliferador do peroxissoma alfa) são reguladas, coativando fatores de transcrição e receptores nucleares promotores da biogênese mitocondrial, fosforilação oxidativa e β-oxidação de AG, visando neutralizar o estresse metabólico induzido pela escassez de alimentos (PICCININ; VILLANI; MOSCHETTA, 2019). A função do PGC-1α é principalmente estimulada pela Sirtuina-1 (SIRT-1), que regula o metabolismo energético celular através da desacetilação de fatores de transcrição e cofatores do metabolismo energético de acordo com o estado nutricional, de modo que a redução de SIRT-1 é associada a obesidade e o aumento a perda de peso (KOSGEI et al., 2020).

As proteínas de ligação do elemento regulador de esterol (SREBP) são fatores de transcrição que regulam a expressão de genes envolvidos na síntese de COL (SREBP-2), AG (SREBP-1c) ou ambos (SREBP-1b). Estudos mostram que a insulina ativa a SREBP-1c que, por sua vez, estimula a transcrição dos genes lipogênicos (ACC, FAS e SCD-1) e por consequência, a lipogênese hepática (FORETZ et al., 1999; AZZOUT-MARNICHE et al., 2000). Outro fator de transcrição, a proteína de ligação do elemento responsivo à carboidratos (ChREBP) induzido pela glicose, independente da insulina, estimula a expressão de genes glicolíticos, tais como a piruvato quinase hepática (LPK), a glicose-6-fosfatase (G6Pase), a glicoquinase (GCK) e genes lipogênicos (DENTIN et al., 2004; ISHII et al., 2004). O receptor X do fígado (LXR), ativa a transcrição direta dos genes lipogênicos via ChREBP, e indiretamente através da SREBP-1c (CHA; REPA, 2007). Outro membro da família de receptores X é o *farnesoid X receptor* (FXR), importante regulador na homeostase glicêmica e lipídica no fígado (FORMAN et al., 1995; LU et al., 2000; ZHANG; KAST-WOELBERN; EDWARDS, 2003). Sua ativação realiza a redução de TG por diversos mecanismos, sendo os principais: redução da expressão de SREBP-1c e LXR, com consequente redução da lipogênese e indução da β-oxidação através do aumento da expressão do receptor ativado pelo proliferadores de peroxissomo-α (PPAR-α) (MODICA; GADALETA; MOSCHETTA, 2010; TEODORO; ROLO; PALMEIRA, 2011), o principal regulador da β-oxidação dos AGCL nas mitocôndrias e nos peroxissomos (HUANG et al., 2012).

Situações adversas como a privação de nutrientes, variações no nível de cálcio, estresse oxidativo e inibição da glicosilação podem levar ao acúmulo de proteínas mal enoveladas no lúmen do retículo endoplasmático (RE). Este acúmulo pode causar estresse que, por sua vez, pode ativar vias de transdução de sinal intracelulares na tentativa de manter a homeostase celular, conhecida como *Unfolded Protein Response* (UPR) (SONG; MALHI, 2019). As principais proteínas sensoras de estresse do RE que estão envolvidas na indução da UPR são: IRE1 (*Inositol-requiring enzyme* 1), PERK (*PKR-like ER kinase*) e ATF6 (*activating transcription factor* 6). No estado basal, estas três proteínas estão inativadas pela associação com a chaperona BiP mas, durante o estresse de RE, BiP dissocia-se de seus ligantes para auxiliar no dobramento de proteínas, o que permite a ativação de IRE1, PERK e ATF6 (GHEMRAWI; BATTAGLIA-HSU; ARNOLD, 2018). A IRE1 sofre autofosforilação e induz *splicing* de XBP1 (*X Box Protein 1*), que estimula genes que codificam a maquinaria de dobramento de proteínas, genes ERAD (*Endoplasmic Reticulum Associated Degradation*) e vias de síntese de lipídios. A PERK fosforila eIF2α (fator de iniciação da tradução eucariótica 2α) que suprime a tradução de mRNA levando à atenuação da síntese de proteínas. O ATF4 é

traduzido seletivamente e estimula vários alvos transcricionais, incluindo CHOP (C/EBP-Homologous Protein). O ATF6α clivado, denominado ATF6N, estimula a transcrição dos genes alvo UPR. De modo geral, a ativação dessas proteínas aumenta a expressão de chaperonas, para evitar a agregação e facilitar o correto dobramento das proteínas; em paralelo, inibe a tradução de novas proteínas e aumenta a degradação das proteínas mal enoveladas por meio da degradação associada ao RE. No entanto, quando o estresse se torna mais severo ou persistente, a UPR não consegue reestabelecer a homeostase da célula e pode ativar mecanismos de morte celular, principalmente por apoptose (MOHAN, S.; BROWN, L.; AYYAPPAN, 2019).

Outra via importante para a manutenção da homeostase lipídica do fígado é a macroautofagia, capaz de reciclar organelas e substratos reduzindo o estresse celular (CZAJA, 2016). Essa via pode ser ativada pelo estresse de retículo e oxidativo (MAIERS; MALHI, 2019) e envolve o recrutamento de uma cascata enzimática que forma um fagossomo responsável por sequestrar a estrutura a ser degradada. Os componentes celulares alvos para a degradação autofágica são reconhecidos e transportados para a degradação por meio de proteínas adaptadoras responsáveis pela conexão entre essas duas etapas (GALLUZZI; GREEN, 2019). A primeira proteína com função adaptadora identificada foi a proteína p62 (SQSTM1), que apresenta um domínio UBA (domínio de ligação com proteínas ubiquitinadas) que se liga aos alvos de degradação e o domínio LIR (domínio de ligação à LC3), responsável por entregar os alvos à degradação, desempenhando um papel importante para o fluxo autofágico (LEE et al. 2020). Por fim, a degradação autofágica finaliza quando o autofagossomo se funde aos lisossomos e as enzimas lisossomais proporcionam o ambiente de degradação e reciclagem do conteúdo entregue (NAKATOGAWA, 2020).

Alterações nos processos descritos acima, que levam ao desequilíbrio na captação e eliminação de TG nos hepatócitos, podem levar ao acúmulo de TG no citoplasma e, consequentemente, ao desenvolvimento da doença hepática gordurosa não alcoólica (DHGNA) (ÂNGULO, 2002; MCCULLOUGH, 2006; ZÁMBÓ et al., 2013; BERLANGA et al., 2014). A DHGNA ou NAFLD, do inglês *Nonalcoholic Fatty Liver Disease* é caracterizada pela deposição de lipídeos nos hepatócitos cujo quadro patológico se assemelha ao da lesão hepática induzida pelo álcool, porém ocorre em indivíduos sem histórico de consumo abusivo da droga (DIEHL et al., 1999; ÂNGULO, 2002; MARCHESINI et al., 2003).

A DHGNA inclui um espectro de doenças hepáticas crônicas variando de simples esteatose hepática, onde o indivíduo apresenta apenas acúmulo de lipídeos, até esteato-hepatite não alcoólica (ENGIN, 2017; SCHUPPAN; SURABATTULA; WANG, 2018). Nesse caso, além do acúmulo de lipídeos, ocorre a presença de inflamação e degeneração hepatocelular,

que pode estar acompanhada do aparecimento de fibrose e até mesmo evoluir para a cirrose hepática e carcinoma hepatocelular (CHALASANI et al., 2012; WILLEBRORDS et al., 2015). Essa doença permaneceu pouco conhecida até a década de 1980, mas atualmente está entre as doenças hepáticas crônicas mais comuns, ocupando a primeira posição em países ocidentais (TOLMAN; DALPIAZ, 2007). A sua prevalência mundial tem aumentado, abrangendo de 6 a 35% da população em geral de diferentes países (VERNON; BARANOVA; YOUNOSSI, 2011). Assim espera-se que a DHGNA se torne a principal causa de doença hepática terminal, transplante de fígado e carcinoma hepatocelular da próxima década (CALZADILLA BERLOT; ADAMS, 2016).

O mecanismo molecular subjacente à progressão da DHGNA não é completamente compreendido, sua patogênese tem sido interpretada pela hipótese de "duplo-*hit*". O "primeiro *hit*" inclui o acúmulo de lipídeos no fígado, seguido pelo "segundo *hit*", onde mediadores pró-inflamatórios induzem a inflamação, lesão hepatocelular e fibrose (DAY; JAMES, 1998).

A característica do "primeiro *hit*" da DHGNA é o acúmulo de TG no citoplasma dos hepatócitos em decorrência do desequilíbrio entre a entrada e a saída de lipídeos. Ocorre o aumento na absorção hepática de AGL derivados da circulação, devido ao aumento da lipólise do tecido adiposo e/ou a partir da dieta em forma de quilomicron. Ocorre também o aumento na concentração de glicose e insulina em resposta à ingestão de carboidratos, que promovem a lipogênese *de novo*. Esses eventos são acompanhados pela diminuição da oxidação mitocondrial de AGL e diminuição na secreção hepática de TG na forma de VLDL. Ou seja, em pacientes com DHGNA o aumento na captação de AG e lipogênese *de novo*, não são compensados pela oxidação de AGL e produção de VLDL (ANSTEE; GOLDIN, 2006; BERLANGA et al., 2014).

O "segundo *hit*" é geralmente atribuído ao estresse oxidativo, que causa peroxidação lipídica na membrana do hepatócito, e também a produção de citocinas que são, em parte, responsáveis pela progressão de esteatose hepática até esteato-hepatite não alcoólica e cirrose. Toxinas bacterianas, hiperprodução de citocinas, especialmente, o fator de necrose tumoral-α, alteração dos estoques de ATP e da atividade da enzima citocromo P450 Cyp2E1 parecem ser gatilhos importantes para a progressão da doença e fibrogênese (DAY; JAMES, 1998; ÂNGULO, 2002; SASS; CHANG; CHOPRA, 2005).

A disfunção hepática é um achado comum em diversos modelos de programação metabólica, possivelmente em decorrencia das alterações metabólicas apresentadas pelo modelo experimental, mas também por mecanismos adaptativos que podem ocorrer no fígado em resposta ao insulto perinatal (WESOLOWSKY et al., 2017). Durante o desenvolvimento

fetal e ao longo dos primeiros dias de vida, o fígado é muito vulnerável às alterações fisiológicas materna, além de ser um órgão essencial no metabolismo de carboidratos, lipídios e proteínas (DHILLON; STEADMAN, 2012). Os modelos experimentais alvos desta Tese apresentam disfunções metabólicas sugestivas de alterações hepáticas que ainda não foram completamente elucidadas.

A literatura relata vários efeitos da restrição protéica durante a gestação e a lactação sobre o fígado; esses incluem alterações no conteúdo de TG e alterações na atividade de enzimas do metabolismo de ácidos graxos, produção e uso de energia (NICOLÁS-TOLEDO et al., 2018; MENDEZ-GARCIA et al., 2019; SIMÕES-ALVES et al., 2019; OKE; SOHI; HARDY, 2020). Entretanto, nosso grupo observou que a oferta de dieta contendo 8% de proteinas às mães, exclusivamente durante a lactação, não promove alterações no peso ou morfologia do fígado dos filhotes adultos (CORRÊA et al., 2011). Todavia, devido às alterações metabólicas observadas nestes animais programados, acreditamos que as vias hepáticas de manutenção da homeostase glicêmica e lipídica podem estar funcionando de modo alterado para suprir as necessidades do organismo. Dessa forma, nos propusemos estudar mais detalhadamente, a função hepática de machos adultos e, de modo inédito, das fêmeas.

Também temos reportado a relação existente entre o desmame precoce e alterações hepáticas. Tanto o modelo de desmame precoce farmacológico (BRO) quanto o não farmacológico (DP) promovem aumento de triglicerídeos hepáticos, entretanto, o modelo DP promove aumento de estresse oxidativo e, consequente, microsteatose (FRANCO et al., 2013), enquanto o modelo BRO promove o aumento da atividade das enzimas antioxidantes, mantendo preservada a arquitetura do hepatócito (PEIXOTO-SILVA et al., 2014). Portanto, apesar da disfunção metabólica detectada no modelo BRO, a bromocriptina pode prevenir danos hepáticos durante a vida, possivelmente por melhorar o status redox da prole macho (BONOMO et al., 2007; MOURA et al., 2009; LIMA et al., 2011; NOBRE et al., 2012).

O estresse oxidativo, causado pela administração aguda de nicotina pode ser observado em diferentes tecidos como cardíaco (ZHOU et al., 2010), adiposo 3T3L1 (AN et al., 2007), aórtico (XIAO et al., 2011), pancreático (BRUIN et al., 2008) e hepático (HALIMA et al., 2010). De fato, o fígado é um alvo da nicotina, pois seu metabolismo ocorre principalmente neste órgão através do citocromo CYP2A6 (BENOWITZ et al., 2009), contribuindo assim para a produção de espécies reativas de oxigênio (ROS) (KIRBY et al., 2011). A análise dos efeitos hepáticos tardios da exposição materna crônica à nicotina exclusivamente durante a amamentação mostrou que os ratos machos apresentam aumento do conteúdo de triglicerídeos

hepáticos, estresse oxidativo e microesteatose na vida adulta (CONCEIÇÃO et al., 2015). Neste modelo, o fenótipo das fêmeas permanecia desconhecido até a conclusão deste trabalho.

Assim, considerando as evidências de alterações hepáticas nestes três modelos de programação metabólica exclusivamente causados por *imprinting* na lactação, nesta Tese nos propusemos investigar a morfologia hepática e fatores chaves de vias de sinalização envolvidas no controle da homeostase hepática e controle do metabolismo glicêmico e lipídico (gliconeogênese, lipogênese, β-oxidação e montagem de VLDL), cujas adaptações poderiam justificar uma suscetibilidade ao desenvolvimento de esteatose hepática. A prole fêmea adulta foi estudada pela primeira vez pelo nosso grupo, tendo em vista a possibilidade de respostas distintas associadas ao gênero.

1 **OBJETIVOS**

1.1 **Objetivo geral**

Investigar os principais mecanismos relacionados ao desenvolvimento ou não da esteatose hepática em ratos Wistar em modelos de programação metabólica para sobrepeso (exposição materna à nicotina, desmame precoce) e baixo peso (restrição proteica materna), cujo insulto ocorreu durante o período de lactação.

1.2 **Objetivos específicos**

Estudar os efeitos da exposição materna à nicotina (120 e 180 dias de vida), desmame precoce farmacológico e não farmacológico (180 dias de vida) e dieta materna pobre em proteínas (180 dias de vida) nas proles de ambos os sexos sobre:

a) Resposta hepática à administração intraperitoneal de piruvato;
b) Perfil lipídico plasmático e hepático;
c) Morfologia hepática;
d) Expressão protéica de enzimas e fatores transcricionais envolvidos nos principais mecanismos relacionados ao metabolismo de lipídeos e glicose, e manutenção da homeostase celular no fígado.

2 **MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO**

Esse trabalho está organizado sob a forma de 3 manuscritos, sendo dois publicados e um a ser submetido.

2.1 **Modelo experimental I: Exposição materna à nicotina na lactação**

Os itens “Material e Métodos”, “Resultados” e “Discussão” referentes a este modelo de programação estão descritos no artigo abaixo:

2.1.1 Artigo 1 - Programming of hepatic lipid metabolism in a rat model of postnatal nicotine exposure e sex-related differences

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Programming of hepatic lipid metabolism in a rat model of postnatal nicotine exposure – Sex-related differences☆

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Lipids

ABSTRACT

Maternal nicotine exposure during lactation induces liver damage in adult male rats. However, the mechanism in males is unknown and females have not been tested. Here, we determined the liver lipid composition and lipogenic enzymes in male and female offspring at two ages in a model of postnatal nicotine exposure. Osmotic minipumps were implanted in lactating Wistar rat dams at postnatal day (PND) 2 to release 6 mg/kg/day of nicotine (NIC group) or saline (CON group) for 14 days. Offspring received a standard diet from weaning until euthanasia at PND120 (1 pup/litter/sex) or PND180 (2 pups/litter/sex). At PND120, NIC males showed lower plasma triglycerides (TG), steatosis degree 1, higher hepatic cholesterol (CHOL) ester, free fatty acids, monoacylglycerol content as well as acetyl-coa carboxylase-1 (ACC-1) and fatty acid synthase (FAS) protein expression in the liver compared to CON males. At this age, NIC females had preserved hepatocytes architecture, higher plasma CHOL, higher CHOL ester and lower total CHOL content in the liver compared to CON females. At PND180, NIC males showed steatosis degrees 1 and 2, higher TG, lower free fatty acids and total CHOL content in the liver and an increase in ACC-1 hepatic protein expression. NIC females had higher plasma TG and CHOL levels, no change in hepatic morphology, lower CHOL ester and free fatty acids in the liver, which also showed higher total ACC-1 and FAS protein expression. Maternal nicotine exposure induces long-term liver dysfunction, with an alteration in hepatic cytoarchitecture that was aggravated with age in males. Concerning females, despite unchanged hepatic cytoarchitecture, lipid metabolism was compromised, which deserves further attention.

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1. Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is an inclusive term for a group of non-alcohol-related progressive liver disorders (Angulo, 2002) that has become a serious public health problem worldwide, affecting more than half billion people worldwide (Younossi et al., 2018) and currently being the main cause of liver transplantation and carcinoma (Pais et al., 2016; Michelotti et al., 2013). Steatosis is the first manifestation of NAFLD, which is characterized by excessive accumulation of triglycerides (TG). In normal situations, this accumulation is not hepatotoxic and represents a defensive mechanism to balance free fatty acids excess (Koliwad et al., 2010; Yamaguchi et al., 2007). However, increased TG concentration happens simultaneously with toxic metabolites generation, lipotoxicity, liver injury, activation of inflammatory cascades and fibrogenesis (Neuschwander-Tetri, 2010). In this case, steatosis

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progress to non-alcoholic steatohepatitis (NASH) (Berlanga et al., 2014), which may progress to cirrhosis and hepatocellular carcinoma (Charlton et al., 2011).

Dietary lipids, circulating lipids and *de novo* lipogenesis dysregulations in the liver are the three main sources of fatty acids that contribute to excessive TG (Postic and Girard, 2008; Jensen-urstad and Semenkovich, 2012), so other pathways of hepatic lipid disposal, such as impaired fatty acid oxidation, reduced synthesis/secretion of very-low density lipoprotein (VLDL) have less importance in determining lipid accumulation and lipotoxicity in NAFLD (Bertolani and Marra, 2008; Lambert et al., 2014). The underlying mechanism for the development and progression of this disease is complex and multifactorial, where genetic or epigenetic factors affect liver lipid content, enzymatic activity and inflammatory markers, thus influencing the risk of steatosis progression to inflammation and fibrosis, or persistence in a stable stage of NAFLD (Buzzetti et al., 2016).

Several epidemiological and animal studies in the developmental origins of health and disease (DOHaD) concept address the relationship between environmental changes within a critical window of development and alterations in metabolism in adult life (Almeida et al., 2019). Breastfeeding is one of these critical periods, as important cognitive, neurological, and metabolic tissue developments occur, such as that of the liver, providing a unique window of opportunity within the 'first 1000 days' to intervene and reduce lifelong metabolic disease risk, like those associated with NAFLD (Lynch et al., 2017; Ellsworth et al., 2018).

Maternal smoking is associated with adverse fetal, obstetric and developmental outcomes; despite this, approximately 9%–20% of mothers worldwide smoke during pregnancy, an even higher proportion smokes after delivery (Hammoud et al., 2005; Einarson and Riordan, 2009; Warren et al., 2014; Meernik and Goldstein, 2015; Al-Sahab et al., 2010; Dhalwani et al., 2013; Tong et al., 2013). Among all the chemicals that are present in cigarette smoke, nicotine is the major psychoactive one (Wickstrom, 2007). Nicotine is an important environmental pollutant, capable of propagating to soil and atmosphere (Selmar et al., 2018), being found in bottled mineral waters (Alonso et al., 2012) and hair of pregnant woman (Li et al., 2017). Also, nicotine is considered the main component of cigarette capable of disturbing fetal development (Yildiz, 2004). In this context, our group has already demonstrated that rat male offspring exposed to nicotine only during the lactation period show greater body mass gain, higher adiposity and insulin resistance at adulthood (Oliveira et al., 2009), while the female offspring are eutrophic and have normal visceral fat depot (Pinheiro et al., 2011). Regarding the liver, increased hepatic TG content, oxidative stress and microsteatosis were observed in male offspring at PND180 (Conceição et al., 2015); the adult female offspring phenotype remains unknown. Also, it is unknown if the steatosis is secondary to the obesity observed at this age or whether it is programmed simultaneously to the development of the obese phenotype. NAFLD is now considered a multifaceted complex systemic condition (Petta et al., 2016; Hardy et al., 2016), which exhibits a sexual dimorphism (Ballestri et al., 2017) since it is more common in men and postmenopausal women (Browning et al., 2004; Turola et al., 2015; Ballestri et al., 2017). The mechanism by which nicotine causes lipid dysmetabolism and hepatic fat accumulation is poorly understood (Dangana et al., 2019) as is the mechanism by which maternal nicotine exposure during breastfeeding induces long-term liver dysfunctions. Therefore, it is relevant to assess the mechanisms that contribute to the development of manifestations of liver steatosis in the adult progeny at distinct ages and in both sexes in this programming model.

In this context, considering the scarce knowledge regarding the late effects of nicotine exposure via the milk of lactating dams on the liver metabolism of the offspring; the difficulty in determining if the cause of programmed steatosis is an epigenetic phenomena acting on hepatic lipid metabolism or if it is secondary to obesity; and the lack of information on possible sex-differences on the programming effects on lipid metabolism, the present study was designed to address the long lasting effects of maternal nicotine exposure upon the hepatic lipid profile and associated hepatic *de novo* fatty acid biosynthesis markers, hepatocyte morphology and lipid plasma content in 120- (an age at which animals were not overweight) and 180-days-old male and female rats.

2. Material and methods

2.1. Animals

Experiments were carried out in accordance with the protocols approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/015/2017) and in accordance with the Brazilian Law no. 11.794/2008. Wistar rats (3-months old) were housed in a temperature-controlled vivarium (23 ± 1 °C) with artificial light/dark cycles (lights on: 7:00 a.m. to 7:00 p.m.) and free access to water and standard chow (Nuvilab, Sogorb, São Paulo, SP, Brazil). During two weeks, mating was carried out using 2 females per male. After pregnancy detection (done through monitoring of the estrous cycle), dams were housed in individual cages. In the first postnatal day (PND0), litters were adjusted to six pups per dam (3 males and 3 females).

2.2. Experimental model of maternal nicotine exposure only during breastfeeding

On PND2, twenty lactating rats were randomly divided into two groups: 1) Nicotine group (NIC, n = 10), lactating dams were anaesthetized with thiopental (30 mg/kg) for the s.c. implantation of osmotic minipumps (2ML2; Alzet, Cupertino, CA, USA) on their backs. Pumps were prepared with nicotine free-base diluted in a saline solution (NaCl 0.9%) to deliver an initial dose rate of 6 mg/kg/day of nicotine until PN16, as previously published (Oliveira et al., 2009; Pinheiro et al., 2011; Younes-Rapozo et al., 2013; Lisboa et al., 2015). Cotinine content is directly associated with nicotine exposure levels and due to the short half-life of nicotine, it is considered the most reliable measure (Lichtensteiger et al., 1988) of nicotine exposure level. In this experimental design, cotinine levels were detected in the milk and serum of dams at PND15, with levels close to 230 ng/mL, while NIC pups showed levels of 20 ng/mL (Oliveira et al., 2010); these levels are found in typical smokers; 2) Control group (CON, n = 10), lactating dams were implanted with osmotic minipumps containing saline solution.

After weaning (PND21), male and female offspring had free access to water and standard rodent chow (Nuvilab, Sogorb, São Paulo, SP, Brazil) until euthanasia at PN120 (1 pup/sex/litter) and PN180 (2 pups/sex/litter). One month prior to euthanasia, the estrous cycles of all female offspring were monitored daily (at 8:00 a.m.) using a vaginal swab technique (Chuffa et al., 2011, which showed regular cycles (4–5 days). Euthanasia occurred during diestrus.

2.3. Biometric and plasma parameters

Body mass (BM) was measured throughout development. For the euthanasia at PND120 or PND180, rats were anaesthetized with thiopental (i.p. 150 mg/kg of body mass) followed by cardiac puncture. White adipose tissues that constitute the visceral fat

mass – VFM (retroperitoneal, perigonadal, mesenteric) were collected, weighted and expressed as g/100g of body mass. The levels of plasma CHOL and TG were assessed using a commercial kit in accordance with the manufacturer's instructions (Bioclin, Brazil).

2.4. Liver histology

At PND120 and PND180, a liver sample was removed and fixation was performed by 4% paraformaldehyde for 24 h, followed by dehydration in alcohol using progressively higher concentrations and then diaphanization in xylene. The materials were embedded in histological Paraplast® (Sigma-Aldrich MO, USA) and non-consecutive slices (5 μm thickness) were prepared for staining with hematoxylin and eosin (H&E). Ten photomicrographs per animal were randomly acquired with an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan), and projected on a high-resolution LCD flat-panel monitor (LG Electronics, Seoul, South Korea). For volume density of hepatic steatosis (Vv[steatosis, liver]) determination, a test system consisting of 36 test points was used, in accordance with Catta-Preta et al. (2011). Values were classified in accordance with the magnitude of the steatosis, using Brunt's classification: 0 (none to 5% of hepatocytes affected); 1 (>5%–30% affected); 2 (>30%–60% affected) and 3 (>60% affected) (Brunt, 2001).

At PND180, fresh liver samples were immersed in Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) in aluminum molds, frozen quickly in liquid nitrogen, and stored at −80 °C. Non-consecutive 10-μm cryosections were stained by Oil red-O (ORO) and then Harris's hematoxylin was used for counterstain. Photomicrographs (10 per animal) were randomly acquired and the percentage of lipid droplets per analyzed area was determined using the software Image Pro Plus, version 7.01 for Windows (Media Cybernetics, Silver Springs, MD, USA) (Catta-Preta et al., 2011).

2.5. High-performance thin layer chromatography (HPTLC)

Lipid extraction from hepatic samples (±1 mg) was performed using the method of Bligh and Dyer (1959) with modifications, based on chloroform-methanol extraction. Total neutral lipids were assessed by HPTLC using a DC Silica gel 60 plate (Merck Millipore, Germany) immersed in a charring solution (3% $CuSO_4$ and 8% $H3PO_4$ v/v) (Ruiz and Ochoa, 1997). HPTLC plates were analyzed and the quantification of the lipids was obtained by densitometry using Image Master software (Total Lab, New Zealand).

2.6. Western blotting

A fragment of the liver (±30 mg) was homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; SDS 0.1%; 5 mM EDTA; 50 mM NaF; 30 mM $Na_4P_2O_7$; 1 mM Na_3VO_4) containing protease inhibitors cocktail (La Roche Ltd., SW). After centrifugation (7500×g/15 min/4 °C), total protein content of the supernatant was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA). 20 μg of the samples were denatured in sample buffer containing beta-mercaptoethanol for SDS gel electrophoresis with a molecular weight standard (Kaleidoscope, Bio-Rad™ USA) and then transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, UK). These membranes were incubated with the appropriate primary antibodies (overnight) and with secondary antibodies (90 min) conjugated to HRP (Table 1). Visualization of specific protein bands was carried out using chemiluminescent reagents (Bio-Rad™, USA) and then by using the ImageQuant Las 500 (GE Healthcare, UK) image capture system. Band intensities were quantified by optical densitometry using the ImageJ software (Media Cybernetics, MD, USA) for protein expression determination. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control and the results were expressed as percentages relative to the control group.

Table 1
Antibodies used for Western Blotting.

	Primary antibodies	
Specificity	Company	Dilution
ACC-1	Cell Signaling Technology MA, USA	1:500
pACC	Cell Signaling Technology MA, USA	1:500
FAS	Cell Signaling Technology MA, USA	1:500
SREBP-1c	Santa Cruz Biotechnology CA, USA	1:1000
GAPDH	Cell Signaling Technology MA, USA	1:1000
Secondary antibodies		
Specificity	Company	Dilution
Anti-rabbit	Santa Cruz Biotechnology CA, USA	1:7000

ACC-1, acetyl-coa carboxylase-1; pACC, phosphorylated acid-acetyl-coa carboxylase; FAS, fatty acid synthase; SREBP-1c, sterol regulatory element-binding protein-1c; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.7. Statistical analyses

Data are shown as mean ± SEM. Differences were considered significant when $p < 0.05$. Comparisons were performed using Student's unpaired *t*-tests. Since sex was not considered as a factor; only the programming effect (NIC and CON) was considered in the analyses. The statistical program GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the graphics and analyses.

3. Results

3.1. PND120

3.1.1. Body and plasma parameters of male and female offspring

PND120 males and females showed similar body mass and VFM between groups (Table 2). NIC males had a decrease in TG levels (+53% *vs* CON; Table 2) while CHOL levels were not changed. NIC females showed an increase in CHOL levels (+98% *vs* CON; Table 2), however TG was not altered between groups.

3.1.2. Liver parameters of male offspring

The liver mass was not altered between the experimental groups (Table 2). Histological analysis, according to the Brunt's NAFLD score, revealed that 75% of NIC animals displayed hepatic fatty deposition graded at 1 (Fig. 1C), mainly characterized by microsteatosis (Fig. 1B). NIC animals also had an increase in CHOL ester, TG, free fatty acids and monoacylglycerol content in the liver (+41%, +43%, +118% and +50%, respectively) when compared to CON animals (Fig. 3A). Total CHOL and phospholipids contents were not different between the groups (Fig. 3A).

In NIC animals, the hepatic expression of total ACC-1 and FAS enzymes were 66% and 92% higher than in CON animals (Fig. 4A); with a reduction of 51% in the inactive/active ACC form (pACC/ACC), which indicates that the ACC-1 enzyme was more expressed in NIC animals when compared to CON animals (Fig. 4A).

3.1.3. Liver parameters of female offspring

Females of both groups showed similar liver mass (Table 2). As depicted in Fig. 1F, at PND120, all females showed absence of steatosis (degree 0), with preserved hepatocyte architecture (Fig. 1E). Regarding the liver lipid composition, NIC animals showed an increase of 29% in CHOL ester content and a reduction of 46% in total CHOL content when compared to CON animals (Fig. 3B). TG,

Table 2
Plasma and biometric parameters of adult male and female offspring programmed by postnatal nicotine exposure.

	Male		Female	
	CON	NIC	CON	NIC
PND120				
Body mass (g)	492.0 ± 14.0	468.0 ± 8.7	260.0 ± 6.6	248.0 ± 4.5
VFM (g/100g body mass)	4.7 ± 0.3	5.3 ± 0.3	4.7 ± 0.4	4.1 ± 0.3
Liver (g/100g body mass)	3.0 ± 0.1	3.2 ± 0.1	2.5 ± 0.1	2.6 ± 0.1
Plasma TG (mg/dL)	45.0 ± 8.7	21.0 ± 6.4*	11.3 ± 2.0	13.8 ± 2.5
Plasma CHOL (mg/dL)	18.0 ± 3.3	24.6 ± 3.3	9.3 ± 1.8	18.4 ± 2.8*
PND180				
Body mass (g)	542.0 ± 6.8	574.0 ± 7.6**	294.0 ± 4.3	299.4 ± 4.4
VFM (g/100g body mass)	5.90 ± 0.02	6.70 ± 0.03*	5.60 ± 0.03	6.30 ± 0.04
Liver (g/100g body mass)	3.1 ± 0.1	3.5 ± 0.1*	2.7 ± 0.1	2.8 ± 0.1
Plasma TG (mg/dL)	48.5 ± 4.5	45.6 ± 9.0	27.3 ± 4.2	42.5 ± 4.0*
Plasma CHOL (mg/dL)	65.8 ± 1.8	66.4 ± 7.8	68.2 ± 4.7	90.0 ± 8.7*

Visceral fat mass (VFM); triglycerides (TG); cholesterol (CHOL). Groups: CON – offspring of dams that were saline-exposed during lactation; NIC – offspring of dams that were nicotine-exposed during lactation. Values represent mean ± SEM of 1/sex/litter/group. Student's unpaired *t*-test. *$p < 0.05$ and **$p < 0.01$ – NIC *vs* CON.

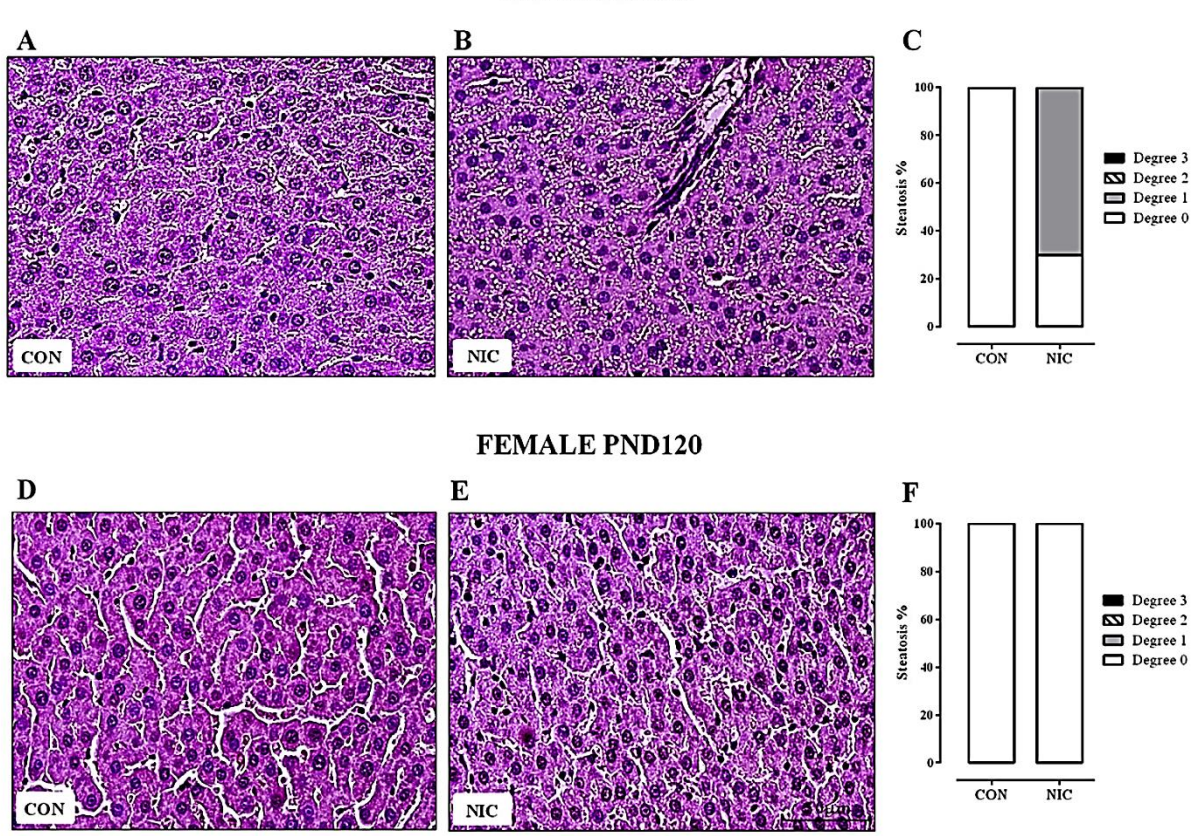

Fig. 1. Long-term effects of maternal nicotine exposure during breastfeeding on hepatic morphology of male and female offspring at PND120.

free fatty acids, monoacylglycerol and phospholipids levels were unchanged in both groups (Fig. 3B). The expression of key enzymes involved in hepatic *de novo* fatty acid biosynthesis was similar between the groups (Fig. 4B).

3.2. PND180

3.2.1. Body and plasma parameters of male and female offspring

PND180 NIC males showed an increase of 6% in body mass and 14% in VFM compared to CON males (Table 2). Body mass and VFM were similar between the female groups (Table 2). NIC males had no change in TG and CHOL levels (Table 2). NIC females showed an increase in TG (+56% *vs* CON; Table 2) and CHOL levels (+32% *vs* CON; Table 2).

3.2.2. Liver parameters of male offspring

NIC males had a 13% increase in liver mass when compared to CON males (Table 2). The histological analysis of the liver samples stained with H&E revealed that 80% of NIC animals showed a steatosis degree of 2 and 20% showed a degree of 1 (Fig. 2C), mainly characterized by microvacuolar steatosis (Fig. 2B), when compared to CON animals (Fig. 2A). After quantification of lipid-specific labeling by Oil Red-O staining, NIC males showed a 31-fold-increase compared to CON animals (Fig. 2F) as indicated in Fig. 2E.

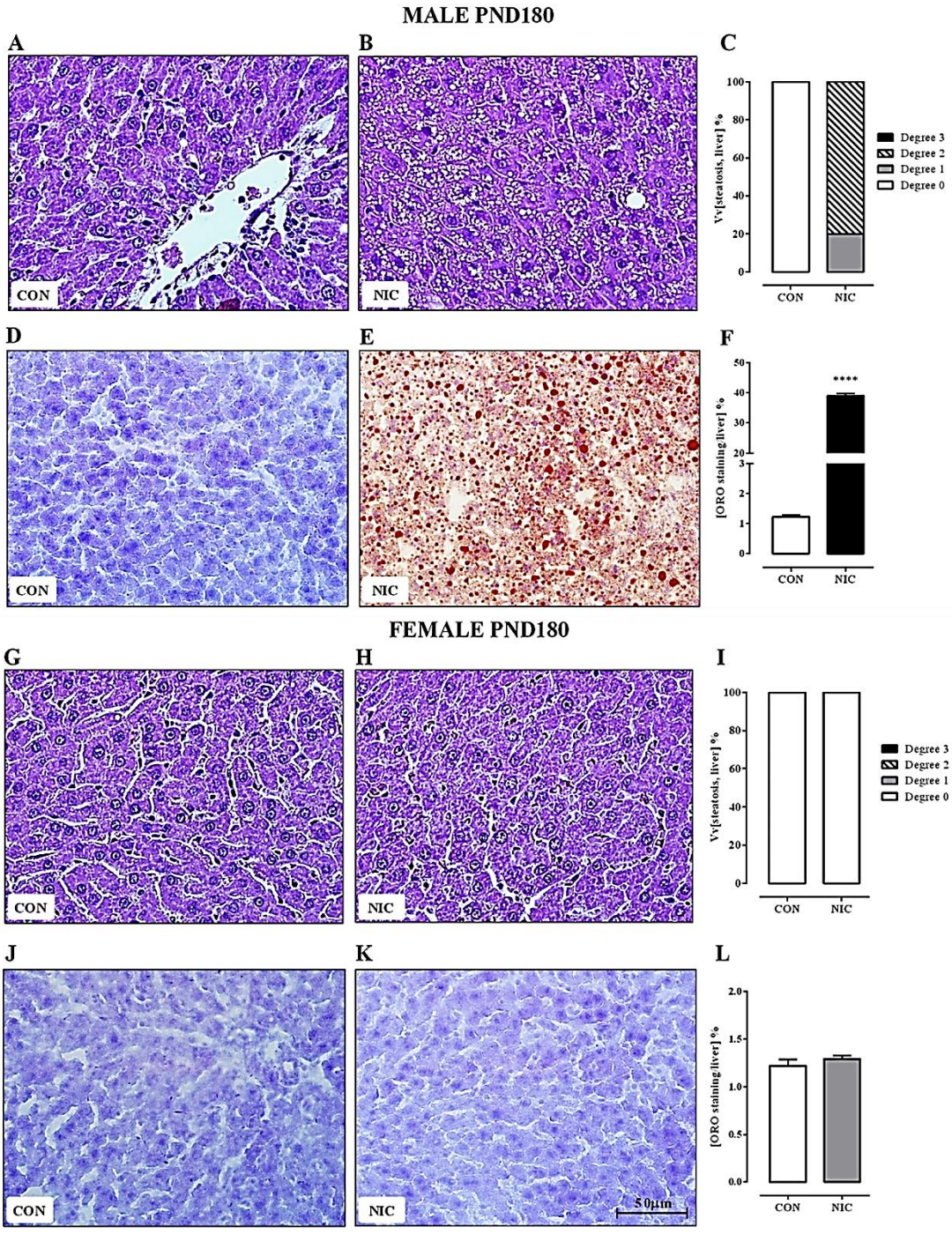

Fig. 2. Long-term effects of maternal nicotine exposure during breastfeeding on hepatic morphology of male and female offspring at PND180.

NIC males displayed higher TG levels (+39% *vs* CON) and a decreased content of free fatty acids and total CHOL (−64% and −43% *vs* CON, respectively; Fig. 3C). CHOL ester, monoacylglycerol and phospholipids contents were not affected (Fig. 3C). Regarding protein expression, total ACC-1 enzyme showed an increase of 43% in NIC animals when compared to CON animals (Fig. 4C) and a reduction of inactive/active ACC form (−35% *vs* CON), indicative of increased expression of this enzyme (Fig. 4C).

3.2.3. Liver parameters of female offspring

The liver mass and morphology of females remained unchanged at PND180 (Table 2; Fig. 2G–L). Levels of CHOL ester and free fatty acids decreased in NIC females compared to CON females (−29% and −55%, respectively; Fig. 3D). TG, total CHOL, monoacylglycerol and phospholipids were not altered (Fig. 3D). NIC females also showed higher total ACC-1 and FAS expression (+41% and +17% *vs* CON, respectively; Fig. 4D).

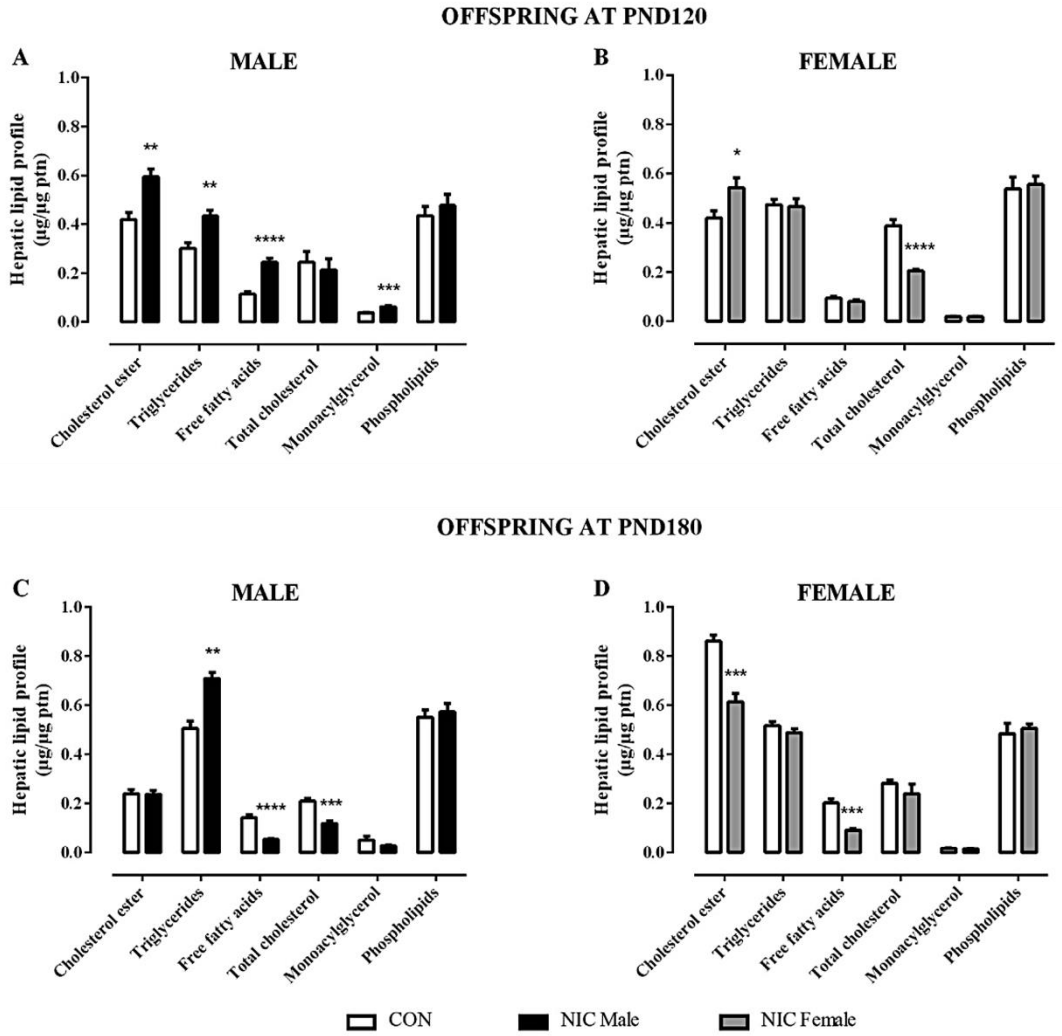

Fig. 3. Long-term effects of maternal nicotine exposure during breastfeeding on hepatic lipid profile of male and female offspring at PND120 and PND180.

4. Discussion

The results regarding hepatic lipid metabolism showed that males and females have different profiles. Here, we demonstrated in detail, for the first time, the hepatic lipid profile exhibited by both sexes at different ages at adulthood. Only in males did TG accumulation at PND120 and PND180 corroborate the liver histological analyzes that indicated steatosis degrees 1 and 2, respectively, in line with the fact that TG accumulation is the main feature of hepatic steatosis (Kim et al., 2017).

Total TG levels arise from 3 sources: *de novo* hepatic lipogenesis, diet and white adipose tissue turnover (Hodson and Gunn, 2019). Since here the animals received only standard chow throughout life and, at PND120, did not show changes in body mass or VFM, the impairment in lipid metabolism is possibly related to a nicotine programming effect instead of a secondary effect of obesity. At this age, NIC males showed reduced plasma TG, which corroborate the lipid accumulation in less than 5% of the hepatocytes, which is a characteristic of the observed steatosis (EASL, 2016). In addition, the 120-day-old male NIC offspring also had hepatic free fatty acids and monoacylglycerol accumulation accompanied by increased FAS protein expression and the active form of ACC-1 (pACC/ACC), two of the major proteins of *de novo* fatty acid biosynthesis (Kim et al., 2017).

The retention of intrahepatic TG is closely related to impaired mitochondrial β-oxidation and exportation and/or assembly of VLDL by compromising the action of key enzymes in these processes, such as CPT1α (Carnitine Palmitoyltransferase1α) and MTTP (Microsomal triglyceride transfer protein), respectively (Bessone et al., 2019). This mechanism might occur in NIC males at PND120. Besides, this group has high levels of CHOL ester. Proteins such as ACAT1 (Acyl Coa: cholesterol acyltransferase1) and HMGCR (3-hydroxy-3methyl-glutaryl-CoA reductase), which are involved in CHOL esterification and biosynthesis pathways, respectively (Ikonen, 2008), are probably increased in our model. However, further analysis will be necessary to confirm this possibility. NIC males seem to be programmed to increase *de novo* fatty acid synthesis, TG synthesis, CHOL esterification and VLDL assembly and/or export. Thus, the hepatic steatosis observed in NIC males happened first than and independent of overweight and insulin resistance already described at PND180 (Oliveira et al., 2009; De Oliveira et al., 2010). From PND120 to PND180, VLDL assembly and/or exportation processes appear to be normalized in NIC males since plasma TG was normalized, despite the increased hepatic TG biosynthesis and

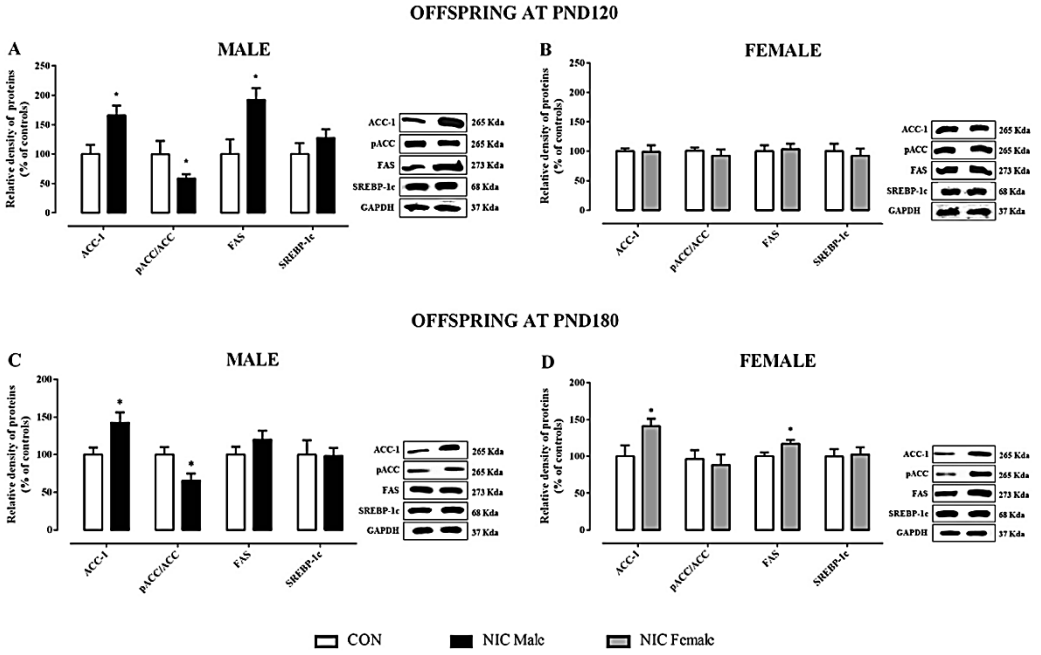

Fig. 4. Long-term effects of maternal nicotine exposure during breastfeeding on hepatic protein expression of male and female offspring at PND120 and PND180.

steatosis progresses to degree 2. The slight difference in liver mass between the experimental groups may be related to this increase in the liver lipid accumulation. As NIC males at PND180 had epididymal and inguinal adipocytes hypertrophy (De Oliveira et al., 2010), we also suggest that the excess of plasma TG may be captured by adipocytes.

Regarding fatty acids synthesis, only the active form of ACC-1 was higher in NIC males at PND180, at which point FAS expression was not affected. Literature reports increased hepatic FAS expression in the adult male offspring of dams exposed to nicotine during pregnancy and lactation (Fan et al., 2016; Ma et al., 2014). The reduction of hepatic free fatty acids content at PND180 may be related to a normalization of FAS expression. It may also suggest that proteins in the oxidation pathway may be induced in an attempt to reduce the excess of hepatic TG (Kim et al., 2017). However, hepatic lipotoxicity since PND120 could activate other pathways of lipid accumulation, such as the oxidative stress one. The increased stress of the endoplasmic reticulum could contribute to the worsening of the steatosis (Svegliati-Baroni et al., 2019).

In NIC males, we also observed different age-dependent profiles in the accumulation of esterified CHOL and total CHOL in the liver. At PND180, we detected lower total CHOL without alterations in CHOL ester content. It is possible that HMGCR is reduced or the oxidation pathways are activated in an attempt to reduce the availability of CHOL ester that was observed at PND120. Therefore, it seems that CHOL biosynthesis and/or oxidation pathways are modulated in our model; further analyses will be needed to confirm either possibility. Thus, we believe that the increase in the oxidative stress markers observed at PND180 by Conceição et al. (2015) in addition to the progressive accumulation of TG in the hepatocytes observed in this study, are due to the changes in lipid metabolism programmed by nicotine. These alterations are independent of the overweight state since, at PND120, animals were neither obese nor insulin resistant, since Conceição et al. (2015) have already described that insulin signaling pathway in the liver was normal at PND180.

The hepatic lipid metabolism of female offspring of dams exposed to nicotine during lactation has not been previously described in the literature. In contrast to males, PND120 female offspring did not show alterations in the protein expression of the *de novo* fatty acid biosynthesis pathway (active form of ACC-1; FAS; SREBP-1c), which justify the unaltered TG and free fatty acids, as well as the absence of microsteatosis. The metabolism of CHOL was, however, affected in females. Females showed a reduction in total CHOL and an increase in CHOL ester (with no changes in other lipid fractions), possibly due to a compensatory reduction in HMGCR activity to reduce the excess of CHOL esterification by ACAT1. This deregulation may involve an impairment in the uptake of CHOL by the liver, possibly due to alterations in the hepatic low-density lipoprotein receptor (LDLR) expression, since NIC females had higher plasma CHOL and no alterations in body mass and VFM at PND120. Further analysis will be necessary to support our hypothesis.

The PND180 NIC females had a reduction in CHOL ester and free fatty acids, with no changes to the other lipid fractions and an absence of steatosis, which would indicate an improvement in the hepatic lipid profile. However, an increase in FAS protein expression was observed, which explains the increase in fatty acid production and the higher levels of plasma TG and CHOL. So, these lipids may be reduced in hepatocytes due to their release to the circulation. It seems that these lipids are not being captured by the adipose tissue, keeping them in the plasma, increasing the risk for atherosclerosis (Wang et al., 2018).

The Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) regulates fatty acid synthetic genes (Knebel et al., 2018). Similarly, the Liver X Receptor (LXR) is a key nuclear receptor involved in the transcriptional regulation of *de novo* triglyceride synthesis (Ballestri et al., 2016). In mice, the oral administration of an LXRα agonist results in elevated plasma TG levels concomitant with increased gene activity of the key synthetic enzymes ACCα, FAS and Stearoyl-CoA Desaturase-1 (SCD-1) (Schultz et al., 2000). These effects are

lost in studies involving LXRα/β−/− mice (Horton et al., 2003). Interestingly, nicotine exposure during pregnancy and lactation led to an increase in hepatic LXRα protein expression of 180-days-old male rats (Ma et al., 2014) and we showed no change of hepatic SREBP-1c content, which suggests the LXRα involvement in the regulation of lipogenic enzymes described here. A limitation of this study was that the LXRα levels of expression were not determined.

Regarding CHOL metabolism, the Sterol Regulatory Element-binding Protein-2 (SREBP-2) seems fundamental to the biosynthesis control through the regulation of the expression of genes such as HMGCR and of CHOL uptake, by regulating LDLR expression (Ye and DeBose-Boyd, 2011). Thus, we suggest that the regulation of CHOL levels and TG metabolism is being performed by different factors in our programming model in a sex-specific manner.

In addition to the mechanisms described above, we cannot discard the possible involvement of transporter-mediated hepatic fatty acid uptake, such as fatty acid transport proteins (FATPs), fatty acid binding proteins (FABPs) and CD36, in NAFLD pathogenesis (Jiang et al., 2019). The increased expression of FATP2 is strongly related to NAFLD progression (Krammer et al., 2011), while the decreased expression of fatty acid transport protects mice from NAFLD development (Falcon et al., 2010). Hepatic FABP1 expression is upregulated in NAFLD patients (Higuchi et al., 2011) and animal models (Baumgardner et al., 2008), while downregulation ameliorate steatosis and hepatic injury (Wu et al., 2017). CD36 increases liver TG accumulation (Hui et al., 2015) and a hepatocyte-specific deletion attenuates NAFLD in high-fat diet-fed mice (Wilson et al., 2016). Acute or chronic nicotine exposure effects on transporter-mediated hepatic fatty acid uptake were poorly described; this is also a limitation of the current study.

Our data suggest that hepatic lipid metabolism may be an important nicotine target, even after withdrawal, in the adult offspring of dams that were exposed to nicotine only during breastfeeding. Both male and female offspring developed hepatic changes in the biosynthesis pathways and, probably, in the oxidation and lipid exportation pathways, which result in microsteatosis and/or lipid accumulation in the plasma and other tissues, a possibility that still need to be investigated.

5. Conclusion

Here we evidence the late effects of early postnatal nicotine exposure on lipid metabolism. Male offspring were programmed to develop alterations in TG metabolism, with the accumulation being preferentially intrahepatic, independently of overweight and insulin resistance. Female offspring showed alterations mainly in CHOL metabolism. Unlike males, lipid accumulation in females occurred preferentially in the plasma and female hepatocyte architecture was preserved. The same sexually dimorphic pattern was observed in humans with NAFLD at a reproductive age, with a higher risk in men, probably due to sex steroids effects. This study helps to elucidate the initial steps of liver microsteatosis in male NIC offspring and expand knowledge regarding the female phenotype. New studies are being carried out in our laboratory to evaluate other lipid metabolic pathways to better understand the consequences of lipotoxicity programmed by neonatal exposure to nicotine.

Declaration of competing interest

The authors declare that there are no conflict of interest.

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Representative photomicrographs of the liver stained with Hematoxylin-Eosin (× 40 magnification) of control (A) and nicotine (B) male offspring; control (D) and nicotine (E) female offspring; hepatic steatosis degree of male (C) and female (F) offspring in accordance with Brunt's classification. Values represent mean ± SEM (n = 6–7; same-sex siblings were not allocated to the same experimental group). Student's unpaired *t*-test.

Representative photomicrographs of the liver stained with Hematoxylin-Eosin (× 40 magnification) of control (A) and nicotine (B) male offspring; control (G) and nicotine (H) female offspring; hepatic steatosis degree of male (C) and female (I) offspring in accordance with Brunt's classification. Representative photomicrographs of the liver stained with Oil Red-O (× 40 magnification) of control (D) and nicotine (E) male offspring; control (J) and nicotine (K) female offspring; Oil Red-O quantification of male (F) and female (L) offspring. Values represent mean ± SEM (n = 6–7; same-sex siblings were not allocated to the same experimental group). Student's unpaired *t*-test. $^{**}p < 0.01$ - NIC *vs* CON.

(A and B) HTPLC, high-performance thin layer chromatography in males and females at PN120 and PN180 (C and D). Values represent mean ± SEM (n = 5–6; same-sex siblings were not allocated to the same experimental group). Student's unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ - NIC vs CON.

(A and B) Hepatic lipogenic enzymes expression of male and female offspring at PN120 and PN180 (C and D). Values represent mean ± SEM (n = 7–8; same-sex siblings were not allocated to the same experimental group). Student's unpaired *t*-test. $^{*}p < 0.05$ - NIC vs CON.

CRediT authorship contribution statement

Iala Milene Bertasso: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Carla Bruna Pietrobon:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Bruna Pereira Lopes:** Investigation, Methodology, Writing - review & editing. **Thamara Cherem Peixoto:** Investigation, Methodology, Writing - review & editing. **Patrícia Novaes Soares:** Methodology, Writing - review & editing. **Elaine Oliveira:** Funding acquisition, Writing - review & editing. **Alex Christian Manhães:** Formal analysis, Writing - review & editing. **Maria Lucia Bonfleur:** Methodology, Writing - review & editing. **Sandra Lucinei Balbo:** Methodology, Writing - review & editing. **Suellen Silva Cabral:** Investigation, Methodology, Writing - review & editing. **George Eduardo Gabriel Kluck:** Investigation, Methodology, Writing - review & editing. **Georgia Correa Atella:** Investigation, Methodology, Writing - review & editing. **Egberto Gaspar de Moura:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Patrícia Cristina Lisboa:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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2.2 **Modelo experimental II: Desmame precoce farmacológico e desmame precoce não farmacológico**

Os itens “Material e Métodos”, “Resultados” e “Discussão” referentes a este modelo de programação estão descritos no artigo abaixo:

2.2.1 Artigo 2 - Hepatic lipid metabolism in adult rats using early weaning models: sex-related differences

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Hepatic lipid metabolism in adult rats using early weaning models: sex-related differences

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Abstract

Non-pharmacological early weaning (NPEW) induces liver damage in male progeny at adulthood; however, pharmacological early weaning (PEW) does not cause this dysfunction. To elucidate this difference in liver dysfunction between these two models and determine the phenotype of female offspring, *de novo* lipogenesis, β-oxidation, very low-density lipoprotein (VLDL) export, and gluconeogenesis in both sexes were investigated in the adult Wistar rats that were weaned after a normal period of lactation (control group) or early weaned either by restriction of access to the dams' teats (NPEW group) or by reduction of dams' milk production with bromocriptine (PEW group). The offspring received standard diet from weaning to euthanasia (PN180). NPEW males had higher plasma triglycerides and TyG index, liver triglycerides, and cholesterol by *de novo* lipogenesis, which leads to intracellular lipids accumulation. As expected, hepatic morphology was preserved in PEW males, but they showed increased liver triglycerides. The only molecular difference between PEW and NPEW males was in acetyl-CoA carboxylase-1 (ACC-1) and stearoyl-CoA desaturase-1 (SCD-1), which were lower in PEW animals. Both early weaning (EW) females had no changes in liver cholesterol and triglyceride contents, and the hepatic cytoarchitecture was preserved. The expression of microsomal triglyceride transfer protein was increased in both the female EW groups, which could constitute a protective factor. The changes in hepatic lipid metabolism in EW offspring were less marked in females. EW impacted in the hepatic cytoarchitecture only in NPEW males, which showed higher ACC-1 and SCD-1 when compared to the PEW group. As these enzymes are lipogenic, it could explain a worsened liver function in NPEW males.

Introduction

The liver is a fundamental organ in the regulation of body energy metabolism, and the synchronism of glycemic and lipid metabolism is destabilized in situations of nutritional changes, predisposing individuals to excessive accumulation of lipids in the hepatocyte and the consequent development of non-alcoholic fatty liver disease (NAFLD).[1] NAFLD includes a group of chronic liver diseases, ranging from simple hepatic steatosis to non-alcoholic steatohepatitis and hepatocellular carcinoma. The molecular mechanism underlying NAFLD progression is not completely understood; its pathogenesis has been interpreted by the hypothesis of "two-hits." The "first hit" concerns the accumulation of greater than 5.5% lipids in hepatocytes in individuals with no history of excessive alcohol consumption, resulting in steatosis due to the excessive lipid influx or impaired lipid efflux.[2] Hepatocytes activate metabolic pathways to minimize lipotoxicity in response to elevated hepatic lipid uptake, thus steatosis develops when lipid influx overwhelms these protective mechanisms (β-oxidation, ketogenesis, and VLDL export), mainly in response to fasting, high-fat diet (HFD), or lipodystrophies.[1–3] In numerous experimental animal models of hepatic steatosis, including HFD, fructose or ethanol, and genetic lipodystrophy, fatty liver is associated with peripheral metabolic dysfunction.[4] In addition to the accumulation of lipids, there is inflammation and hepatocellular degeneration featuring the "second hit."[5] Bacterial toxins, hyperproduction of cytokines, tumor necrosis factor α, changes in adenosine triphosphate stocks, and cytochrome P450 activity appear to be important triggers for this progression, which can be accompanied by the appearance of fibrosis and even progress to liver cirrhosis and carcinoma.[6–8]

Epidemiological studies show that the environment in critical periods, including gestation, breastfeeding, and adolescence, exerts a strong influence on the development of an individual and is associated with the onset of diseases throughout life, in particular, obesity and its

comorbidities.[9] This phenomenon, known as metabolic programming, is related to the developmental origins of health and disease hypothesis.[10–12] In humans, exclusive breastfeeding during the first six months of life is usually recommended, since shortening this period has been linked to adverse metabolic effects later in life.[13,14] In rodents, the shortening of the standard period of breastfeeding through experimental early weaning (EW) can program the offspring for metabolic disturbances later in life.[15–17]

In an attempt to mimic the effects caused by EW in humans and to increase the understanding concerning the metabolic programming mechanisms involved in this process, animal models of EW have been developed. In this context, our research group has previously demonstrated that EW caused by pharmacological (PEW) or non-pharmacological (NPEW) inhibition of breastfeeding in the last three days of lactation promotes metabolic changes in rats throughout the life.[15–18] This short period for a suckling rat corresponds to 1 month for a human baby,[19] i.e., this experimental model mimics a child weaned at the 5th month of age. The PEW experimental model decreases milk consumption through maternal administration of a type 2 dopaminergic agonist (bromocriptine (BRO)) that inhibits the synthesis of prolactin (PRL),[20] whereas the NPEW experimental model uses an adhesive bandage to prevent pup's access to maternal nipples, with consequent deprivation of breastfeeding.[17] When adults, both NPEW and PEW male rats are overweight and have metabolic syndrome, although some parameters in liver function differ between models. NPEW offspring have hepatic microsteatosis accompanied by an increase in oxidative stress,[21] while PEW offspring show improved redox status and absence of steatosis,[22] perhaps by a protection against NAFLD induced by BRO.[23] This phenotype is not stress-dependent, since NPEW males do not have alterations in serum corticosterone concentrations at weaning and the dams continued to groom and lick their pups, minimizing any stress effect caused by the maternal adhesive bandage.[24]

BRO can be found in both placenta and milk[25] and does not completely block milk production.[26] In rats, BRO-treated mothers (1 mg/kg per day) showed lower serum PRL at the end of lactation, which caused a significant failure in milk production, although milk PRL of the BRO dams and serum PRL of the BRO pups were not altered.[27] Peixoto-Silva *et al.* (2017) showed that BRO treatment of the pups during the last 3 days of lactation prevents the hepatic fat accumulation associated with an improvement in adiposity at adulthood, but in a dose 4 times higher than the administrated to the dams (4 mg/kg per day).[28] Thus, in the PEW model, both maternal lower PRL levels and BRO (even in small quantities) could be responsible for the programming of the liver function in the adult male offspring.

Therefore, considering the unexpected effects of BRO on the liver function of male offspring programmed by EW during the critical window of breastfeeding, in the present study, our primary aim was to assess morphological parameters of the liver tissue as well as main molecular pathways associated with hepatic metabolism to better understand the underlying mechanisms responsible for the altered function in each of the EW models (PEW and NPEW) used in our laboratory. We hypothesize that liver energy metabolism is programmed by different mechanisms in each model since, as previously reported, only PEW protected male rat offspring against liver steatosis. Furthermore, considering that the effects of EW could be sex-dependent, we also investigated, for the first time, in both EW models, the female offspring's phenotype. We therefore studied the main molecular pathways related to energy hepatic metabolism (lipogenesis, β-oxidation, VLDL export, and gluconeogenesis) in male and female adult rat offspring that were submitted either to the non-pharmacological model of EW (teat blockage by adhesive bandage) or to the pharmacological one (BRO treatment).

Experimental methods

Animals

All experimental procedures were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/014/2017), which was based on its decision on Brazilian Law 11.794/2008, which regulates the procedures for the scientific use of animals.

Wistar female rats 3 months of age were housed under controlled temperature (23 °C ± 1 °C) and artificial light/dark cycles (lights on at 07:00 a.m. and lights off at 07:00 p.m.). The mating period covered 2 weeks (two females: one male), and after pregnancy confirmation by estrous cycle monitoring, pregnant females were housed in individual maternity cages. During pregnancy and breastfeeding, rat dams received free access to water and standard chow (Nuvilab, Sogorb, São Paulo, Brazil). Diet composition is described in Table 1. In the first postnatal day (PN0), litters were adjusted to six pups per dam (three males and three females).

Experimental design of NPEW and PEW

On the last 3 days of the breastfeeding period (PN18–PN20), the lactating rats were randomly divided into three groups (n = 10 dams/group): (1) NPEW group, dams were anesthetized with xylazine (9-mg/kg body mass; Xilazin®, Paulinia, SP, Brazil) and ketamine (90-mg/kg body mass; Cetamin®, Paulinia, SP, Brazil) and then wrapped with an adhesive bandage that covered all the teats, thereby restricting pups' access to them[29]; (2) PEW group, dams received 0.5-mg BRO (Parlodel; Novartis, São Paulo, Brazil) intraperitoneally (i.p), two times per day[24] to inhibit PRL synthesis; and (3) control group (CON), dams whose weaning of the pups occurred at PN21 (standard period). Both NPEW and PEW pups were also separated from their respective dams at PN21. After separation, male siblings were kept separated from female siblings. From PN21 to PN180, offspring from the three groups received water and standard chow (Nuvilab). We randomly evaluated one offspring/sex/litter, and the other pups in each litter were used for other studies.

One month before euthanasia, which occurred at PN180, the estrous cycle of the female offspring of all three groups was evaluated every day (08:00 a.m.) by vaginal smears collected in a solution containing 0.9% NaCl. The female offspring showed regular estrous cycles (4–5 days). They were killed during diestrus.

Intraperitoneal pyruvate tolerance test

To analyze the gluconeogenesis rate of adult rats, the intraperitoneal pyruvate tolerance test (iPTT) was performed in one animal/sex/litter at PN175. Pyruvate was used because it is the common intermediary metabolite in the gluconeogenic pathways, being generated from lactate, amino acids, and glycerol. The animals were fasted for 12 h, and blood samples were taken from the tip of the tail vein to obtain fasting glycemia (time 0) using a handheld glucometer (Accu-Chek Advantage; Roche Diagnostics, Mannheim, Germany). Subsequently, the animals received an i.p. injection of 2-g/kg body mass of pyruvate, and additional blood samples were recorded at 15, 30, 60, and 120 min.[30]

Table 1. Macronutrients and micronutrients composition of standard rat chow

	Standard chow
Carbohydrate (g/kg)	660.0
% kJ	68.0
Protein (g/kg)	220.0
% kJ	22.7
Fat (g/kg)	40.0
% kJ	9.2
Vitamins and minerals	
Ca (g/kg)	10.0–14.0
P (mg/kg)	8000.0
Na (mg/kg)	2700.0
Fe (mg/kg)	50.0
Mn (mg/kg)	60.0
Zn (mg/kg)	60.0
Cu (mg/kg)	10.0
I (mg/kg)	2.0
Se (mg/kg)	0.05
Co (mg/kg)	1.5
F (mg/kg)	80.0
Vitamin A (IU/kg)	13000.0
Vitamin D3 (IU/kg)	2000.0
Vitamin E (IU/kg)	34.0
Vitamin K3 (mg/kg)	3.0
Vitamin B1 (mg/kg)	5.0
Vitamin B2 (mg/kg)	6.0
Vitamin B3 (mg/kg)	60.0
Vitamin B5 (mg/kg)	20.0
Vitamin B6 (mg/kg)	7.0
Vitamin B7 (mg/kg)	0.05
Vitamin B9 (mg/kg)	1.0
Vitamin B12 (mcg/kg)	22.0
Choline (mg/kg)	1900.0
Amino acids	
Lysine (mg/kg)	12000.0
Methionine (mg/kg)	4000.0
BHT (mg/kg)	100.0

BHT, butylated hydroxytoluene.
Standard rat chow (Nuvilab, Sogorb, São Paulo, SP, Brazil). Composition of diet: whole corn, soybean bran, wheat bran, calcium carbonate, dicalcium phosphate, sodium chloride, vitamin A, vitamin D3, vitamin E, vitamin K3, vitamin B1, vitamin B2, vitamin B6, vitamin B12, niacin, calcium pantothenate, folic acid, biotin, choline chloride, iron sulfate, manganese sulfate, zinc sulfate, copper sulfate, calcium iodate, sodium selenite, cobalt sulfate, lysine, methionine.

Euthanasia and tissues

Prior to euthanasia (PN180), animals were fasted for 12 h. After measurement of fasting glycemia, the rats were anesthetized with xylazine (100-mg/kg body mass; Xilazin®, Paulinia, SP, Brazil) and ketamine (50-mg/kg body mass; Cetamin®, Paulinia, SP, Brazil) and euthanized by cardiac puncture. The retroperitoneal, perigonadal, and mesenteric white adipose tissues (visceral fat mass (VFM)), and the liver were collected and weighed. Blood samples were centrifuged (1500 × g for 20 min at 4 °C) to obtain plasma, which was kept at −20 °C until use. Tissue samples were frozen at −80 °C until analysis.

Plasma and hepatic levels of cholesterol and triglycerides

The quantifications of total cholesterol (CHOL), high-density lipoprotein-cholesterol (HDL-c), and triglycerides (TGs) were performed using a commercial kit in accordance with the manufacturer's instructions (Bioclin, Rio de Janeiro, RJ, Brazil). Left-lobe hepatic samples of approximately 100 mg were removed to determine total lipids using the Folch method.[31] The extract was evaporated and then diluted in isopropanol. The CHOL and TG liver content were measured using a commercial kit in accordance with the manufacturer's instructions (Bioclin, Rio de Janeiro, RJ, Brazil). Additionally, the TG and glucose index (TyG index) were calculated (Ln[fasting TG (mg/dL) × fasting glycemia (mg/dL)/2) and used to evaluate insulin resistance.[32]

Hepatic histology

A liver sample was removed from the left lobe of each animal in the direction transverse to the board. The material was fixed in 4% paraformaldehyde for 24 h, then dehydrated in alcohol of increasing concentration, and, after diaphanization in xylene, was embedded in histologically Paraplast (Sigma-Aldrich, St. Loius, MO, USA). Slices of 5-µm thickness were prepared for staining with hematoxylin and eosin (H&E). For this, photomicrographs (10 per animal, ×60 magnification) were randomly acquired with an Olympus DP71 camera coupled to an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan). Images were displayed on a high-resolution liquid crystal display flat-panel monitor (LG Electronics, Seoul, South Korea). For volume density of hepatic steatosis (Vv[steatosis, liver]) determination, a test system consisting of 36 test points was used.[33] The values were classified in accordance with Brunt's classification of steatosis magnitude. Thus, steatosis was graded as follows: 0 (none to 5% of hepatocytes affected); 1 (>5%–30% affected); 2 (>30%–60% affected); and 3 (>60% affected).[34]

Hepatic protein expression

To determine protein expression, 30 mg from the liver was homogenized in radioimmunoprecipitation assay buffer (50-mM Tris-HCl, pH 7.4; 150-mM NaCl; 1% Triton X-100; sodium dodecyl sulfate (SDS) 0.1%; 5-mM ethylenediaminetetraacetic acid; 50-mM NaF; 30-mM $Na_4P_2O_7$; 1-mM Na_3VO_4,) containing a complete cocktail of protease inhibitors (La Roche Ltd., Basel, Canton of Basel-Stadt, SW). After centrifugation (7500 × g/15 min/4 °C), the total protein content of the supernatant was measured using a Pierce bicinchoninic acid protein assay kit (Thermo Scientific, San Jose, CA, USA). For SDS gel electrophoresis, 30 µg of the samples were initially denatured in sample buffer containing beta-mercaptoethanol and then transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Amersham, Bucks, UK) along with the molecular weight standard (Kaleidoscope; Bio-Rad™, USA). The membranes were incubated overnight with the appropriate primary antibodies and then, for 90 min, with the secondary antibodies conjugated to horseradish peroxidase (Table 2). Visualization of the specific protein bands was performed by chemiluminescence (Bio-Rad™) using an ImageQuant Las 500

Table 2. Antibodies used for Western blotting

	Primary antibodies		Secondary antibodies		
	Company	Dilution	Company	Dilution	Specificity
ACC-1	Cell Signaling Technology, Boston, MA, USA	1:500	Santa Cruz Biotechnology, Paso Robles, CA, USA	1:7000	Anti-rabbit
pACC	Cell Signaling Technology	1:500	Santa Cruz Biotechnology	1:7000	Anti-rabbit
FAS	Cell Signaling Technology	1:500	Santa Cruz Biotechnology	1:7000	Anti-rabbit
SCD-1	Abcam, Cambridge, MA, USA	1:500	Sigma-Aldrich, St. Loius, MO, USA	1:1000	Anti-mouse
CPT-1α	Santa Cruz Biotechnology	1:1000	Santa Cruz Biotechnology	1:7000	Anti-rabbit
MTTP	Thermo Fisher Scientific, Waltham, MA, USA	1:500	Santa Cruz Biotechnology	1:7000	Anti-rabbit
SREBP-1c	Santa Cruz Biotechnology	1:1000	Santa Cruz Biotechnology	1:7000	Anti-rabbit
PPAR-α	Santa Cruz Biotechnology	1:500	Santa Cruz Biotechnology	1:7000	Anti-rabbit
PPAR-γ	Santa Cruz Biotechnology	1:500	Santa Cruz Biotechnology	1:7000	Anti-rabbit
G6Pase-α	Santa Cruz Biotechnology	1:300	Santa Cruz Biotechnology	1:7000	Anti-rabbit
PEPCK-1	Abcam	1:1000	Santa Cruz Biotechnology	1:7000	Anti-rabbit
AMPK-α	Cell Signaling Technology	1:1000	Santa Cruz Biotechnology	1:7000	Anti-rabbit
pAMPK-α	Cell Signaling Technology	1:1000	Santa Cruz Biotechnology	1:7000	Anti-rabbit
α-Tubulin	Sigma-Aldrich	1:1000	Sigma-Aldrich	1:10000	Anti-mouse

ACC-1, acetyl-CoA carboxylase-1; pACC, phosphorylated acid-acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase-1; CPT-1α, carnitine palmitoyl-transferase-1α; MTTP, microsomal triglyceride transfer protein; SREBP-1c, sterol regulatory element-binding protein-1c; PPAR-α, peroxisome proliferator-activated receptor alpha; PPAR-γ, peroxisome proliferator-activated receptor gamma; G6Pase-α, glucose 6-phosphatase-α; PEPCK-1, phosphoenolpyruvate carboxykinase-1; AMPK-α, AMP-activated protein kinase-α; pAMPK, phosphorylated AMP-activated protein kinase.

(GE Healthcare, Chalfont St Giles, Bucks, UK) image capture system. The band intensities were quantified by optical densitometry using the ImageJ software (Media Cybernetics, Rockville, MD, USA). α-Tubulin was used as an internal control, and the results were expressed as percentages relative to the control group.

Statistical analysis

The Kolmogorov–Smirnov one sample test (K–S) was used to assess the normality of the distributions of each of the variables. Data are compiled as means and standard errors of the means (SEM). Only one animal per sex per litter was used in the analyses. Univariate analyses of variance were used to analyze all the data except those pertaining to the pyruvate-response assessment. Group and sex were used as the between-subjects factors. Repeated measures analyses of variance (rANOVA) were used to analyze the pyruvate-response data. Group and sex were used as the between-subjects factors, and interval was used as the within-subjects factor. Whenever the sphericity assumptions appeared to be violated (Mauchly's test) in the rANOVAs, an adjustment to the numerator and denominator degrees of freedom was made by using parameter ε.[35] Lower order ANOVAs and Fisher Protected Least Significant Difference tests were used *post hoc*. Significance is assumed at the level of $P < 0.05$. For interactions at $P < 0.10$ (two-tailed), we also examined whether lower order main effects were detectable after subdivision of the interactive variables.[36]

Results

Body composition

As indicated in Table 3, body mass gain was increased between PN21 and PN180 in both EW models, irrespective of sex, when compared to controls ($F_{2,52} = 12.3$, $P < 0.001$). NPEW animals had 13% more ($P = 0.006$) body mass gain than controls, while PEW animals had 20% more ($P < 0.001$). No differences were observed between NPEW and PEW in this parameter. Males had higher body mass gain (+122%) than females ($F_{1,52} = 558$, $P < 0.001$). The same pattern of results was observed regarding VFM at PN180. Both EW models, irrespective of sex, showed higher VFM ($F_{2,51} = 18.7$, $P < 0.001$): NPEW +55% ($P < 0.001$) and PEW +64% ($P < 0.001$) *vs.* controls. Males had higher VFM (+17.6%) than females ($F_{1,51} = 6.2$, $P = 0.016$). All these data are in accordance with our previous published studies.[15,17,37]

Plasma parameters of both sexes

The Intraperitoneal Pyruvate Tolerance Test (iPPT) analysis at PN175 indicated that all groups showed variations in plasma glucose levels throughout the test (interval: $F_{2.7,\ 128.4} = 64.3$, $P < 0.001$) (Fig. 1). The profiles of these variations were affected both by group ($F_{2,47} = 7.4$, $P = 0.002$) as well as by sex (interval × sex: $F_{2.7,\ 128.4} = 4.7$, $P = 0.005$). Given these results, the analyses proceeded separated by sex. Regarding females (Fig. 1a), all three groups showed that similar increases were present from 0 to 15 min and that levels remained relatively stable from 15 to 30 min. It is at 60 ($F_{2,23} = 4.5$, $P = 0.023$) and 120 ($F_{2,23} = 4.0$, $P = 0.031$) min that differences become apparent: PEW females show higher values than controls at both time points (60 min: +18%, $P = 0.014$; 120 min: +24%, $P = 0.009$) and higher than NPEW at 60 min (+18%, $P = 0.014$). As for males (Fig. 1b), significant differences between groups were observed from 30 min onwards: PEW males show higher values than controls at 30 (+22%, $P = 0.009$) and 60 (+26%, $P = 0.001$) min; NPEW males show higher values than controls at 60 (+18%, $P = 0.013$) and 120 (+23%, $P = 0.005$) min. The area under curve (AUC) of iPTT showed an effect of group both in females ($F_{2,23} = 5.4$, $P = 0.012$) and males ($F_{2,24} = 4.5$, $P = 0.020$) (Fig. 1c). PEW females had higher values

Table 3. Somatic and plasma data separated by sex and group in both early weaning models at PN180

	Female			Male		
Parameters	CON	NPEW	PEW	CON	NPEW	PEW
Body mass gain (g)	103.6 (4.0)	121.0 (4.0)	129.0 (6.4)	234.0 (6.9)	275.8 (9.0)	278.7 (11.7)
VFM (g/100g body mass)	2.9 (2.7)	4.4 (0.3)	4.5 (0.3)	3.3 (0.2)	5.1 (0.3)	5.5 (0.5)
Plasma glucose (mg/dL)	94.7 (2.9)	94.2 (2.6)	94.4 (2.0)	84.5 (3.2)	80.8 (2.5)	79.3 (1.8)
Plasma TG (mg/dL)	32.9 (4.8)	45.7 (6.1)	41.4 (6.4)	34.6 (4.3)	58.9 (6.0)	49.2 (8.1)
Plasma CHOL (mg/dL)	57.6 (5.2)	72.8 (4.6)	61.9 (5.7)	63.8 (4.5)	68.6 (6.2)	67.0 (2.9)
Plasma HDL-c (mg/dL)	18.4 (0.1)	6.0 (1.6)	7.1 (1.7)	22.9 (1.4)	15.7 (0.9)	13.3 (1.2)
TyG index	7.2 (0.2)	7.6 (0.1)	7.5 (0.2)	7.3 (0.2)	7.8 (0.1)	7.5 (0.2)

VFM, visceral fat mass; BM, body mass; TG, triglycerides; CHOL, cholesterol; HDL-c, high-density lipoprotein; TyG index, plasma triglycerides-glucose index. Values represent mean (SEM) of 8–10 animals per group from different litters. Significance is assumed at the level of $P < 0.05$.

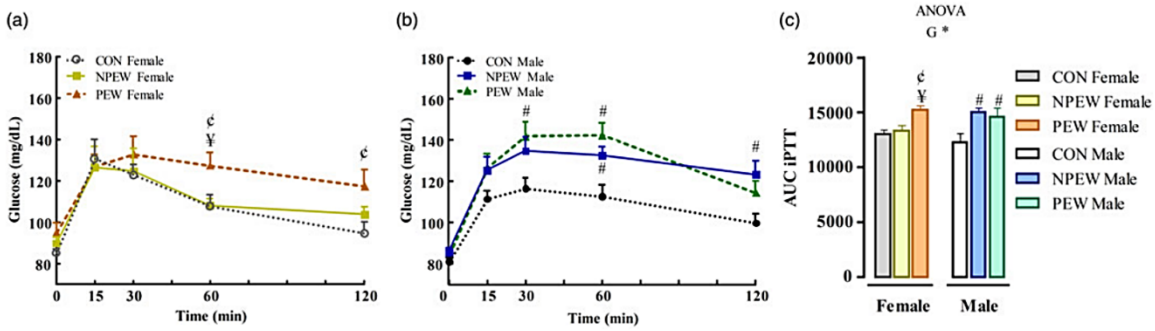

Fig. 1. iPTT of female (a) and male (b) offspring at PN175. (c) iPTT AUC. Values are mean ± SEM (n = 8–10). G, group factor. ¢ PEW females vs. CON females; ¥ PEW females vs. NPEW females; # NPEW or PEW males vs. CON males. Significance is assumed at the level of $P < 0.05$.

than control (+17%, $P = 0.005$) and NPEW (+14%, $P = 0.013$) females. Both PEW (+20%, $P = 0.010$) and NPEW (+23%, $P = 0.024$) males had higher values than controls.

At PN180, fasting plasma glucose (Table 3) showed no group effect, but females, irrespective of group assignment, had higher values (+16%) than males ($F_{1,50} = 39.8$, $P < 0.001$). Regarding TG levels ($F_{2,44} = 4.5$, $P = 0.016$) and the TyG index ($F_{2,44} = 39.8$, $P = 0.014$), only group effects were present (Table 3): NPEW had higher values than controls (TG: +57%, $P = 0.014$; TyG: +6%, $P = 0.014$). Plasma total CHOL showed no effects or interactions (Table 3). As for HDL-c, the effects of group ($F_{2,29} = 30.8$, $P < 0.001$) and sex ($F_{2,29} = 32.8$, $P < 0.001$) were identified: Both EW groups showed lower levels than control animals (PEW vs. C: −52%, $P < 0.001$; NPEW vs. C: −51%, $P < 0.001$); females had lower levels than males (−43%).

Liver parameters

At PN180, liver mass (Fig. 2a) was affected both by the weaning procedure and by the sex of the offspring (group: $F_{2,51} = 8.9$, $P < 0.001$; sex: $F_{1,51} = 75.4$, $P < 0.001$; group × sex: $F_{2,51} = 4.7$, $P = 0.013$). In females, both PEW (−14%, $P < 0.001$) and NPEW (−15%, $P < 0.001$) animals had lower liver masses than control ones. No differences were observed among male groups. As for the liver CHOL content (Fig. 2b), it was also observed that both factors affected the outcome (sex: $F_{1,42} = 4.4$, $P = 0.043$; group × sex: $F_{2,42} = 4.5$, $P = 0.017$). In males, both EW groups showed higher contents than controls (PEW vs. C: +31%, $P = 0.010$; NPEW vs. C: +36%, $P = 0.022$). No differences were observed among female groups. Regarding liver TG content (Fig. 2c), it was affected both factors (group: $F_{2,42} = 10.5$, $P < 0.001$; sex: $F_{1,42} = 88.3$, $P < 0.001$; group × sex: $F_{2,42} = 4.4$, $P = 0.018$). In males, both EW groups showed higher contents than controls (PEW vs. C: +78%, $P = 0.002$; NPEW vs. C: +80%, $P = 0.001$). No differences were observed among female groups.

The liver histomorphologic analysis showed hepatocytes with preserved architecture, arranged in rows, delimited by connective tissue containing sinusoidal capillaries and lipid droplets in less than 5% of the analyzed area in all female groups (Fig. 2d; Table 4) as well as in CON and PEW male groups (Fig. 2e; Table 4); this is considered absence of steatosis (degree 0). Only the NPEW male group had hepatocytes with several cytoplasmic lipid droplets in approximately 25% of the analyzed area on 75% of samples, indicating a profile of microvesicular hepatic steatosis that is considered degree 1 (Fig. 2e; Table 4).

The evaluation of protein contents of key enzymes involved in hepatic lipid metabolism is depicted in Fig. 3. Total acetyl-CoA carboxylase-1 (ACC-1) content (Fig. 3a) showed a group effect ($F_{2,38} = 7.8$, $P < 0.001$): NPEW animals had a higher content than control (+28%, $P < 0.001$) and PEW (+18%, $P < 0.001$) ones. Fatty acid synthase (FAS) content (Fig. 3b) showed a clear sex ($F_{1,44} = 11.4$, $P = 0.002$) effect (females had less (−29%) than males) as well as a group × sex interaction that approached significance ($F_{2,44} = 2.9$, $P = 0.063$). After subdivision by sex, EW males showed higher contents than control ones (PEW vs. C: +31%,

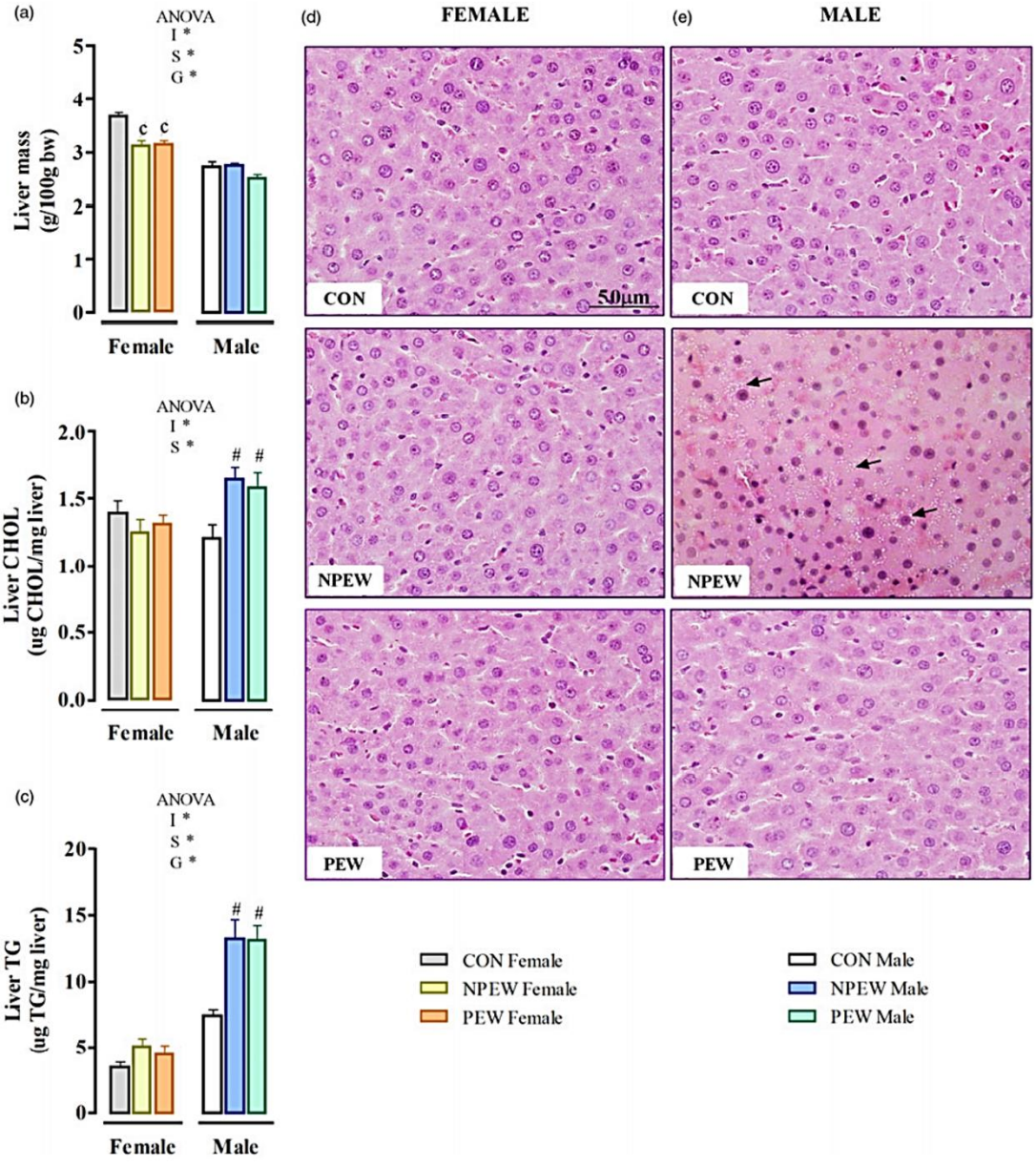

Fig. 2. Liver parameters of female and male offspring at PN180. (a) Liver mass. (b) CHOL, and (c) TG liver contents. Representative photomicrographs of the liver (×60 magnification) of female (d) and male (e) rats (bar: 50 µm). Values are mean ± SEM (n = 8–10). Arrows indicate microvesicular liver steatosis in NPEW males at PND180. I, intersection; S, sex factor; G, group factor. ¢ NPEW or PEW females vs. CON females; # NPEW or PEW males vs. CON males. Significance is assumed at the level of $P < 0.05$.

$P = 0.037$; NPEW vs. C: +40%, $P = 0.010$). Stearoyl-CoA desaturase-1 (SCD-1) content (Fig. 3c) showed a pattern of results that was similar to those observed for the ACC-1 context: A group effect was present ($F_{2,48} = 5.4$, $P = 0.008$) and this effect is explained by the fact that NPEW animals had a higher content than control (+10%, $P = 0.010$) and PEW (+10%, $P = 0.006$) ones. As for microsomal triglyceride transfer protein (MTTP) (Fig. 3d), its context was affected by both factors (group: $F_{2,41} = 7.8$, $P = 0.001$; sex: $F_{1,41} = 4.9$, $P = 0.033$). Overall, both EW groups had higher contents than the control one (PEW vs. C: +48%, $P = 0.001$; NPEW vs. C: +39%, $P = 0.004$). A planned comparison separated by sex further indicated that both EW females had higher contents than the control ones (PEW vs. C: +73%, $P = 0.022$; NPEW vs. C: +49%, $P = 0.022$); no significant differences were observed among the male groups. All representative western blot bands are shown in Fig. 3e.

Table 4. Degree of steatosis (Brunt's classification) in rats' liver in both early weaning models at PN180

	Female			Male		
	CON (%)	NPEW (%)	PEW (%)	CON (%)	NPEW (%)	PEW (%)
Degree 0 (0%–5% hepatocytes affected)	100	100	100	100	25	100
Degree 1 (>5%–30% hepatocytes affected)	0	0	0	0	75	0
Degree 2 (>30%–60% hepatocytes affected)	0	0	0	0	0	0
Degree 3 (>60% hepatocytes affected)	0	0	0	0	0	0

Eight animals from different litters per group.

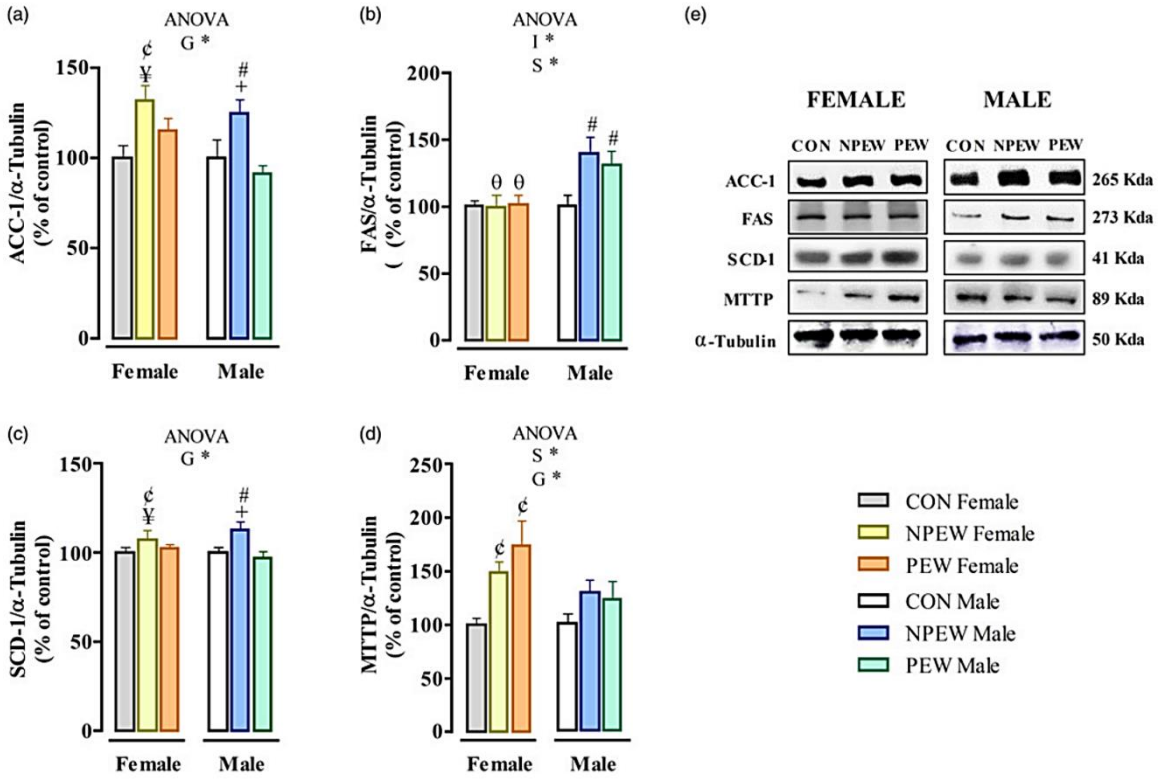

Fig. 3. Expression, in male and female offspring at PN180, of total ACC-1 (a), FAS (b), SCD-1 (c), and MTTP (d). These enzymes are involved in hepatic metabolism. Representative Western blot bands for each protein are shown in (e). Values are mean ± SEM (n = 8–10). I, intersection; S, sex factor; G, group factor. ¢ NPEW or PEW females vs. CON females; ¥ NPEW females vs. PEW females; # NPEW or PEW males vs. CON males; + NPEW males vs. PEW males; θ Females vs. respective males. Significance is assumed at the level of $P < 0.05$.

The following proteins showed no effects or interaction: ACC-1/phosphorylated acid-acetyl-CoA carboxylase (pACC), carnitine palmitoyl-transferase-1α (CPT-1α), sterol regulatory element-binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor (PPAR) α (PPAR-α), PPAR-γ, glucose 6-phosphatase-α (G6Pase-α), phosphoenolpyruvate carboxykinase-1 (PEPCK-1), total Adenosine Monophosphate (AMP)-activated protein kinase (AMPK), and phosphorylated AMP-activated protein kinase (pAMPK)/AMPK) (Supplementary Fig. 1).

Discussion

The two EW models seem to mimic the two main situations of human early interruption of exclusive breastfeeding. The most common situation is that of the mother gradually reducing the frequency of breastfeeding, which could be best reproduced by the PEW model, which reduces the amount of milk that is made available to the baby but does not totally cease breastfeeding, while the NPEW model reproduces a complete interruption.

Corroborating previously published data, we demonstrated here the occurrence of hepatic steatosis among adult males depending on the type of EW. In addition, in the present study, the enzymatic alterations, which were studied for the first time here, partially help explaining this process. Intriguingly, the adult females that were programmed by EW, despite having a higher accumulation of abdominal fat, did not display steatosis, which may be related to the protective action of estrogen on the liver of these animals, a possibility that will be discussed later.

EW programs adult male rats for greater body mass gain and VFM. Since pyruvate is preferentially used by hepatocytes as a substrate for gluconeogenesis, the pyruvate challenge is a good tool to examine gluconeogenic activity *in vivo*. In the first glucose measurement (15 min after injection), EW animals of both sexes showed unchanged levels. Corroborating this finding, PEPCK and G6Pase, which catalyze the rate-limiting step of gluconeogenesis, were similar among groups.

At 30 min, PEW males showed higher glucose levels only when compared to the CON group. At this moment, increased glycemia under iPTT can be due to an impairment of glycogen synthesis by lower insulin action in the liver as well as to lower peripheral sensitivity to this hormone, reducing glucose uptake by muscle and adipose tissue. At 60 min, males of both EW models had impaired maintenance of the glycemic homeostasis. At 120 min, PEW males showed a drop in glucose levels, while NPEW glycemia remained high. Despite the differences in the iPPT profiles, the AUCs of both EW groups were increased. Pietrobon *et al.* (2020) reported that both EW groups have insulin resistance in the skeletal muscle,[38] which is responsible for the uptake of 70%–80% of the plasma glucose.[39] Thus, EW seems to program muscle insulin resistance prior to the liver one. To reinforce this idea, the content in the liver made of the proteins that play key functions in the lipid metabolism homeostasis, which is insulin-regulated, was not altered in EW animals (SREBP-1c, PPAR-α and PPAR-γ, CPT-1α, pACC/ACC, and pAMPK/AMPK).

The hepatic steatosis characteristic of NPEW males seems to be related to other factors that are independent of insulin action on this tissue. The high hepatic TG observed in this group may be due to an increase in *de novo* lipogenesis (increased ACC-1, FAS, and SCD-1). Also, NPEW males can have higher diacylglycerol O-acyltransferase 1 (DGAT-1), a key enzyme for TG biosynthesis, and compromised TG uptake, possibly by transporter-mediated hepatic fatty acid uptake alterations, for example,[40] since this lipid was higher in hepatocytes and plasma. A limitation of our study in this regard was that we have not analyzed these parameters. The increase in TG synthesis concurrently with unaltered mitochondrial β-oxidation and TG exportation through VLDL, as shown in this work, can promote hepatic lipid accumulation and increased oxidative stress, which has already been demonstrated in the NPEW model.[21]

Plasma CHOL was not altered in the EW models. However, this lipid content was higher in the liver of males, which could be related to dysfunctions in synthesis and/or esterification caused by, respectively, alterations in 3-hydroxy-3methyl-glutaryl-CoA reductase or Acyl CoA: CHOL acyltransferase1.[41] Despite normal total CHOL in plasma, EW animals showed lower HDL-c, which indicates a higher atherogenesis risk[42] and suggests that EW females and males have a higher risk of developing cardiovascular diseases.

PEW males had normal ACC-1 and SCD-1, absence of steatosis, higher TG and CHOL in the liver, but unchanged in the plasma. Compared to NPEW males, PEW males had reduced SCD-1, which can be an attempt to contain TG and fatty acids synthesis (higher FAS). However, higher TG in the hepatocytes of PEW males suggests that DGAT-1 was higher.

As previously reported by our research group, BRO appears to have a long-term protective effect on the hepatic functions of EW male rats.[22] Thus, we hypothesize that the protective role of BRO is related to an increase in Sirtuin 1, an enzyme involved in NAFLD inhibition.[22] Despite the higher TG in the liver, PEW males had normal TG in the plasma. This lipid could be uptaken by other tissues, such as pancreas and muscle. Indeed, our group have previously reported the impairment of insulin secretion at higher glucose concentrations and muscle insulin resistance in EW males.[38] Thus, impairment of glycemic homeostasis could be due to lipotoxicity related to steatosis development in other tissues.

Clinical studies demonstrate that NAFLD manifestations occur with a higher prevalence in men than in women.[43] In fact, the accumulation of hepatic lipids and the consequent development of liver steatosis are twice as common in the postmenopausal period compared to the reproductive age.[44–46] In addition, mice fed with a diet deficient in choline and methionine for two weeks show steatosis, an increase in hepatic transaminases, and an increased expression of inflammatory markers with these changes more pronounced in males,[47] corroborating our present findings where EW females did not have alterations in liver morphology or in serum and hepatic CHOL and TG levels.

In the literature, it is well known that estrogens play an important role in the regulation of energy homeostasis in different organs, especially suppressing ingestion and increasing energy expenditure,[48] as changes in the synthesis and/or action of 17β-estradiol predispose to obesity, metabolic syndrome, and type 2 diabetes.[49] The biological effects of this hormone are mainly mediated by nuclear estrogen receptors α (ER-α) and β (ER-β)[50,51] and also by the G protein coupled receptor.[52,53] ER-α appears to be the mediator of the protective anti-inflammatory effects of estrogen in several tissues, such as the liver.[54] According to Barsalani *et al.* (2010), the accumulation of hepatic lipids observed in ovariectomized rats is not strictly related to the accumulation of adipose tissue and hyperphagia caused by the absence of estradiol but is also due to the dysregulation of the lipid metabolism of the organ itself.[55,56] In Sprague–Dawley rats, the reduction of estrogenic activity in the liver promoted an increased expression of lipogenic genes, such as SREBP-1c, ACC-1, FAS, and SCD-1, and a reduced gene expression of proteins related to hepatic lipid oxidation.[57–59] Additionally, the activation of only one of the ER-α domains (Activation Function 1/ Activation Function 2) is sufficient to prevent obesity, steatosis, and insulin resistance in mice submitted to HFD.[60] The replacement of estradiol levels in ovariectomized females improved cholesterolemia, reduced liver damage due to reduced macrophage infiltration and the expression of pro-inflammatory genes,[61] and reduced the expression of lipogenic genes in the liver of obese mice (ob/ob), which improved the glucose tolerance and response to insulin[62] and demonstrates the protective role of estrogen on the development of NAFLD.[55,63] Considering that the increase in the biological effects of estradiol can cause the reduction of adipose tissue and that, in our study, NPEW and PEW females had a greater increase of visceral adiposity, we suggest a greater estrogenic action specifically at the liver level regardless of the EW model. A study of ER in liver and adipose tissues is necessary to confirm this hypothesis.

The role of estrogens on the maintenance of normal lipid accumulation in the hepatocytes is also related to the regulation of VLDL production. Ovariectomized rats have decreased VLDL in plasma, indicating that the reduction in the hepatic production of this molecule may be responsible for increased lipid accumulation in the absence of estrogen.[55] The assembly and secretion of VLDL are complex mechanisms that mainly involve the MTTP[62] whose activity is a limiting factor for this process.[64–66] Ovariectomized rats showed lower gene and protein expression of MTTP,[57,67] which was recovered with the administration of estrogen,[55] demonstrating that estrogens are involved in the regulation of all lipid pathways in the liver (*de novo* lipogenesis, lipid

oxidation, and export). In our study, despite higher liver ACC-1 and SCD-1 in the NPEW group, both EW females showed an increase in MTTP protein expression, without showing changes in oxidative enzymes, possibly due to a protective effect of estradiol preventing excessive accumulation of lipids in hepatocytes by new metabolic challenges, for example, as the supply of HFD throughout life.

Additionally, in the pyruvate challenge, PEW females had higher glucose levels only at 60 and 120 min after injection, which were responsible for the higher AUC observed in this group. This finding could be related to a higher uptake by peripheral tissues since hepatic content of G6Pase and PEPCK enzymes was unchanged and isolated insulin secretion was lower.[38] It is possible that, in challenging situations, the maintenance of normal levels of glycemia is compromised in PEW females. Of note, the activities or metabolites of the studied enzymes were not evaluated, which is a limitation of the present research.

It is possible that females developed an adaptive mechanism during evolution to deal with insults during lactation better than males as these mechanisms are related to the need for satisfactory reproduction. In this sense, MTTP could be a key enzyme in protecting the liver from steatosis and its grave consequences.

Conclusion

Taken together, our findings demonstrate that the alterations in the liver cytoarchitecture only in NPEW males are explained by the differences in the expression of FAS and SCD-1. Unlike males, the adult female offspring from both EW models did not develop hepatic steatosis but showed increased MTTP content, which may have a role in improving the outcomes of the EW programming.

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Conflicts of interest. None.

Ethical standards. The experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/014/2017). Experiments were carried out in accordance with the Brazilian Law 11.794/2008, which regulates the procedures for the scientific use of animals.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174420000495

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2.3 **Modelo experimental III: Exposição materna à dieta hipoprotéica na lactação**

Os itens “Material e Métodos”, “Resultados” e “Discussão” referentes a este modelo de programação estão descritos no artigo abaixo:

2.3.1 Artigo 3 - Low protein diet during lactation programs hepatic metabolism in adult male and female rats

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LOW PROTEIN DIET DURING LACTATION PROGRAMS HEPATIC METABOLISM IN ADULT MALE AND FEMALE RATS

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Short title: Liver function after maternal protein restriction diet.

Abstract

The liver is an essential regulator of energy metabolism, which is destabilized by nutritional alterations. Liver development continues during breastfeeding; hence, important nutritional challenges during this period predispose patients to diseases throughout subsequent life. A maternal protein-restricted (PR) diet during lactation promotes a reduction in the body weight, adiposity and plasma glucose and insulin, leptin resistance and an increase in corticosterone and catecholamines in male rat offspring in adulthood. The present study investigated hepatic metabolism in the offspring of both sexes from control dams receiving standard chow containing 23% protein and PR dams receiving an 8% protein diet during lactation. The offspring of both sexes were separately evaluated at 6 months of age. PR males had no liver steatosis and manifested a reduction in the lipids in hepatocytes adjacent to vascularization. These animals had lower levels of esterified cholesterol in hepatocytes, suggesting higher biliary excretion, unchanged glycolysis and gluconeogenesis and lower contents of the markers of mitochondrial redox balance and endoplasmic reticulum (ER) stress response and oestrogen receptor alpha. PR females manifested normal hepatic morphology associated with higher uptake of cholesterol esters, normal glycolysis and gluconeogenesis and lower ER stress parameters without changes in the key markers of redox balance. Additionally, these animals had a lower content of oestrogen receptor alpha and higher content of androgen receptor. The maternal PR diet during lactation did not program hepatic lipid accumulation in adult progeny; however, several repair homeostasis pathways were altered in males and females, possibly compromising maintenance of normal liver function.

Key words: Metabolic programming, lactation, maternal diet, protein restriction, liver.

1. Introduction

Concerns about the short-term effects of child malnutrition on morbidity and mortality have evolved to include the late effects on chronic noncommunicable diseases [1]. Epidemiological evidence during the periods of global crisis, such as the Dutch Hunger Winter (1944-1945) or the great Chinese famine (1950-1960), highlighted the associations between maternal undernutrition, low birth weight and subsequent adult diseases, such as obesity, diabetes and hyperlipidaemia [2–4]. The 'thrifty phenotype' hypothesis explains this phenomenon; foetal undernutrition leads to metabolic adaptations that optimize the utilization of limited nutrient availability to thus augment the survival opportunities under poor conditions after birth [5]. However, these adaptations increase the risk of a disease when an individual is exposed to a nutrient-rich environment in postnatal life [6].

Despite nutritional transition in recent decades, various studies on maternal diabetes and maternal obesity have demonstrated that excessive energy intake during gestation and childhood programmes the foetus in a way similar to maternal undernutrition [7–11]. Epidemiological studies demonstrated that environment during critical periods, including gestation, breastfeeding and adolescence, exerts a strong influence on individual development and is associated with the onset of diseases throughout later life, in particular, obesity and its comorbidities. This phenomenon is known as metabolic programming and is related to the developmental origins of health and disease (DOHaD) hypothesis [12,13].

Since 2009, the world has been affected by numerous pandemics, including H1N1, SARS, MARS, Ebola and COVID-19 [14]. The latter disease is expected to particularly strongly influence nutritional and health security of vulnerable groups, including young children and pregnant and lactating women, further aggravating social and health

differences [15,16]. Global evidence shows that increasing food insecurity is a serious public health problem and may be associated with suboptimal infant feeding practices, which are possibly related to inadequate breastfeeding [17–20]. Thus, the immediate effects of COVID-19 primarily target the adults; however, the legacy of this pandemic in unborn babies and children is profound [21]. In low-income populations, the replacement of proteins by carbohydrates is a common practice; adding fortifying carbohydrates to bottle-feeding reduces the protein content of the milk [22].

Animal studies by our and other research groups demonstrated that a protein-restricted (PR) diet during lactation alters maternal metabolism [23–26] and milk composition [27–29] and modifies offspring metabolism at weaning [23,24,30] and in adulthood [30–33]. The milk yielded by PR mothers is low in protein [23], and their pups manifest hypoalbuminemia, which confirms malnutrition of these animals during lactation [30]. PR pups have lower body weight at weaning and remain underweight through adulthood despite a lack of changes in food intake in adult life, supporting the hypothesis of hypermetabolic status programming [23].

The liver is a critically important organ for the regulation of body energy metabolism and is vulnerable to maternal nutritional changes [34,35] because its development continues during breastfeeding [36,37]. Maternal protein undernutrition (8% protein) throughout pregnancy [38] or at the last third of pregnancy (4% protein) [39] promotes higher hepatic lipid inclusion in the offspring in adulthood. Souza-Melo et al. [40] demonstrated that maternal protein malnutrition (5% protein) throughout gestation and the first half of lactation causes a significant decrease in hepatocyte number and increases the rate of hepatic steatosis in 6-month-old rats, and a post-weaning high-fat diet maximizes these alterations in both sexes. However, we have previously shown that adult rodents whose mothers were submitted to PR (8% protein) exclusively during

lactation had normal hepatic morphology [33] despite metabolic and hormonal alterations, such as reduced body weight and adiposity [23,30,32,33], reduced serum cholesterol [30], leptin resistance in the hypothalamus [31], reduced plasma glucose and insulin, higher muscle glycogen and higher corticosterone and catecholamine levels [30,32]. Notably, these findings were detected in male progeny. Thus, the phenotype of female PR offspring in adulthood requires investigation since several parameters altered in PR males can be influenced by sex steroids, as demonstrated in other models [41,42].

In this context, we hypothesized that lactation can be a critical window that determines future liver function. The hypothesis of the present study is that the hepatic pathways of glucose-lipid metabolism can be altered in a programming model by protein restriction during lactation and are different between males and females. Therefore, we aimed to investigate the long-term effects of a maternal PR diet exclusively during lactation on liver lipid inclusion (1), key biomarkers of glycolysis (2), gluconeogenesis (3), *de novo* lipogenesis (4), lipid oxidation and export (5), mitochondrial redox balance (6), endoplasmic reticulum stress (7), macroautophagy (8) and sexual steroid receptors (9) in adult male and female offspring.

2. Materials and Methods

All procedures were approved and performed following the recommendations by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/024/2019) in accordance with Brazilian Law no. 11.794/2008. Wistar rats were housed in a temperature-controlled vivarium (23 ± 1°C) with an artificial light/dark cycle (lights from 7:00a.m. to 7:00p.m.) and free access to water and standard chow (Nuvilab-NUVITAL Nutrientes LTDA, Paraná, Brazil). For two weeks, 3-month-old virgin female rats were cohabitated with adult male rats for mating at a proportion of

126 2 females:1 male. The day of conception was determined by examination of a vaginal
127 smear for the presence of sperm on the day after mating. The pregnant rats were allowed
128 to deliver spontaneously in individual maternity cages, and only animals with a litter size
129 of 10 pups were used to avoid the influence of litter size on the programming effect. In
130 the first postnatal day (PND), the litters were adjusted to 6 pups per dam (3 males and 3
131 females) to maximize lactation performance and to assure standardized nutrition until
132 weaning [23].
133
134 *2.1 Experimental design of programming by a maternal low-protein diet during lactation*
135 At birth, dams and offspring were randomly assigned to one of two groups fed an
136 isocaloric diet *ad libitum* (n = 10 dams/group): PR (protein-restricted group was fed an
137 8% protein diet) and CTL (control group was fed a 23% standard rodent diet) for the
138 duration of lactation (3 weeks). As shown in Tab. 1, the PR diet was produced in our
139 laboratory using a standard commercial diet for rodents (Nuvilab-NUVITAL Nutrientes
140 LTDA, Paraná, Brazil) by replacing a part of the protein in the diet with cornstarch as
141 described previously [23]. The amount of the starch was calculated to compensate for a
142 decrease in energy content due to a reduction in protein. Vitamin and mineral mixtures
143 were formulated according to the AIN-93 G recommendation for rodent diets [43]. At
144 weaning, the male and female offspring were weighed and fed a standard diet containing
145 23% protein until 6 months of age. We randomly evaluated biometric parameters in a
146 single offspring/sex/litter (a total of 10 animals per group), and other pups in the litters
147 were used for other tests. For molecular, morphological and in vivo tests, we used 7-8
148 animals of different litters/sex per group.
149
150 *2.2 Oral glucose and intraperitoneal pyruvate tolerance test – OGTT/iPTT*

151 The OGTT was performed at PND175. After 12 hours of fasting, a blood sample
152 was collected to determine basal glycaemia (time 0) using a glucometer (ONETOUCH
153 ULTRA®, Johnson & Johnson, São Paulo, Brazil). Then, glucose (50%) was
154 administered in sterile saline (0.9% NaCl) via an oral probe at 2 g/kg of BW. The level
155 of glucose was evaluated 15, 30, 60 and 120 min after the administration [44].
156 To analyse the rate of gluconeogenesis in adult rats, iPTT was performed in a
157 single animal/sex/litter at PND178. Pyruvate is a common intermediary metabolite of the
158 gluconeogenic pathways and is generated from lactate, amino acids and glycerol; thus
159 pyruvate challenge is used in this test. The animals were fasted for 12 hours, and blood
160 samples were taken from the tip of the tail vein to determine fasting glycaemia (time 0)
161 using a handheld glucometer (Accu-Chek Advantage; Roche Diagnostics, Mannheim,
162 Germany). Subsequently, the animals received an i.p. injection of 2 g/kg of body mass of
163 pyruvate, and blood samples were assayed after 15, 30, 60, 120 and 180 min [42].
164
165 *2.3 Biometric and plasma parameters*
166 The body weight and food intake were measured throughout development. At 6
167 months of age, the rats were euthanized by decapitation, and total blood was collected.
168 The liver and white adipose tissues from visceral fat mass (VFM; retroperitoneal,
169 perigonadal and mesenteric) were collected and weighed, and the weight was recalculated
170 as g/100 g of body weight. The levels of total cholesterol (CHOL), high-density
171 lipoprotein cholesterol (HDL-c) and total triglycerides (TGs) in the plasma were
172 determined using commercial kits according to the manufacturer's instructions (Bioclin,
173 Brazil).
174
175 *2.4 Liver morphology*

Transversely dissected liver samples were obtained at 6 months of age. The samples were fixed with 4% paraformaldehyde for 24 hours, followed by dehydration in ascending alcohol concentrations and diaphanization in xylene. The materials were embedded in histological Paraplast® (Sigma-Aldrich MO, USA), and non-consecutive slices of 4 µm thickness were prepared for staining with haematoxylin and eosin (H&E). Ten photomicrographs per animal were randomly acquired by an Olympus DP71 camera and Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan) and projected on a high-resolution LCD flat panel monitor (LG Electronics, Seoul, South Korea).

Additionally, fresh liver samples were immersed in Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) in aluminium moulds, quickly frozen in liquid nitrogen, and stored at −80°C. Non-consecutive cryosections of 10 µm thickness were stained with Oil red O (ORO), and Harris's haematoxylin was used for counterstaining. Photomicrographs (15/animal) were randomly acquired, and the percentage of lipid droplets per analysed area was evaluated using Image-Pro Plus software version 7.01 for Windows (Media Cybernetics, Silver Springs, MD, USA) (Catta-Preta et al., 2011). The values were classified in accordance with the magnitude of steatosis using Brunt's classification: 0 (none to 5% of hepatocytes affected), 1 (> 5% to 30% affected), 2 (> 30% to 60% affected) and 3 (> 60% affected) [45].

2.5 High-Performance Thin Layer Chromatography (HPTLC)

Lipids were extracted from hepatic samples (±1 mg) with chloroform-methanol by the method of Bligh and Dyer [46] with modifications. Total neutral lipids were developed by HPTLC using a DC silica gel 60 plate (Merck Millipore, Germany) immersed in a charring solution (3% CuSO4 and 8% H3PO4, v/v) [47]. HPTLC plates were analysed, and the hepatic contents of free fatty acids (FFAs), triglycerides (TGs),

monoacylglycerol (MAG), phospholipids (PLs), total cholesterol (t-CHOL) and esterified cholesterol (e-CHOL) were obtained by densitometry using Image Master software (Total Lab, New Zealand).

2.6 Western blotting

A fragment of the liver (±30 mg) was homogenized in RIPA buffer containing a complete protease inhibitor cocktail (La Roche Ltd., SW). After centrifugation (13,000 rpm for 15 minutes at 4°C), the total protein content of the supernatant was measured using a Pierce BCA protein assay kit (Thermo Scientific, USA). The samples (20 µg) were denatured in sample buffer containing beta-mercaptoethanol, subjected to SDS gel electrophoresis with molecular weight standards (Kaleidoscope, Bio-Rad™, USA), and then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, UK). The membranes were incubated with appropriate primary (overnight) and secondary antibodies (60 minutes) conjugated to biotin and streptavidin (60 minutes) (Tab. 2). Visualization of specific protein bands was performed by chemiluminescence reagents (Bio-Rad™) using an ImageQuant Las 500 image capture system (GE Healthcare, UK). The intensities of the bands were quantified by optical densitometry using ImageJ software (Media Cybernetics, MD, USA) and were used to assess protein expression. Cyclophilin-β (Cyclo-β), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used as internal controls according to protein molecular weight. The results are expressed as the percentages relative to the control group.

2.7 Statistical analyses

The data are presented as the mean ± SEM, and GraphPad Prism 6.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used for graphics and

226 analyses. Only a single pup per litter was randomly used for analysis. Comparisons were
227 performed using Student's unpaired *t*-tests. Since sex was not considered a factor, only
228 the programming effect (PR and CON) was used for the analyses. The differences were
229 considered significant at $P < 0.05$.
230
231 **3. Results**
232 *3.1 Effects of a maternal protein restriction diet (8%) during lactation on the male*
233 *offspring*
234 As shown in Tab. 3, PR males had lower body weight than that of control males
235 (-33%, $P < 0.0001$) at weaning (3-week-old), and the body weight remained lower until
236 adulthood (-6%, $P = 0.0021$). At 6 months of age, VFM and total plasma CHOL were
237 reduced in PR males compared with those in the control group (-16%, $P = 0.0019$; - 19%,
238 $P = 0.0478$, respectively), and body weight gain, food intake, liver weight, plasma TG
239 and HDL were similar in the groups.
240 Fig. 1 shows the data related to FA and TG metabolism, including liver
241 photomicrographs and the percentage of lipid inclusions. PR males had higher FFA
242 content in the liver (+43%, $P = 0.0024$; Fig. 1A) than the controls. The total and
243 phosphorylated ACC-1 contents were unchanged, and the pACC/ACC ratio was
244 increased in PR males (+29%, $P = 0.0071$; Fig. 1B), indicating a reduction in the active
245 ACC form. FAS and SREBP-1c contents were similar between the groups. The liver
246 contents of TG, MAG and PL were unchanged in PR males (Fig. 1C). With regards to
247 enzyme and transcription factor contents, PR males had higher level of DGAT-1 (+58%,
248 $P = 0.0052$; Fig. 1D) and lower level of PGC-1α (-44%, $P = 0.0102$; Fig. 1D) than the
249 controls. PLIN-2, CPT-1α, MTTP, PPAR-α, PPAR-γ and SIRT-1 contents were similar
250 in the groups. The results of histomorphological analysis of the liver using H&E staining

251 indicated that hepatocytes had normal architecture and were arranged in rows delimited
252 by connective tissue containing sinusoidal capillaries (Fig. 1F and G). Evaluation of
253 various lobular regions stained with ORO demonstrated a reduction in lipid inclusions in
254 hepatocytes adjacent to the vascularization (-68%, $P = 0.0013$; Fig. 1L), i.e., zone 1 (Fig.
255 1H and I). Hepatocytes distal to the vascularization were designated zone 2 (Fig. 1J and
256 K) and had lipid inclusions similar to those in the controls (Fig. 1L). Lipid inclusions in
257 all groups affected less than 5% of hepatocytes in the analysed area, showing the absence
258 of steatosis in accordance with Brunt's NAFLD score (Fig. 1L).

259 The data on hepatic CHOL metabolism are shown in Fig. 2 (A and B). PR males
260 had unchanged t-CHOL (Fig. 2A) and HMGCR contents and the levels of enzyme
261 synthesis (Fig. 2B). The content of e-CHOL was lower (-42%, $P = 0.0039$; Fig. 2A), and
262 the content of the key esterification enzyme ACAT-1 was unchanged (Fig. 2B).

263 The main glycolytic enzymes were unaltered (Fig. 2C). Regarding the
264 gluconeogenesis pathway, PR males had higher PEPCK-1 content (+191%, $P < 0.0001$;
265 Fig. 2D) and no changes in G6Pase-α content (Fig. 2D), responses to glucose and the
266 results of the pyruvate tolerance tests (Fig. 2E-H).

267 The data on the key enzymes related to hepatic redox balance are shown in Fig.
268 2I. PR males had lower contents of anti-oxidant enzymes, including SOD-2 (-36%, $P =$
269 0.0063) and catalase (-37%, $P = 0.0060$), and the GPx-1/2 content was unchanged. The
270 content of 4-HNE, a main marker of lipid oxidative damage, was similar in the groups.

271 The data on two pro-inflammatory cytokines are shown in Fig. 2J. PR males had
272 lower level of TNF-α (-71%, $P = 0.0052$; Fig. 2J) than the controls, and the level of IL-6
273 was similar in the groups.

274 The data on the main markers of the unfolded protein response and
275 macroautophagy pathway are shown in Fig. 2K. CHOP content was lower in PR males (-

21%, P = 0.0205; Fig. 2K), and the levels of eIF, peIF and sXBP-1 were similar in the groups. The contents of enzymes related to macroautophagy (LC3 I/II and p62) was not altered.

PR males had lower ER-α content (-44%, $P = 0.0331$; Fig. 2L), and the levels of ER-β and AR were unchanged.

3.2 Effects of a maternal protein restriction diet (8%) during lactation on female offspring

As shown in Tab. 3, females had decreased body weight at 3 weeks of age (-32%, $P < 0.0001$) and 6 months of age (-9%, $P < 0.0001$). In adulthood, PR females had lower VFM (-15%, $P = 0.0428$) than control females, and body weight gain, cumulative food intake and liver weight were unchanged. The levels of total plasma CHOL (-32%, $P = 0.0490$) and TG (-29%, $P = 0.0323$) were reduced, without alterations in the HDL levels.

Fig. 3 shows the results related to FA and TG metabolism, including photomicrographs and lipid inclusions in the liver. PR females had higher FFA content (+103%, $P < 0.0001$; Fig. 3A) and lower total ACC-1 content (-29%, $P = 0.0126$; Fig. 3B). The content of pACC (inactive form) and the ratio of pACC/ACC, FAS and SREBP-1c were similar in the groups (Fig. 3B).

PR females had lower levels of TG in the liver (-51%, $P = 0.0058$; Fig. 3C), and the levels of MAG and PL were unaltered. Regarding the key enzymes involved in TG metabolism, the DGAT-1 (+38%, $P = 0.0335$; Fig. 3D) and CPT-1α contents (+37%, $P = 0.0211$; Fig. 3D) were higher in PR females than those in the controls. These animals also had lower PGC-1α (-43%, $P = 0.0109$; Fig. 3D) and PPAR-α contents (-39%, $P = 0.0011$; Fig. 3D), and the contents of PLIN-2, MTTP, PPAR-γ and SIRT-1 were unchanged (Fig. 3D). The results of morphological liver analyses demonstrated similar

300 structures and lipid inclusions in hepatocytes in the female groups, both in the absence of
301 steatosis (Fig. 3F-L).

302 Evaluation of liver CHOL metabolism is shown in Fig. 4. PR females had
303 unchanged t-CHOL levels (Fig. 4A), and the HMGCR content was lower than that in the
304 controls (-42%, P = 0.0012; Fig. 4B). PR females also had higher levels of e-CHOL
305 (+46%, P = 0.0147; Fig. 4A), and the ACAT-1 content was unchanged (Fig. 4B).

306 Regarding glucose metabolism, PR females had a higher GCK content (+51%, P
307 = 0.0098; Fig. 4C), and the level of GLUT-2 was unchanged. PR females had a higher
308 PEPCK-1 content (+17%, P = 0.0087; Fig. 4D) and the G6Pase-α content, response to
309 OGTT and results iPTT were similar to those in the controls (4E-H).

310 The levels of the main mitochondrial redox balance markers were similar in the
311 groups (Fig. 4I). PR females had a lower TNF-α content (-37%, P = 0.0052; Fig. 4J) than
312 the controls, and the IL-6 content was unchanged.

313 The contents of the key markers of the unfolded protein response and
314 macroautophagy pathway are shown in Fig. 4K. PR females had lower contents of peIF
315 (-47%, P = 0.0299), sXBP-1 (-47%, P = 0.0221), CHOP (-37%, P = 0.0346) and p62 (-
316 49%, P = 0.0350) than the controls, and the levels of eIF and LC3 I/II were unchanged.

317 PR females had a lower ER-α content (-44%, P = 0.0138; Fig. 4L) and a higher
318 AR content (-47%, P = 0.0380; Fig. 4L), and the level of ER-β was similar to that in the
319 controls.

320

321 **4. Discussion**

322 The present study investigated the implications of low-protein milk on liver
323 homeostasis in adult offspring of both sexes. Males programmed by PR do not develop
324 hepatic steatosis [33]. However, the present study performed original characterization of

a dysregulation of metabolic pathways in the liver caused by maternal PR at a critical window for development of hepatic tissue; moreover, the present study is the first to describe the phenotype of female offspring. The changes in the percentage of protein offered in the maternal diet and various periods (pregnancy or lactation) investigated using the PR model may be associated with the development or absence of steatosis [33,48], which should be compared with the data of the literature.

Fatty acids (FAs) present in the liver originate from the diet, plasma and/or *de novo* FA synthesis [49]. Inclusion of the standard diet components contributed to the lack of alterations in the main markers of *de novo* FA synthesis (SREBP-1c, active ACC and FAS), suggesting that an increase in intracellular FFAs observed in PR males was due to an increase in hepatic influx. Furthermore, unaltered pathways, such as *de novo* lipogenesis, β-oxidation and VLDL assembly, could have contributed to high availability of FFAs in hepatocytes.

Considering high plasma levels of lipolytic hormones (catecholamines, corticosterone and thyroid hormones) [30,32,50–52] and a reduction in VFM and in the size of visceral and subcutaneous adipocytes in PR males [32,53], WAT lipolysis may be the main source of FFAs that are taken up by hepatocytes. An increase in the levels of fatty acid transport proteins (FATPs), fatty acid binding proteins (FABPs) and CD36 [54] may be associated with increased hepatic uptake of FFAs in our model; however, the expression of these biomarkers was not evaluated in the present study.

Apparently, an increase in the DGAT-1 content detected in PR males was not associated with an increase in the product of this enzyme, i.e., TGs. The level of TGs in hepatocytes and the plasma was normal; thus, inactive fraction of DGAT-1 could have been increased. To confirm this suggestion, it is important to assess the enzyme activity. Since the MTTP content and serum levels of VLDL were normal [30], the assembly and

350 export of lipoproteins was preserved, indicating adequate functioning of the TG synthesis
351 pathway in the liver of males programmed by PR.

352 Liver zoning is important for proper metabolic functioning of this organ.
353 Hepatocytes in the vicinity of the portal triad have greater oxidative activity, such as
354 gluconeogenesis and glycogenesis, and hepatocytes close to the central vein have higher
355 *de novo* TG synthesis and lower β-oxidation [55]. Considering that TG stored as lipid
356 droplets can be recruited for the formation of VLDLs [49], a reduction in lipid inclusions
357 in the region of higher lipogenic activity in the liver of PR males (zone 1) suggested that
358 lipid droplets are hydrolysed to maintain the assembly and export of VLDLs,
359 complementing *de novo* TG synthesis.

360 *De novo* CHOL synthesis was not programmed by maternal PR during lactation,
361 since the contents of t-CHOL and its synthetic enzyme (HMGCR) were similar to those
362 in the control group. However, the e-CHOL content was reduced despite the normal
363 content of the esterification enzyme ACAT-1, suggesting increased biliary excretion
364 possibly caused by the hyperthyroid state of PR males. To confirm this hypothesis,
365 evaluation of ACAT-1 activity is necessary. A decrease in the plasma CHOL levels may
366 indicate an increase in tissue uptake, especially for the synthesis of steroid hormones.

367 The glycolytic pathway was not programmed by maternal PR during lactation
368 because the levels of GLUT-2 and GCK were not altered in PR males. Regarding
369 gluconeogenesis, an increase in PEPCK-1 does not reflect the formation of new glucose,
370 since the content of the enzyme that regulates glucose release (G6Pase-α) was not altered,
371 and the gluconeogenic response to high concentrations of pyruvate was normal. Gene
372 expression of PEPCK-1 is influenced by several hormones, including lower level of
373 insulin and higher levels of corticosterone and thyroid hormones in PR males [56]. A
374 maternal PR diet during lactation may influence oxidative balance in the male offspring.

375 PR males had unchanged 4-HNE content but the contents of SOD-2 and catalase were
376 reduced, suggesting a reduction in the antioxidant system. This phenotype can predispose
377 the liver of these animals to an insufficient antioxidant response to certain insults,
378 promoting oxidative stress that can cause the development of steatosis [57]. Therefore,
379 individuals submitted to moderate protein restriction during breastfeeding may manifest
380 liver dysfunction, for example, facing an unbalanced diet. On the other hand, we cannot
381 neglect a possibility that lower oxidative stimuli and inflammatory insult may be
382 responsible for lowered antioxidant profile.

383 The changes in endoplasmic reticulum homeostasis lead to the activation of the
384 unfolded protein response (UPR) [58]. PR males had a lower content of CHOP, which is
385 one of the enzymes that integrate the UPR. A failure of the activation of this pathway
386 points to an impairment of the endoplasmic reticulum response, which can indicate
387 inefficient repair pathways in case of liver injury [59].

388 As mentioned above, we investigated adult females programmed by maternal PR
389 exclusively during lactation. The results indicated the lack of accumulation of the lipids
390 in the liver of PR females; however, the assayed levels of the markers of lipid metabolism
391 predisposed these animals to considerable hepatic alterations due to nutritional challenges
392 throughout life. PR females had low body weight from weaning to adulthood and had also
393 reduced VFM, plasma CHOL and TG levels in adulthood. Similar to PR males, females
394 did not manifest changes in weight gain; thus, these findings were independent of
395 compensatory enhanced growth.

396 Hepatic accumulation of FFAs in females suggested that maternal PR promoted
397 the mobilization of these lipids from WAT independently of sex, considering that both
398 standard diet intake and *de novo* FA synthesis were unchanged (SREBP-1c, active ACC
399 and FAS). Similar to males, PR females could have manifested WAT lipolysis due to

400 lower body weight, VFM and normophagia; however, the hormonal profile of these
401 animals remains unknown. Higher β-oxidative activity related to high content of CPT-1α
402 may indicate a mechanism of FFA reduction in hepatocytes of PR females.

403 Maternal PR during lactation programmed a decrease in hepatic and plasma TG
404 in female offspring. Higher content of DGAT-1 was not related to availability of TG in
405 hepatocytes and plasma in PR females. Similar to males, an increase in *de novo*
406 lipogenesis machinery was product-independent. The proportion of inactivated fraction
407 of DGAT-1 could have been increased. In addition, a reduction in plasma TG suggested
408 that the production and export of VLDLs was also reduced since the total plasma TG
409 content mainly comprises VLDLs. Thus, unchanged MTTP content suggested that this
410 protein was available but was used according to availability of TG. Interestingly, no
411 changes in the percentage of lipid infiltrations were detected in the tested liver regions.
412 Therefore, unlike males, PR females did not mobilize TG from the lipid droplets.
413 Evaluation of the activities of these enzymes is important to confirm this suggestion.

414 A reduction in the plasma levels of CHOL and increased levels of e-CHOL in the
415 liver suggested that PR females had increased hepatic LDL uptake. A reduction in hepatic
416 HMGCR content associated with unchanged t-CHOL and increased esterified fraction
417 levels could have been related to allosteric control over HMGCR. Unaltered ACAT-1
418 content suggested that e-CHOL could have caused an increase in the LDL receptors in
419 the liver (LDLR), reflecting a reduction in the plasma levels of total CHOL, given that
420 HDL levels were not altered. Thyroid hormones increase LDLR; thus, PR females could
421 have had hyperthyroidism, as was previously shown in PR males [51,52].

422 Due to the key role of GCK in glycolysis, the enzyme is considered a hepatic
423 glucose sensor highly responsive to insulin [60]. The content of this enzyme was
424 increased in females with PR; however, the content of GLUT-2, which acts independently

425 of insulin, was normal [61]. Similar to males, the content of PEPCK-1 (gluconeogenic
426 enzyme) was increased in PR females without an increase in gluconeogenesis after
427 pyruvate overload. In this context, an increase in the content of enzymes related to
428 glycolysis and gluconeogenesis may be due to hormonal stimulation (low insulin or high
429 thyroid hormones) of gene expression without detectable increase in enzymatic activity.
430 The content of the key enzymes of oxidative balance was not different in PR
431 females; however, the repair pathways of the endoplasmic reticulum could have been
432 compromised. Autophagy is an important pathway activated by the reticulum and/or
433 oxidative stress [62] that promotes lipid oxidation and maintenance of homeostasis,
434 reducing cellular stress due to recycling of the organelles and substrates, such as lipids
435 [62,63]. The ubiquitin-binding protein p62 is continuously produced and degraded,
436 reflecting autophagic flow [64]. Thus, a reduction in the key enzymes of the endoplasmic
437 reticulum repair pathway (sXBP-1 and CHOP) and autophagy pathway (p62) may
438 indicate a reduced stress response and contribute to impaired liver homeostasis in the case
439 of various hepatic injuries [63].
440 The liver is the largest non-reproductive organ targeted by sex steroids [65], which
441 act as sensors of the metabolic state and play a role in the adaptation of hepatic energy
442 homeostasis, regulating the genes involved in various metabolic processes [66]. In males,
443 testosterone signalling via AR and oestrogen via ER-α inhibit the development of hepatic
444 steatosis, inflammation, lipogenesis and gluconeogenesis while stimulating FA oxidation
445 and lipid export [66]. In females, testosterone promotes hepatic steatosis, inflammation
446 and gluconeogenesis, while oestrogen protects against the development of hepatic
447 steatosis, inflammation and gluconeogenesis [66]. A maternal PR diet during gestation
448 and lactation programs the adult female offspring for increased plasma levels of
449 progesterone and oestradiol without altering plasma testosterone [67]. Protein

450 malnutrition during lactation leads to an increase in ER-α expression in the uterus in
451 adolescent female offspring despite normal oestradiol and testosterone levels [68].
452 Maternal PR during pregnancy does not change the plasma testosterone levels in adult
453 male offspring [69]; during pregnancy and lactation, PR causes a reduction in testosterone
454 [70], and malnutrition exclusively during the lactation period increases testosterone and
455 reduces oestradiol [71]. There are no data on the effects of oestrogens and androgens on
456 the liver of the offspring in models of PR during pregnancy and/or lactation and on the
457 distribution of steroid receptors in the liver tissue. In the present study, maternal PR
458 programmed a reduction in the ER-α content in the offspring of both sexes. An increase
459 in the AR content exclusively in PR females may be related to various alterations because
460 both sex steroids are intrinsically involved in hepatic metabolism. The profile of steroid
461 receptors in programmed females (low ER-α and high AR) may favour the development
462 of hepatic steatosis.

463 In summary, a maternal low-protein diet exclusively during lactation programs
464 the offspring of both sexes for lower weight associated with reduced adiposity and for
465 normophagia without the development of steatosis, suggesting hyperactivation of
466 lipolysis and a consequent increase in availability of FFAs for the liver. The use of lipids
467 from WAT as preferred energy substrate for the liver may explain the absence of
468 compensatory growth. Apparently, the liver is prepared for increased synthesis; however,
469 the enzymatic machinery does not reflect increased functionality, since an increase in the
470 key enzymes of gluconeogenesis and triglyceride synthesis does not reflect an increase in
471 the corresponding products in either sex. Interestingly, an increase in β-oxidation and
472 LDL uptake and a reduction in TG availability suggested greater adaptation of lipid
473 metabolism in females, which may be an adaptive mechanism for a reduction in intra-
474 and extra-hepatic lipid contents. Maternal PR also programs the response to endoplasmic

475 reticulum and/or mitochondrial stress, and the effect of a reduction in the repair pathways
476 of endoplasmic reticulum homeostasis appears to be more severe in females. The
477 phenotype of PR females may be related to the combined effect of ER-α reduction and
478 AR increase; however, this suggestion requires further studies. One of the main lessons
479 learned from this programming model is that an underweight individual has a healthy
480 liver (no fat accumulation), which can hide unregulated machinery with steatotic
481 potential. Therefore, the practice of adding farinaceous products to thicken the milk in
482 the baby bottles, which results in protein dilution, should be avoided, and an adequate
483 protein intake by mothers during breastfeeding should be encouraged.
484
485 **Author Contributions**
486 IMB, EGM and PCL designed the study. IMB and PCL wrote the manuscript. IMB and
487 CBP were responsible for animal programming. IMB was responsible for *in vivo* tests,
488 liver morphology and WB analysis. SSC, GEGK and GCA were responsible for HPTLC
489 analysis. EGM and PCL were responsible for resources and funding acquisition. PCL
490 were responsible for supervision and project administration. All authors contributed to
491 and approved the final manuscript.
492
493 **Declaration of Competing Interest**
494 The authors have no competing interest to declare.
495
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501

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505

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774 **Figure legends**

775 ***Fig. 1. Effects of maternal low-protein diet during lactation in key-markers content of***
776 ***FA and TG metabolism of male offspring.*** **(A)** FFA content and **(B)** enzymes related to
777 FA biosynthesis. **(C)** TG, MAG, PL content and **(D)** enzymes related to TG metabolism.
778 **(E)** Western blotting representative bands. **(F)** Representative photomicrographs of the
779 liver stained with Hematoxylin & Eosin (×40 magnification) of CTL and **(G)** PR males
780 (bar: 50 μm). **(H)** Representative photomicrographs of Zone 1 hepatocytes (near to
781 vascularization) stained with Oil Red-O (×40 magnification) of CTL and **(I)** PR male
782 offspring. **(J)** Representative photomicrographs of Zone 2 hepatocytes (distal to
783 vascularization) of CTL and **(K)** PR male offspring. **(L)** Percentage of lipid inclusion in
784 hepatocytes after ORO staining. Values are mean ± SEM (n = 7 – 8, different litters/sex
785 per group). Significance is assumed at the level of $P < 0.05$.
786
787 **Fig. 2.** ***Effects of maternal low-protein diet during lactation in key-markers content of***
788 ***CHOL metabolism, glucose metabolism, redox balance, pro-inflammatory cytokines,***
789 ***unfolded protein response, macroautophagy pathway and sex steroids receptors of male***
790 ***offspring.*** **(A)** Total and esterified CHOL. **(B)** Enzymes related to CHOL biosynthesis
791 and esterification. **(C)** Key-enzymes of glycolysis and **(D)** gluconeogenesis pathway. **(E)**
792 Oral glucose tolerance test (OGTT) of male offspring at PN175 and **(F)** area under curve.
793 **(G)** Intraperitoneal pyruvate tolerance test (iPTT) of male offspring at PN178 and **(H)**
794 area under curve. **(I)** Redox balance key-markers. **(J)** Pro-inflammatory cytokines. **(K)**
795 Unfolded protein response and macroautophagy pathway. **(L)** Sexual steroids receptors
796 and **(M)** western blotting representative bands. Values are mean ± SEM (n = 7 – 8,
797 different litters/sex per group). Significance is assumed at the level of $P < 0.05$.
798

799 **Fig. 3.** ***Effects of maternal low-protein diet during lactation in key-markers content of***
800 ***FA and TG metabolism of female offspring***. **(A)** FFA content and **(B)** enzymes related
801 to FA biosynthesis. **(C)** TG, MAG, PL content and **(D)** enzymes related to TG
802 metabolism. **(E)** Western blotting representative bands. **(F)** Representative
803 photomicrographs of the liver stained with Hematoxylin & Eosin (×40 magnification) of
804 CTL and **(G)** PR females (bar: 50 µm). **(H)** Representative photomicrographs of Zone 1
805 hepatocytes (near to vascularization) stained with Oil Red-O (×40 magnification) of CTL
806 and **(I)** PR female offspring. **(J)** Representative photomicrographs of Zone 2 hepatocytes
807 (distal to vascularization) of CTL and **(K)** PR female offspring. **(L)** Percentage of lipid
808 inclusion in hepatocytes after ORO staining. Values are mean ± SEM (n = 7 – 8, different
809 litters/sex per group). Significance is assumed at the level of $P < 0.05$.
810
811 **Fig. 4.** ***Effects of maternal low-protein diet during lactation in key-markers content of***
812 ***CHOL metabolism, glucose metabolism, redox balance, pro-inflammatory cytokines,***
813 ***unfolded protein response, macroautophagy pathway and sex steroids receptors of***
814 ***female offspring.*** **(A)** Total and esterified CHOL. **(B)** Enzymes related to CHOL
815 biosynthesis and esterification. **(C)** Key-enzymes of glycolysis and **(D)** gluconeogenesis
816 pathway. **(E)** Oral glucose tolerance test (OGTT) of female offspring at PN175 and **(F)**
817 area under curve. **(G)** Intraperitoneal pyruvate tolerance test (iPTT) of female offspring
818 at PN178 and **(H)** area under curve. **(I)** Redox balance key-markers. **(J)** Pro-inflammatory
819 cytokines. **(K)** Unfolded protein response and macroautophagy pathway. **(L)** Sexual
820 steroids receptors and **(M)** western blotting representative bands. Values are mean ± SEM
821 (n = 7 – 8, different litters/sex per group). Significance is assumed at the level of $P < 0.05$.
822

Figure 1

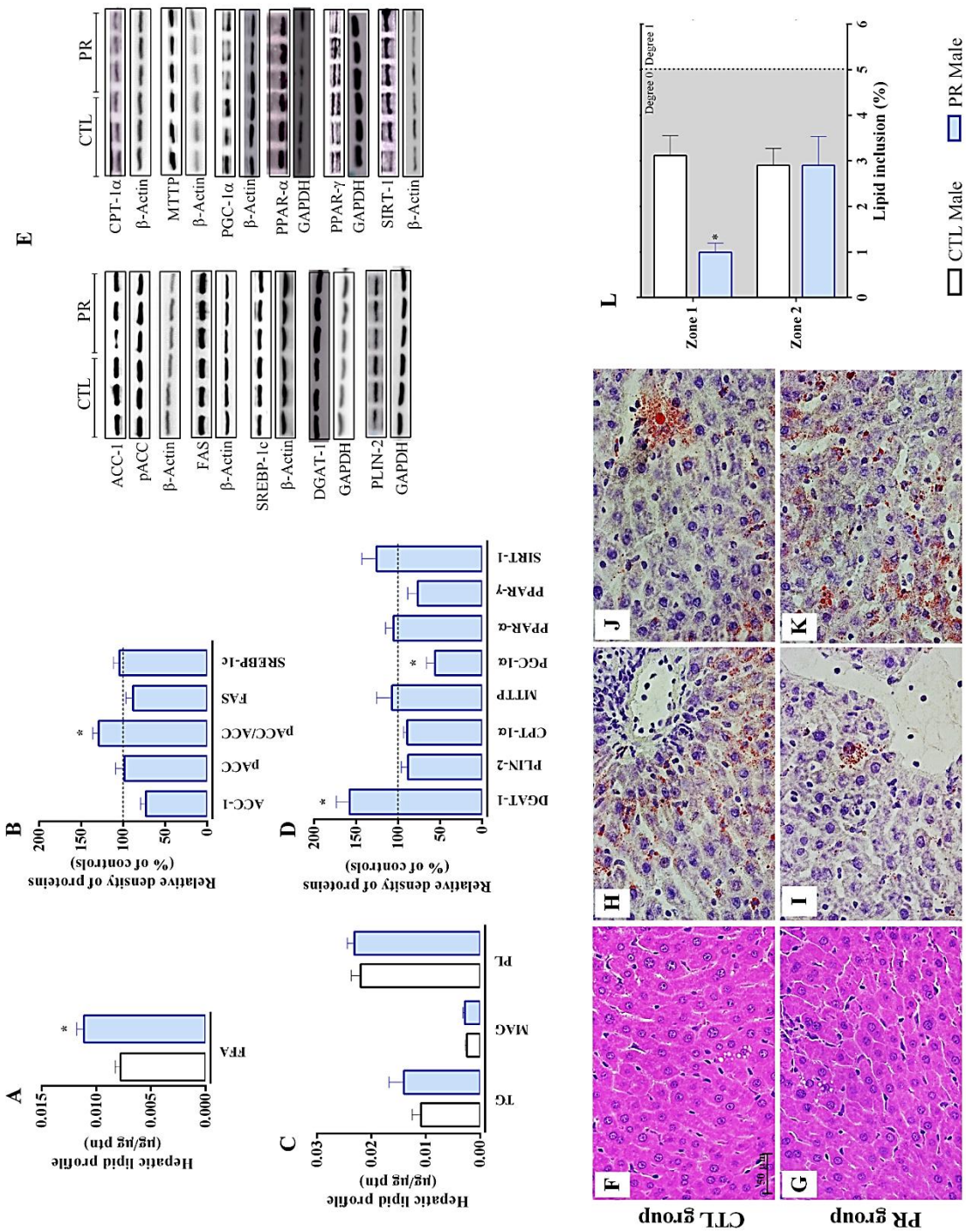

A
Hepatic lipid profile (μg/μg ptn)
FFA
B
Relative density of proteins (% of controls)
ACC-1
pACC
pACC/ACC
FAS
SREBP-1c
C
Hepatic lipid profile (μg/μg ptn)
TG
MAG
PL
D
Relative density of proteins (% of controls)
DGAT-1
PLIN-2
CPT-1α
MTTP
PGC-1α
PPAR-α
PPAR-γ
SIRT-1
E
CTL
PR
ACC-1
pACC
β-Actin
FAS
SREBP-1c
DGAT-1
GAPDH
PLIN-2
CPT-1α
MTTP
PGC-1α
PPAR-α
PPAR-γ
SIRT-1
F
G
H
I
J
K
CTL group
PR group
L
Degree 0
Degree 1
Lipid inclusion (%)
Zone 1
Zone 2
CTL Male
PR Male

Figure 2

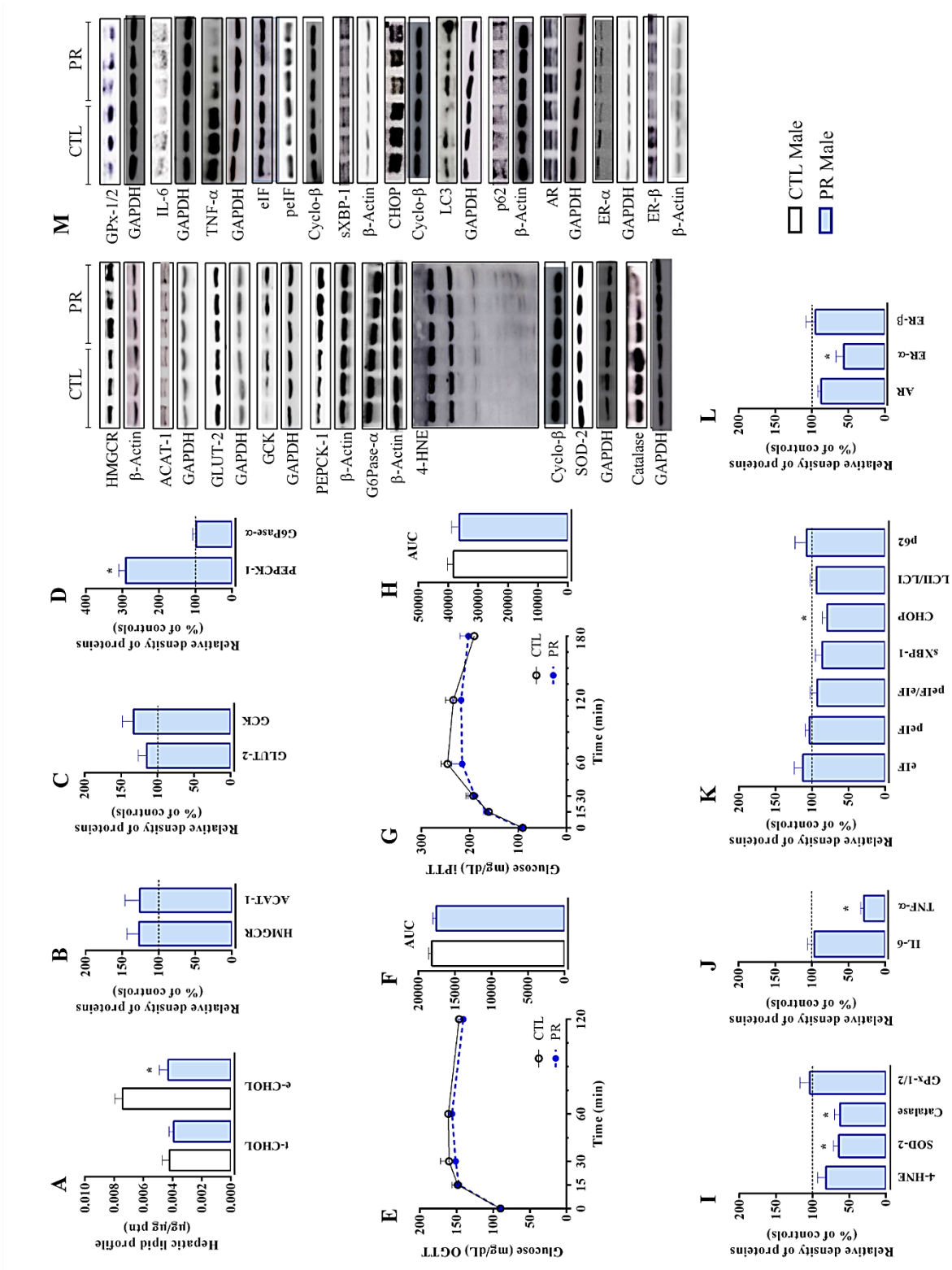

CTL Male
PR Male
Hepatic lipid profile (µg/µg ptn)
t-CHOL
e-CHOL
Relative density of proteins (% of controls)
HMGCR
ACAT-1
GLUT-2
GCK
PEPCK-1
G6Pase-α
Glucose (mg/dL) OGTT
Glucose (mg/dL) iPTT
Time (min)
AUC
CTL
PR
AR
ER-α
ER-β
eIF
peIF
peIF/eIF
sXBP-1
CHOP
LCII/LCI
p62
4-HNE
SOD-2
Catalase
GPx-1/2
IL-6
TNF-α
HMGCR
β-Actin
ACAT-1
GAPDH
GLUT-2
GCK
PEPCK-1
G6Pase-α
Cyclo-β
SOD-2
Catalase
ER-β
eIF
peIF
Cyclo-β
sXBP-1
CHOP
LC3
p62
AR
ER-α
ER-β
IL-6
TNF-α
4-HNE
SOD-2
Catalase
GPx-1/2

Figure 3

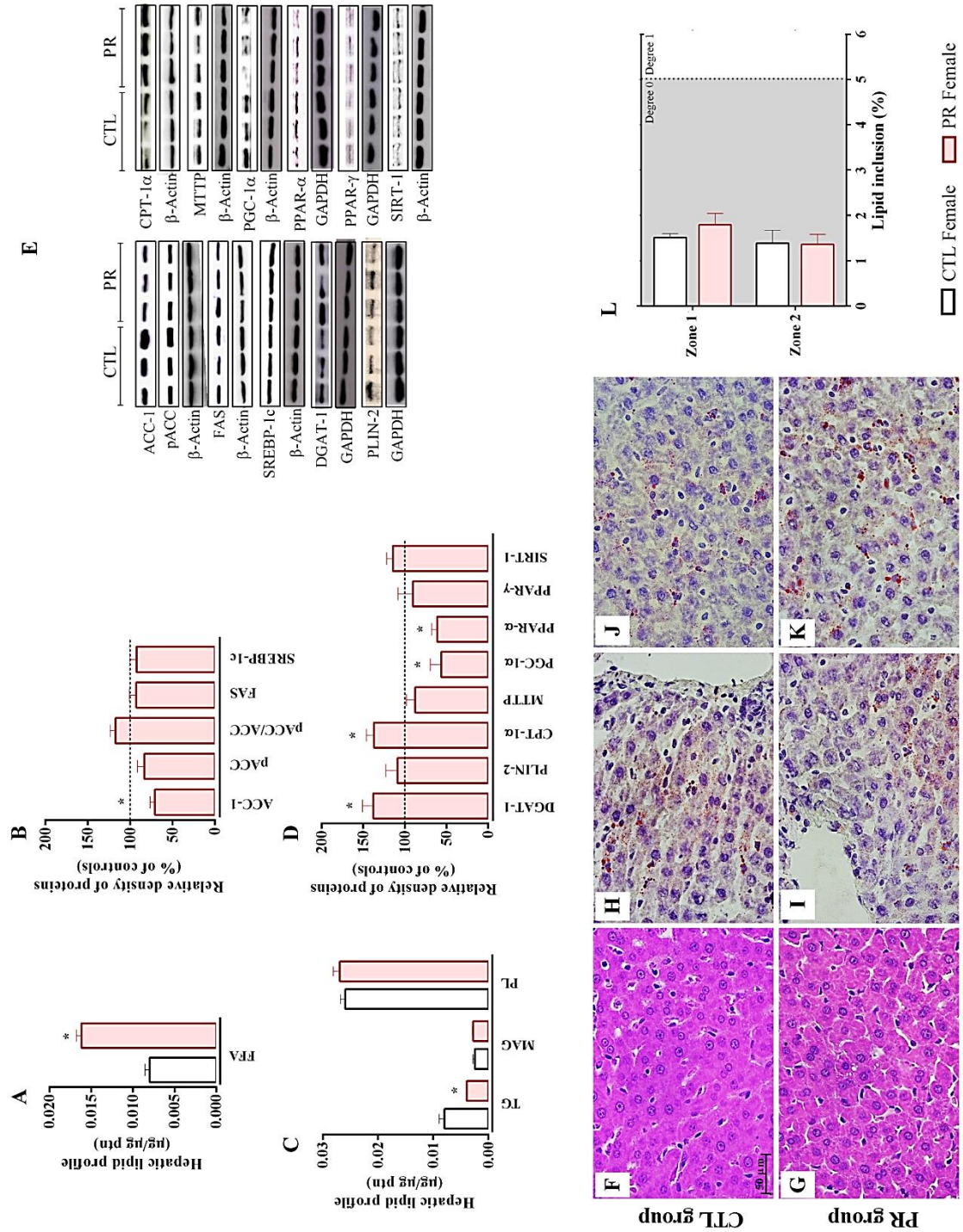

A
Hepatic lipid profile (μg/μg ptn)
FFA
B
Relative density of proteins (% of controls)
ACC-1
pACC
pACC/ACC
FAS
SREBP-1c
C
Hepatic lipid profile (μg/μg ptn)
TG
MAG
PL
D
Relative density of proteins (% of controls)
DGAT-1
PLIN-2
CPT-1α
MTTP
PGC-1α
PPAR-α
PPAR-γ
SIRT-1
E
CTL
PR
ACC-1
pACC
β-Actin
FAS
SREBP-1c
DGAT-1
GAPDH
PLIN-2
CPT-1α
MTTP
PGC-1α
PPAR-α
PPAR-γ
SIRT-1
L
Degree 0
Degree 1
Lipid inclusion (%)
Zone 1
Zone 2
CTL Female
PR Female
CTL group
PR group
F
G
H
I
J
K

Figure 4

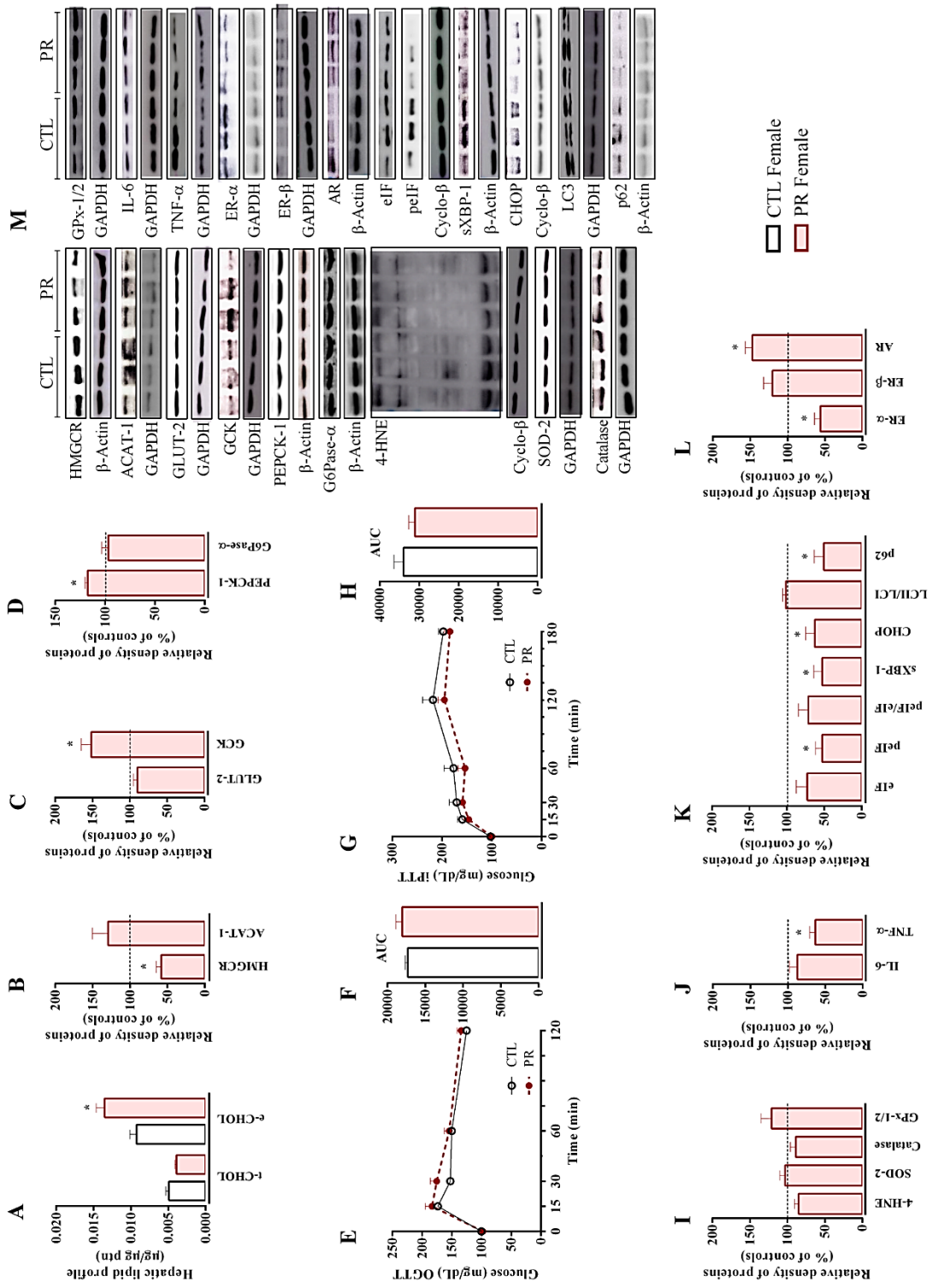

A
Hepatic lipid profile (µg/µg pth)
t-CHOL
e-CHOL
B
Relative density of proteins (% of controls)
HMGCR
ACAT-1
C
Relative density of proteins (% of controls)
GLUT-2
GCK
D
Relative density of proteins (% of controls)
PEPCK-1
G6Pase-α
E
Glucose (mg/dL) OGTT
Time (min)
CTL
PR
F
AUC
G
Glucose (mg/dL) iPTT
Time (min)
CTL
PR
H
AUC
I
Relative density of proteins (% of controls)
4-HNE
SOD-2
Catalase
GPx-1/2
J
Relative density of proteins (% of controls)
IL-6
TNF-α
K
Relative density of proteins (% of controls)
eIF
peIF
peIF/eIF
sXBP-1
CHOP
LCII/LCI
p62
L
Relative density of proteins (% of controls)
ER-α
ER-β
AR
CTL Female
PR Female
M
CTL
PR
HMGCR
β-Actin
ACAT-1
GAPDH
GLUT-2
GAPDH
GCK
GAPDH
PEPCK-1
β-Actin
G6Pase-α
β-Actin
4-HNE
Cyclo-β
SOD-2
GAPDH
Catalase
GAPDH
GPx-1/2
GAPDH
IL-6
GAPDH
TNF-α
GAPDH
ER-α
GAPDH
ER-β
GAPDH
AR
β-Actin
eIF
peIF
Cyclo-β
sXBP-1
β-Actin
CHOP
Cyclo-β
LC3
GAPDH
p62
β-Actin

Table 1. Composition of the diets used during lactation period.

	Control[a]	**Low protein**[b]
Ingredients (g/kg)		
Soybean + wheat	230.0	80.0
Corn starch	676.0	826.0
Soybean oil	50.0	50.0
Vitamin mix[c]	4.0	4.0
Mineral mix[c]	40.0	40.0
Macronutrient composition (%)		
Protein	23.0	8.0
Carbohydrate	66.0	81.0
Fat	11.0	11.0
Total energy (kJ/kg)	17,038.7	17,038.7

[a]Standard Standard diet for rats (Nuvilab-NUVITAL Nutrientes LTDA, Paraná, Brazil). [b]The low-protein diet was prepared in our laboratory using the control diet and replacing part of its protein with cornstarch. The amount of the latter was calculated so as to make up for the decrease in energy content due to protein reduction. [c]Vitamin and mineral mixtures were formulated according to the AIN-93G recommendation for rodent diets (Reeves, Nielsen, Fahey, 1993).

Table 2. Antibodies used for Western Blotting.

Primary antibodies			Secondary antibodies		
Antibody	Catalogue number/distributed by	Dilution	Catalogue number/distributed by	Dilution	Specificity
4-HNE	ab46545/Abcam MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:10000	Anti-rabbit
ACAT-1	G-15 sc-161307/Santa Cruz Biotechnology CA, USA	1:500	#61-1620 Thermo Fisher Scientific MA, USA	1:10000	Anti-goat-HRP
ACC-1	#4190/Cell Signaling Technology MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
AR	C-19 sc-815/Santa Cruz Biotechnology CA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
β-Actin*	A2228/Sigma-Aldrich MO, USA	1:1000	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
Catalase	H-300 sc-50508/Santa Cruz Biotechnology CA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
Cyclophilin-β*	PA1-027A/Thermo Fisher Scientific MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
CHOP	L63F7 #2895/Cell Signaling Technology MA, USA	1:1000	B8520 Sigma-Aldrich MO, USA	1:1000	Anti-mouse
CPT-1α	8F6AE9 ab128568/Abcam MA, USA	1:500	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
DGAT-1	A-5 sc-271934/Santa Cruz Biotechnology CA, USA	1:500	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
eIF-2α	L57A5 #2103/Cell Signaling Technology MA, USA	1:800	B8520 Sigma-Aldrich MO, USA	1:1000	Anti-mouse
ER-α	#04-1564 Clone F3-A/Millipore Corporation CA, USA	1:1000	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
Er-β	#04-824 Clone 68-4/ Millipore Corporation CA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
FAS	C20G5 #3180/Cell Signaling Technology MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
GAPDH*	DI6H11 xp #5174/Cell Signaling Technology MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
G6-Pase-α	H-60 sc-25840/Santa Cruz Biotechnology CA, USA	1:300	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
GCK	G-6 sc-17819/Santa Cruz Biotechnology CA, USA	1:500	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
GLUT-2	#720238/Thermo Fisher Scientific MA, USA	1:200	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
GPx-1/2	D-12 sc-133152/Santa Cruz Biotechnology CA, USA	1:1000	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
IL-6	ab83339/ Abcam MA, USA	1:200	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
HMGCR	EPR1685N ab174830/Abcam MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
LC3- β	#2775/Cell Signaling Technology MA, USA	1:600	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
MTTP	#PA5-42391/Thermo Fisher Scientific MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
p62/ SQSTM1	#5114/ Cell Signaling Technology MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
pACC	#3661/Cell Signaling Technology MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
peIF-2α	Cell Signaling (D9G8) #3398	1:800	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
PCK-1	ab70358/Abcam MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:10000	Anti-rabbit
PGC1-α	ab54481/Abcam MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
PLIN-2	#PA5-87206/Thermo Fisher Scientific MA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
PPAR-α	H-98 sc-9000/Santa Cruz Biotechnology CA, USA	1:500	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
PPAR-γ	H-100 sc-7196/Santa Cruz Biotechnology CA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
SirT-1	D1D7 #9475/Cell Signaling Technology MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
SOD-2	A-2 sc-133134/Santa Cruz Biotechnology CA, USA	1:500	B8520 Sigma-Aldrich MO, USA	1:10000	Anti-mouse
SREBP-1c	C-20 sc-366/Santa Cruz Biotechnology CA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
TNF-α	52B83 sc-52746/Santa Cruz Biotechnology CA, USA	1:300	B7389 Sigma-Aldrich MO, USA	1:7000	Anti-rabbit
XBP-1s	D2C1F #12782/Cell Signaling Technology MA, USA	1:1000	B7389 Sigma-Aldrich MO, USA	1:10000	Anti-rabbit

*Used as internal control. 4-HNE, 4-hydroxynonenal; ACAT-1, acetyl-Coenzyme A acetyltransferase-1; ACC-1, acetyl-Coenzyme A carboxylase-1; AR, androgen receptor; CHOP, C/EBP homologous protein; CPT-1α, carnitine palmitoyl-transferase-1α; DGAT-1, acyl-Coenzyme A:diacylglycerol acyltransferase-1; eIF-2α, eukaryotic initiation factor-2α; ER-α, estrogen receptor α; ER-β, estrogen receptor-β; FAS, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6-Pase-α, glucose 6-phosphatase-α; GCK, glucokinase; GLUT-2, glucose transporter-2; GPx-1/2, glutathione peroxidase-1/2; IL-6, Interleukin-6; HMGCR, hydroxy-3-methylglutaryl Coenzyme A reductase; LC3-β, light chain 3-β; MTTP, microsomal triglyceride transfer protein; p62/SQSTM1, sequestome-1; pACC, phosphorylated acid-acetyl-Coenzyme A carboxylase; peIF-2α, phosphorylated eukaryotic initiation factor-2α; PCK-1, phosphoenolpyruvate carboxykinase-1; PGC1-α, peroxisome proliferator-activated receptor gamma coactivator 1-α; PLIN-2, Perilipin-2; PPAR-α, peroxisome proliferator-activated receptor alpha; PPAR-γ, peroxisome proliferator-activated receptor gamma; SirT-1,Sirtuin-1; SOD-2, superoxide dismutase-2; SREBP-1c, sterol regulatory element-binding protein-1c; TNF-α, tumor necrosis factor-α; XBP-1s, X-box binding protein-1s.

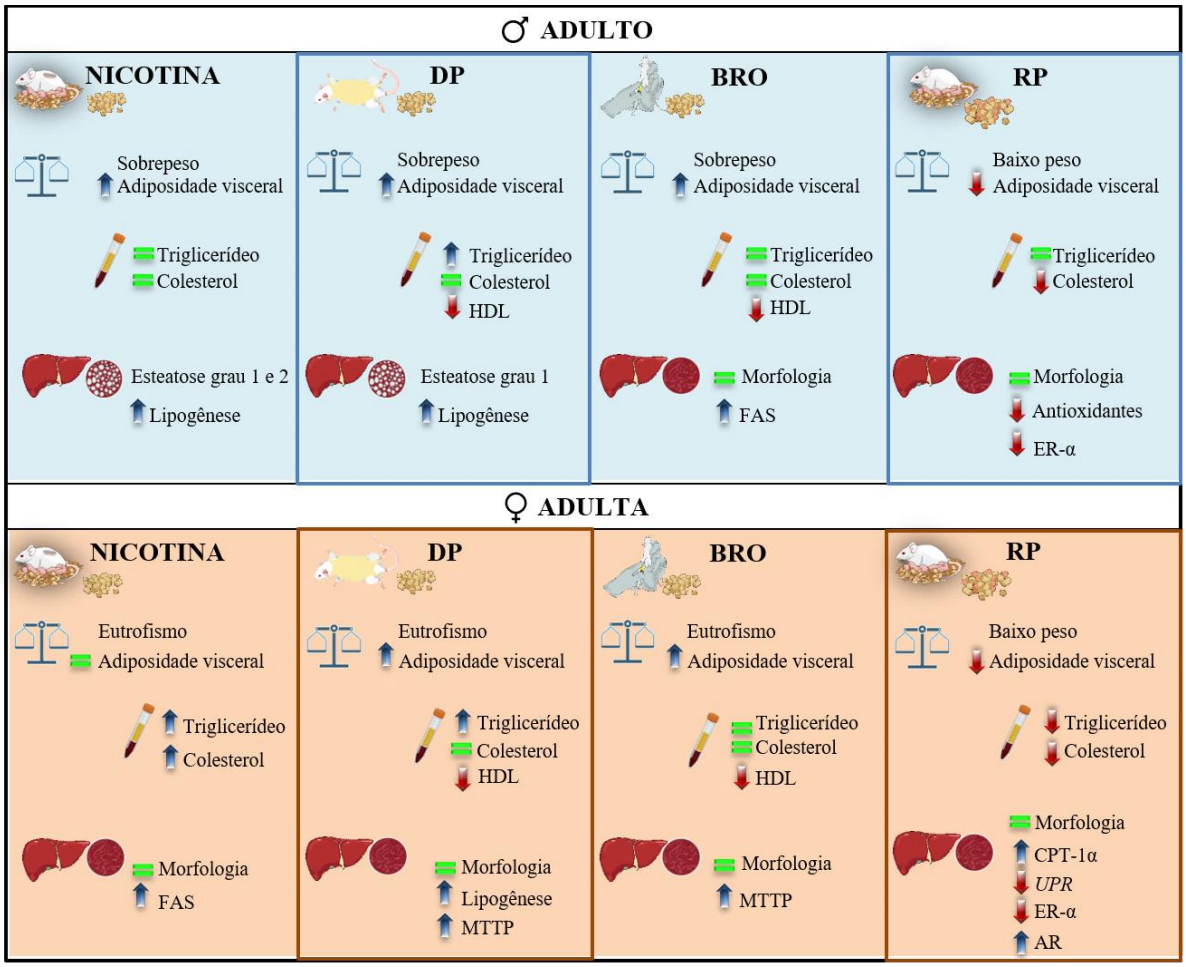
Table 3. Long-term effects of maternal low protein diet during breastfeeding on biometric and plasma profile of male and female rat progeny.

	MALE		FEMALE	
	CTL	**PR**	**CTL**	**PR**
Body weight 3-week-old (g)	61.3 ± 1.0	41.0 ± 0.8*	59.4 ± 0.6	40.6 ± 0.7*
Body weight 6-month-old (g)	544.7 ± 7.4	513.6 ± 6.2*	299.6 ± 5.0	273.6 ± 4.2*
Body weight gain (g)	483.3 ± 7.3	472.6 ± 5.9	240.2 ± 4.9	233.0 ± 4.1
Food intake (g)	1193.0 ± 6.1	1171.0 ± 9.5	871.9 ± 5.9	853.0 ± 7.5
VFM (g/100g bw)	5.4 ± 0.2	4.6 ± 0.2*	5.0 ± 0.2	4.3 ± 0.3*
Liver (g/100g bw)	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
Plasma cholesterol (mg/dL)	62.3 ± 2.8	50.4 ± 4.4*	63.0 ± 6.1	42.9 ± 7.1*
Plasma triglycerides (mg/dL)	58.7 ± 4.1	56.4 ± 6.8	49.3 ± 5.0	34.8 ± 3.2*
Plasma HDL (mg/dL)	48.2 ± 4.8	52.7 ± 5.5	62.1 ± 3.8	62.5 ± 4.1

CTL – control, PR – protein restriction; AUC – area under curve, VFM – visceral fat mass, bw – body weight, HDL – high density lipoprotein. The food intake is expressed as the cumulative consumption from weaning to adulthood. Values represent mean ± SEM of 7-10 animals from different litter per sex per group; Significance is assumed at the level of $P < 0.05$.

3 RESUMO DOS RESULTADOS

Figura 8 - Principais resultados obtidos nos três modelos experimentais



Legenda: **AR:** Receptor de androgênio**; BRO:** Bromocriptina; **CPT-1α:** Carnitina palmitoil-transferase-1alfa; **DP:** Desmame precoce; **ER-α:** Receptor de estrogênio alfa; **FAS:** Ácido graxo sintase; **HDL:** Lipoproteína de alta densidade; **MTTP:** Proteína de transferência de triglicerídeos microssomal; ***UPR*: *Unfolded Protein Response.***

Fonte: A autora, 2021.

CONSIDERAÇÕES FINAIS

Com o avanço científico, atualmente admite-se que as doenças crônicas não-transmissíveis que preocupam o cenário mundial, compartilham de origens comuns. Insultos (nutricionais, ambientais e psicossociais) em períodos de maior susceptibilidade a alterações são capazes de adaptar o organismo do indivíduo para a sobrevivência a curto prazo, entretanto, a longo prazo essa programação pode torná-lo propenso para o desenvolvimento de doenças. Nesse contexto, o período gestacional foi de imediato o mais investigado, principalmente por seu papel organogênico. Entretanto, a lactação tem sido cada vez mais estudada devido a continuidade do desenvolvimento de órgãos, como o fígado, e a importância da comunicação mãe-filho via leite. O ambiente fetal adverso e o período pós-natal inicial estão relacionados a maior suscetibilidade ao desenvolvimento e progressão da DHGNA. Contudo, muitas questões ainda permanecem não respondidas acerca da lactação como janela de programação. Nesse sentido, nos dedicamos a estudar neste trabalho, a influência da exposição à fatores ambientais (exposição materna à nicotina) e alteração de fatores nutricionais através da redução da oferta do leite (desmame precoce) ou alteração em sua composição (dieta materna restrita em proteínas) sobre o metabolismo lipídico hepático de ratos adultos machos e fêmeas.

Mesmo com a tentativa árdua da OMS em divulgar as consequências do tabagismo, o cigarro é a droga mais comum entre gestantes, sendo que a maioria interrompe este hábito mas retorna durante os primeiros seis meses pós-parto, expondo o neonato a fumaça e a nicotina via leite materno. Neste primeiro modelo animal experimental, evidenciamos os efeitos tardios da exposição precoce à nicotina durante a lactação sobre o metabolismo lipídico do fígado. Os machos foram programados para o desenvolvimento de alterações no metabolismo de triglicerídeos, sendo o acúmulo preferencialmente intra-hepático e independente de sobrepeso e resistência à insulina. De modo oposto, o acúmulo de lipídios nas fêmeas ocorreu preferencialmente no plasma e a arquitetura do hepatócito foi preservada, com alterações principalmente no metabolismo de colesterol.

A amamentação exclusiva nos primeiros meses de vida é considerada a intervenção mais potente contra a morbidade e mortalidade infantil e uma meta de saúde pública global. Apesar dos diversos benefícios relacionados ao aleitamento materno nos primeiros seis meses de vida, o mundo passa por uma “epidemia de desmame”, que está associada à redução do desempenho intelectual, bem como a alterações no perfil lipídico, desenvolvimento cardiovascular, pressão arterial, DM2, sobrepeso e obesidade ao longo da vida. Neste contexto, no segundo modelo

animal desta Tese, abordamos a importância do aleitamento materno exclusivo durante os 6 primeiros meses de vida, mimetizando o desmame precoce farmacológico e não-farmacológico.

Tomados em conjunto, nossos resultados demonstram que as alterações na citoarquitetura do fígado apenas em machos desmamados precocemente do modelo não-farmacológico são associadas às diferenças na expressão da FAS e SCD-1. Interessantemente, independente ao tipo de desmame, as fêmeas não desenvolvem esteatose hepática, embora apresentem aumento de MTTP, o que pode influenciar no melhor perfil lipídico observado. É possível que as fêmeas tenham desenvolvido um mecanismo adaptativo para lidar melhor com os insultos durante a lactação do que os machos, pois esses mecanismos estariam relacionados à necessidade de reprodução satisfatória. Nesse sentido, o MTTP poderia ser uma enzima chave na proteção do fígado contra a esteatose e suas graves consequências.

Em contrapartida, a alteração de apenas um dos macronutrientes programa os descendentes para um fenótipo oposto. Em nosso terceiro modelo experimental, a dieta materna hipoprotéica exclusivamente durante a lactação programa machos e fêmeas para o baixo peso associado à redução da adiposidade e normofagia, sem o desenvolvimento de esteatose, sugerindo hiperativação da lipólise e consequente aumento da disponibilidade de lipídeos para o fígado. Aparentemente, o fígado está preparado para aumentar a síntese de lipídeos, mas a maquinaria enzimática não reflete o aumento da funcionalidade, uma vez que o aumento das enzimas-chave da gliconeogênese e da síntese de triglicerídeos não parece elevar os respectivos produtos. Curiosamente, o aumento da β-oxidação, captação de LDL e redução da disponibilidade de TG sugerem maior adaptação das fêmeas em relação ao metabolismo lipídico, o que pode ser um mecanismo adaptativo para redução do conteúdo lipídico intra e extra-hepático. A restrição de proteínas da dieta materna também programa a resposta ao retículo endoplasmático e/ou estresse mitocondrial, e o efeito de redução das vias de reparo da homeostase do retículo parece ser mais intenso em fêmeas. O fenótipo das fêmeas PR pode estar relacionado ao efeito combinado da redução do ER-α e aumento do AR, porém mais estudos são necessários. Uma das principais lições aprendidas com esse modelo de programação é que um indivíduo abaixo do peso possui um fígado aparentemente saudável (sem acúmulo de gordura), mas que pode ocultar vias metabólicas desreguladas com potencial esteatótico. Portanto, deve-se evitar a prática de adição de produtos farináceos para engrossar o leite da mamadeira, o que resulta na diluição das proteínas, bem como estimular a ingestão adequada de proteínas pelas mães durante a amamentação e, o cumprimento do direito à alimentação adequada e saudável garantido pela Constituição Federal e reafirmado pelo

Estatuto da Criança e do Adolescente (ECA, Lei nº 8.069, de 13 de julho de 1990) por parte do poder público.

Os modelos de indução ao sobrepeso abordados neste trabalho (nicotina e desmame) mostraram que machos armazenam os lipídeos no fígado de modo preferencial comparado às fêmeas, mas o que inicialmente é visto como algo bom pode não ser, já que fêmeas NIC (PIETROBON et al., 2021) e DP/BRO (dados ainda não publicados) desenvolvem esteatose pancreática. Em relação ao modelo de baixo peso (dieta materna RP), animais de ambos os sexos não desenvolvem esteatose, mas apresentam diversas alterações camufladas pelo consumo exclusivo de dieta padrão. Sendo assim, o rápido ganho de peso após o desmame está associado ao desenvolvimento de sobrepeso e suas implicações a longo prazo, entretanto, o baixo peso observado nesses animais também não é sinônimo de boa saúde. Podemos inferir que a exposição a um novo insulto ao longo da vida, como dietas desequilibradas, pode romper o sincronismo mantido pelo fígado e, evoluir para o estado de injúria hepática.

Em seres humanos com DHGNA, há o mesmo padrão de dimorfismo sexual que verificamos neste trabalho, esta doença é mais prevalente entre homens em idade reprodutiva e em mulheres após a menopausa, por influência dos esteróides sexuais. As intervenções para doenças crônicas, como a DHGNA, geralmente são iniciadas mais tarde na vida, quando a resposta aos tratamentos pode ser subótima. O período neonatal é uma janela crítica de plasticidade do desenvolvimento, quando pequenas mudanças podem ter um grande impacto na predisposição às doenças, destacando este período como alvo para intervenções. Podemos concluir que para a interrupção do ciclo vicioso ao qual estamos inseridos, o estímulo ao aleitamento exclusivo, combate ao tabagismo e a garantia de segurança alimentar à população deve cursar com políticas públicas eficazes para a prevenção de doenças e não apenas contenção de danos.

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ANEXO A – Aprovação do Comitê de Ética do Modelo Experimental I

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

ibrag Instituto de Biologia Roberto Alcantara Gomes - ESTADO

CERTIFICADO

Certificamos que a proposta intitulada "**Função hepática em ratos wistar programados pela exposição a fumaça de cigarro e a nicotina na lactação**", registrada com o nº 015/2017, sob a responsabilidade de **Nayara Peixoto Silva** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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, 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), e foi aprovada pela COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 28/03/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	28/03/2021
Espécie/linhagem/raça	Rato Wistar
Nº de animais	200
Peso/Idade	45-500 g / 21 – 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 28 de Março de 2017.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

ANEXO B – Aprovação do Comitê de Ética do Modelo Experimental II

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

ibrag Instituto de Biologia Roberto Alcantara Gomes - ESTADO

CERTIFICADO

Certificamos que a proposta intitulada "**Alterações metabólicas e hormonais nos modelos de desmame precoce: relação com disfunções no fígado e no tecido adiposo**", registrada com o nº 014/2017, sob a responsabilidade de **Nayara Peixoto Silva** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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, 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), e foi aprovada pela COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 28/03/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	28/03/2021
Espécie/linhagem/raça	Rato Wistar
Nº de animais	420
Peso/Idade	45-500 g / 21 – 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 28 de Março de 2017.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

ANEXO C – Aprovação do Comitê de Ética do Modelo Experimental III

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

ibrag Instituto de Biologia Roberto Alcantara Gomes - ESTADO

CERTIFICADO

Certificamos que a proposta intitulada "**Funcao hepatica em animais programados pela exposicao a dieta hipoproteica durante o aleitamento**", registrada com o nº 024/2019, sob a responsabilidade de **Patricia Cristina Lisboa da Silva** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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, 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), e foi aprovada pela COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 26/11/2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	26/11/2023
Espécie/linhagem/raça	Rato Wistar
Nº de animais	180
Peso/Idade	200-600 g / 2-6 meses
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 26 de Novembro de 2019.

Prof. Dr. Alex C. Manhães
Coordenador
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Profa. Dra. Patricia C. Lisboa
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ANEXO D - Comprovantes de publicação de artigos científico como primeira autora e em coautoria durante o período do doutorado

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Programming of hepatic lipid metabolism in a rat model of postnatal nicotine exposure – Sex-related differences☆

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ABSTRACT

Maternal nicotine exposure during lactation induces liver damage in adult male rats. However, the mechanism in males is unknown and females have not been tested. Here, we determined the liver lipid composition and lipogenic enzymes in male and female offspring at two ages in a model of postnatal nicotine exposure. Osmotic minipumps were implanted in lactating Wistar rat dams at postnatal day (PND) 2 to release 6 mg/kg/day of nicotine (NIC group) or saline (CON group) for 14 days. Offspring received a standard diet from weaning until euthanasia at PND120 (1 pup/litter/sex) or PND180 (2 pups/litter/sex). At PND120, NIC males showed lower plasma triglycerides (TG), steatosis degree 1, higher hepatic cholesterol (CHOL) ester, free fatty acids, monoacylglycerol content as well as acetyl-coa carboxylase-1 (ACC-1) and fatty acid synthase (FAS) protein expression in the liver compared to CON males. At this age, NIC females had preserved hepatocytes architecture, higher plasma CHOL, higher CHOL ester and lower total CHOL content in the liver compared to CON females. At PND180, NIC males showed steatosis degrees 1 and 2, higher TG, lower free fatty acids and total CHOL content in the liver and an increase in ACC-1 hepatic protein expression. NIC females had higher plasma TG and CHOL levels, no change in hepatic morphology, lower CHOL ester and free fatty acids in the liver, which also showed higher total ACC-1 and FAS protein expression. Maternal nicotine exposure induces long-term liver dysfunction, with an alteration in hepatic cytoarchitecture that was aggravated with age in males. Concerning females, despite unchanged hepatic cytoarchitecture, lipid metabolism was compromised, which deserves further attention.

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1. Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is an inclusive term for a group of non-alcohol-related progressive liver disorders (Angulo, 2002) that has become a serious public health problem worldwide, affecting more than half billion people worldwide (Younossi et al., 2018) and currently being the main cause of liver transplantation and carcinoma (Pais et al., 2016; Michelotti et al., 2013). Steatosis is the first manifestation of NAFLD, which is characterized by excessive accumulation of triglycerides (TG). In normal situations, this accumulation is not hepatotoxic and represents a defensive mechanism to balance free fatty acids excess (Koliwad et al., 2010; Yamaguchi et al., 2007). However, increased TG concentration happens simultaneously with toxic metabolites generation, lipotoxicity, liver injury, activation of inflammatory cascades and fibrogenesis (Neuschwander-Tetri, 2010). In this case, steatosis

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Hepatic lipid metabolism in adult rats using early weaning models: sex-related differences

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Abstract

Non-pharmacological early weaning (NPEW) induces liver damage in male progeny at adulthood; however, pharmacological early weaning (PEW) does not cause this dysfunction. To elucidate this difference in liver dysfunction between these two models and determine the phenotype of female offspring, *de novo* lipogenesis, β-oxidation, very low-density lipoprotein (VLDL) export, and gluconeogenesis in both sexes were investigated in the adult Wistar rats that were weaned after a normal period of lactation (control group) or early weaned either by restriction of access to the dams' teats (NPEW group) or by reduction of dams' milk production with bromocriptine (PEW group). The offspring received standard diet from weaning to euthanasia (PN180). NPEW males had higher plasma triglycerides and TyG index, liver triglycerides, and cholesterol by *de novo* lipogenesis, which leads to intracellular lipids accumulation. As expected, hepatic morphology was preserved in PEW males, but they showed increased liver triglycerides. The only molecular difference between PEW and NPEW males was in acetyl-CoA carboxylase-1 (ACC-1) and stearoyl-CoA desaturase-1 (SCD-1), which were lower in PEW animals. Both early weaning (EW) females had no changes in liver cholesterol and triglyceride contents, and the hepatic cytoarchitecture was preserved. The expression of microsomal triglyceride transfer protein was increased in both the female EW groups, which could constitute a protective factor. The changes in hepatic lipid metabolism in EW offspring were less marked in females. EW impacted in the hepatic cytoarchitecture only in NPEW males, which showed higher ACC-1 and SCD-1 when compared to the PEW group. As these enzymes are lipogenic, it could explain a worsened liver function in NPEW males.

Introduction

The liver is a fundamental organ in the regulation of body energy metabolism, and the synchronism of glycemic and lipid metabolism is destabilized in situations of nutritional changes, predisposing individuals to excessive accumulation of lipids in the hepatocyte and the consequent development of non-alcoholic fatty liver disease (NAFLD).[1] NAFLD includes a group of chronic liver diseases, ranging from simple hepatic steatosis to non-alcoholic steatohepatitis and hepatocellular carcinoma. The molecular mechanism underlying NAFLD progression is not completely understood; its pathogenesis has been interpreted by the hypothesis of "two-hits." The "first hit" concerns the accumulation of greater than 5.5% lipids in hepatocytes in individuals with no history of excessive alcohol consumption, resulting in steatosis due to the excessive lipid influx or impaired lipid efflux.[2] Hepatocytes activate metabolic pathways to minimize lipotoxicity in response to elevated hepatic lipid uptake, thus steatosis develops when lipid influx overwhelms these protective mechanisms (β-oxidation, ketogenesis, and VLDL export), mainly in response to fasting, high-fat diet (HFD), or lipodystrophies.[1–3] In numerous experimental animal models of hepatic steatosis, including HFD, fructose or ethanol, and genetic lipodystrophy, fatty liver is associated with peripheral metabolic dysfunction.[4] In addition to the accumulation of lipids, there is inflammation and hepatocellular degeneration featuring the "second hit."[5] Bacterial toxins, hyperproduction of cytokines, tumor necrosis factor α, changes in adenosine triphosphate stocks, and cytochrome P450 activity appear to be important triggers for this progression, which can be accompanied by the appearance of fibrosis and even progress to liver cirrhosis and carcinoma.[6–8]

Epidemiological studies show that the environment in critical periods, including gestation, breastfeeding, and adolescence, exerts a strong influence on the development of an individual and is associated with the onset of diseases throughout life, in particular, obesity and its

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Effects of maternal bisphenol A on behavior, sex steroid and thyroid hormones levels in the adult rat offspring

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ABSTRACT

Aims: Bisphenol A (BPA), an endocrine disruptor used in industrial applications, has been detected in both placenta and milk. We studied the effects of BPA exposure during pregnancy and lactation on body composition, palatable food intake, biochemical, hormonal and behavioral profiles of young and adult Wistar rat offspring.
Main methods: Female rats were divided into: control, BPA10 (10 μg/kg/day) and BPA50 (50 μg/kg/day). BPA was administered by gavage to dams from gestation until the end of lactation. Euthanasia occurred at weaning [postnatal day (PN) 21] or adulthood (PN180).
Key findings: At weaning, BPA10 female pups had higher plasma cholesterol and triacylglycerol. BPA10 male pups showed lower plasma T3. BPA10 pups of both sexes had higher plasma progesterone, testosterone and estradiol. At adulthood, females of both BPA groups had lower food intake and higher insulinemia, whereas males had lower visceral fat, lower progesterone and testosterone concentrations. BPA10 females and males had lower T4 levels, while only males showed lower estradiol. BPA50 females showed lower fat mass, higher lean mass and lower corticosteronemia, while males had lower food intake. In the feeding study, BPA10 males ate more fat at 30 min, while BPA10 females and males ingested less fat after 12 h. BPA10 females showed hyperactivity while both groups showed less exploration.
Significance: Maternal exposure to BPA during gestation and lactation, even at low doses, induces life-long changes in the regulation of metabolic homeostasis of the progeny, affects sex steroids and thyroid hormones levels, compromises behavior, but does not lead to obesity or dyslipidemia.

1. Introduction

Adverse events during the intrauterine and postnatal periods can modify gene expression and lead to permanent changes in metabolism and hormonal regulation [1,2]. Maternal exposure, during pregnancy and breastfeeding, to insults such as inadequate nutrition, social stress and environmental pollutants can induce obesity and its comorbidities later in life [3–5]. Studies in humans have identified that environmental pollutants are potential risk factors in the development of several diseases [6,7]. Substances defined as endocrine disrupting chemicals (EDCs) or "exogenous agents" have been associated with numerous health problems, such as reproductive dysfunction, obesity, diabetes, among others disturbances [8].

Bisphenol A (BPA) is one of the most used EDCs worldwide since it is a starting material for the synthesis of plastics (such as toys, tools, flame retardants, antioxidants and pesticides). It has bioaccumulation characteristics [8–10] and an annual production that exceeds 3.5 million tons [11]. The U.S. Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) established a BPA reference dose of 50 μg/kg BW/day [12,13], while the European Food Safety Authority (EFSA) estimated as tolerable a daily intake (TDI) of 4 μg BPA/kg BW/day [14].

BPA is considered a xenoestrogen, capable of binding to endogenous estrogen receptors [15,16]. Besides, it can bind to receptors of testosterone [17,18], thyroid hormones [19,20] and glucocorticoids [21]. BPA has been detected in amniotic fluid, neonatal blood, placenta,

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Short and long-term effects of bisphenol S (BPS) exposure during pregnancy and lactation on plasma lipids, hormones, and behavior in rats☆

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ABSTRACT

Bisphenol S (BPS) has replaced bisphenol A (BPA), a known non-persistent endocrine disrupting chemical, in several products. Considering that little is known regarding BPS effects, especially during critical windows of ontogenetic development, and that BPA, which is quite similar to BPS, is know to be transferred to the offspring via the placenta and milk, in the present study we investigated the behavioral, biochemical and endocrine profiles of Wistar rats born from dams that were BPS-exposed [groups: BPS10 (10 μg/kg/day), BPS50 (50 μg/kg/day)] during pregnancy and lactation. Due to the non-monotonic dose-response effect of bisphenol, the data of both BPS groups were directly compared with those of the controls, not to each other. Males and females were analyzed separately. At weaning, male BPS50 offspring had hypotriglyceridemia and hyperthyroxinemia, whereas BPS50 females showed higher 25(OH)D levels. At adulthood, BPS offspring of both sexes had lower food intake. BPS males showed lower visceral adiposity. BPS50 females had smaller fat droplets in brown adipocytes. BPS males showed higher anxiety and higher locomotor activity, while BPS10 females showed lower exploration. During a food challenge test at adulthood, BPS males consumed more high-fat diet at 30 min. BPS10 females initially (at 30 min) consumed more high-fat diet but, after 12 h, less of this diet was consumed. BPS50 males had hypertriglyceridemia and lower plasma T3, while BPS females showed lower plasma T4. BPS10 females had lower progesterone, whereas BPS50 females had higher plasma 25(OH)D. Maternal BPS exposure has adverse effects on the triacylglycerol, hormones levels and behavior of the progeny. Furthermore, the increased preference for the fat-enriched diet suggests an increased risk for obesity and its health consequences in the long term.

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1. Introduction

One of the most studied non-persistent Endocrine Disrupting Chemicals (EDCs) is bisphenol A (BPA), initially used as a synthetic estrogen, but currently used in the manufacture of polyvinyl-chloride films (PVC), food packaging (Dziewirska et al., 2018; Novo et al., 2018; Son et al., 2018), metallic cans and plastic packaging of food and beverage, medical devices, dental materials, computers, thermal papers, among others (Zbucka-Kretowska et al., 2018). BPA acts as an endogenous estrogen by interacting with estrogen receptors (Alonso-Magdalena et al., 2008). BPA may be associated with a higher risk of developing diabetes, obesity, cardiovascular disease and sexual dysfunction (Bernier and Vandenberg, 2017), even at levels equal to or lower than 50 μg BPA/kg body weight/day for humans, a level used as reference for daily human BPA consumption by the EPA (Environmental Protection Agency) (Altamirano et al., 2015; García-Córcoles et al., 2018) and FDA (Food Drug Administration). However, the European Food Safety Authority (EFSA) has estimated an acceptable daily intake of only 4 μg

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Endocrine Research

Thieme

Body Adiposity and Endocrine Profile of Female Wistar Rats of Distinct Ages that were Early Weaned

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ABSTRACT

Early weaning (EW) is a risk factor for metabolic syndrome. Male rats that were precociously weaned present neonatal malnutrition and, in adulthood, developed overweight, accumulation of body fat, dyslipidemia, changes in glycemic homeostasis, hyperleptinemia, and increase of vitamin D. As metabolic profile of early-weaned females is not known, we investigated the endocrine-metabolic parameters in adolescence and adult female rats of 2 different EW models. Wistar lactating rats and pups from both sexes were separated into 3 groups: non-pharmacological EW (NPEW), dams were involved with a bandage interrupting suckling in the last 3 days of lactation; pharmacological EW (PEW), dams were bromocriptine-treated (0.5 mg/twice a day via intraperitoneal injection) for 3 days before weaning; and control, dams whose pups ate milk throughout lactation. At 21 days-old, NPEW and PEW females had lower body weight. At 180 days-old, NPEW and PEW females showed higher feed efficiency, weight gain, body fat percentage, and greater accumulation of gonadal and retroperitoneal fat depots associated with adipocyte hypertrophy. NPEW females also showed hyperphagia. Only NPEW females presented hyperleptinemia. Plasma thyroid hormones and vitamin D were unchanged among EW females. Regarding sex hormones, at 45 days-old, no change was found in EW females, while at 180 days-old, PEW females had hypoestrogenemia. EW increases the risk for obesity in female rats in adulthood, as already demonstrated for males, although through distinct mechanisms involving some hormones.

Introduction

Exclusive breastfeeding in the first months of life is considered as the most potent intervention against child morbidity and mortality, and a global public health goal [1–3]. Despite several benefits related to breastfeeding during the first six months of life, exclusive breastfeeding during this period occurs in only 41 % of cases worldwide and in 38.6 % of Brazilian's population [3]. The "weaning epidemic" is associated with reduced intellectual performance as well as changes in lipid profile, cardiovascular development, blood pressure, type 2 diabetes mellitus, overweight, and obesity throughout life regardless of gender [4–10].

Considering the nutritional status and its important correlation with development, Hales and Barker [11] proposed the theory of the "thrifty phenotype" suggesting that the development of the fetus is sensitive to the nutritional environment. Therefore, according to the hypothesis of the developmental origins of health and disease (DOHaD), the metabolism of an individual has the capacity to adapt to different environmental factors, leading to a great genetic variability and survival capacity to adverse conditions [12, 13]. Such metabolic programming by insults during the critical windows of development, promotes permanent changes throughout the life [14, 15]. Among the critical developmental periods, lactation presents great programming potential, since it is characterized by the neurological and cognitive development of the child, suggesting that adverse conditions at this stage of life

* CBP and IMB contributed equally to this study.

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ORIGINAL ARTICLE

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Early weaning leads to specific glucocorticoid signalling in fat depots of adult rats

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Abstract

Purpose Early weaning (EW) is a stressful condition that programmes a child to be overweight in adult life. Fat mass depends on glucocorticoids (GC) to regulate adipogenesis and lipogenesis. We hypothesised that the increased adiposity in models of EW was due to a disturbed HPA axis and/or disrupted GC function.

Methods We used two experimental models, pharmacological early weaning (PEW, dams were bromocriptine-treated) and non-pharmacological early weaning (NPEW, dams' teats were wrapped with a bandage), which were initiated during the last 3 days of lactation. Offspring from both genders was analysed on postnatal day 180.

Results Offspring in both models were overweight with increased visceral fat mass, but plasma corticosterone was increased in both genders in the PEW group but not the NPEW group. NPEW males had increased GRα expression in visceral adipose tissue (VAT), and GRα expression decreased in PEW males in subcutaneous adipose tissue (SAT). Females in both EW groups had increased 11βHSD1 expression in SAT. PEW males had increased C/EBPβ expression in SAT. PEW females had lower PPARγ and FAS expression in VAT than the NPEW females. We detected a sex dimorphism in VAT and SAT in the EW groups regarding 11βHSD1, GRα and C/EBPβ expression.

Conclusions The accumulated adiposity induced by EW exhibited distinct mechanisms depending on gender, specific fat deposition and GC metabolism and action. The higher proportion of VAT/SAT in both sets of EW males may be related to the action of GC in these tissues, and the higher conversion of GC in SAT in females may explain the differences in the fat distribution.

Keywords Early weaning · Glucocorticoids · Adipogenesis and lipogenesis

Introduction

The interruption of exclusive breastfeeding is related to a susceptibility to chronic diseases in adulthood, such as obesity and associated comorbidities. This phenomenon is called "metabolic programming", and it describes the influence of early nutritional, hormonal and environmental insults during critical phases of development; the phases include gestation and lactation period, and disruptions at these points could predispose individuals to long-term non-communicable diseases [1, 2].

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Breastfeeding inhibition deprives children of the natural bioactive factors present in milk, including hormones, growth factors and essential nutrients [3], and it induces several dysfunctions [4]. The World Health Organisation [5] suggests the initiation of complementary feeding at 6 months of age. The interruption of lactation before this period is considered "early weaning" (EW), which is a stressful status that is prejudicial to babies primarily because it is associated with gastrointestinal and airways diseases, under-nutrition, obesity and diabetes [6].

Our group has two well-established models of programming based on EW, in which milk yield is reduced during the last 3 days of lactation via the pharmacological inhibition of prolactin using administration of a type 2 dopaminergic receptor agonist, bromocriptine [7], or the non-pharmacological interruption of lactation using a bandage that covers all of the mothers' teats [7, 8], which causes malnutrition in pups and leads to several distinct

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Early weaning induces short- and long-term effects on pancreatic islets in Wistar rats of both sexes

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Key points

- The World Health Organization recommends exclusive breastfeeding until 6 months of age as an important strategy to reduce child morbidity and mortality.
- Studies have associated early weaning with the development of obesity and type 2 diabetes in adulthood.
- In our model, we demonstrated that early weaning leads to increased insulin secretion in adolescent males and reduced insulin secretion in adult offspring.
- Early weaned males exhibit insulin resistance in skeletal muscle.
- Early weaning did not change insulin signalling in the muscle of female offspring.
- Taking into account that insulin resistance is one of the primary factors for the development of type 2 diabetes mellitus, this work demonstrates the importance of breastfeeding in the fight against this disease.

Abstract Early weaning (EW) leads to short- and long-term obesity and diabetes. This phenotype is also observed in experimental models, in which early-weaned males exhibit abnormal insulinaemia in adulthood. However, studies regarding the effect of EW on pancreatic islets are rare. We investigated the mechanisms by which glycaemic homeostasis is altered in EW models through evaluations of insulin secretion and its signalling pathway in offspring. Lactating Wistar rats and their pups were divided into the following groups: non-pharmacological EW (NPEW): mothers were wrapped with an adhesive bandage on the last 3 days of lactation; pharmacological EW (PEW): mothers received bromocriptine to inhibit prolactin (1 mg/kg body mass/day) on the

Carla Bruna Pietrobon has a Bachelor's in Biology. She obtained her MSc in 2017 at the State University of Western Paraná, Brazil. She is currently a PhD student in the Bioscience Program of the State University of Rio de Janeiro, Brazil. She investigates different models of metabolic programming in rats and the impact on the offspring metabolism in adult life, with emphasis on insulin secretion by pancreatic β-cells and the peripheral action of insulin. Part of the current study was awarded at the 3rd Meeting of Ibero-American DOHaD (Developmental Origins of Health and Disease), 2018, in the Basic Science Research Category.

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Nicotine exposure during breastfeeding reduces sympathetic activity in brown adipose tissue and increases in white adipose tissue in adult rats: Sex-related differences

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ABSTRACT

Nicotine transfer via breast milk induces obesity in the adult offspring. We hypothesize that sympathetic nervous system (SNS) activity, brown adipose tissue (BAT) thermogenesis and white adipose tissue (WAT) lipogenesis/adipogenesis are altered in adult rats that were exposed to nicotine exclusively during the breastfeeding period. Lactating Wistar rats were separated into two groups: nicotine (NIC), dams implanted with osmotic minipumps containing 6 mg/kg of nicotine at postnatal day (PN) 2; control, dams were implanted with saline-containing minipumps. Euthanasia occurred at PN120 or PN180. NIC offspring had lower BAT SNS activity and higher BAT lipid content. NIC males showed lower UCP1, β3-AR and CPT1a, while NIC females showed lower UCP1, TRα1, CPT1a, suggesting lower thermogenesis. NIC males showed higher WAT SNS activity, WAT β3-AR, adrenal catecholamine, FAS, PPARγ and adipocytes area, while NIC females showed higher ACC, FAS, CEBPβ and PPARγ. These findings indicate increased lipogenesis/adipogenesis in both sexes, with a possible compensatory sympathetic activated-lipolysis in males. NIC males had higher hypothalamic pAMPK/AMPK, explaining the lower BAT sympathetic activity. Neonatal nicotine exposure reduces BAT SNS activity and thermogenesis, and, only in males, increases WAT adipogenesis/lipogenesis, despite higher WAT SNS activity. These alterations can be associated with obesogenesis in this programming model.

1. Introduction

Obesity prevalence is increasing worldwide (Engin, 2017). Different conditions may lead to obesity and other related chronic diseases, such as high calorie intake, physical inactivity and genetic conditions (Rao et al., 2019; Penninx and Lange, 2018). According to clinical and pre-clinical data, environmental and nutritional insults during fetal or perinatal life can program epigenetic changes that contribute to the onset of different metabolic disorders later in life, such as obesity, dyslipidemias, type 2 diabetes mellitus, hypertension, hormonal and behavioral changes (Fraga-Marques et al., 2010; Hanley et al., 2010; Lisboa et al., 2017; Almeida et al., 2019; Krzeczkowski et al., 2019). In this sense, both experimental and epidemiological studies have shown that smoking during breastfeeding may increase the likelihood of the offspring developing obesity as adults (Lisboa et al., 2017; Mizutani et al., 2007; Oken et al., 2008; Santos-Silva et al., 2013). Some women stop smoking during gestation but resume it during breastfeeding, believing it does not affect their babies (Hannöver et al., 2008; McBride and Pirie, 1990). However, nicotine, the main additive molecule of the cigarette, is transferred by breast milk and is capable of promoting several short- and long-term adverse effects. In rodents, maternal nicotine exposure exclusively during the lactation period programs for higher body and visceral fat mass, hyperleptinemia, hyperinsulinemia, hypercorticosteronemia and hypothyroidism in the male offspring (Oliveira et al., 2009; de Oliveira et al., 2010). The female offspring in this programming model show no changes in body mass or plasma hormones (Pinheiro et al., 2011). Our group has previously shown that exposure to cigarette smoke exclusively during lactation increased visceral fat mass, food intake and circulating leptin in the progeny of both sexes, while hyperthyroidism was observed only in males (higher TSH, T4 and T3) and increased serum T4 was observed only in females (Santos-Silva et al., 2013; Lisboa et al., 2017).

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ORIGINAL CONTRIBUTION

Early weaning alters the thermogenic capacity of brown adipose tissue in adult male and female rats

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Abstract

Purpose Early weaning (EW) is a risk factor for obesity development. Brown adipose tissue (BAT) hypofunction is related to obesity onset. Here, we evaluated whether sympathetic nervous system (SNS) activity in BAT and the thermogenic function of BAT are decreased in adulthood in obese rats from two EW models.

Methods At the time of birth, lactating Wistar rats and their pups (three males and three females) were separated into three groups: the control group, in which pups consumed milk throughout lactation; the non-pharmacological EW (NPEW) group, in which suckling was interrupted with a bandage during the last 3 days of lactation; and the pharmacological EW (PEW) group, in which dams were treated with bromocriptine (0.5 mg/twice a day) 3 days before weaning. The offspring were sacrificed on PN180.

Results Adult male rats from both EW models exhibited lower BAT SNS activity. Female rats from the PEW group showed a decrease in BAT SNS activity. The protein levels of UCP1 were lower in the NPEW males, while PGC1α levels were lower in both PEW and NPEW males. Both groups of EW females showed reductions in the levels of β3-AR, TRβ1, and PGC1α. The UCP1 protein level was reduced only in the NPEW females. The EW groups of both sexes had lower AMPK protein levels in BAT. In the hypothalamus, only the PEW females showed an increase in AMPK protein levels. In both groups of EW males, adrenal catecholamine was increased and tyrosine hydroxylase was decreased, while in EW females, adrenal catecholamine was decreased.

Conclusions Early weaning alters the thermogenic capacity of BAT, which partially contributes to obesity in adulthood, and there are sex-related differences in these alterations.

Keywords Breastfeeding · Early weaning · Bromocriptine · Developmental plasticity · Metabolic syndrome · Autonomic function

Introduction

Environmental and nutritional changes during periods of great plasticity, such as intrauterine life and/or lactation, can cause metabolic disorders in offspring. This phenomenon is known as metabolic programming [1–4]. Early weaning can lead to metabolic programming. Epidemiological data have shown that exclusive breastfeeding up to 6 months of age is protective against the development of obesity in adulthood [5–8]. In rats, we have shown the long-term adverse effects of breast milk deprivation, which include programming for metabolic syndrome [9–12]. In a pharmacological early weaning (PEW) model, bromocriptine, a type 2 dopamine agonist that inhibits prolactin production/secretion, is administered to mothers during the last 3 days of lactation, and this programs adult male offspring for obesity, hyperleptinemia, insulin resistance, dyslipidaemia, central hypothyroidism, and high concentrations of adrenal hormones [9, 10]. Lima et al. [11, 12] developed a non-pharmacological early weaning (NPEW) model in which a bandage is applied to mothers during the last 3 days of lactation. They found that adult males are programmed for obesity, higher body fat, insulin resistance, hyperleptinemia, and hypertriglyceridemia [11, 12]. The aforementioned metabolic and hormonal alterations

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Late effects of early weaning on food preference and the dopaminergic and endocannabinoid systems in male and female rats

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Abstract

Early weaning (EW) is associated with obesity later in life. Here, using an EW model in rats, we investigated changes in feeding behavior and the dopaminergic and endocannabinoid systems (ECS) in the adult offspring. Lactating Wistar rats were divided into two groups: EW, dams were wrapped with a bandage to interrupt suckling during the last 3 days of breastfeeding; CONT; dams fed the pups throughout the period without hindrances. EW animals were compared with CONT animals of the same sex. At PN175, male and female offspring of both groups could freely self-select between high-fat and high-sugar diets (food challenge test). EW males preferred the high-fat diet at 30 min and more of the high-sugar diet after 12 h compared to CONT males. EW females did not show differences in their preference for the palatable diets compared to CONT females. Total intake of standard diet from PN30-PN180 was higher in both male and female EW animals, indicating hyperphagia. At PN180, EW males showed lower type 2 dopamine receptor (D2r) in the nucleus accumbens (NAc) and dorsal striatum, while EW females had lower tyrosine hydroxylase in the ventral tegmental area and NAc, D1r in the NAc, and D2r in the prefrontal cortex. In the lateral hypothalamus, EW males had lower fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, whereas EW females showed lower N-arachidonoyl-phosphatidylethanolamine phospholipase-D and increased FAAH. Early weaning altered both the dopaminergic and ECS parameters at adulthood, contributing to the eating behavior changes of the progeny in a sex-dependent manner.

Introduction

Epidemiological and animal studies have demonstrated that environmental and nutritional changes during pregnancy and lactation may initiate permanent changes in metabolism that lead to several diseases later in life.[1–3] This phenomenon, known as metabolic programming, involves epigenetic changes during critical windows of development and plays an important role, for example, in the onset of obesity.[4]

Breastfeeding is considered a critical period of development since milk is the first source of nutrition for the baby after birth. A child is considered exclusively breastfed only when breast milk is ingested with no intake of any other food or drink for the first 6 months of life.[5] Introduction of any food before 6 months of age characterizes as early weaning.[6] Studies have shown that alterations during the lactation period, even if transitory, are related to late-emerging metabolic disturbances.[7–9] Our research group developed an animal model of early weaning and observed, in males, hyperphagia, overweight, increased visceral adiposity, and resistance to leptin in the adult offspring.[10–12] In addition, hepatic alterations were detected, with increased triglycerides and microsteatosis associated with increased oxidative stress.[13] Early-weaned females also exhibited hyperphagia and higher adiposity, despite having normal body mass.[14] A sexual dimorphism seems to be involved in these phenotypes, since we have already shown that females differ from males in parameters such as hormonal profiles, hepatic lipid metabolism, brown adipose tissue function, and fat deposit distribution.[15]

It is known that obesity is a multifactorial disease that may be associated with the development of several other metabolic diseases and is considered a major epidemic in the 21st century.[16] Alterations in the endocannabinoid system (ECS) have been seen as one of the mechanisms capable of regulating aspects related to hyperphagia and greater storage of calories, playing a crucial role in the regulation of energy balance.[17,18] Likewise, the dopaminergic system can control the search for palatable food and reward[19,20]; the excess of fat and sugar in foods can activate central pathways that are similar to those triggered by drug addiction.[20] Therefore,

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ORIGINAL ARTICLE

Pancreatic steatosis in adult rats induced by nicotine exposure during breastfeeding

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Abstract

Purpose Maternal nicotine exposure negatively impacts offspring's health and metabolism, leading to obesity and insulin resistance. Here we investigated the pancreatic islet function, glycemic homeostasis, and insulin signaling in adult rat offspring that were nicotine-exposed during breastfeeding.

Methods For this, lactating Wistar rat dams were divided into two groups: Nicotine (implanted with osmotic minipumps containing 6 mg/Kg, NIC) and Control (saline, CON). Solutions were released from postnatal (PN) day 2–16. At PN110 and PN170, 10 offspring per litter/sex/group were submitted to the oral glucose tolerance test (OGTT). PN180 offspring were killed and glycemia, insulinemia, adiponectinemia, pancreas morphology as well as pancreatic islet protein expression (related to insulin secretion) and skeletal muscle (related to insulin action) were evaluated. Males and females were compared to their respective controls.

Results Adult NIC offspring of both sexes showed glucose intolerance in the OGTT. Despite normoglycemia, NIC males showed hyperinsulinemia while females, although normoinsulinemic, had hyperglycemia. Both sexes showed increased IRI, reduced adiponectin/visceral fat mass ratio and higher ectopic deposition of lipids in the pancreatic tissue adipocytes. In pancreatic islets, NIC males showed lower PDX-1 expression while females had higher PDX-1 and GLUT2 expressions plus lower α2 adrenergic receptor. In the muscle, NIC offspring of both sexes showed reduction of GLUT4 expression; NIC males also had lower insulin receptor and pAKT expressions.

Conclusions Thus, glycemic homeostasis and peripheral insulin signaling in adult offspring of both sexes are affected by nicotine exposure through the milk, increasing the risk for type 2 diabetes development.

Keywords Glycemia · Lactation · Nicotine · Pancreatic beta cell · Programming

Introduction

Type 2 diabetes mellitus is considered a pandemic, mainly caused by insulin resistance (IR), which is closely associated with obesity [1]. However, in recent years, experimental and epidemiological evidence have shown that the growing incidence of type 2 diabetes and the obesity pandemic may also be due to insults during critical early life periods [2]. In the 1980s, Barker raised the "thrifty phenotype hypothesis" to explain the relationship between the fetal environment and the diseases found in adulthood [3]. This concept is now known as "Developmental Origins of Health and Disease" (DOHaD), which postulates that specific environmental insults during a critical period of early life can induce developmental plasticity by programming the individual for disease development [4].

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