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ANA PAULA DA FONSECA ARCOVERDE CABRAL DE MELLO

REPERCUSSÕES DE UMA DIETA PÓS-NATAL RICA EM ÁCIDOS GRAXOS SATURADOS SOBRE A FUNÇÃO HEPÁTICA E MODULAÇÃO AUTONÔMICA DE PROLES SUBMETIDAS À DESNUTRIÇÃO PROTEICA PERINATAL

Vitória de Santo Antão
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Dissertação apresentada ao Programa de Pós-Graduação em Nutrição, Atividade Física e Plasticidade Fenotípica da Universidade Federal de Pernambuco, como requisito para a obtenção do título de mestre em Nutrição, Atividade Física e Plasticidade Fenotípica.

Área de concentração: Bases experimentais e clínicas da plasticidade Fenotípica.

Orientador: Prof. Dr. João Henrique da Costa Silva

Coorientador: Prof. Dr. Francisco Carlos Amanajás de Aguiar Júnior

Coorientadora: Profa. Dra. Karla Patrícia de Sousa Barbosa Teixeira

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Aprovada em: 18/02/2020.

BANCA EXAMINADORA

_____

Prof. Dr. João Henrique da Costa Silva (Orientador)
Universidade Federal de Pernambuco

_____

Profa. Dra. Mariana Fernandes Pinheiro (Examinadora Interna)
Universidade Federal de Pernambuco

_____

Prof. Dr. Leucio Duarte Vieira Filho (Examinador Externo)
Universidade Federal de Pernambuco

_____

Prof. Dr. José Luiz de Brito Alves (Examinador Externo)
Universidade Federal da Paraíba

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“O maior espetáculo do pobre da
atualidade é comer”.

(Carolina Maria de Jesus, 1914-1977).

RESUMO

Deficiências nutricionais ou excessos alimentares durante estágios críticos de desenvolvimento podem influenciar o fenótipo metabólico na idade adulta e parecem ser impactados por divergências nutricionais entre o ambiente perinatal e pós-natal. Nosso objetivo foi avaliar a repercussão de uma dieta hiperlipídica pós-desmame rica em ácidos graxos saturados na função hepática e na modulação autonômica cardiorrespiratória da prole após desnutrição protéica perinatal. Foram utilizadas ratos wistar, cujas mães receberam dieta controle (C: proteína de 19%) ou dieta hipoproteica (LP: proteína 8%) durante a gestação e lactação, passaram a consumir dieta controle (C: 13% de lipídios) ou dieta hiperlipídica rica em ácidos graxos saturados (HL: 32% lipídio) após o desmame (21 dias após o nascimento) e até 90 dias de vida. Três grupos foram formados: controle: C (n = 5-12), LP-C (n = 5-12) e LP-HL (n = 5-12). Avaliamos os triglicerídeos e colesterol séricos, peso relativo do fígado, conteúdo sérico de AST e ALT, histologia hepática, expressão gênica de IL-1β, TNFα, NFκβ e fatores de transcrição PPARα, ChREBp, FOXO3 e SREBp1 no tecido hepático, bem como a modulação autonômica no controle cardiorrespiratório. O consumo de dieta hiperlipídica enriquecida com ácidos graxos saturados não promoveu alterações morfológicas, danos teciduais ou alterações da modulação autonômica cardiorrespiratória aos 90 dias de idade. A desnutrição perinatal aumentou a expressão do gene PPARα e diminuiu a expressão do gene FOXO3. A dieta hiperlipídica rica em ácidos graxos saturados pós-desmame induziu aumento da expressão do gene SREBp1 e expressão de genes pró-inflamatórios no tecido hepático. Nossos resultados sugerem que a desnutrição perinatal induz adaptações que mantém a normalidade do metabolismo lipídico e a modulação autonômica, mesmo diante de um segundo insulto nutricional com consumo pós-natal de dieta hiperlipídica. No entanto, o aumento da expressão gênica do metabolismo e de fatores inflamatórios sugerem que a maior ingestão de ácidos graxos saturados por animais previamente desnutridos acarretam em resposta inflamatória em nível molecular, que pode levar a danos ao fígado em longo prazo.

Palavras-chave: Transição nutricional. Desnutrição. Dieta hiperlipídica. Doenças metabólicas. Modulação autonômica.

ABSTRACT

Nutritional deficiencies or food excesses during critical stages of development may influence the metabolic phenotype in adulthood, and appear to be impacted by nutritional divergences between the perinatal and postnatal environment. To evaluate the repercussion of a post-weaning high-fat diet rich in saturated fatty acids on liver function and cardiorespiratory autonomic modulation of offspring after perinatal protein malnutrition. Wistar rats, in whom mothers received a control diet (C: 19% protein) or low protein diet (LP: 8% protein) during pregnancy and lactation, started to consume a control diet (C: 13% lipid) or a high-fat diet rich in saturated fatty acids (HL: 32% lipid) after weaning (21 days after birth) up to 90 days of life. Three groups were formed: control: C (n = 5-12), LP-C (n= 5-12) and LP-HL (n = 5-12). Serum triglyceride and cholesterol, liver relative weight, liver βhad and FAS enzymatic activity, AST and ALT serum content, liver histology, gene expression of IL-1β, TNFα, NFκβ and transcription factors PPARα, ChREBp, FOXO3 and SREBp1 in liver tissue were measured, autonomic modulation on cardiorespiratory control by spectral analyses. The consumption of a high-fat diet enriched with saturated fatty acids did not promote morphological alterations, tissue damage or cardiorespiratory autonomic modulation changes at 90 days of age. However, perinatal malnutrition increased PPARα gene expression and decreased FOXO3 gene expression. A post-weaning high-fat diet induced increased SREBp1 gene expression and expression of proinflammatory genes in liver tissue. Our results suggest that perinatal malnutrition induces adaptations that maintain normal lipid metabolism and autonomic modulation, even in the face of a second nutritional insult with postnatal consumption of a high-fat diet. However, the increased gene expression of metabolism and inflammatory factors suggests that higher intake of saturated fatty acids by previously malnourished animals induces an inflammatory molecular response, which may lead to liver damage in the long term.

Keywords: Transition nutrition. Malnutrition. High-fat diet. Metabolic desease. Autonomic modulation.

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1 APRESENTAÇÃO

A transição nutricional tem se consolidado mundialmente, principalmente entre as populações de países em desenvolvimento (KOSAKA; UMEZAKI, 2017). Os índices de desnutrição relacionados ao déficit de peso e baixa estatura têm sido substituídos por alta prevalência da obesidade (SOUSA; OLINDA; PEDRAZA, 2016). Uma ocorrência intrínseca da transição nutricional é a passagem dos sujeitos de estados de desnutrição durante períodos críticos de desenvolvimento (como a gestação e lactação), para estágios de hipernutrição, com consumo excessivo de alimentos processados e com alto teor de ácidos graxos saturados, açúcares e calorias (KEDING, 2016). A desnutrição perinatal parece promover uma espécie de programação do metabolismo para condições de escassez nutricional (ZHU; CAO; LI, 2019). Dessa forma, a ocorrência da hipernutrição, precedida pela desnutrição, estabelece um ambiente nutricional pós-natal divergente do ambiente perinatal, correspondendo a uma sobrecarga metabólica que pode acarretar em repercussões deletérias sobre a homeostase energética dos indivíduos (ZHU; CAO; LI, 2019).

O ambiente nutricional a que os seres vivos são submetidos é determinante para sua saúde e monitorado por sensores nutricionais altamente sensíveis, que respondem, inclusive, à redução no conteúdo de aminoácidos (CHANTRANUPONG; WOLFSON; SABATINI, 2015). As alterações metabólicas associadas a um ambiente perinatal escasso podem estar relacionadas à programação epigenética (MISKA; FERGUSON-SMITH, 2016), que modula a expressão de enzimas metabólicas (PLANQUÉ *et al.*, 2018). O controle metabólico é realizado pela interação entre órgãos e vias com função altamente regulada (DESAI; JELLYMAN; ROSS, 2015). O fígado é um dos órgãos chave para o controle metabólico, e demonstra sensibilidade a insultos nutricionais, reagindo, entre outras formas, a partir de ajustes no metabolismo glicídico e lipídico (JONES, 2016). Desbalanços entre os mecanismos lipolíticos e lipogênicos podem direcionar o tecido hepático a condições fisiopatológicas (FABBRINI; MAGKOS, 2015).

Os hepatócitos são sensíveis à escassez de aminoácidos, cujos níveis séricos reduzidos se correlacionam com o adoecimento hepático (NISHI *et al.*, 2018). Em um modelo experimental de desnutrição proteica, hepatócitos isolados de ratos alimentados com 5% de proteína caseína durante 14 dias apresentaram síntese de triglicerídeos aumentada (TAGUCHI *et al.*, 2017). Um estudo usando modelo de desnutrição materna, com consumo de 8% de proteínas durante gestação e lactação, identificou que as proles adultas apresentavam aumento do colesterol hepático em um mecanismo regulado epigeneticamente (SOHI *et al.*, 2011). Avaliando o gasto energético de animais submetidos a uma dieta reduzida em proteínas (5, 10

e 15% de proteínas) e rica em lipídios (33% das calorias originadas de lipídios), Pezeshki *et al.* (2016) demonstraram que a expressão gênica das enzimas Ácido Graxo Sintase (FAS) estava aumentada e β-hidroxiaxil-CoA desidrogenase (β-HAD) estava reduzida, o que poderia sugerir uma aumento na capacidade lipolítica e lipogênica, dependente do percentual de redução proteico da dieta. Foi notado ainda que a dieta hiperlipídica acentuou a ação lipogênica.

As dietas hiperlipídicas têm sido relacionadas a diversas doenças que compõem a síndrome metabólica (MORENO-FERNÁNDEZ *et al.*, 2018). Os ácidos graxos saturados apresentam alto potencial inflamatório, com ativação de respostas imunes crônicas de baixo grau, mediadas por hiperatividade de receptores *tool like*, ciclooxigenases e estimulação da via dos lipopossacarídeos, desde a digestão até a incorporação dos ácidos graxos (ROCHA *et al.*, 2016; ROGERO; CALDER, 2018). Fatores de transcrição, como a proteína de ligação do elemento de resposta sensível a carboidratos (ChREBp), receptores ativados por proliferador de peroxissoma (PPAR) e a proteína de ligação ao elemento regulador de esterol (SREBp) respondem ao consumo excessivo de lipídios, controlam a homeostase energética e podem ativar vias relacionadas a processos inflamatórios e degenerativos no tecido hepático (MELLO; MATEROZZI; GALLI, 2016; SHIMANO; SATO, 2017; LI; JIANG; XU, 2019). O metabolismo energético, a expressão gênica e a ativação de vias inflamatórias parecem ter como elo a atividade do sistema nervoso autonômico.

Em recente revisão, Taher, Farr e Adeli (2017) registraram que o sistema nervoso central se comunica com o fígado por vias autonômicas aferentes e eferentes e regula o metabolismo lipídico e a produção tecidual de lipoproteínas. Distúrbios que compõem a síndrome metabólica estão relacionados a uma regulação alterada do sistema nervoso simpático (STUCKEY *et al.*, 2014). A variabilidade da frequência cardíaca, assim como a da frequência respiratória, tem sido utilizada para avaliar desbalanços simpatovagais, onde valores reduzidos se correlacionam a sensibilidade à insulina prejudicada, dislipidemias e hipertensão arterial (SAITO *et al.*, 2017; ALVES; COSTA-SILVA, 2018a). Contudo, os efeitos metabólicos hepáticos e a relação com a modulação autonômica em animais previamente desnutridos ainda não estão esclarecidos. Dessa forma, avaliamos as repercussões de uma dieta hiperlipídica rica em ácidos graxos saturados, ofertada do pós-desmame até os 90 dias de vida da prole, sobre a função hepática e a modulação autonômica de proles submetidas à desnutrição proteica perinatal, buscando auxiliar na compreensão dos fatores subjacentes ao desenvolvimento de doenças cardiometabólicas em populações submetidas à transição nutricional.

1.1 Nutrição, plasticidade e as origens evolutivas da saúde e da doença

Em seu percurso histórico, a ciência tem se debruçado incessantemente para desvendar as origens da saúde e da doença e, desde a década de cinquenta, teorias têm sido debatidas, buscando relacionar as condições ambientais ao desenvolvimento de doenças (BARKER; OSMOND, 1986; NEEL, 1999; LAW *et al.*, 2002; BHARGAVA *et al.*, 2004; ALEXANDER; DASINGER; INTAPAD, 2015). A alimentação e nutrição constituem-se em requisitos básicos para a promoção e proteção da saúde, possibilitando a afirmação plena do potencial de crescimento e desenvolvimento humano, com qualidade de vida (BRASIL, 2012).

Em 1962 teve início uma série de estudos prospectivos e observacionais que relacionaram aspectos nutricionais materno-fetais e características populacionais como peso e estatura, com possíveis conexões epidemiológicas entre essas variáveis e o surgimento de doenças na vida adulta, tendo como pano de fundo o período de escassez delimitado pela "fome holandesa" (ROSEBOOM *et al.*, 2001). Carências ou excessos nutricionais, modificações no ambiente perinatal (durante gestação e lactação) e nos demais estágios críticos do desenvolvimento, como a infância e adolescência, têm sido considerados como fatores de risco para o desenvolvimento de doenças crônicas na vida adulta (ALVES; COSTA-SILVA, 2018b). Dietas com carências ou excessos significativos de nutrientes estão amplamente relacionadas a alterações morfológicas, metabólicas e fisiológicas, para as quais tem-se buscado origens em processos disfuncionais (BASSI *et al.*, 2012).

Os achados científicos inicialmente relacionados com a teoria de um "genótipo poupador" (NEEL, 1999) receberam atualizações advindas de estudos observacionais, prospectivos e clínicos, que incluíam populações de países desenvolvidos e em desenvolvimento e se contrapunham ao conceito de modificação genotípica (WEST-EBERHARD, 2005). A partir da década de 80, Barker *et al.* lançaram um novo olhar sobre as teorias preditivas, que reforçava a relação entre o ambiente e o sujeito, em seus estágios iniciais do desenvolvimento, cujas alterações morfológicas e fisiológicas advinham de insultos ou estímulos durante estágio críticos, e se configuravam por respostas adaptativas advindas do fenótipo e não do genótipo (BARKER, 2007).

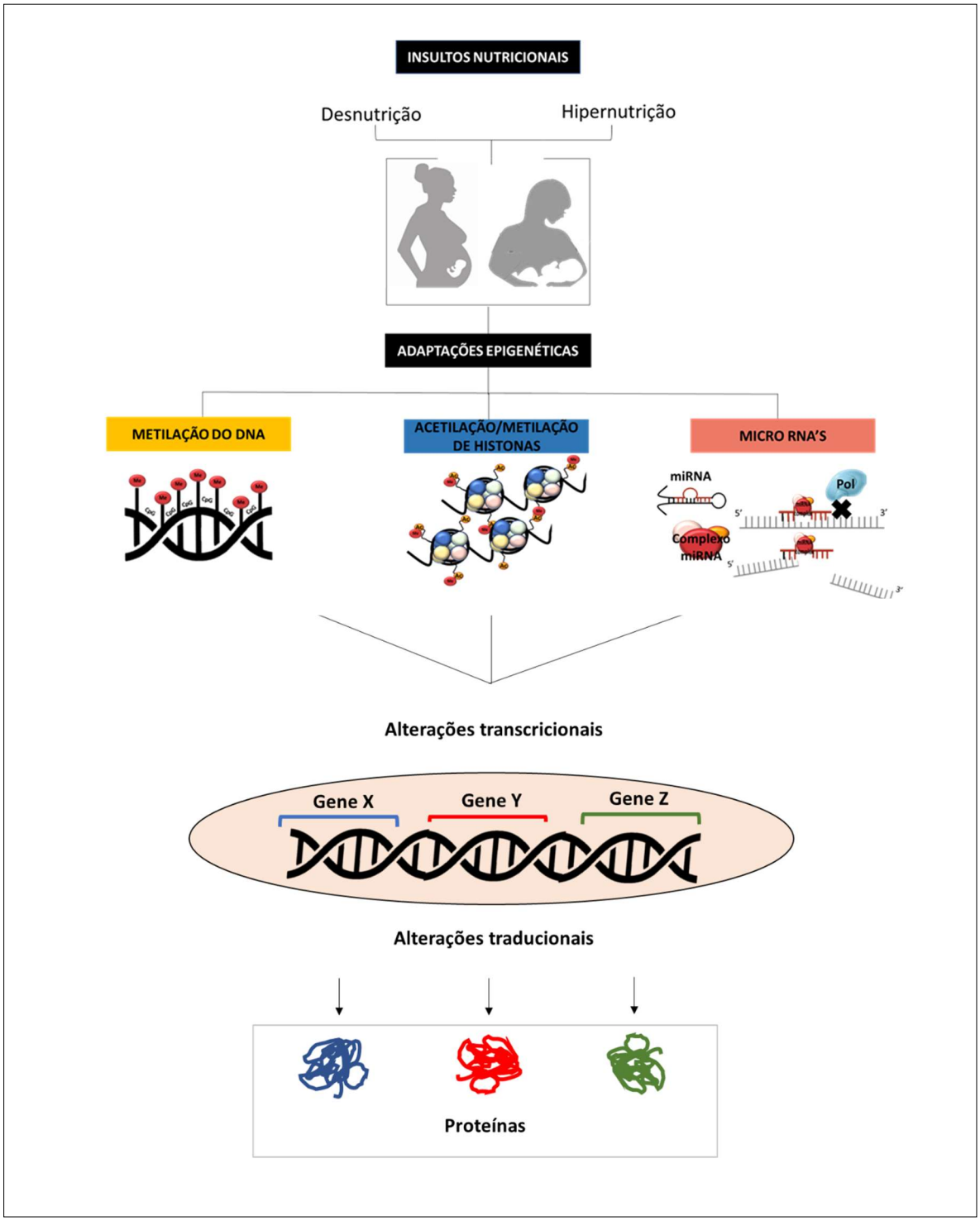
A "hipótese de Barker" foi a mola propulsora para inúmeras pesquisas em todo o mundo com foco sobre as origens fetais do adoecimento (CORREIA-BRANCO; KEATING; MARTEL, 2014; FUKUOKA, 2015; COSTA-SILVA; SIMÕES-ALVES; FERNANDES, 2016). A Teoria das Origens Desenvolvimentistas da Saúde e da Doença (DOHaD), anteriormente conhecida como Hipótese das Origens Fetais (FOAD), propõe que perturbações no ambiente perinatal promoveriam adaptações fenotípicas, que influenciam mecanismos

endócrinos e metabólicos de maneira permanente, com impactos sobre processos fisiológicos, cognitivos e motores a longo prazo (HOFMAN *et al.*, 2006; BARKER, 2007; DALZIEL *et al.*, 2007). A DOHaD obteve ampliação conceitual, com prolongamento temporal do período compreendido como "origem", que passa a se estender desde o período pré-natal até todo o período de desenvolvimento dos indivíduos (UAUY; CORVALAN; KAIN, 2011).

A partir das observações da DOHaD, do conceito de desenvolvimento plástico e das pesquisas que nela se apoiaram, surge o conceito de plasticidade fenotípica (WEST-EBERHARD, 2005), que tem sido amplamente utilizada para caracterizar uma série de heranças não genéticas surgidas a partir de fenótipos gerados em resposta a alterações ambientais - adaptações durante os diversos estágios do desenvolvimento, com o objetivo de garantir a sobrevivência e eficiência do indivíduo em ambientes hostis (HANSON; GLUCKMAN, 2014).

No campo da plasticidade, as respostas adaptativas preditivas, orientam para uma programação metabólica das proles, em resposta ao ambiente fetal: quando encontram resposta positiva do meio às suas predições, as adaptações fenotípicas tornam-se aparelhagem vantajosa para a sobrevivência dos indivíduos (MCMILLEN; ROBINSON, 2005). Antagonicamente, um ambiente pós-natal divergente do perinatal parece representar uma sobrecarga metabólica que pode acarretar em adoecimento (MCMILLEN; ROBINSON, 2005). As repercussões do ambiente nutricional materno sobre a prole têm sido amplamente observadas a partir da observação de alterações de origem epigenética (GERAGHTY *et al.*, 2016).

Figura 1 - Vias sensíveis à programação metabólica nutricional perinatal.



Fonte: Elaborada pela autora.

A epigenética, em ampla expansão, tem possibilitado o esclarecimento de cada vez mais mecanismos intrincados na resposta fenotípica às alterações ambientais, e tem demonstrado que

a interação entre um conjunto de genes e o ambiente atuam para produzir várias expressões fenotípicas, seja por diferenciação, variação e/ou adaptação (PARK *et al.*, 2017). Os fatores causais que, originalmente, iniciaram com o estudo do estado nutricional durante a gravidez, evoluíram para incluir fatores ambientais externos e internos, que incluem alimentos, poluentes, fármacos, ondas sonoras e eletromagnéticas, microbiota intestinal e uma ampla gama de inter-relações entre o organismo e o seu meio, e que pode direcionar para a saúde ou para o adoecimento (HAUGEN *et al.*, 2015; ROSENFELD, 2016).Nesse contexto, a plasticidade fenotípica tem como base molecular as alterações epigenéticas, como a metilação do DNA, acetilação da histona e expressão do microRNA (WELLS, 2011). Essas modificações "epigenéticas" foram primeiramente descritas por Conrad Waddigton em 1940 e estudam a relação entre causa e efeito nos genes para produzir um fenótipo (JABLONKA; LAMB, 2002). Atualmente, esse conceito é empregado para descrever o processo de expressão gênica e sua ligação a modificações na estrutura da cromatina sem alterar a sequência de DNA (CHONG; WHITELAW, 2004; EGGER *et al.* 2004).

A metilação do DNA está relacionada à adição de grupos metil nos resíduos de citosina do DNA, seguida pelo resíduo de guanina (dinucleotídeos CpG) e adenina, que podem produzir inibição da expressão gênica por prejudicar a ligação do fator transcricional (WATERLAND; MICHELS 2007; MANSEGO *et al.*, 2013; CHANGO; POGRIBNY, 2015; MITCHELL *et al.*, 2016). A acetilação da histona diminui a afinidade eletrostática entre as proteínas das histonas e o DNA e, assim, promove uma estrutura de cromatina que é mais permissiva à transcrição de genes, regulada pela ação das histona acetil transferases (HATs), enquanto a remoção de grupos acetila é catalisada pela histona desacetilase (HDACs) (GRAFF; TSAI, 2013). MicroRNAs são pequenas moléculas de RNA endógenas não codificantes envolvidas na regulação de genes e estão localizadas em íntrons de genes que codificam proteínas, íntrons de genes não codificadores ou exons de genes não codificadores; eles foram implicados em muitos processos celulares, incluindo proliferação, apoptose, diferenciação, senescência e respostas ao estresse e estímulos imunológicos (D'IPPOLITO; IORIO, 2013).

Nesse contexto, tem sido investigado como o aspecto nutricional pode induzir essas modificações epigenéticas. Essas marcas epigenéticas são estabelecidas no início do desenvolvimento e podem persistir por um longo período de tempo e modificam as cascatas que complementam o dogma central da biologia, repercutindo em processos de transcrição e tradução alterados, e reforçam a hipótese de que as modificações epigenéticas são amplamente um mecanismo abrangente que liga a nutrição materna a fenótipos metabólicos de saúde na prole (MAZZIO; SOLIMAN, 2014; SZARC VEL SZIC *et al.,* 2015).

À luz da epigenética, nutrigenômica e metabolômica, o estudo da influência da nutrição na saúde passa a englobar as interconexões entre o nutrição e o gene (GERAGHTY *et al.*, 2016). Cada nutriente, através de múltiplas ações a nível molecular, contribui para a adaptação aos estímulos ou insultos sofridos durante o desenvolvimento e busca manter o organismo em homeostase e, assim, garantir a sobrevivência (ROSENFELD, 2016). Dessa forma, compreender os efeitos da quantidade, qualidade e duração dos insumos nutricionais ou estímulos do ambiente pode apontar para caminhos que levam ao estabelecimento da seguridade à saúde relacionada à alimentação e nutrição (GLUCKMAN; HANSON, 2008; LANGLEY-EVANS, 2015).

1.2 Desnutrição perinatal e distúrbios cardiometabólicos na vida adulta

A desnutrição é conceituada como uma condição clínica provocada por um aporte insuficiente de calorias ou macro e micronutrientes, que tem como causas básicas as condições socioeconômicas de um indivíduo, sua família e/ou população na qual está inserido, ou ainda, a condições absortivas patológicas (MONTEIRO; CONDE; POPKIN, 2002). Apesar do importante declínio desse fenômeno, observado desde a segunda metade do século passado, a desnutrição ainda é um grande problema em muitos países e voltou a crescer em 2016, atingindo 804,2 milhões de pessoas em todo o mundo. Estima-se que em 2017, esse número tenha se elevado para 820 milhões (WORLD HEALTH ORGANIZATION, 2017a). O panorama da FAO (Food and Agriculture Organization of United Nations) relata que a desnutrição crônica ainda afeta 94 milhões de crianças e, juntamente com o continente africano, as populações da América do Sul representam a maior contribuição para essa conjuntura (WORLD HEALTH ORGANIZATION, 2017a).

Evidências epidemiológicas sugerem que a exposição a escassez nutricional, tanto no útero quanto na vida pós-natal imediata (considerados como o período perinatal), relacionam-se a uma maior propensão para distúrbios cardiometabólicos em estágios mais tardios da vida (GLUCKMAN; HANSON, 2008; LANGLEY-EVANS, 2015). Essa propensão pode ser explicada por respostas hipotalâmicas alteradas à ação da leptina e de glicocorticoides, com efeitos duradouros sobre a ingestão de alimentos, o gasto energético e o metabolismo (ALVES *et al.*, 2017). O aporte proteico durante os estágios críticos do desenvolvimento - desde o período embrionário, lactação e primeira infância - é fundamental para garantir o crescimento e funcionamento pleno de órgãos e sistemas, a partir da manutenção adequada da síntese proteica e expressão gênica (ALVES *et al.*, 2014; NISHI *et al.*, 2018).

Estudos epidemiológicos reforçam os indicativos e evidenciam forte correlação entre o binômio baixo peso ao nascer/déficit de estatura na infância (manifestações clínicas marcantes da desnutrição) e risco de comorbidades na vida adulta como a síndrome metabólica, diabetes tipo II, hipertensão, além de retardos no desenvolvimento motor e cognitivo (RAVELLI; STEIN; SUSSER, 1976; SAWAYA; ROBERTS, 2003; THORN *et al.*, 2011) No caso da desnutrição perinatal, as respostas adaptativas podem apresentar desde medidas metabólicas de economia energética, como diminuição da insulina plasmática, do fator de crescimento semelhante à insulina (IGF-1), de reservas nutricionais e do fluxo sanguíneo, a alterações na regulação dos centros de saciedade e na secreção e recepção de reguladores do apetite (BENTO-SANTOS *et al.*, 2012). Diferentes modelos de escassez proteica materna relataram alterações morfológicas, fisiológicas e funcionais nos mais diversos sistemas, como cardiovascular (AUBIN *et al.*, 2008; COSTA-SILVA; SIMÕES-ALVES; FERNANDES, 2016), renal (GLASTRAS *et al.*, 2016) e muscular (ALVES *et al.*, 2017), que se relacionam com o desenvolvimento de doenças cardiometabólicas.

A desnutrição proteica refere-se ao consumo de proteínas inferior às necessidades do indivíduo e, em modelos animais de roedores, está caracterizado pelo consumo de proteínas inferior a 17% (REEVES *et al.*, 1993). Modelos animais de desnutrição buscam avaliar os efeitos do baixo consumo materno de proteínas durante a gestação e lactação sobre a prole de roedores. Alguns modelos experimentais de desnutrição utilizam a redução do consumo de proteínas materna (6 a 9% durante gestação e lactação) com o objetivo de investigar a influência da desnutrição proteica em períodos considerados críticos para desenvolvimento de sistemas vitais (METGES, 2009). A oferta de 8% de proteínas ao invés de 20%, no período perinatal, por exemplo, promoveu baixo peso ao nascer (BAYOL *et al.*, 2004; FALCÃO-TEBAS *et al.*, 2011; BARROS *et al.*, 2015b), redução do balanço simpatovagal (BARROS *et al*., 2015) e hipertensão (ALVES *et al.*, 2014; BARROS *et al.*, 2015b; LANGLEY-EVANS, 2015). Outros modelos relatam redução da capacidade de sucção (PINE *et al.*, 1994), alterações no músculo esquelético (ALVES; DÂMASO; DAL PAI, 2008) e resistência à insulina (THORN *et al.*, 2011; BENTO-SANTOS *et al.*, 2012).

Estudos prévios de nosso laboratório demonstraram que a desnutrição proteica perinatal (consumo materno de dieta com 8% de proteínas durante a gestação e lactação) induziu ao aumento da pressão arterial em animais que consumiram ração padrão, do pós-desmame até os 90 dias de vida (ALVES *et al.*, 2014). Este resultado parece estar relacionado a um desbalanço na modulação autonômica (BARROS *et al.*, 2015b) e maior ativação simpática (BRITO-ALVES *et al.,* 2015). Em um modelo de desnutrição proteica materna (10% de proteínas

durante gestação e/ou lactação), Sosa-Larios *et al.* (2017) identificaram que animais desnutridos super expressam enzimas e fatores de transcrição relacionadas ao metabolismo lipídico tanto no tecido hepático quanto no tecido adiposo, com o objetivo de garantir a homeostase energética.

A influência da desnutrição proteica perinatal sobre as respostas biológicas na vida adulta possui amplos relatos científicos, porém, os mecanismos envolvidos ainda não estão totalmente esclarecidos. De fato, modelos animais de programação metabólica incluem dietas de baixa proteína em estágios críticos do desenvolvimento. As adaptações que citamos anteriormente neste trabalho, podem estar relacionadas à plasticidade fenotípica. Os avanços em estudos epigenéticos conquistados nas últimas décadas têm contribuído fortemente para a compreensão de alguns fatores envolvidos na inter-relação nutrição-saúde-doença, mas ainda há muito a se avançar, inclusive com relação aos impactos da transição nutricional observada em todo o mundo.

1.3 Transição nutricional e consumo de dietas hiperlipídicas

A partir do século XX, o adoecimento e morte das populações apresentaram causas diferentes do modelo prevalente na primeira metade do século. A alta incidência de doenças infecciosas e parasitárias vem sendo substituída pela alta prevalência de doenças crônicas e degenerativas não transmissíveis, que acompanham a intensa transição demográfica e de costumes (POPKIN, 2015). Concomitante às revoluções industriais e tecnológicas, foi possível observar profundas alterações no comportamento e nos hábitos alimentares das famílias em todo o mundo (BATISTA FILHO; BATISTA, 2010). Paralelo ao declínio da desnutrição endêmica, que caracterizou a realidade de muitos países no século passado e arrastava junto a si as doenças *carenciais*, a incidência de obesidade no mundo tem-se elevado significativamente (WORLD HEALTH ORGANIZATION, 2018).

Segundo dados da Organização Mundial da Saúde, 804 milhões de pessoas ainda encontram-se desnutridas em todo o mundo (WORLD HEALTH ORGANIZATION, 2017a). Dicotomicamente, desde 1975, a obesidade mundial quase triplicou, alcançando 650 milhões de pessoas. O excesso de peso atingiu mais de 1,9 bilhão de adultos com 18 anos ou mais, até 2016. Atualmente, o sobrepeso e a obesidade matam mais pessoas que a desnutrição (WORLD HEALTH ORGANIZATION, 2017b). O aumento da obesidade se espalha em praticamente todos os continentes do globo, inclusive em países com alta insegurança alimentar. Esse

panorama nos aponta para a alarmante coexistência de ambos os agravos nutricionais, desnutrição e obesidade, numa mesma população, uma das características do fenômeno da transição nutricional. A interação entre mudanças epidemiológicas, socioeconômicas e demográficas determina a natureza e a velocidade da transição nutricional, e consequentemente, a gravidade de suas repercussões (BLACK *et al.*, 2013)

A transição nutricional é normalmente caracterizada pela redução na prevalência da desnutrição em suas diversas dimensões (energética e de macro ou micronutrientes), acompanhada por consumos excessivos de alimentos hipercalóricos e ultra processados, com consequente aumento do peso corporal (ABDULLAH, 2015). A última década foi marcada por uma série de publicações baseadas em estudos epidemiológicos que alertam para a transição nutricional e seus impactos: relatos de manutenção de déficits de estatura paralelos ao sobrepeso e obesidade, com suas múltiplas comorbidades, coexistindo na mesma população, principalmente nos países mais pobres e em desenvolvimento (BLACK *et al.*, 2013; KROKER *et al.*, 2014; SARMIENTO *et al.*, 2014; ABDULLAH, 2015; WINICHAGOON, 2015; KEDING, 2016; SOUSA; OLINDA; PEDRAZA, 2016).

Em um recente levantamento global, realizado em 30 países de baixa renda, Abdulah *et al.* (2015) relataram que o padrão de ganho de peso corporal da população de países em desenvolvimento é quase idêntico ao encontrado em países desenvolvidos e a estimativa de sobrepeso ao longo do tempo foi maior nos grupos de menor renda e menor escolaridade. A coexistência de crianças desnutridas e mães obesas numa mesma residência é realidade no México (KROKER *et al.*, 2014), na Colômbia (SARMIENTO *et al.*, 2014), na China (FENG *et al.*, 2015), na África Subsaariana (STEYN; MCHIZA, 2014) e também no Brasil (SOUSA; OLINDA; PEDRAZA, 2016). A baixa estatura e obesidade podem refletir uma nutrição inadequada e uma dieta de baixa qualidade nos primeiros dois anos de vida, seguida por excessos na ingestão de energia mais tarde na infância (ABDULLAH, 2015).

A desnutrição infantil está relacionada a atrasos no desenvolvimento psicomotor, a menor desempenho escolar, maior suscetibilidade a doenças infecciosas e desenvolvimento de doenças crônicas, enquanto a obesidade precoce é um fator de risco para problemas respiratórios, *diabetes mellitus* tipo 2, doenças cardiovasculares e outros distúrbios que compõem a síndrome metabólica (SOUSA; OLINDA; PEDRAZA, 2016). A coexistência dessas comorbidades advindas de fatores nutricionais tem sido denominada de “dupla carga da má nutrição” onde a desnutrição perinatal e infantil se segue a uma hipernutrição calórico/lipídica de baixo valor nutricional, com repercussões no ganho ponderal dos indivíduos, sobrepeso e obesidade (KEDING, 2016).

Embora evidentes, os mecanismos subjacentes à relação entre carências e excessos nutricionais precoces e obesidade e outras doenças crônicas tardias ainda não estão bem esclarecidos. Isso exige atenção, do ponto de vista das ciências da saúde e das ações de saúde pública, no intuito de compreensão e políticas de prevenção e minimização de danos. Enquanto a desnutrição perinatal sinaliza para doenças metabólicas na vida adulta (CASAS-AGUSTENCH; IGLESIAS-GUTIÉRREZ; DÁVALOS, 2015), o consumo de dietas hiperlipídicas associadas ao sobrepeso e obesidade aumentam o risco de diabetes tipo 2, síndrome metabólica e doença cardiovascular (O'SULLIVAN, 1982). As evidências científicas reforçam que o ambiente nutricional intrauterino desempenha importante papel na programação de fenótipos que afetam a saúde ao longo da vida (FERNANDEZ-TWINN; CONSTÂNCIA; OZANNE, 2015).

Coortes populacionais e estudos clínicos randomizados estabelecem relações determinantes entre um alto consumo de lipídios pela população e o aumento de distúrbios metabólicos (ROCHA *et al.*, 2016; ROGERO; CALDER, 2018). Em modelos de roedores, a hipernutrição lipídica se caracteriza pelo consumo de 30 a 60% do componente calórico total constituído de lipídios (KAKIMOTO; KOWALTOWSKI, 2016). Nesse contexto, Wong *et al.* (2016) observaram que doze semanas de dieta com alto teor lipídico promoveu o desenvolvimento de síndrome metabólica em ratos, com aumento da circunferência abdominal, pressão arterial, intolerância à glicose e dislipidemia, em relação a um grupo que recebeu apenas dieta padrão.

Os distúrbios podem estar relacionados a uma atividade imune aumentada, característico do alto consumo de dietas hiperlipídicas, e favorece a manutenção de processos inflamatórios crônicos, tanto no tecido hepático quanto no tecido adiposo, e extravasa para tecidos adjacentes. A resposta imune é mediada, entre outros fatores, pelas vias PPARγ e NFkβ. Essa rede de sinalização parece desencadear a redução da sensibilidade à insulina (ANDRÉA *et al.*, 2008; CALDER, 2012) e alterar o perfil lipídico plasmático e tecidual (RALSTON *et al.*, 2017), além de promover desordens cardiovasculares (WANG; HU, 2017). Contudo, em humanos, os resultados parecem ser distintos em função do tipo e da quantidade de ácidos graxos ingeridos (FINUCANE *et al.*, 2013; O'REILLY *et al.*, 2016; SIMOPOULOS, 2016; IGGMAN, 2011).

Em um modelo animal de roedores, Hernández-Rodas *et al.* (2017) submeteram camundongos a 12 semanas de dieta hiperlipídica com 60% de calorias vindas dos lipídios e 25% dos lipídios constituídos de ácidos graxos saturados. Os animais desenvolveram estresse oxidativo, esteatose hepática e resistência à insulina. Em uma metanálise publicada em 2018, que reuniu dezesseis coortes prospectivas realizadas em doze países, Imamura *et al.*

identificaram que altos níveis plasmáticos de ácidos graxos saturados com 15 e 17 carbonos na cadeia estavam associados com baixo risco de desenvolvimento de diabetes tipo 2 (IMAMURA *et al.*, 2018). Contudo, os resultados são conflitantes. O comprimento da cadeia carbônica e o tipo do ácido graxo parece realmente exibir efeitos distintos sobre a saúde humana e estudos mais profundos são necessários para compreender e estabelecer diretrizes confiáveis (IGGMAN, 2011).

1.4 Metabolismo lipídico e impactos dos ácidos graxos saturados sobre o tecido hepático

As dietas ocidentais, características da transição nutricional, são consideravelmente ricas em carboidratos simples, alimentos ultraprocessados e gorduras, principalmente em países em desenvolvimento (ABDULLAH, 2015), sendo as gorduras saturadas as mais presentes na dieta humana (IGGMAN, 2011). O metabolismo dos lipídios assimilados pela dieta tem o fígado como órgão chave e corresponde a um processo altamente regulado tanto fisiológica quanto molecularmente.

Os lipídios presentes na dieta encontram-se principalmente em forma de triglicerídeos e colesterol. A participação do fígado se inicia já na primeira etapa necessária para digestão dos lipídios: a bile produzida pelo fígado promove a emulsificação dos lipídios ingeridos, que são hidrolisados no lúmen e absorvidos pelo epitélio intestinal (IGGMAN, 2011). As células epiteliais do intestino ressintetizam os ácidos graxos que são transportados pelos quilomícrons através da circulação linfática e sanguínea, até atingir o fígado onde são metabolizados (HARVEY, 2012).

Os ácidos graxos livres no fígado podem ser reesterificados a triacilgliceróis ou embalados em partículas de lipoproteínas de muito baixa densidade (VLDL) e transportados para outros órgãos. O transporte de ácidos graxos para o interior das células é realizado por proteínas transportadoras específicas, como a proteína de transporte de ácidos graxos (FATP) e translocase de ácidos graxos (FAT / CD36) e proteína de ligação a ácidos graxos (FABP), em particular, de cadeia longa. Os fluxos de carboidratos e lipídios são coordenados pela disponibilidade de nutrientes e regulados pela ação, em partes, dos hormônios glucagon e insulina, dentre outros (JONES, 2016).

Durante estados de jejum, o uso dos ácidos graxos como substrato energético ocorre por meio da β-oxidação, que ocorre principalmente em ambiente mitocondrial (REDDY; RAO, 2006). Nesse processo, duplas de unidades de carbono são oxidados sucessivamente com acetil-CoA, com a ação de enzimas chave como acil-CoA desidrogenase, enoil-CoA hidroxilase e β-

hidroxiacil CoA-desidrogenase (JONES, 2016), que são reguladas pelo fator de transcrição PPARα. Falhas na atuação de alguma dessas enzimas pode afetar prejudicialmente a oxidação lipídica celular e induzir a acumulação de triglicerídeos hepáticos (HASHIMOTO *et al.*, 2000).

O aumento dos níveis energéticos induz a redução da atividade oxidativa mitocondrial. Altas concentrações intracelulares de ATP inibem a oxidação do citrato no Ciclo de Krebs por inibição da isocitrato desidrogenase. No citoplasma, o acetil-CoA é incorporado a ácido graxo com ação principal do complexo multifuncional enzimático ácido graxo sintase (FAS), capaz de sintetizar ácidos graxos de cadeia longa como o palmitato, usando acetil-CoA como iniciador, o malonil-CoA como doador de dupla de carbonos e NADPH como redutor (HARVEY, 2012). O silenciamento do gene precursor da FAS no tecido hepático reduziu o estabelecimento de esteatose em modelo experimental de murinos (KUHLA *et al.*, 2015).

Níveis elevados de insulina e glicose regulam a síntese de triacilgliceróis, estimulando a atividade de fatores de transcrição como a proteína de ligação do elemento de resposta sensível a carboidratos (ChREBp), proteína de ligação ao elemento regulador de esterol (SREBp) e receptores ativados por proliferador de peroxissoma (PPAR) e o fator *Forkhead* 01 (FOXO1). Esses fatores aumentam a expressão de enzimas lipogênicas como acetil-coenzima A carboxilase (ACC) e ácidos graxos sintase (FAS) (CANBAY; BECHMANN; GERKEN, 2007). Desbalanços nos mecanismos de transporte ou na ativação de fatores de transcrição podem então induzir ao acúmulo excessivo de triglicerídeos no interior dos hepatócitos e desencadear em adoecimento hepático. Na condição de diabetes tipo 2, a insulina não consegue suprimir a produção de glicose hepática ao passo que mantém a síntese lipídica - esse desbalanço desencadeia hiperglicemia e hipertrigliceridemia (TITCHENELL; LAZAR; BIRNBAUM, 2017).

Os ácidos graxos podem ser classificados pelo comprimento das cadeias carbônicas que os compõem, ou pela presença e número de ligações duplas presentes na cadeia. Dessa forma, podem se apresentar como insaturados, saturados, monossaturados ou poli-insaturados. E ainda, podem possuir cadeia de carbono curta, média ou longa (HARVEY, 2012). De maneira geral, a dieta ocidental, que marca o hábito alimentar das populações em transição nutricional, é predominantemente composta por ácidos graxos saturados (AGS's), principalmente do tipo palmítico, um AGS de cadeia longa (IGGMAN D, 2011). Uma coorte holandesa com 35.597 voluntários foi recrutada entre 1993 e 1997 e acompanhada por 12 anos, para avaliar a correlação entre os tipos de ácidos graxos consumidos e o risco de isquemia cardíaca. Os pesquisadores observaram que o consumo de ácidos graxos saturados não foi capaz de influenciar o número de casos da doença (PRAGMAN *et al.,* 2017).

Um estudo prospectivo acompanhou homens de aproximadamente 50 anos de idade, por uma média de 33,7 anos, com o objetivo de avaliar a relação entre biomarcadores de gordura na dieta, índices de dessaturase e mortalidade. A atividade dessaturase pode prever a mortalidade total, enquanto a proporção de ácido linoléico sérico foi inversamente relacionada, assim como maiores níveis de ácido palmítico, palmitoleico e dihomo-gama-linolênico se relacionaram diretamente à mortalidade total e cardiovascular (WARENSJÖ *et al.*, 2008).

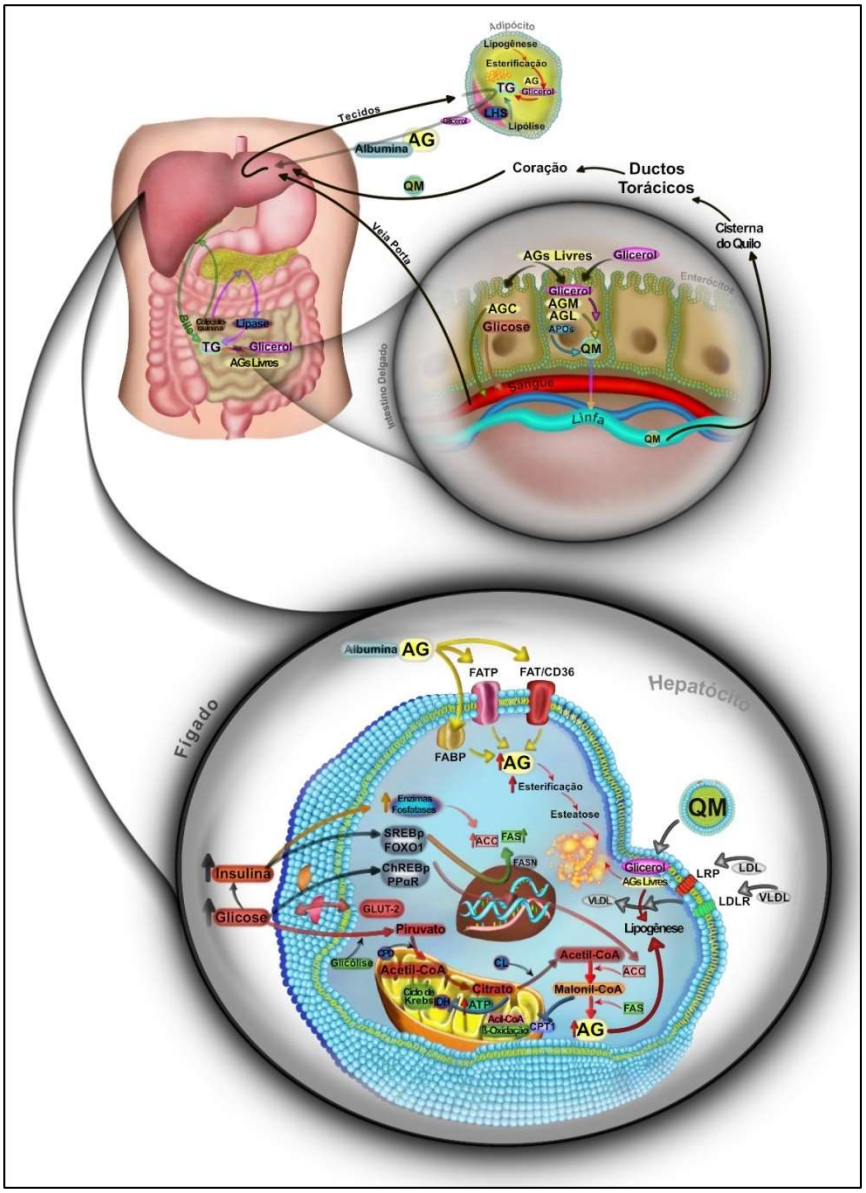
Em um estudo clínico com mulheres em menopausa e hipercolesterolêmicas, um tratamento com ácido palmítico manteve valores de colesterol e LDL-c mais elevados, do que quando tratadas com ácido oleico ou esteárico, enquanto o HDL manteve-se mais elevado (MENG *et al.*, 2019). Outro estudo, com homens e mulheres adultos saudáveis, que consumiram três diferentes dietas contendo 40% de lipídios enriquecidas em ácido palmítico, láurico ou oleico, por seis semanas, considerou que as dietas enriquecidas com ácidos palmíticos e láuricos apresentam efeito hipercolestolêmico (TEMME; MENSINK; HORNSTRA, 1996).

Segundo Eding A., a esteatose hepática não alcóolica é o componente hepático da síndrome metabólica, e fortemente influenciado pelos fatores nutricionais (2017). Em um modelo experimental com ratos submetidos à programação metabólica perinatal por desnutrição materna (8% de proteínas durante gestação e lactação), seguida por 70 dias de consumo de dieta hiperlipídica (32% de lipídios) pós desmame, rica em ácidos graxos, Simões-Alves *et al.* (2019) identificaram que o duplo insulto nutricional é capaz de prejudicar a bioenergética mitocondrial das células hepáticas. Outro estudo com programação fetal também em ratos wistar (5% de proteína materna durante gestação e metade da lactação), seguida de dieta hiperlipídica (30% de lipídios) após o desmame e até os 180 dias de vida, promoveu redução do número de hepatócitos (SOUZA-MELLO; MANDARIM-DE-LACERDA; AGUILA, 2007).

Por sua vez, no mesmo modelo utilizado por Simões-Alves, estudo recém publicado por nosso grupo identificou que a dieta hiperlipídica não foi capaz de potencializar danos promovidos pela desnutrição no perfil glicolipídico e proteico sérico, bem como sobre parâmetros somáticos ou respiratórios (BARBOSA *et al.*, 2020). As variáveis que ainda envolvem o consumo de lipídios e as repercussões dos diferentes ácidos graxos, seu comprimento de cadeia e saturação são delicados em compreensão, uma vez que recebem interferências diversas em vias metabólicas convergentes. Por sua vez, o impacto da programação metabólica perinatal sobre o metabolismo lipídico, quando submetido a um

segundo insulto com dieta hiperlipídica e excesso de ácidos graxos saturados, requerem estudos aprofundados dos mecanismos subjacentes, tanto fisiológicos quanto moleculares.

Figura 2 - Vias do metabolismo lipídico e impactos dos ácidos graxos saturados sobre o tecido hepático.



Fonte: Elaborada pela autora.

1.5 Nutrição, distúrbios cardiometabólicos e modulação autonômica

Uma nutrição balanceada é um dos contribuintes essenciais para a prevenção de doenças relacionadas à síndrome metabólica (ALBERTI *et al.*, 2009). O sistema nervoso central é sensível a ingestão nutricional e apresenta controle significativo da homeostase energética, em

uma via dupla de comunicação, onde transitam informações advindas da periferia e comandos para diversos órgãos e sistemas (KUWAHARA *et al.*, 2011). Parte integrante do sistema nervoso central, o sistema nervoso autônomo é composto pelo sistema nervoso simpático e sistema nervoso parassimpático, que juntos regulam a manutenção da homeostase (KENNEY; GANTA, 2014).

O sistema nervoso autônomo controla inúmeras funções, inclusive as consideradas vitais para a sobrevivência. Os sistemas cardiovascular, respiratório, digestivo, endócrino são inervados por vias aferentes e eferentes advindas tanto do sistema nervoso simpático quanto do parassimpático que compõem o balanço simpatovagal (KENNEY; GANTA, 2014; SEOANE-COLLAZO *et al.*, 2015). A frequência cardíaca, frequência respiratória, secreção de insulina e motilidade gastrointestinal são coordenadas pelas fibras autonômicas, pela ação da leptina e atividade do corpúsculo carotídeo, cuja ingestão de alimentos é capaz de influenciar (KUWAHARA *et al.*, 2011).

Em um estudo com homens adultos jovens, Kaneno *et al.* (1995) identificaram que uma refeição rica em calorias é capaz de aumentar a atividade nervosa simpática quando comparada com uma refeição com menor valor calórico. Outro estudo com seis homens adultos que consumiram isoladamente dietas controle, hiperlipídica ou hiperglicícida, identificou que o maior teor de carboidratos está correlacionado com aumento da modulação simpática (MILLIS *et al.*, 2009). Distúrbios que compõem a síndrome metabólica, como a diabetes tipo 2 e a hipertensão arterial, estão relacionados a uma regulação alterada do sistema nervoso autonômico (STUCKEY *et al.*, 2014), com aumento da modulação autonômica simpática e uma redução da modulação parassimpática (ROSSI *et al.*, 2015).

A programação metabólica promovida pela desnutrição materna induz desbalanço simpatovagal que pode culminar em hipertensão. Estudo realizado por Brito-Alves *et al.* (2014) observou que ratos cujas mães foram alimentadas com deita de baixa proteína (8% durante gestação e lactação), desenvolveram aumento da frequência respiratória precoce e hipertensão arterial em estados mais tardios. Posteriormente, Barros *et al.* (2015b), utilizando o mesmo modelo experimental, identificaram que os animais desnutridos durante gestação e lactação apresentavam maior atividade simpática do que os submetidos à dieta controle (18% de proteínas). As alterações respiratórias têm demonstrado relação com desenvolvimento de hipertensão arterial e representam um elo entre a hipertensão e hiperativação simpática precoce (ZOCCAL *et al.*, 2008; MORAES *et al.*, 2016), que parece mediada por disfunções em quimiorreceptores (ZOCCAL, 2015).

As dietas hiperlipídicas induzem o aumento da ATP disponível e a secreção de insulina bem como de leptina, com o objetivo de estimular os centros de saciedade. A leptina, hormônio secretado principalmente pelo tecido adiposo em estados alimentados, possui funcionalidade respiratória importante, uma vez que atua sobre o corpo carotídeo, promovendo a despolarização e induzindo o aumento da sinalização simpática com aumento da frequência respiratória (PORZIONATO *et al.*, 2011). Em um modelo experimental animal, Ribeiro *et al.* (2018) ofertaram três semanas de dieta controle (10% de lipídios) ou hiperlipídica (34% de lipídios) a ratos adultos e observaram que os animais que consumiram dieta hiperlipídica desenvolveram síndrome metabólica com aumento da frequência respiratória. Contudo, quando submetidos à infusão de leptina, a responsividade respiratória dos animais era reduzida. Esse achado demonstra a estreita conexão entre hormônios nutricionalmente responsivos e a ativação simpática.

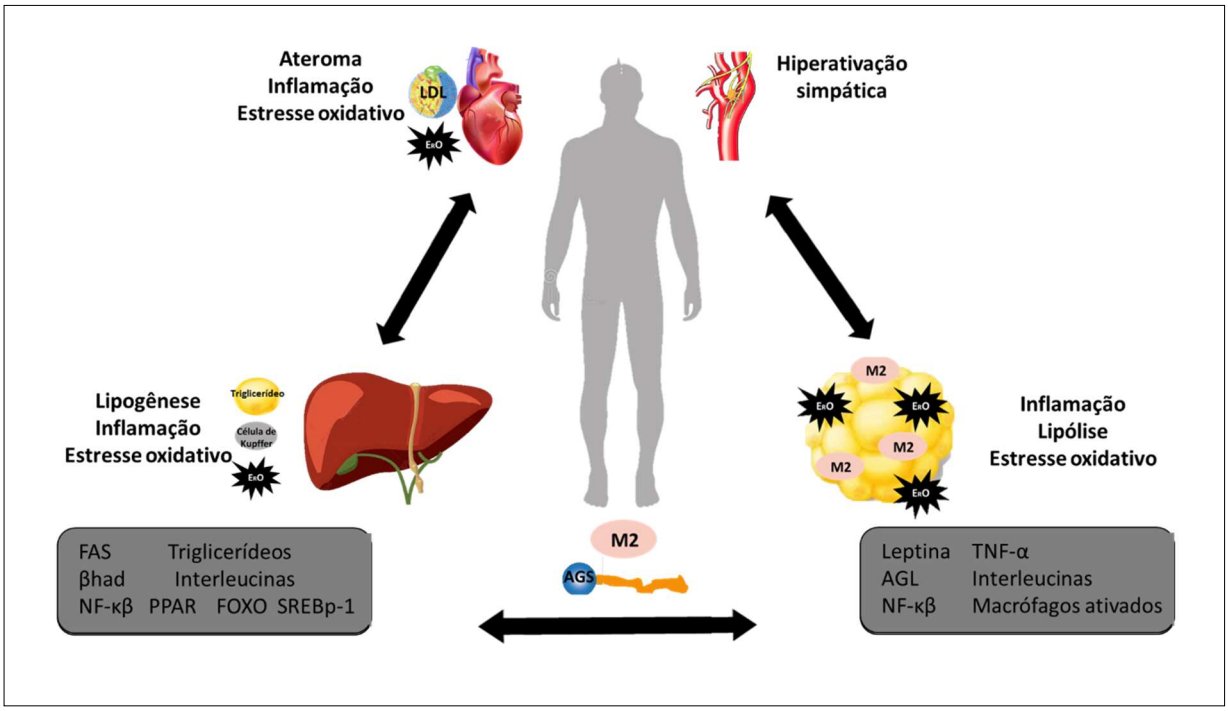
O sistema nervoso central se comunica com o fígado pelas vias autonômicas aferentes e eferentes que podem regular o metabolismo lipídico nesse tecido (TAHER; FARR; ADELI, 2017). Em um modelo de roedores transfectados para hiperexpressar aminoácidos no tecido hepático, Uno *et al.* (2015) identificaram aumento da lipogênese e dos níveis séricos de triglicerídeos, induzidos por ativação simpática e redução da atividade vagal. A via neuronal "fígado-cérebro-adiposo", proposta por Uno *et al.* (2006), descreve a ativação do sistema nervoso autonômico por meio do ramo vagal na percepção do acúmulo excessivo de gorduras hepáticas e a ativação do sistema nervoso simpático para promover a lipólise no tecido adiposo branco. Em um modelo experimental de esteatose hepática induzida por dieta hiperlipídica, tanto a ablação farmacológica quanto a remoção da inervação simpática do fígado de camundongos promoveu melhorias nas vias de acúmulo de triglicerídeos hepáticos (HURR *et al.*, 2019), demonstrando que, na condição de obesidade, a inervação simpática hepática modula vias de aquisição de lipídios hepáticos.

A variabilidade da frequência cardíaca, assim como da frequência respiratória, tem sido utilizada para avaliar desbalanços simpatovagais, onde valores reduzidos sinalizam para sensibilidade à insulina prejudicada, dislipidemias e hipertensão arterial (SAITO *et al.*, 2017). Metanálise realizada por Benichou *et al.* (2018) identificou que a variabilidade da frequência cardíaca está reduzida em pessoas com diabetes tipo 2. Outro estudo identificou que em uma corte de crianças brasileiras com desnutrição, a variabilidade da frequência cardíaca se apresentava reduzida, mesmo com altos valores de frequência cardíaca (BARRETO *et al.*, 2016).

A gordura da dieta também pode influenciar a variabilidade da frequência cardíaca. Soares-Miranda *et al.* (2012) avaliaram duas cortes isoladas (homens de 19 e de 72 anos) e observaram que o consumo de gorduras trans estava relacionado a menores valores de variabilidade cardíaca. Um estudo realizado com 220 pessoas, com idades entre 23 e 70 anos, de ambos os sexos, relacionou fatores de risco para síndrome metabólica com alterações na variabilidade cardíaca. A pesquisa identificou que mulheres com síndrome metabólica apresentam variabilidade cardíaca prejudicada (STUCKEY *et al.*, 2015). Em outro estudo com mulheres, a presença de ovários policistos e alto consumo de ácidos graxos estavam relacionados a menor variabilidade da frequência cardíaca (GRAFF *et al.*, 2017).

Dessa forma, a literatura tem relatado que a redução da ativação parassimpática e maior ativação simpática, com redução da variabilidade da frequência cardíaca, está relacionada com disfunções que podem acarretar em hipertensão, diabetes tipo 2 e esteatose hepática. Os mecanismos subjacentes relacionados a este fenômeno ainda não estão esclarecidos, mas parecem ser sensíveis a dietas desbalanceadas. Da mesma forma, apesar da relação entre desnutrição, hipernutrição e desbalanços simpatovagais ser corroborada, os efeitos da programação metabólica perinatal seguida por um alto consumo de ácidos graxos saturados ainda não estão esclarecidos. A Figura 2 traz uma representação ilustrativa da interação entre a modulação autonômica, o metabolismo energético hepático e o adoecimento cardiometabólico.

Figura 3 – Imagem ilustrativa da interação entre modulação autonômica, metabolismo energético hepático e adoecimento cardiometabólico.



Fonte: Elaborada pela autora.

2 HIPÓTESE

Uma dieta hiperlipídica rica em ácidos graxos saturados, ofertada do pós-desmame até os 90 dias de vida, é capaz de exercer uma sobrecarga metabólica em animais previamente desnutridos no período perinatal, acarretando desordens sobre o metabolismo lipídico, inflamação hepática e desbalanço da modulação autonômica cardiorrespiratória.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar as repercussões de uma dieta pós-desmame rica em ácidos graxos saturados Uma dieta hiperlipídica rica em ácidos graxos saturados, ofertada do pós-desmame até os 90 dias de vida, sobre a função hepática e modulação autonômica de proles submetidas à desnutrição proteica perinatal.

3.2 Objetivos específicos

3.2.1 No soro:

- Analisar como a desnutrição proteica perinatal e a exposição pós-desmame a uma dieta HL repercutem sobre os níveis séricos de marcadores do metabolismo lipídico;
- Avaliar o impacto da dieta HL pós-natal sobre marcados séricos de dano tecidual hepático.

3.2.2 No tecido hepático:

- Registrar o peso do tecido;
- Investigar os efeitos dietéticos sobre a morfologia;
- Analisar a responsividade à dieta de fatores de transcrição relacionados ao metabolismo lipídico e de genes pró-inflamatórios;

3.2.3 Sobre a frequência cardíaca, pressão arterial e frequência respiratória

- Avaliar a modulação autonômica e o balanço simpatovagal;

4 MATERIAIS E MÉTODOS

4.1 Questões éticas

Este projeto foi autorizado pelo Comitê de Ética para Uso Animal (CEUA) da Universidade Federal de Pernambuco (UFPE), sob protocolo Nº 23076.046459/2018-17, e seguiu as normas e padrões de manipulação e boas práticas no manejo de animais de laboratório, conforme determinações internacionais e orientações do Conselho Nacional de Controle de Experimentação Animal (CONCEA).

4.2 Animais

Para geração das proles, foram utilizadas 16 ratas albinas, nulíparas, com idade entre 90 e 100 dias de vida e peso entre 230 e 250g, e ratos reprodutores machos, adultos, ambos da linhagem wistar, provenientes do biotério de Fisiologia e Farmacologia da Universidade Federal de Pernambuco. As ratas foram acasaladas com os machos na proporção 3:1, durante o ciclo escuro (noturno) dos animais. A observação da presença de espermatozoides no esfregaço vaginal foi usada para definir o 1° dia de prenhez. A partir da determinação da prenhez, as ratas foram alojadas em gaiolas individuais e agrupadas aleatoriamente de acordo com as dietas experimentais correspondentes, como descrito no ítem 5.3 – manipulação nutricional. A temperatura e a umidade do ambiente foram mantidas dentro dos limites adequados, de 22 a 25 °C e 55 a 65%, respectivamente. Ao nascimento, as proles foram reduzidas, por eutanásia, a oito machos por ninhada, e quando as ninhadas foram compostas por menos de oito machos, fêmeas foram mantidas para padronização do tamanho da prole e adequação do número de filhotes ao número de tetas funcionais das mães. A alocação dos filhotes nos grupos foi realizada por randomização, para garantir a minimização de efeitos advindos de caracteres genéticos.

4.3 Manipulação nutricional

4.3.1 Dietas

As dietas utilizadas foram confeccionadas no Laboratório de Técnica Dietética do Centro Acadêmico de Vitória, dentro das especificações e padrões estabelecidos pelo *American*

Institute of Nutrition (AIN – 93), e das normas de manipulação da Agência Nacional de Vigilância Sanitária (ANVISA). As dietas foram compostas pelos ingredientes descritos na Tabela 1, e a composição centesimal, bem como a distribuição de ácidos graxos das dietas pós desmame estão descritas nas Tabelas 2 e 3, respectivamente.

Tabela 1 – Composição e análise centesimal das dietas experimentais quanto aos ingredientes utilizados nas formulações.

Ingrediente (g por 100g de dieta)	***Normoproteica/ Normolipídica (AIN-93G)***	***Hipoproteica (AIN-93)***	***Hiperlipídica AGS +***
Amido de milho	39,7	50,3	15
Amido dextrinizado	13,2	13,2	-
Farinha de trigo	-	-	12
Biscoito maisena	-	-	5
Farinha de soja	-	-	6
Banha de porco	-	-	5,3
Creme de leite	-	-	4
Margarina (65% lipídios)	-	-	4
Caseína (>85%)	20	9,4	20
Goma guar	-	-	0,5
Sacarose	10	10	18
Óleo de soja	7	7	4
Fibra (celulose)	5	5	2
Vitaminas	1	1	0,7
Mineral mix	3,5	3,5	2,5
DL-metionina	0,3	0,3	0,25
Bitartarato de colina	0,25	0,25	0,25
BTH	0,0014	0,0014	0,014
Glutamato monossódico 12,3%	-	-	0,2
Cloreto de sódio	-	-	0,3
Total (g)	100	100	100
Kcal /100g	369	364	446
Macronutrientes (g/100g)			
Umidade	3,5	8,1	4,4
Proteínas	18,6	8,7	22,5
Lipídios	6,1	5,1	15,3
Carboidratos	68,6	75,8	54,7
Cinzas	3,3	2,3	3,1

Descrição dos ingredientes e dos macronutrientes que compõem as dietas utilizadas para a manipulação nutricional dos animais. As dietas normoproteica/normolipídica (controle) e hipoproteica foram adaptadas das orientações descritas pelas AIN-93, publicada por Reeves, Nielsen e Fahey (1993). A dieta hiperlipídica foi adaptada do modelo desenvolvido por Ferro-Cavalcante *et al.* (2013). A análise centesimal foi realizada no Laboratório de Bromatologia do Centro Acadêmico de Vitória (CAV-UFPE).

Tabela 2 – Distribuição percentual de ácidos graxos, quanto à presença ou não de dupla ligação na cadeia carbônica das dietas experimentais formuladas.

Ácidos graxos	Normoproteica/ Normolipídica (AIN-93G)	Hiperlipídica AGS +
Saturados		
Ácido octanoico (C8:0)	0	0
Ácido decanoico (C10:0)	0	0
Ácido láurico (C12:0)	0	0
Ácido mirístico (C14:0)	0	1,09
Ácido pentadecanoato (C15:0)	0	0
Ácido palmítico (C16:0)	11,97	19,1
Ácido heptadecanoato (C17:0)	0	0
Ácido esteárico (C18:0)	4,62	9,4
Ácido araquídico(C20:0)	0,36	0
Ácido behênico(C22:0)	0,37	0
Total	**17,32**	**29,59**
Monoinsaturados		
Ácido miristoleico (C14:1)	0	0
Ácido palmitoleico (C16:1)	0	0
Ácido heptadecanoico (C17:1)	0	0
Ácido oleico (C18:1)	29,93	34,77
Ácido eicosenoico (C20:1)	0	0
Total	**29,93**	**34,77**
Poliinsaturados		
Ácido linolênico (C18:3)	3,89	5,11
Ácido linoleico (C18:2)	48,87	30,53
Total	**52,76**	**35,64**
Ômega 3(g/100g)	0,27	0,78
Ômega 6(g/100g)	3,42	4,66
Razão ômega 6:3	**12,66**	**5,97**

Descrição do percentual de ácidos graxos das dietas pós-desmame. A dieta normoproteica/normolipídica (controle) foi baseada na AIN-93, publicada por Reeves, Nielsen e Fahey (1993). A dieta hiperlipídica foi adaptada do modelo desenvolvido por Ferro-Cavalcante *et al.* (2013). Os ácidos graxos foram identificados segundo padrão externo (FAME Supelco™ mix C4-C24, Bellefonte, PA, USA) e o percentual calculado conforme normalização das áreas dos picos pelo método de cromatografia gasosa no Laboratório de Fitoquímicos e Processos, do Centro de Tecnologias e Estratégias do Nordeste – CETENE. A partir da determinação percentual de ácido linolênico (ômega 3) e linoleico (ômega 6) foram calculadas as quantidades (g/100g de dieta) desses ácidos graxos e obteve-se a razão de ômega 6 / 3.

4.3.2 Indução da desnutrição proteica

A indução da desnutrição proteica materna foi baseada em manipulação nutricional, com utilização de dois tipos de dieta durante todo o período de gestação e lactação (até 21 dias após o parto), sendo:

- Grupo controle (C), cujas mães receberam dieta normoproteica/normolipídica (AIN-93G);

- Grupo desnutrido (LP), cujas mães receberam dieta hipoproteica (AIN-93).

As dietas confeccionadas foram isocalóricas entre si. A desnutrição foi confirmada pela constatação do menor peso e/ou tamanho corporal ao nascer (dia 1) e ao final da lactação (21 dias de vida), bem como pela redução do conteúdo sérico de proteínas dos filhotes ao desmame.

4.3.3 Indução da transição nutricional

Após o desmame, os filhotes foram mantidos em gaiolas coletivas com 3 a 4 animais cada, com balanceamento do número de filhotes por gaiola por grupo, com água e dietas normoproteica/normolipídica (C) ou hiperlipídica (HL) *ad libitum*.

4.4 Desenho experimental

O protocolo experimental materno se iniciou a partir da identificação da prenhez por esfregaço vaginal. As ratas prenhes foram submetidas à uma nutrição balanceada ou à desnutrição proteica no período de gestação e lactação, recebendo dieta controle (C, 18% de caseína) ou dieta com baixo teor de proteínas (LP, 8% de caseína). As proles foram reduzidas a 8 filhotes por ninhada ao nascimento, dando preferência aos filhotes machos; as fêmeas foram utilizadas apenas para complementar o tamanho da ninhada, quando não houveram 8 machos em uma mesma prole. Os filhotes machos foram submetidos ao desmame aos 21 dias de vida, passando a receber dieta C ou hiperlipídica (HL, 32% de lipídios + 65% de AGS). Assim, foram formados os seguintes grupos experimentais de filhotes:

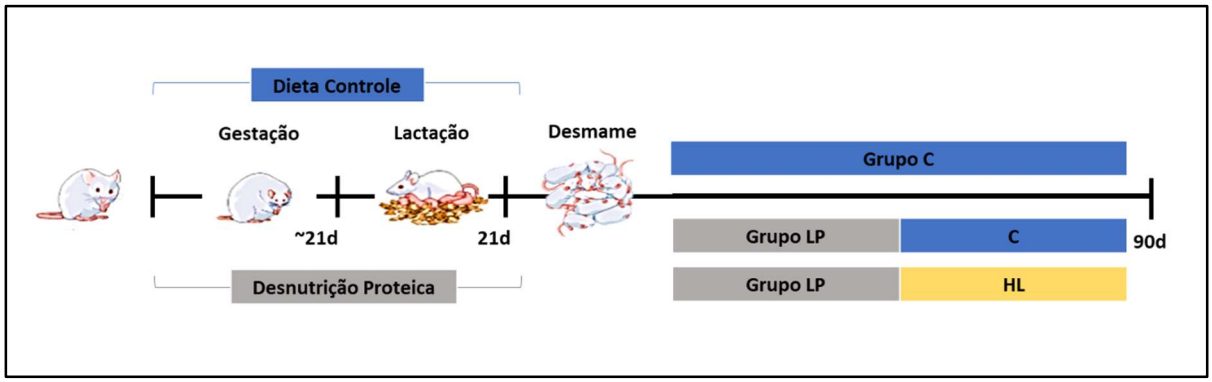
- Grupo C: cuja mãe do filhote recebeu dieta controle durante a gestação e lactação e o filhote recebeu dieta controle após o desmame e até os 90 dias de vida;

- Grupo LP-C: cuja mãe do filhote recebeu dieta hipoproteica durante a gestação e lactação e o filhote recebeu dieta controle após o desmame e até os 90 dias vida;

- Grupo LP-HL: cuja mãe do filhote recebeu dieta hipoproteica durante a gestação e lactação e o filhote recebeu dieta hiperlipídica após o desmame e até os 90 dias de vida;

Os animais experimentais foram acompanhados até os 90 dias de vida, quando foram eutanasiados. A Figura 1 traz uma representação esquemática do desenho experimental utilizado.

Figura 3 – Desenho experimental para modelo de transição nutricional com programação metabólica por desnutrição e sobrecarga com dieta hiperlipídica pós-desmame



Desenho experimental para modelo de transição nutricional, a partir de desnutrição proteica materna durante gestação e lactação, seguida por hipernutrição lipídica rica em ácidos graxos saturados após-desmame. **C** – grupo controle, cujas mães receberam apenas dieta controle durante gestação e lactação, e as proles consumiram dieta controle após o desmame (n=5-12); LP-C -grupo cuja mãe foi submetida à desnutrição proteica durante a gestação e lactação da prole, e a prole consumiu dieta controle após o desmame (n=5-12); LP-HL – grupo cuja mãe recebeu dieta de baixa proteína durante durante gestação e lactação e a prole consumiu dieta HL após o desmame (n=5-12).
Fonte: Elaborada pela autora.

4.5 Peso corpóreo e consumo alimentar

A massa corporal dos animais foi acompanhada diariamente em balança de precisão (1 – 500g) AS-1000 (Marte, São Paulo, Brasil), assim como o consumo alimentar, determinado a partir da diferença entre a quantidade de dieta ofertada no dia e o rejeito pesado no dia posterior, conforme descrito por Brito-Alves *et al*. (2014):

Consumo alimentar diário (g)= [peso da dieta ofertada no dia – peso do rejeito de dieta no dia subsequente à oferta]

As análises foram realizadas no início do período escuro dos animais (18h). A eficiência alimentar foi calculada a partir da fórmula descrita por Novelli *et al*. (2007):

$$CEA\% = \frac{[\text{média do ganho de massa corporal do período x 100 (g)}}{\text{consumo alimentar ou calórico do período (g)}]}$$

O crescimento somático foi avaliado a partir do comprimento naso-anal nos dias 1, 21 e aos 90 dias de vida, conforme descrito previamente por Bayol *et al*., (2004).

4.6 Coleta de amostras

4.6.1 Amostras de soro

Aos 21, 30 e 90 dias de vida, os animais foram anestesiados por via intraperitoneal, com ketamina (80 mg/kg i.p.) e xilazina (10 mg/kg i.p). O plexo orbital foi então rompido por tubo capilar sem heparina (Precision, TC500). O sangue coletado foi acondicionado em tubos sem anti-coagulante e centrifugados a 4ºC e 3500 RPM por 10 minutos, em centrífuga refrigerada (Mikro 220R, Hettich, Alemanha), para obtenção do soro. O soro foi acondicionado em freezer a -20ºC até as análises bioquímicas.

4.6.2 Amostras de tecido hepático

Aos 90 dias de vida, os animais foram anestesiados conforme descrito no Ítem *4.6.1* foram eutanasiados por decapitação. O tecido hepático foi dissecado e seccionado. Parte foi fixado em formalina tamponada a 10%, para procedimentos histológicos posteriores. Outras secções foram armazenadas em ultrafreezer a -80ºC até a utilização.

4.7 Análises bioquímicas séricas

As amostras de soro armazenadas a -20ºC foram analisadas em analisador bioquímico automático (PW-2280, PIOWAY, China) para conteúdo sérico de albuminas, proteínas totais, colesterol total, triglicerídeos e glicose (aos 21, 30 e 90 dias), e para aspartato aminotransferase (AST) e alanina aminotransferase (ALT) apenas aos 90 dias, utilizando-se respectivos kits comerciais (Biotecnica, Brasil).

4.8 Quantificação do peso do fígado

Após dissecção, o peso total do fígado coletado foi quantificado em balança de alta precisão para 0,001g (JÁ3003N, CELTAC, Alemanha) e corrigido pelo peso corporal do animal, conforme fórmula:

$$Peso\ \% = \frac{peso\ total\ do\ animal\ x\ peso\ do\ tecido}{100}$$

4.9 Análises histológicas hepáticas

4.9.1 Processamento e coloração

Um fragmento do tecido hepático de cada animal, medindo aproximadamente 1 cm³ foi clivado do lobo lateral esquerdo e mergulhado em solução de formalina neutra tamponada a 37% (NBF) em diluição de 10x, permanecendo na solução por um período de 24 horas. Após esse procedimento, o fragmento foi conservado em álcool etílico ao 70% até o dia do processamento, em que foi submetido a concentrações crescentes a partir de 70% e até 100%, diafanizados por xilol, impregnados em aparelho histotécnico automático (San Pietro, O Patologista) e incluídos em parafina. Os blocos obtidos foram seccionados em micrótomo (RM 2245, Leica, Alemanha), ajustado para 4µm. Os cortes obtidos foram colocados em lâminas untadas com albumina, desparafinizados em estufa regulada à temperatura de 60ºC, por 24 horas e em seguida, coradas por Hematoxilina e Eosina, conforme descrito por Dorigan Neto (2012)

4.9.2 Visualização e captura de imagens

As lâminas foram visualizadas em microscópio de luz, sob foco fixo e clareza de campo. As imagens histológicas foram capturadas por meio de câmera digital (MOTICAM 2.300) acoplada a um microscópio biológico óptico (Nikon E-100, China), obtendo-se 20 campos por lâmina, em aumento final de 400x. As fotomicrografias serão avaliadas através do software ImageJ versão 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA).

4.9.3 Análise dos dados histológicos

No tecido hepático, foram quantificados: o número de hepatócitos monoclueados e binucleados (identificados a partir da presença de núcleo envolvido em citoplasma e membrana celular), o número de células de Kupffer e de células endoteliais em cada campo. O total de cada tipo celular registrado nas imagens obtidas foi somado para cada animal.

4.10 Análise da expressão gênica

4.10.1 Extração de mRNA, transcrição reversa e PCR quantitativo (qPCR)

O mRNA total foi extraído de um fragmento de tecido hepático previamente congelado a -80 ºC (~50mg) com reagente Tripure (Roche, Meylan, France), de acordo com as instruções do fabricante. Utilizamos 10µL de Trizol por mg de tecido hepático e a suspensão resultante foi homogeneizada usando o kit Precellys Lysing (Bertin, Montigny-du-Brettonneux, France). Após homogeneização, foi acrescentado clorofórmio na proporção de ¼ do volume da solução, e essa preparação foi misturada em vórtex em 3 passagens de 15 segundos, incubado por 5 minutos e centrifugado por 15 minutos em centrífuga refrigerada a 15.000 x g a 4 ºC (Mikro 220R, Hettich, Alemanha). Foi adicionado isopropanol (Carlo Erba reagentes, Val-de-Reiul, France), na proporção de metade do volume da solução e novamente centrifugado conforme descrito acima. Os pellets com mRNA formados foram sequencialmente lavados com etanol (Carlo Erba reagentes, Val-de-Reiul, France) a 70% e 95%, secos, e dissolvidos em 100 µL de água Rnase-free.A concentração e a pureza do mRNA foi determinada por espectofotômetro Nanodrop® 2000/2000c (Thermo Scientific, USA).

A transcrição reversa foi realizada usando um kit RT-TAKARA (Primescript TM, Dalian, China) para geração de cDNA para o PCR Tempo Real. A amplificação quantitativa foi medida (qPCR) por Rotor Gene Real Time System (Labgene Scientific Instruments, Archamps, France). Todos os resultados foram representados como unidades arbitrárias (A.U.), derivadas de uma curva de calibração padrão, baseada das amostras de referência. O PCR para cada amostra foi corrido em para todos os genes e TBP foi utilizada como controle endógeno. As sequências de genes utilizados para o estudo estão descritas na Tabela 4.

Tabela 3 - Sequência de primers utilizados para as análises de RT-PCR.

Gene	Fow/Rev	Sequência 5'-3'	TmºC	Tamanho	RefSec NCBI
Tbp	F	TGGTGTGCACAGGAGCCAAG	62 ºC	139 pb	NM_001004198
	R	TTCACATCACAGCTCCCCAC			
NFκβ	F	GTGGAGTACGACAACATCTC	62 ºC	176pb	NM_008689
	R	CAAGAGTCGTCCAGGTCATA			
TNF-α	F	CCAGACCCTCACACTCAGATC	58°C	78pb	NM_012675
	R	CTTGGTGGTTTGCTACGACG			
IL-1β	F	CTCTCCACCTCAATGGACAG	60°c	181pb	NM_031512
	R	TTGGGATCCACACTCTCCAG			
PPARα	F	GAGAATCCACGAAGCCTAC	60°c	196pb	NM_013196
	R	GAAGAATCGGACCTCTGCCTC			
ChREBp	F	AGTCCTTCAATGGGATGGTG	58°C	267pb	NM_133552
	R	GACGTGTCCACAAGTTGTTC			
FOXO3	F	GAGAGCAGATTTGGCAAAGG	58°C	182pb	NM_001106395
	R	CCTCATCTCCACACAGAACG			
SREBp1	F	ACGGAGCCATGGATTGCACA	60ºC	210pb	XM_213329 1c
	R	AAGGGTGCAGGTGTCACCTT			

4.11 Avaliação da modulação autonômica

A modulação autonômica foi avaliada a partir da variabilidade da frequência respiratória, da pressão arterial e da frequência cardíaca.

4.11.1 Análise dos variáveis respiratórias

4.11.1.1 Registro da frequência respiratória

A frequência respiratória foi registrada por pletismografia de corpo inteiro, segundo protocolo adaptado do descrito por Malan (1973), registrado durante o período basal, em ciclo claro (período diurno) dos animais não anestesiados, sem movimentação corporal dentro da caixa, sem diferenças comportamentais evidentes entre os grupos. Foram mantidas condições ambientais semelhantes para todos os ratos analisados. Cada rato foi colocado dentro de uma câmara pletismográfica acrílica (de 1 a 3 litros), seguido de um período de aclimatação de 30

min antes de iniciar os registros. Uma injeção de 1 ml de ar foi usada para a calibração do volume e foram obtidos no mínimo 3 registros sem oscilações anormais, considerados viáveis, por animal. Durante a realização de cada medida respiratória, o fluxo de ar foi interrompido e a câmara do animal permaneceu totalmente vedada por aproximadamente 2 minutos. As oscilações causadas pela respiração do animal foram captadas por um dispositivo conectado à câmara, que contém um transdutor diferencial de pressão e um amplificador de sinais (ML141 spirometer, PowerLab, ADInstruments), conectado ao sistema de aquisição de dados (LabChartTM Pro, PowerLab, ADInstruments). No intervalo entre os registros, o fluxo de ar da caixa foi mantido por uma bomba de ar e uma válvula de circulação. Para análise, foram selecionados 10 segundos contínuos do registro mais estável entre os três realizados, nas idades de 1, 21 e 90 dias de vida.

4.11.1.2 Análise da variabilidade respiratória

A fim de avaliar a influência simpática e parassimpática sobre a frequência respiratória, foi analisada a variabilidade respiratória em um trecho contendo entre 180 e 200 ciclos respiratórios completos, para captar os valores da respiração-a-respiração (BB_n) e da respiração em intervalo subsequente (BB_{n+1}). Um gráfico de Poincaré foi utilizado para representar a relação BB_n *versus* Bb_{n+1}. A variabilidade de curto prazo (SD1) e a variabilidade de longo prazo (SD2) foi calculada como índices de variabilidade respiratória (PENG *et al.*, 2011).

4.11.2 Análise dos variáveis cardíacas

4.11.2.1 Procedimento cirúrgico

Aos 89 dias de vida, os animais foram anestesiados com ketamina (80 mg/kg i.p.) e xilazina (10 mg/kg i.p), e em seguida, foi realizada canulação da artéria femoral, através da inserção de um cateter de polietileno conforme descrito anteriormente por Barros *et al.* (BARROS *et al.*, 2015a). A cânula foi posicionada subcutaneamente pelo dorso do animal e exteriorizada no dorso do animal, entre as escápulas. Os animais receberam uma dose de anti-inflamatório cetoprofeno (5 mg/kg i.m.) e foram acomodados em caixas individuais até o momento do registro.

4.11.2.2 Registro pressórico e da frequência cardíaca

O registro pressórico foi realizado 24 horas após o procedimento cirúrgico, aos 90 dias de vida dos animais, para que os animais se recuperassem totalmente da anestesia. Para confirmar a recuperação, foram observados o comportamento motor dos ratos, sua resposta estímulos sonoros e táteis, movimentação das vibrissas e ainda, a existência de sinais de estresse pós-cirúrgico ou sofrimento animal, como piloereção e porfirina. A pressão arterial pulsátil foi registrada por 60 minutos, através da conexão da cânula da artéria femoral a um transdutor mecanoelétrico de pressão e um amplificador de sinal (ML866/P, ADInstruments, Power Lab, Bella Vista, NSW, Australia). Os registros foram armazenados em computador equipado com software apropriado (LabChart® Pro, ADInstruments, Bella Vista, NSW, Austrália) e analisados posteriormente. A pressão arterial média e frequência cardíaca foram obtidas como derivações da pressão arterial pulsátil, calculado pelo sistema de aquisição e análise.

4.11.2.3 Análise espectral da variabilidade cardíaca

A avaliação da modulação autonômica cardíaca foi realizada através variabilidade da pressão arterial e da frequência cardíaca, através dos métodos espectrais no domínio do tempo e da frequência, com o uso dos programas Cardioseries (Cardioseries®2.4, São Paulo, Brasil) e do pluggin HRV do programa LabChart®Pro, 8.1 (ADInstruments, Bella Vista, NSW, Austrália).

A avaliação no domínio da frequência foi realizada em um trecho do registro pressórico pulsátil estável, de 10 minutos de duração, e gerou um gráfico de densidade de potência espectral, que pode ser descomposto nos seguintes componentes (ROSÁRIO, 2018):

- Componente de alta frequência (*High Frequency* – HF, ondas de 0,75 a 3 Hz), que indica atuação do SNA parassimpático;
- Componente de baixa frequência (*Low Frequency* – LF, ondas de 0,2 a 0,75 Hz), que indica atuação do SNA simpático;
- Componente de muito baixa frequência (*Very Low Frequency* – VLF, ondas de 0,003 a 0,04 Hz), que sugere modulações hormonais, possivelmente oriundas de atividades termorreguladoras ou dependentes de renina;
- Razão LF/HF, que representa o índice de balanço simpatovagal;

- Componente simbólico, representado por 0V (atuação parassimpática) e 2V (atuação simpática)
- Resposta barorreflexa espontânea pelo Índice de Efetividade do Barorreflexo (BEI) e pelo Ganho Médio.

Para as análises no domínio do tempo, utilizamos os valores integrados normais das ondas QRS, obtendo os seguintes componentes a partir do intervalo RR:

- SDRR: desvio padrão dos intervalos RR - índice que representa a variabilidade geral da frequência cardíaca;
- RMSSD: raiz quadrada da média dos quadrados das diferenças entre os intervalos RR adjacentes, em um intervalo de tempo – componente de curta duração da VFC, que representa a atividade vagal parassimpática
- Variabilidade baseada em alterações de curto prazo (SD1) e de longo prazo (SD2).

4.12 Análise dos dados

Os resultados foram expressos como média ± e.p.m. (erro padrão da média). A análise de normalidade da amostra foi realizada por meio do teste de *Shapiro-wilk*. Para os dados considerados paramétricos, a comparação entre os grupos foi realizada por meio de análise de variância (ANOVA) de 1 via, e quando houve significância entre os grupos, utilizamos o pós-teste de Newman-Keuls para identificá-las. Para os dados não paramétricos, foi utilizado o teste de Kruskal-Wallis, seguido de pós-teste de Dunn. As análises foram realizadas em GraphPad Prism (GraphPad Software Corporation®, versão 7.04, 2018), e o nível de significância considerado foi de $p<0,05$.

5 RESULTADOS

ARTIGO 01 – **EFFECTS OF A HIGH-FAT DIET ENRICHED WITH SATURATED FATTY ACIDS ON LIVER FUNCTION AND AUTONOMIC MODULATION IN RATS PREVIOUSLY MALNOURISHED DURING THE PERINATAL PHASE**

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Ana Paula Fonseca Arcoverde Cabral de Mello[1], Aiany Cibelle Simões-Alves[1], Viviane de Oliveira Nogueira[1], Francisco Carlos Amanajás de Aguiar Júnior[2], Karla Patrícia Barbosa Luciano Pirola[3], Beatrice Morio[3], , João Henrique Costa-Silva[1].

[1]Laboratory of Nutrition, Physical Activity and Phenotypic Plasticity, Department of Physical Education and Sport Sciences, Universidade Federal de Pernambuco, UFPE, Vitória de Santo Antão – PE, 55608-680, Brazil.

[2]Laboratory of Biotechnology and Pharmaceutical, Department of Biology, Universidade Federal de Pernambuco, UFPE, Vitória de Santo Antão – PE, 55608-680, Brazil.

[3]Laboratoire de Recherche en Cardiovasculaire, Métabolisme, Diabétologie et Nutrition (CarMeN), INSERM U1060, INRA U1397, Université Claude Bernard Lyon1, Oullins, Lyon, France.

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Corresponding author:

João Henrique Costa-Silva

Núcleo de Educação Física e Ciências do Esporte – CAV - UFPE

Rua Alto do Reservatório, S/N, Bela Vista, Vitória de Santo Antão, PE.

CEP: 55608-680

Phone/fax: 55 81 31144101

Email: joao.hcsilva@ufpe.br

Abstract

Background: Nutritional deficiencies or food excesses during critical stages of development may influence the metabolic phenotype in adulthood, and appear to be impacted by nutritional divergences between the perinatal and postnatal environment.

Aims: To evaluate the repercussion of a post-weaning high-fat diet rich in saturated fatty acids on liver function and cardiorespiratory autonomic modulation of offspring after perinatal protein malnutrition.

Methods: Wistar rats, in whom mothers received a control diet (C: 19% protein) or low protein diet (LP: 8% protein) during pregnancy and lactation, started to consume a control diet (C: 13% lipid) or a high-fat diet rich in saturated fatty acids (HL: 32% lipid) after weaning (21 days after birth) up to 90 days of life. Three groups were formed: control: C (n = 5-12), LP-C (n= 5-12) and LP-HL (n = 5-12). Serum triglyceride and cholesterol, liver relative weight, liver βhad and FAS enzymatic activity, AST and ALT serum content, liver histology, gene expression of IL-1β, TNFα, NFκβ and transcription factors PPARα, ChREBp, FOXO3 and SREBp1 in liver tissue were measured, autonomic modulation on cardiorespiratory control by spectral analyses.

Results: The consumption of a high-fat diet enriched with saturated fatty acids did not promote morphological alterations, tissue damage or cardiorespiratory autonomic modulation changes at 90 days of age. However, perinatal malnutrition increased PPARα gene expression and decreased FOXO3 gene expression. A post-weaning high-fat diet induced increased SREBp1 gene expression and expression of proinflammatory genes in liver tissue.

Conclusion: Our results suggest that perinatal malnutrition induces adaptations that maintain normal lipid metabolism and autonomic modulation, even in the face of a second nutritional insult with postnatal consumption of a high-fat diet. However, the increased gene expression of metabolism and inflammatory factors suggests that higher intake of saturated fatty acids by previously malnourished animals induces an inflammatory molecular response, which may lead to liver damage in the long term.

Keywords: Transition nutrition. Malnutrition. Maternal malnutrition. Metabolic programming. High fat diet. Metabolic diseases. Autonomic control. Lipid metabolism. Transcription factors.

Introduction

A nutritional transition is consolidated worldwide, especially among the populations of developing countries (1). Older hunger-related malnutrition rates were replaced by a higher prevalence of obesity with overnutrition (2, 3). An intrinsic occurrence of nutritional transition in developing countries is a transition from states of malnutrition during periods of stress (such as pregnancy and lactation) to overnutrition in adulthood? (overconsumption of processed foods, high use of mostly saturated fatty acids, sugars and calories) (4). Perinatal malnutrition seems to promote a sort of metabolism programming for nutritional scarcity conditions (5). Thus, the occurrence of overnutrition preceded by a malnutrition during perinatal phase establishes a divergent postnatal nutritional environment (2), implicating in a metabolic overload that may impact on energetic homeostasis (5).

Metabolic changes associated with a scarce perinatal environment may be related to epigenetic programming (7), which favors the modulation of metabolic enzyme expression to ensure growth and survival (2). Metabolic control is accomplished by interaction between highly regulated organs and pathways (8). The liver, a key organ for metabolic control, is sensitive to nutritional insults, which alters glucose and lipid metabolism (9). Imbalances between lipolytic and lipogenic mechanisms may direct liver to a pathophysiological condition that signal the establishment of metabolic syndrome (10).

Hepatocytes are sensitive to amino acid scarcity (11). In an experimental model of protein malnutrition, isolated hepatocytes from rats underfed with 5% of casein for 14 days showed increased triglyceride synthesis (12). A model of maternal malnutrition with an under protein intake during pregnancy and lactation (8%) suggested that adult offspring increases liver cholesterol in an epigenetically regulated mechanism (13). In addition, animals fed with low protein (5, 10 and 15% protein) and high fat (33% of the calories derived from lipids) increased gene expression of the enzyme Fatty Acid Synthase (FAS) and reduced decreased β-hydroxyiazyl-CoA dehydrogenase (β-HAD), B-had, which could suggest an increase in lipolytic and lipogenic capacity, depending on the percentage of protein reduction in the diet. It was also noted that the hyperlipidic diet accentuated the lipogenic action. (14).

High fat diets have been related to several diseases that make up the metabolic syndrome. (15). Specifically, saturated fatty acids have a high inflammatory potential, with activation of low-grade chronic immune responses (16, 17). Transcription factors, such as carbohydrate-responsive element binding protein (ChREB), sterol regulatory element binding protein (SREBP) and peroxisome proliferator-activated receptors (PPAR), respond to excessive lipid consumption, control energetic homeostasis and may activate pathways related to

inflammatory and degenerative processes in liver tissue (18-20). Energetic metabolism, gene expression and activation of inflammatory pathways seem to be linked to the activity of the autonomic nervous system.

In a recent review, Taher, Farr and Adeli (21) reported that the central nervous system communicates with the liver via afferent and efferent autonomic pathways and regulates lipid metabolism and tissue production of lipoproteins. Disorders that make up the metabolic syndrome are related to altered sympathetic nervous system regulation (22). Cardiac as well as respiratory variability has been used to evaluate sympathetic-vagal imbalances, where reduced values indicate impaired insulin sensitivity, dyslipidemia and hypertension. (23, 24). However, the hepatic metabolic effects and the relationship with autonomic modulation in previously malnourished animals are still unclear.

Thus, we evaluated the effect of a high-fat diet enriched with saturated fatty acid post-weaning on liver function and autonomic modulation of rats offspring submitted to perinatal protein malnutrition.

Methods

Ethical approval, animals and experimental designs

This project was approved by the Animal Use Ethics Committee (CEUA) of the Universidade Federal de Pernambuco (UFPE), under protocol nº 23076.046459 / 2018-17, and followed the rules and standards of handling and good practices in the handling of laboratory animals. For offspring generation, 16 albino and nulliparous female rats, aged between 90 and 100 days old and weighing between 230 and 250g, were used, as well as adult male breeding rats, both wistar. The rats were mated in a 3: 1 ratio and observation of the presence of sperm in the vaginal swab was used to define the 1st day of pregnancy. From the determination of pregnancy, the female rats were housed in individual cages and randomly grouped according to the corresponding experimental diets, as described in the nutritional handling item. Pregnant rats were submitted to balanced nutrition or protein malnutrition during pregnancy and lactation, and the offspring were reduced to 8 puppies per litter at birth. The male pups were weaned at 21 days of age, receiving a control or high-fat diet. Thus, the following experimental groups were formed: group C: whose mother of the puppy received a control diet during pregnancy and lactation and the puppy received a control diet after weaning; group LP-C: whose mother received a low-protein diet during pregnancy and lactation and the puppy

received a control diet after weaning; LP-HF group: whose mother received a low-protein diet during gestation and lactation and the puppy received a high-fat diet after weaning at 90 days of age. The experimental animals were followed until 90 days of age, when they were euthanized.

Nutritional manipulation

Diet

The diets used were prepared in the Laboratory of Dietetic Technique of the Academic Center of Vitória, within the specifications and standards established by the American Institute of Nutrition (AIN - 93), and the rules of manipulation of the National Health Surveillance Agency (ANVISA). The diets ingredients and nutrients are described in **Table 1** and fatty acid distribution of the post weaning diets are described in **Table 2**.

Table 1 – Ingredients list and nutritional composition in formulations of the experimental diets.

Ingredients *(g per 100g of diet)*	***Control diet***	***Low protein diet***	***High-fat diet + SFA***
Corn starch	39.7	50.3	15
Dextrinized Starch	13.2	13.2	-
Wheat flour	-	-	12
Cornmeal cookie	-	-	5
Soy flour	-	-	6
Lard	-	-	5.3
Milk cream	-	-	4
Margarine (65% lipídios)	-	-	4
Casein (>85%)	20	9.4	20
Guar gum	-	-	0.5
Sucrose	10	10	18
Soy oil	7	7	4
Fiber (cellulose)	5	5	2
Vitamins	1	1	0.7
Mineral mix	3.5	3.5	2.5
DL-methionine	0.3	0.3	0.25
Choline Bitartrate	0.25	0.25	0.25
BTH	0.0014	0.0014	0.014
Monosodium Glutamate (12.33%)	-	-	0.2
Sodium Chloride	-	-	0,3
Total (g)	100	100	100
Nutrients *(%)*			
Moisture	3.5	8.1	4.4
Proteins	18.6	8.7	22.5
Lipids	6.1	5.1	15.3
Carbohydrates	68.6	75.8	54.7
Ashes	3.3	2.3	3.1
Kcal /100g	369	364	446

Table 2 - Percentage distribution of fatty acids, as to the presence or absence of double bond in the carbon chain of formulated experimental diets.

Fatty acids	***Control diet***	***High-fatty diet +SFA***
Saturated		
Octanoic Acid (C8: 0)	0	0
Decanoic acid (C10: 0)	0	0
Lauric acid (C12: 0)	0	0
Myristic acid (C14: 0)	0	1.09
Pentadecanoate Acid (C15: 0)	0	0
Palmitic acid (C16: 0)	11.97	19.1
Heptadecanoate Acid (C17: 0)	0	0
Stearic acid (C18: 0)	4.62	9.4
Arachidic acid (C20: 0)	0.36	0
Behenic acid (C22: 0)	0.37	0
Total saturated fatty acids	17.32	29.59
Monounsaturated		
Myristoleic Acid (C14: 1)	0	0
Palmitoleic Acid (C16: 1)	0	0
Heptadecanoic acid (C17: 1)	0	0
Oleic acid (C18: 1)	29.93	34.77
Eicosenoic acid (C20: 1)	0	0
Total monounsaturated fatty acids	29.93	34.77
Polyunsaturated		
Linolenic Acid (C18: 3)	3.89	5.11
Linoleic Acid (C18: 2)	48.87	30.53
Total polyunsaturated fatty acids	52.76	35.64
Omega 3(g/100g)	0.27	0.78
Omega 6(g/100g)	3.42	4.66
Omega 6:3 ratio	*12.66*	*5.97*

Induction of metabolic programming by perinatal protein malnutrition

The maternal protein malnutrition was based on nutritional manipulation offering low protein diet to mothers during pregnancy and lactation. Offspring malnutrition repercussion was confirmed by the finding of lower weight and/or body size at birth (1 days old) and at the weaning (21 days of life), as well as by reducing the serum protein content.

Induction of nutritional transition with metabolic overoad

After weaning, the animals were randomly kept in collective cages balancing the number of animals per cage per group, receiving *ad libitum* control diet or high-fat diet until 90 days old.

Body weight, food intake, food efficiency and body size

The body mass of the animals was monitored daily on a precision scale (1 - 500g) AS-1000 (Mars, São Paulo, Brazil), as well as the food intake, determined from the difference between the amount of diet offered on the day and the heavy tailings on the following day, as described by Brito-Alves *et al*.,(25): daily food intake (g) = [weight of diet offered on day - weight of diet waste on the day following supply]. The analyzes were performed at the beginning of the dark period of the animals (18h). Feed efficiency was calculated from the formula described by NOVELLI *et al.*, (26): CEA% = [average body mass gain of period x 100 (g) food or caloric intake of period (g)]. Somatic growth was assessed from naso-anal length on days 1, 21 and 90 days of life, as previously described by Bayol *et al.*, (27)

Sample collection

Serum samples

At 21, 30 and 90 days of age, the animals were anesthetized intraperitoneally with ketamine (80 mg / kg i.p.) and xylazine (10 mg / kg i.p). The orbital plexus was then ruptured by a heparin-free capillary tube (Precision TC500). The collected blood was placed in tubes without anticoagulant and centrifuged at 4ºC and 3500 RPM for 10 minutes in a refrigerated centrifuge (Mikro 220R, Hettich, Germany) to obtain the serum. The serum was stored in a freezer at -20ºC until biochemical analysis.

Liver tissue samples

At 90 days of age, the animals were anesthetized as described previously and were euthanized by decapitation. Liver tissue was dissected and sectioned. Part was fixed in 10%

buffered formalin for subsequent histological procedures. Other sections were stored in an ultrafreezer at -80 ° C until use.

Serum Biochemical Assays

Serum samples were analyzed on automated biochemical analyzer (PW-2280, PIOWAY, China) for serum albumin, total protein, total cholesterol and triglyceride content (at 21, 30 and 90 days), and for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) only at 90 days using commercial kits (Biotecnica, Brazil).

Liver weight quantification and hepatic histology

After dissection, the total liver weight collected was quantified on a high precision scale to 0.001g (JA3003N, CELTAC, Germany) and corrected for the animal's body weight, according to the formula: weight% = (total animal weight x tissue weight) / 100.

Liver fragments were fixed buffered formalin and prepared for light microscopy, embedded in paraffin (Histopar-Easypath, SP, Brazil), sectioned with a thickness of 5 μm, several non-consecutive slices were cut per block, and twenty random and blinded fields were analyzed for each animal and sections were stained with hematoxylin and eosin. Digital photomicrographs were obtained in a digital cam (MOTICAM 2.300) coupled to a Nikon microscope (Nikon E-100, China) at 400x magnification. Cell types were quantified by the ImageJ program 1.44 version (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA). Was quantified the number of mononucleated hepatocytes, Kupffer cells, and endothelial cells in each field were quantified. An average was obtained from the sum of each cell type recorded in all fields analyzed.

Gene expression analysis

(mRNA extraction, reverse transcription and quantitative PCR)

Total mRNA was extracted from a previously frozen liver tissue fragment at -80 ° C (~ 50mg) with Tripure reagent (Roche, Meylan, France) according to the manufacturer's instructions. We used 10μL Trizol per mg liver tissue and the resulting suspension was homogenized using the Precellys Lysing kit (Bertin, Montigny-du-Brettonneux, France). After homogenization, chloroform was added at a ratio of ¼ volume of the solution, and this

preparation was vortexed in 3 15-second passages, incubated for 5 minutes and centrifuged for 15 minutes in a refrigerated centrifuge at 15,000 xg and 4 ° C (Mikro 220R, Hettich, Germany). Isopropanol (Carlo Erba reagents, Val-de-Reiul, France) was added at half the volume of the solution and centrifuged again as described above. The formed mRNA pellets were sequentially washed with 70% and 95% ethanol (Carlo Erba reagents, Val-de-Reiul, France), dried, and dissolved in 100 µl of RNase-free water. MRNA concentration and purity was determined by a Nanodrop® 2000 / 2000c spectrophotometer (Thermo Scientific, USA).

Reverse transcription was performed using an RT-TAKARA kit (Primescript TM, Dalian, China) for cDNA generation for Real Time PCR. Quantitative amplification was measured (qPCR) by Rotor Gene Real Time System (Labgene Scientific Instruments, Archamps, France). All results were represented as arbitrary units (A.U.), derived from a standard calibration curve based on reference samples. PCR for each sample was run on for all genes and TBP was used as an endogenous control. The gene sequences used for the study are described in **Table 3.**

Table 3. Sequence of primers used for RT-PCR analysis

Gene	Fow/Rev	Sequence 5'-3'	TmºC	Amplicon size	RefSec NCBI
Tbp	F	TGGTGTGCACAGGAGCCAAG	62 ºC	139 pb	NM_001004198
	R	TTCACATCACAGCTCCCCAC			
NFκβ	F	GTGGAGTACGACAACATCTC	62 ºC	176pb	NM_008689
	R	CAAGAGTCGTCCAGGTCATA			
TNF-α	F	CCAGACCCTCACACTCAGATC	58°C	78pb	NM_012675
	R	CTTGGTGGTTTGCTACGACG			
IL-1β	F	CTCTCCACCTCAATGGACAG	60°c	181pb	NM_031512
	R	TTGGGATCCACACTCTCCAG			
PPARα	F	GAGAATCCACGAAGCCTAC	60°c	196pb	NM_013196
	R	GAAGAATCGGACCTCTGCCTC			
ChREBp	F	AGTCCTTCAATGGGATGGTG	58°C	267pb	NM_133552
	R	GACGTGTCCACAAGTTGTTC			
FOXO3	F	GAGAGCAGATTTGGCAAAGG	58°C	182pb	NM_001106395
	R	CCTCATCTCCACACAGAACG			
SREBp1	F	ACGGAGCCATGGATTGCACA	60ºC	210pb	XM_213329 1c
	R	AAGGGTGCAGGTGTCACCTT			

Evaluation of autonomic modulation by respiratory rate, blood pressure and heart rate variability.

Analysis of respiratory parameters

Record of respiratory rate

Respiratory rate was recorded by whole body plethysmography, according to protocol adapted from that described by Malan (32), recorded during the baseline period, in a clear cycle (daytime) of non-anesthetized animals, without body movement inside the box or evident behavioral differences between groups. Similar environmental conditions were maintained for all mice analyzed. Each rat was placed inside an acrylic plethysmographic chamber (1 to 3 liters), followed by a 30 min acclimatization period before starting the recordings. An injection of 1 ml of air was used for volume calibration and at least 3 recordings without abnormal oscillations, considered viable, were obtained per animal. During each respiratory measurement, the airflow was interrupted and the animal's chamber was completely sealed for approximately 2 minutes. The oscillations caused by the animal's breathing were captured by a camera-connected device containing a differential pressure transducer and a signal amplifier (ML141 spirometer, PowerLab, ADInstruments) connected to the data acquisition system (LabChartTM Pro, PowerLab, ADInstruments). Between registers, the box airflow was maintained by an air compressor (Jad SC-7500e, Boyu, Taywan, China) coupled to a three-way circulation valve. For respiratory rate analysis, 10 continuous seconds of the most stable record, that describe by de Brito-Alves *et al*. (33), among the three performed at ages 1, 21 and 90 days of life were selected.

Respiratory rate variability analysis

To evaluate the sympathetic and parasympathetic influence on respiratory rate, respiratory variability was analyzed in a section containing between 180 and 200 complete respiratory cycles to capture breath-to-breath (BBn) and subsequent interval values. (BBn + 1). A Poincaré chart was used to represent the ratio BBn versus Bbn + 1. Short-term variability (SD1) and long-term variability (SD2) were calculated as respiratory variability indices (34).

Analysis of cardiac parameters

Surgical procedure

At 89 days old, the animals were anesthetized with ketamine (80 mg / kg ip) and xylazine (10 mg / kg ip), and then femoral artery cannulation was performed by inserting a polyethylene catheter as described. formerly by Barros and collaborators of Brito Alves, de Oliveira (37), (38). The cannula was positioned subcutaneously by the animal's back and exteriorized on the animal's back between the shoulder blades. The animals received a dose of anti-inflammatory ketoprofen (5 mg / kg i.m.) and were accommodated in individual boxes until the time of registration.

Blood pressure and heart rate record

Blood pressure was recorded 24 hours after the surgical procedure, at 90 days of life, so that the animals could fully recover. Pulsed blood pressure was recorded for 60 minutes by connecting the femoral artery cannula to a mechanical pressure transducer and a signal amplifier (ML866 / P, ADInstruments, Power Lab, Bella Vista, NSW, Australia). The records were stored on a computer equipped with appropriate software (LabChart© Pro, ADInstruments, Bella Vista, NSW, Australia) and analyzed later. Mean arterial pressure and heart rate were derived as pulsatile blood pressure derivations, calculated by the acquisition and analysis system, as described by de Brito-Alves et al.,(25) .

Spectral analysis of cardiac variability

The assessment of cardiac autonomic modulation was performed through blood pressure and heart rate variability, through time and frequency domain spectral methods, using Cardioseries (Cardioseries© 2.4, São Paulo, Brazil) and pluggin HRV programs. LabChartPro©, 8.1 (ADInstruments, Bella Vista, NSW, Australia).

The frequency domain assessment was performed on a 10-minute stable pulsatile pressure record and generated a graph of spectral power density, which can be decomposed into the following components: high frequency component (HF, waves between 0.75 and 3 Hz; low frequency component (LF, waves between 0.2 and 0.75 Hz; very low frequency component (VLF, waves between 0.003 and 0.04 Hz); LF / HF ratio, which represents the sympathovagal balance index; symbolic component, represented by 0V (parasympathetic acting) and 2V (sympathetic acting); spontaneous baroreflex response by Baroreflex Effectiveness Index (BEI) and Gain.

For time domain analysis, we used the normal integrated values of QRS waves, obtaining the following components from the RR interval: SDRR: standard deviation of RR intervals; RMSSD: square root mean square of the differences between adjacent RR intervals, in a time interval - short duration component of HRV, representing parasympathetic vagal activity; variability based on short-term (SD1) and long-term (SD2) changes.

Data analysis

Results were expressed as mean ± e.p.m. (mean standard error). Sample normality analysis was performed using the Shapiro-wilk test. For the data considered parametric, the comparison between the groups was performed by one-way analysis of variance (ANOVA), and when there was significance between the groups, we used the Newman-Keuls post-test to identify them. For nonparametric data, the Kruskal-Wallis test was used, followed by Dunn's post-test. Analyzes were performed on GraphPad Prism (GraphPad Software Corporation©, version 7.04, 2018), and the significance level considered was $p < 0.05$.

RESULTS

Sample characterization

Table 4 presents the sample characterization regarding nutritional status at birth (1 days old) and at weaning (21 days old), confirming the efficiency of malnutrition protocol adopted. At 1 day old, animals whose mothers received a low-protein diet during pregnancy and lactation had lower body weight at birth, although they did not suffer any loss in body length. At 21 days of age, animals from malnourished mothers (LP-C and LP-HL) presented reduced body weight, body length and lower serum protein and albumin, compared to animals from control mothers. At 90 days of age, postnatal high-fat diet recovered somatic and serum parameters compared to control group. However, the body weight and body length of the group that started to consume control diet (LP-C) remained reduced compared to control. Food intake as well as feed efficiency per gram of the diet were assessed at 30 and 90 days of age.

Table 4 – Characterization of the sample regarding the protein nutrition somatic parameters

	Groups						
	C	***n***	***LP-C***	***n***	***LP-HL***	***n***	***P value***
1 day old							
Body weight (g)	7.2 ± 0.2	12	6.4 ± 0.2*	14	6.5 ± 0.2*	12	*0.0056*
Body syze (cm)	5.2 ± 0.1	12	5.1± 0.1	14	5.1 ± 0.1	12	*0.1890*
21 days old							
Body weight (g)	45.1 ± 1.2	12	31.0 ± 1.1*	14	32.3 ± 1.2*	10	*<0.0001*
Body syze (cm)	11.5 ± 0.2	12	10.4 ± 0.1*	14	10.38 ± 0.1*	10	*<0.0001*
Serum protein (g/dL)	4.9 ± 0.1	11	4.7 ± 0.0*	12	4.6 ± 0.1*	9	*<0.0001*
Serum albumin (g/dL)	3.9 ± 0.0	11	3.4 ± 0.0*	12	3.4 ± 0.0*	10	*<0.0001*
30 days old							
Body weight (g)	85.7 ± 2.8	12	63.6 ± 2.5*	14	68.4 ± 2.7*	10	*<0.0001*
Body syze (cm)	14.0 ± 0.2	12	12.3 ± 0.2*	14	13.3 ± 0.1*	10	*<0.0001*
Serum protein (g/dL)	4.8 ± 0.1	11	5.0 ± 0.1	12	5.1 ± 0.1	10	*<0.0001*
Serum albumin (g/dL)	4.1 ± 0.0	11	4.1 ± 0.0	12	4.0 ± 0.1*	10	*0.0272*
Food consumption (g/day)	10.5 ± 0.4	12	8.4 ± 0.4*	12	6.8 ± 0.3*$^{\#}$	12	*<0,0001*
Food efficiency (g/day)	0.6 ± 0.0	12	0.6 ± 0.0	12	0.7 ± 0.0*$^{\#}$	12	*<0,0001*
90 days old							
Body weight (g)	359.1 ± 7.1	12	317.4 ± 5.2*	14	328.9 ± 12.0	12	*0.0047*
Body syze (cm)	23.9 ± 0.3	7	22.9 ± 0.2*	14	23.4 ± 0.3	7	*0.0093*
Serum protein (g/dL)	6.1 ± 0.1	10	5.9 ± 0.1	10	5.8 ± 0.2	10	*0.0940*
Serum albumin (g/dL)	4.5 ± 0.2	7	4.7 ± 0.1	13	4.4 ± 0.1	11	*0.1383*
Food consumption (g/day)	21.3 ± 1.3	5	20.2 ± 0.5	5	15.8 ± 1.2*	5	*0.0187*
Food efficiency (g/day)	0.12 ± 0.0	6	0.15 ± 0.0*	6	0.16 ± 0.0*	6	*0.0202*

Effects of perinatal malnutrition and post-weaning high fat diet on serum markers of lipid metabolism

In **Table 5** showed the effects of malnutrition on serum triglycerides and cholesterol values. Significant differences were not observed between groups at 21 and 90 days old. At 30 days old, both, triglycerides and cholesterol were affected by the postnatal high-fat diet: animals

previously malnourished and who received a postnatal high-fat diet (LP-HF) showed low TGL values and high COL levels compared to malnourished animals only (LP-C).

Table 5 - **Effects of perinatal malnutrition and post-weaning high fat diet on serum markers of lipid metabolism**

	Groups						
	C	***n***	***LP-C***	***n***	***LP-HF***	***n***	***P value***
21 days old							
Triglycerides	150.0 ± 15.4	11	138.0 ± 14.0	15	123.8 ± 8.5	10	*0.6268*
Cholesterol	127.0 ± 4.5	10	150.6 ± 9.9	13	148.4 ± 5.7	10	*0.0564*
30 days old							
Triglycerides	211.5 ± 20.8	12	288.2 ± 25.9	11	183.9 ± 24.4 #	12	*0.0306*
Cholesterol	120.8± 4.7	11	110.8 ± 4.3	10	130.2 ± 4.9 #	12	*0.0455*
90 days old							
Triglycerides	118.2±6.5	10	123.6±8.1	14	106.4±5.0	11	*0.2175*
Cholesterol	93.0±6.2	10	86.1±2.5	14	86.8±2.8	9	*0.4091*

Effects of perinatal malnutrition and post-weaning high fat diet on liver tissue weight, serum markers of damage and liver histopathology

The liver weight not altered neither perinatal malnutrition alone nor the postnatal high-fat diet was able to alter liver weight in the experimental groups (*p = 0.2160*). **Figure 1** presents the analysis of serum levels of inflammatory markers and histopathology analyze in liver tissue. Nutritional manipulation, until the age followed, did not promote changes in serum values when the groups are compared with each other (AST - **Fig. 1A**: *p = 0.1709*; ALT - **Fig. 1B**: *p = 0.4088*; AST/ALT ratio - **Fig. 1C**: *p = 0.5857*). **Fig. 1D** shows who protein malnutrition affected the LP-C and LP-HL groups, increasing the number of mononucleated hepatocytes, even after nutritional recovery (C = 29.2 ± 0.5; LP-C = 32.7 ± 0.6; LP-HL = 32.8 ± 0.6 cells per field; *p <0.0001*). The quantification of Kupffer cells (**Fig. 1E**) showed that only malnutrition differentially affected the LP-C group, with an increase in the number of this cell type when compared to the other groups (CC = 5.1 ± 0.3; LP-C = 10.6 ± 0.4; LP-HL = 6.0 ± 0.3 cells per field; **p <0.0001**). The quantification of hepatic endothelial cells (**Fig. 1F**) showed that LP-C showed a reduction in this type of cell when compared to the control group (C = 1.5 ± 0.1; LP-C = 1.0 ± 0.1; LP -HL = 1.2 ± 0.1 cells per field; **p = 0.005**). **Fig. 1G** shows histological photomicrographs, where not differential damage was observed between tissues.

Effects of perinatal malnutrition and post-weaning high fat diet on the protein expression of inflammatory genes and transcriptional factors genes of liver tissue

Figure 2 represents the evaluation of gene expression of inflammatory markers and transcriptional factors genes related in to lipid metabolism in liver tissue. The NFκβ (**Fig. 2A**) and TNFα (**Fig. 2B**) genes were strongly affected by the postnatal high fat diet, being elevated in the LP-HL group compared to the expression of the other groups (NFκβ: C = 100 ± 0.0; LP-C = 63.3 ± 5.7; LP-HL = 223.8 ± 35.6%; *p = 0.0004*; TNFα: C = 100 ± 0.0; LP- C = 83.5 ± 6.7; LP-HL = 392.1 ± 39.6%; *p = 0.0003*). The IL-1β gene (**Fig. 2C**) was affected by post-weaning high fat diet in malnourished animals (IL-1β: C = 100 ± 0.0; LP-C = 184.2 ± 20.6; LP-HL = 189.9 ± 32.2%; *p = 0.0353*).

The perinatal malnutrition increased PPARα (**Fig. 2D**) gene expression (C = 100 ± 0.0; LP-C = 167.7 ± 23.5; LP-HL = 170.6 ± 43.8%; *p = 0.0024)* and decreased FOXO3 (**Fig. 2F**) gene expression (C = 100 ± 0.0; LP-C = 44.5 ± 2.8; LP-HL = 52.3 ± 13.0%; *p = 0.0011).* ChREB gene expression was not altered by nutritional manipulation (**Fig. 2E**; *p = 0.589*). The post-weaning high fat diet increased SREBp1 gene expression (**Fig. 2G**) when compared with others groups (C = 100 ± 0.0; LP-C = 71.2 ± 9.8; LP-HL = 181.1 ± 32.3%; *p = 0.0042)*.

Effects of perinatal malnutrition and post-weaning high fat diet on autonomic modulation by respiratory frequency variability (RFV)

Figure 3 presents the results of offspring of RFV. **Fig. 3A** showed respiratory frequency (RF). At 1 and 21 days old, we show what respiratory frequency (RF) is increased by protein malnutrition in gestation and lactation (1 day – C = 0.5 ± 0.0; LP-C = 0.6 ± 0.0; LP-HF = 0.7 ± 0.0; *p = 0.0069*. 21 days - (C = 2.3 ± 0.1; LP-C = 3.5 ± 0.2; LP-HF = 3.4 ± 0.2; *p < 0.0001*). In the short term (8 days of consumption), the high-fat diet promoted normalization of the frequency and increased respiratory variability, demonstrating an acute effect capable of attenuating the sympathetic activation promoted by perinatal malnutrition. At 30 days old, LP-HF presents values similar to the control, while LP-C shows a higher respiratory rate (C = 1.2 ± 0.1; LP-C = 1.9 ± 0.2; LP-HF = 1.6 ± 0.1 b.p.m / g of total body weight; *p = 0.0014*). At 90 days old, no have differences (*p=0.1130*).

Fig. 3B demonstrate short-term respiratory variability (SD1) and **Fig. 3C** demonstrate long-term respiratory variability. At 1-day old RFV not affected by the low-protein diet during pregnancy (SD1, *p = 0.1915* and SD 2, *p = 0.7935).* At 21 days old, perinatal malnutrition

promote reduction respiratory variability (SD1, C = 116.8 ± 25.9; LP-C = 66.4 ± 6.3; LP-HL = 54.0 ± 7.7 ms; *p = 0.0037*; SD2, C = 203.0 ± 41.1; LP-C = 117.1 ± 10, 9; LP-HL = 93.6 ± 17.5 ms; *p = 0.0062*) and at 30 days old, the short-term (SD1, C = 25.0 ± 2.7; LP-C = 32.9 ± 3.1; LP-HF = 47.6 ± 4.9 ms; *p = 0.0023*) and long-term variability (SD2, C = 36.9 ± 5.9; LP-C = 45.8 ± 4.3; LP-HF = 74.5 ± 8.4 ms; *p = 0.0012*) of the LP-HF group was greater both when compared to the control group and when compared to the LP-C. At 90 days old of offspring, marking the middle-term effect of post-weaning diets. We demonstrate what the recovery of protein malnutrition, observed by the normalization of somatic and serum parameters, reflected in the regularization of heart rate and respiratory variability, independently of post-natal diet. (SD1, *p = 0.9017*; SD2, *p = 0.8389*). In **Fig. 3D**, **Fig. 3E, Fig. 3F and 3G** we demonstrate a Poincaré plot of breath to breath (BBn) and subsequent breath variability (BBn + 1) ratio.

Effects of perinatal malnutrition and post-weaning high fat diet on heart rate, blood pressure and cardiac autonomic modulation

Figure 4 presents the results of mean arterial pressure (MAP - **Fig. 4A**) and heart rate (HR - **Fig. 4B**). In **Fig. 4C**, we can see the representative records of offspring pulsatile blood pressure at 90 days old. Perinatal malnutrition nor a second nutritional insult with a high-fat diet were able to modify the cardiac parameters evaluated (MAP, *p = 0.9719*; RR, *p = 0.8080*).

Effects of perinatal malnutrition and post-weaning high fat diet on cardiac interval time domain HRV

In the time domain, heart rate variability was altered only in the RR interval standard deviation (SDRR), shown in **Figure 4D**, with reduction of SDRR in the LP-C group when compared to the control group (C = 8.5 ± 0 1.8 LP-C = 5.0 ± 0.6; LP-HL = 6.3 ± 0.5 ms; p = 0.0072). The other parameters, RMSSD (p = 0.6939 - **Fig. 4E**), SD1 (p = 0.7468, **Fig. 4F**) and SD2 (p = 0.4467, **Fig. 4G**) were not altered by the diets offered.

Effects of perinatal malnutrition and post-weaning high fat diet on HRV in the frequency domain

Cardiac variability was not altered by the nutritional manipulations performed in the experimental groups, in the frequency domain. **Fig. 5A** shows the performance of the VLF (*p = 0.612*), LF (*p = 0.8439*) and HF (*p = 0.3056*) waves from arterial pressure. **Fig. 5B** represents the relationship between the LF / HF waves at arterial pressure (p = 0.4149). **Fig. 5C** shows the performance of the LF (p = 0.6464) and HF waves (p = 0.7879) cardiac interval pulse. **Fig. 5D** shows the modulation of 0V (p = 0.8469) and 2V (p = 0.9437) variations in symbolic heart rate analysis. **Fig. 5E** represents the spontaneous baroreflex activity parameters by the baroreflex effectiveness index (p = 0.9251) and the average gain (p = 0.6051).

DISCUSSION

In the present study, we investigated whether protein malnutrition during the perinatal period affects liver function and metabolism in adults rats, and whether consumption of the high-fat saturated fat diet is able to regulate lipid metabolism and impair liver function and autonomic modulation of previously malnourished animals. The main results of this study show that protein restriction during perinatal development promotes reduced RRV at the end of lactation and increased number of hepatocytes in adult animals, as well as genetic variation of hepatic transcription factors PPARα and reduction of FOXO3 expression. These findings demonstrate that there is, in fact, a programming on lipid metabolism, with no sense of guarantee or more efficient use of lipids as an energy substrate, likely to preserve protein content, perceived during clinical development and lactation studies.

Regarding the second nutritional insult, with consumption of a high fat diet rich in saturated fatty acids, previously malnourished animals reinforced that lipogenic activity in liver may be increased, with potentiation of increased PPARα and SREBp1 expressions, without, however, altering serum lipid markers or promote liver damage. Recent studies of our research group, it has demonstrated that enzymatic activity of FAS is increased in LP-HL groups in 90 days of life (not published data).

However, when the molecular inflammatory response was evaluated, the increased expression of NFκβ and TNFα were evidenced, demonstrating that when reaching adulthood (90 days of life), the consumption of HF + AGS represents a metabolic overload for liver tissue, even when that there is no evident tissue damage or impairment of autonomic balance.

In murine, the perinatal period (pregnancy and lactation) represents a development window with high availability for actuation of the environment through plasticity. Perinatal malnutrition indicates a reduction in weight and body size and our results are corroborated by

the scientific literature (35-40). Geursen, Carne and Grigor previously demonstrated that the reduction in consumption of maternal proteins decrease of proteins by breast animals (41) and this impairs the supply of proteins and amino acids to a role. This effect is observed in our study, since there was a reduction in the content of proteins and albums in the serum of malnourished puppies, at immediate weaning, and this result is lower weight and body size.

Wykes et al. identified that protein malnutrition reduces the synthesis of tissue proteins as an adaptation mechanism (42), which corroborates with our results regarding the weight and body size of malnourished offspring. The scarcity of amino acids represents a limiting factor for protein synthesis (43) and the increase in the levels of transporter RNAs not loaded with amino acids signal the mTOR pathway (44), which makes adjustments in the gene expression process by negative regulation, reducing the synthesis of non-vital tissues, with the objective of guaranteeing survival (6).

The initial metabolic changes, at 21 and 30 days of life, seem to accompany the changes in autonomic modulation, observed from the reduction of respiratory variability at 21 and 30 days. Our results point to a possible link between increased serum triglyceride content at 30 days, preceded by increased sympathetic-excitation, with reduced respiratory variability in the LP-C group. This result is corroborated by Hurr *et al*., (45), whose study identified that altered sympathetic signaling raises the levels of hepatic triglycerides, with consequences on serum levels. However, this effect seems to have been minimized by the acute consumption of a high-fat diet in the LP-HF group. The reduction in sympathetic signaling by the high-fat diet had already been observed by Ribeiro *et al.* (46), but, the related mechanisms are not yet fully understood, but point to selective mediation of leptin on peripheral chemoreceptors.

In our study, we demonstrated that the studies performed by Qasem *et al.* (47), analyzing the activity of carnitine palmitoyl transferase-1 and medium chain acyl-coA dehydrogenase in the liver of previously malnourished animals, observed a more efficient lipid oxidative system in the liver in these animals. The mechanism of action seems to be related to activation upstream of the transcription factor PPARα, signaling a greater use of hepatic triglycerides as a substrate. In addition, recent studies from our research group observed that enzymatic activity of βhad is elevated in animals that have suffered perinatal malnutrition, and that the post-weaning high-fat diet further enhances the enzyme's functioning, signaling a more efficient lipid oxidation system (Data not published). These data may contribute at least in part to understand the reduced serum triglyceride levels observed in LP-HL animals at 30 days, however, we did not assess PPAR levels at the same age. Herein, we observed that this greater efficiency of the oxidative system is maintained when reaching adult life, even after recovery

from malnutrition and, suggesting a mechanism dependent on PPARα, since both previously malnourished groups had higher levels of this transcription. This mechanism can justify normal values of cholesterol and triglycerides, even with an overload of dietary saturated fatty acids, at 90 days of life.

It has been demonstrated that perinatal protein malnutrition programmed energy metabolism for more efficient ways of using lipids as an energy substrate, both from the increased βhad activity, and from the positively regulated expression of PPARα, which promotes the oxidation of fatty acids in fasting states (48). Our study also observed that maternal malnutrition induces hyperexpression of PPARα in the offspring, regardless of the post-weaning diet. Sengupta *et al*. (49) demonstrated that PPARα is supposedly suppressed in the state fed by a pathway dependent on the activation of mTORC1, which may justify the hyper expression observed in our results. As we saw earlier, as a nutrient-sensitive signaling pathway and regulating protein synthesis in response to nutritional conditions, the mTOR pathway is inhibited during protein malnutrition states (43). In our study, another transcription factor that was programmed from perinatal malnutrition was FOXO3, which showed reduced values even after consuming a recovery diet until adulthood. In hepatocyte culture, Honda et al., (50) show that in an environment low in amino acids, FOXO3 in its phosphorylated form is low expressed and Zhang et al.(51) reported that, not phosphorylated, the transcription factor is translocated to the nucleus, before the activation of its target genes.

As expected, the expression of SREBp1 was increased by consumption of a high-fat diet, accompanying the increase in FAS expression, which demonstrates positive regulation of FAS by SREBp1. In a study by Frederico *et al.* (52), it was demonstrated that wild mice, treated with a high-fat diet, developed hepatic steatosis and overexpressed SREBp-1c, while animals knocked out for SREBp-1c did not present the disease, in a mechanism that involved low regulation of proteins related to lipogenesis, including ACC, SCD-1 and FAS. This demonstrates that a high consumption of fatty acids positively regulates SREBp-1c which induces an increase in FAS. SREBp-1c can also positively regulate mTOR. Our study also demonstrated that the recovery of body weight by malnourished animals occurred only in the group that expressed higher levels of SREBp-1c. However, we can only suggest a relationship, since the levels of mTOR were not measured in our work.

We also demonstrate that the nutritional transition, with high consumption of saturated fatty acids, increases the inflammatory molecular profile. This result can be understood by the overexpression of SREBp-1c. In non-alcoholic fatty liver disease, Engin *et al.* (53) fatty acids activates the SREBp-1c pathway that triggers a cascade effect of NF-κβ, TNF-α activation and

promotes mitochondrial dysfunction. In a previous study by our laboratory, Simões-Alves *et al.* (28) demonstrated that the consumption of a high-fat diet rich in saturated fatty acids induces mitochondrial dysfunction in malnourished animals, in a mechanism based on changes in calcium permeability in the mitochondria. Mitochondrial dysfunction, with increased production of reactive oxygen and nitrogen species, induces the expression of inflammatory interleukins, such as IL-1β (54), which can support our results.

Hemodynamic parameters can be influenced by modulation of macronutrient intake. Previously, our group had demonstrated that perinatal protein malnutrition (8% of proteins in the diet) is able to raise the blood pressure of young rats (25) and change the sympathovagal balance (55), even after consuming a standard diet for rodents , after weaning. With different dietary models, Contreras et al., Using SHR rats (56), and Wexler, using spontaneous hypertension rats (SHR) and Kyoto rats (57), found that reducing carbohydrate consumption with increased lipids in the diet was able to reverse spontaneous hypertension of the animals. Wexler further demonstrated that, when they consumed a control diet, SHR rats developed hypertension, normally. In our model, postnatal diets have reduced carbohydrate content and increased protein and lipid content, when compared to the perinatal diet. For the LP-C group, the reduction in carbohydrates was slight (~ 11%), however, for the LP-HF group, the reduction was significant (~ 38%). Regarding proteins, the increase in protein content more than doubled for the LP-C and LP-HF groups, however, for LP-HF the amount of proteins in the diet was approximately 45% higher than in LP-C. Regarding lipids, the HF diet represented an increase of 200% in relation to the perinatal diet and 150% in relation to the post-weaning normal-lipid diet.

In a clinical study with humans, Apple *et al.* (58) demonstrated that the consumption of a diet with reduced carbohydrates (48%) and higher protein (25%) and lipids (27%) - dietary parameters similar to that used in our HF diet - promotes a reduction in blood pressure, improved lipid levels and reduced estimated cardiovascular risk. In an experimental model of spontaneously hypertensive in rats, Bosse *et al.* (59) demonstrated that the higher consumption of lipids favors the reduction of blood pressure and heart rate. These findings corroborate our hemodynamic results and can be discussed from the perspective of reducing insulin levels and the selective action of leptin on the autonomic nervous system (60). However, analyzes of these variables are a limitation of this study, and will be investigated in ongoing research by our group.

The divergence between the diets of the perinatal and postnatal environment, initially, seems to have favored the metabolic and autonomic functioning. At the physiological level, the

liver, as a fundamental metabolic organ, seems to have been favored by the nutritional transition, since once the tissue weight has not been altered and tissue damage has not been observed, either by histological analysis or by the levels of serum ALT and AST. The HF diet seems had a more prominent protective effect in the LP-HF group, since the cardiac variability of the LP-C was reduced, accompanied by an increase in the number of Kupffer cells, which suggests initial implications for autonomic regulation (61), but, there wasn't damage tissue at analyzed age. Jensen et al. (62) e Lautt (63) indicate that there is a close link between the recruitment of hepatic tissue macrophages by exacerbated sympathetic activation. These findings suggest that the higher consumption of lipids with reduced carbohydrates may be more favorable to reverse the sympathetic programming induced by malnutrition, in young adults rats. However, at the molecular level, the observed in the liver tissue of our previously malnourished animals and submitted to a high-fat diet rich in saturated fatty acids, seems to suggest the beginning of the decline in homeostasis observed up to 90 days. Since important inflammatory factors, related to the development of diseases that make up the hepatic branch of the metabolic syndrome, are hyper expressed in LP-HF, monitoring of repercussions in the longer term is necessary.

CONCLUSION

Perinatal malnutrition may induce metabolic adaptations directed to a better use of lipids as an energy substrate for both oxidation and storage, which maintains the normality of lipid metabolism and autonomic modulation, even after a second nutritional insult with postnatal consumption of a high-fat diet. However, the increased gene expression of lipids metabolism and inflammatory factors suggests that higher intake of saturated fatty acids by previously malnourished animals induces an inflammatory molecular response, which may affect liver function at the long term.

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Captions of figures and tables

Figure 1 – **Effects of perinatal malnutrition and post-weaning high-fat diet on serum markers of liver tissue damage and hepatic histology analyze.** Panel A show aspartate aminotransferase values, panel B show alanine aminotransferase values and panel C show ALT/AST ratio. Panel D show mononucleated hepatocytes numbers, panel E Kupffer cells numbers and Panel F show endothelial cells numbers of liver sample collected at 90 days old. Black bars represented control group (C), whose mothers and puppies received only a control diet (18% protein - n = 12). Dark gray bars represented the LP-C group, whose mothers received a low protein diet (8% protein) during pregnancy and lactation and the pups received a control

diet after weaning (n = 12). Light gray bars represented the LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and high-fat diet (32% lipid ~ 65% + SGA) after weaning (n = 12). Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. Panel G show representative images of each groups. “MH” - mononucleated hepatocyte; “BH” – binucleated hepatocyte; “KC” – Kupffer cell; “EC” – endothelial cell; “VS” – vascular space. Images in 400x magnification. Scale bar 1μm.

Figure 2 – **Effects of perinatal malnutrition and post-weaning high-fat diet on hepatic expression of inflammatory genes and transcription factors genes.** Panel A show NFκβ gene expression, Panel B show TNFα gene expression and Panel C IL-1β gene expression. Panel D show PPARα gene expression, Panel E show ChREBp gene expression, Panel F show FOXO3 gene expression and Panel G show SREBp1 gene expression. The expression was calculated as a percentage of control group values average, in samples by animals at 90 days old. Black bars represented control group (C), whose mothers and puppies received only a control diet (18% protein - n = 5). Dark gray bars represented the LP-C group, whose mothers received a low protein diet (8% protein) during pregnancy and lactation and the pups received a control diet after weaning (n = 5). Light gray bars represented the LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and high-fat diet (32% lipid ~ 65% + SGA) after weaning (n = 5). Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. (*) when compared to C; (**#**) when compared to LP-C.

Figure 3 – **Effects of perinatal malnutrition and post-weaning high-fat diet respiratory frequency variability.** Panel A show respiratory frequency (RF). Panel B show short time respiratory variability (SD1) and Panel C show long time respiratory variability subsequent breath (SD2). Panels D, E, F and G show Poincare plot for the breath-to-breath variability ($BB_{n)}$ and variability in subsequent breath ($BB_{n+1)}$ value. Black bars represented control group (C), whose mothers and puppies received only a control diet (18% protein - n = 5). Dark gray bars represented the LP-C group, whose mothers received low protein diet (8% protein) during pregnancy and lactation and the pups received a control diet after weaning (n = 5). Light gray bars represented the LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and pups received high-fat diet (32% lipid ~ 65% + SGA) after

weaning (n = 5). Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. (*) when compared to C.

Figure 4 – **Effects of perinatal malnutrition and post-weaning high-fat diet on baseline cardiovascular parameters and heart rate variability in time domain at 90 days old.** Panel A show mean arterial pressure (MAP) and Panel B show heart rate (HR) values. Panel C show representative records of the baseline pulsatile arterial pressure (PAP) and heart rate (HR). Panel D show standard error of mean deviation (SDRR) variability values and Panel E show the square root of mean standard deviation (RMSSD) variability values. Panel F show short time variability (SD1) and Panel G show long time variability (SD2). Black bars represented control group (C), whose mothers and puppies received only a control diet (18% protein - n = 5). Dark gray bars represented the LP-C group, whose mothers received low protein diet (8% protein) during pregnancy and lactation and the pups received a control diet after weaning (n = 5). Light gray bars represented the LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and pups received high-fat diet (32% lipid ~ 65% + SGA) after weaning (n = 5). Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. (*) when compared to C.

Figure 5 – **Effects of perinatal malnutrition and post-weaning high-fat diet on heart rate variability in frequency domain at 90 days old.** The Panel shows the occurrence of waves with very low frequency (VLF), low frequency (LF) and high frequency (HF) bands for groups, while panel B presents the variability index from the ratio between the LF / HF waves, from blood pressure. In C, we show it as LF and HF waves from the pulse interval. Panel D presents a symbolic analysis of cardiac variability and Painel E shows spontaneous baroreflex activity. Analyzes applied to pulsatile pressure, using the femoral cannula up to 90 days of life. Dark gray bars represented the LP-C group, whose mothers received low protein diet (8% protein) during pregnancy and lactation and the pups received a control diet after weaning (n = 5). Light gray bars represented the LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and pups received high-fat diet (32% lipid ~ 65% + SGA) after weaning (n = 5). Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$.

Table 1 - **Ingredients list and nutrients composition used in formulations of the experimental diets.** Description of the ingredients that make up the diets used for the

nutritional manipulation of the animals. The control (normal protein / normal lipids) and low protein diets were based on the guidelines described by AIN-93, published by Reeves, Nielsen and Fahey**.** (64). The high-fat diet was adapted from the model developed by Ferro-Cavalcante and collaborators. (65).

Table 2 - **Percentage distribution of fatty acids, as to the presence or absence of double bond in the carbon chain of formulated experimental diets.** Description of fatty acid percentage of post-weaning diets. The control diet (normal protein / normal lipids) was based on AIN-93, published by Reeves, Nielsen and Fahey (64). The high-fat diet was adapted from the model developed by Ferro-Cavalcante and collaborators (65). Fatty acids were identified according to external standard (FAME Supelco™ mix C4-C24, Bellefonte, PA, USA) and the percentage calculated according to peak area normalization by the gas chromatography method in the Phytochemicals and Processes Laboratory, Northeast Center for Technologies and Strategies – CETENE. From the percentage determination of linolenic (omega 3) and linoleic acid (omega 6), the amounts (g / 100g of diet) of these fatty acids were calculated and the omega 6/3 ratio was obtained.

Table 3 - **Sequence of primers used for RT-PCR analysis.**

Table 4 - **Characterization of the sample regarding the protein nutrition somatic parameters**. Characterization of the sample regarding the somatic parameters of malnutrition and nutritional recovery after weaning of offspring submitted to perinatal protein malnutrition. After weaning, the offspring were fed a control or high fat saturated fatty acid diet. C - group whose mothers received only a control diet during pregnancy and lactation, and the offspring consumed a control diet after weaning; LP-C - group whose mother received a low protein diet and offspring consumed a control diet after weaning; LP-HF - group whose mother received low protein diet during pregnancy and lactation and offspring consumed HF diet after weaning. Measurements and serum values obtained from animals at the presented ages. Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. (*) when compared to C; ($^{\#}$) when compared to LP-C.

Table 5 - **Effects of perinatal malnutrition and post-weaning high fat diet on serum markers of lipid metabolism.** C - Control group, whose mothers and puppies received only a control diet (18% protein). LP-C group, whose mothers received a low protein diet (8% protein)

during pregnancy and lactation and the pups received a control diet after weaning. LP-HF group, whose mothers received low-protein diet during pregnancy and lactation (8% protein) and high-fat diet (32% lipid, ~ 65% + SGA) after weaning. Values are mean ± SEM and results obtained by one-way ANOVA analysis with Newman-Keulls post-test, for $p < 0.05$. (**#**) when compared to LP-C.

Figures

Fig. 1

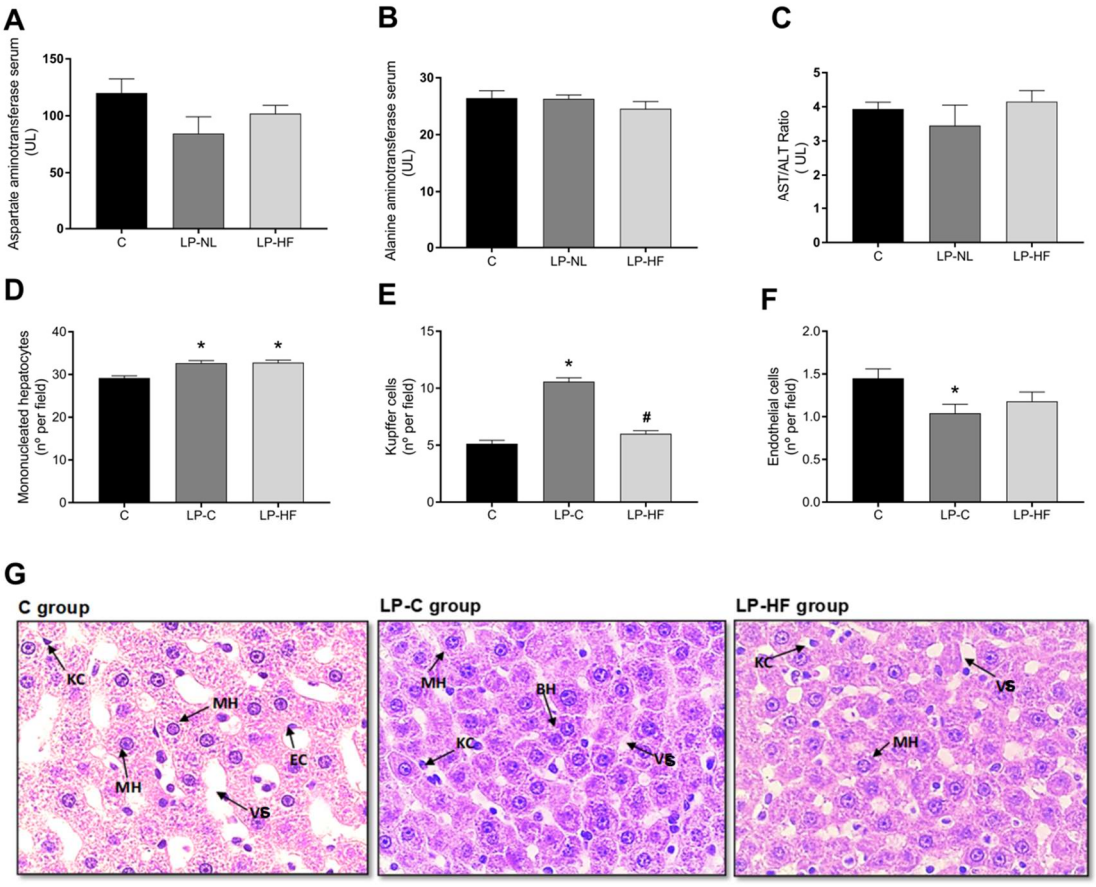


Fig. 2

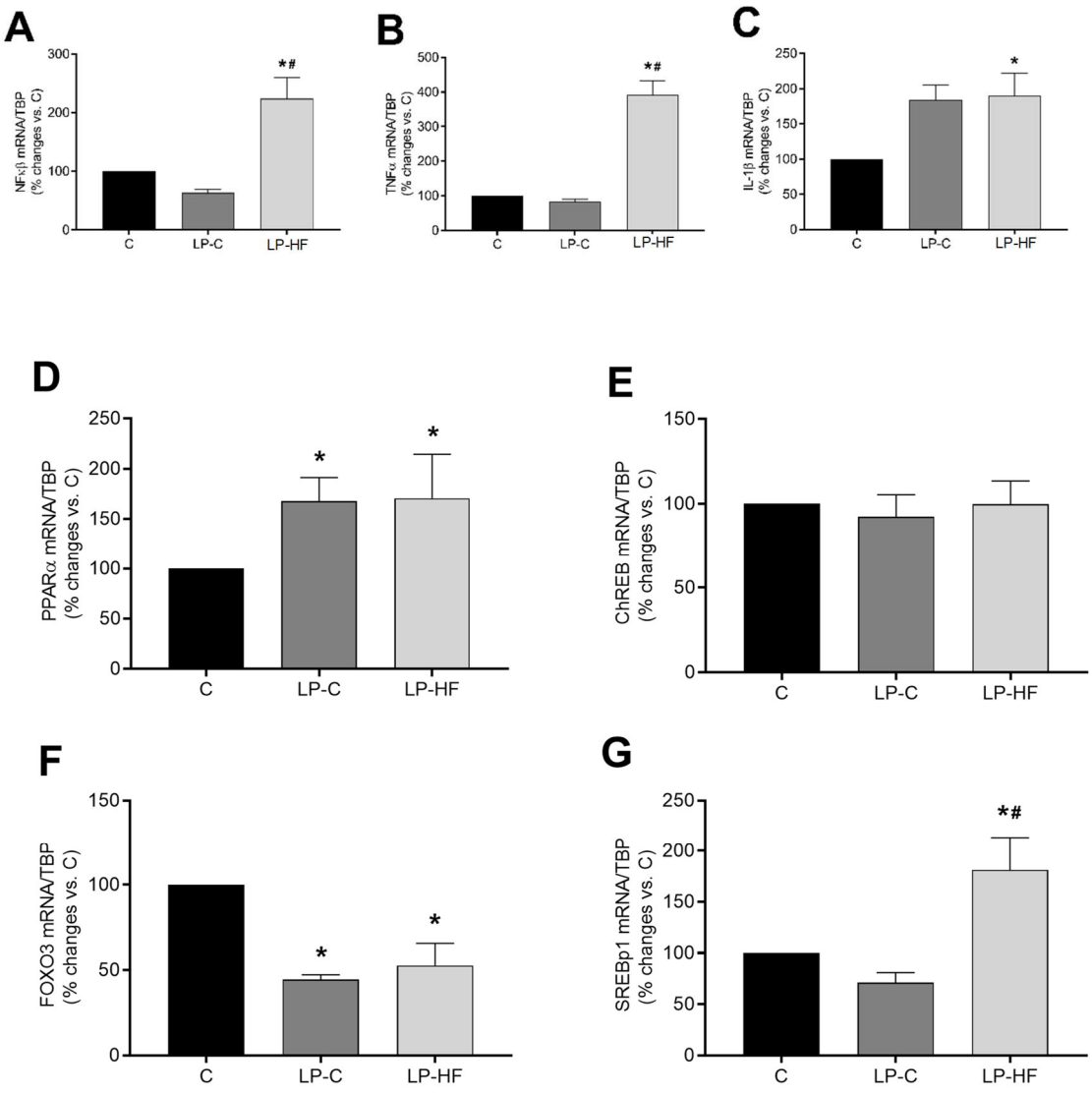

A
NFκβ mRNA/TBP (% changes vs. C)
300
200
100
0
*#
C
LP-C
LP-HF
B
TNFα mRNA/TBP (% changes vs. C)
500
400
300
200
100
0
*#
C
LP-C
LP-HF
C
IL-1β mRNA/TBP (% changes vs. C)
250
200
150
100
50
0
*
C
LP-C
LP-HF
D
PPARα mRNA/TBP (% changes vs. C)
250
200
150
100
50
0
*
*
C
LP-C
LP-HF
E
ChREB mRNA/TBP (% changes vs. C)
150
100
50
0
C
LP-C
LP-HF
F
FOXO3 mRNA/TBP (% changes vs. C)
150
100
50
0
*
*
C
LP-C
LP-HF
G
SREBp1 mRNA/TBP (% changes vs. C)
250
200
150
100
50
0
*#
C
LP-C
LP-HF

Fig. 3

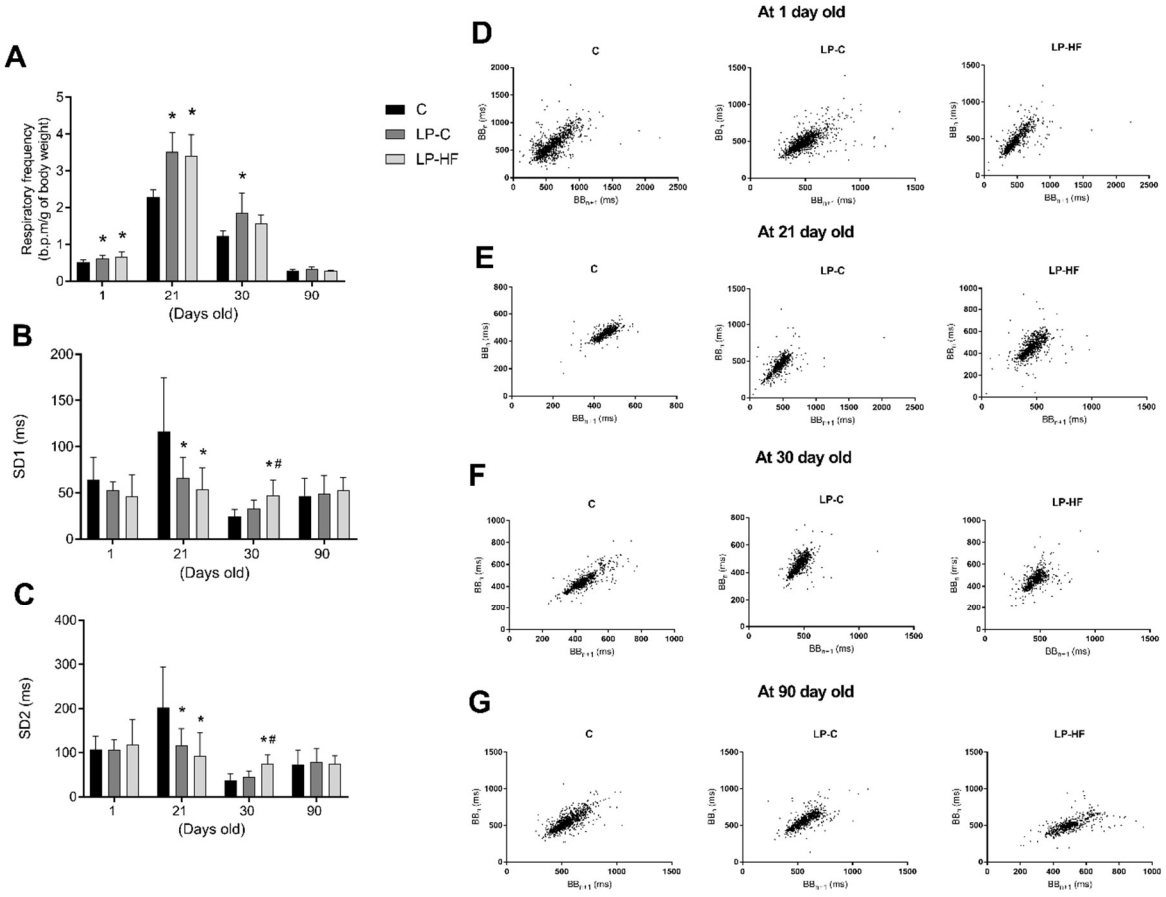

A
B
C
D
E
F
G
C
LP-C
LP-HF
Respiratory frequency (b.p.m/g of body weight)
SD1 (ms)
SD2 (ms)
(Days old)
At 1 day old
At 21 day old
At 30 day old
At 90 day old

Fig. 4

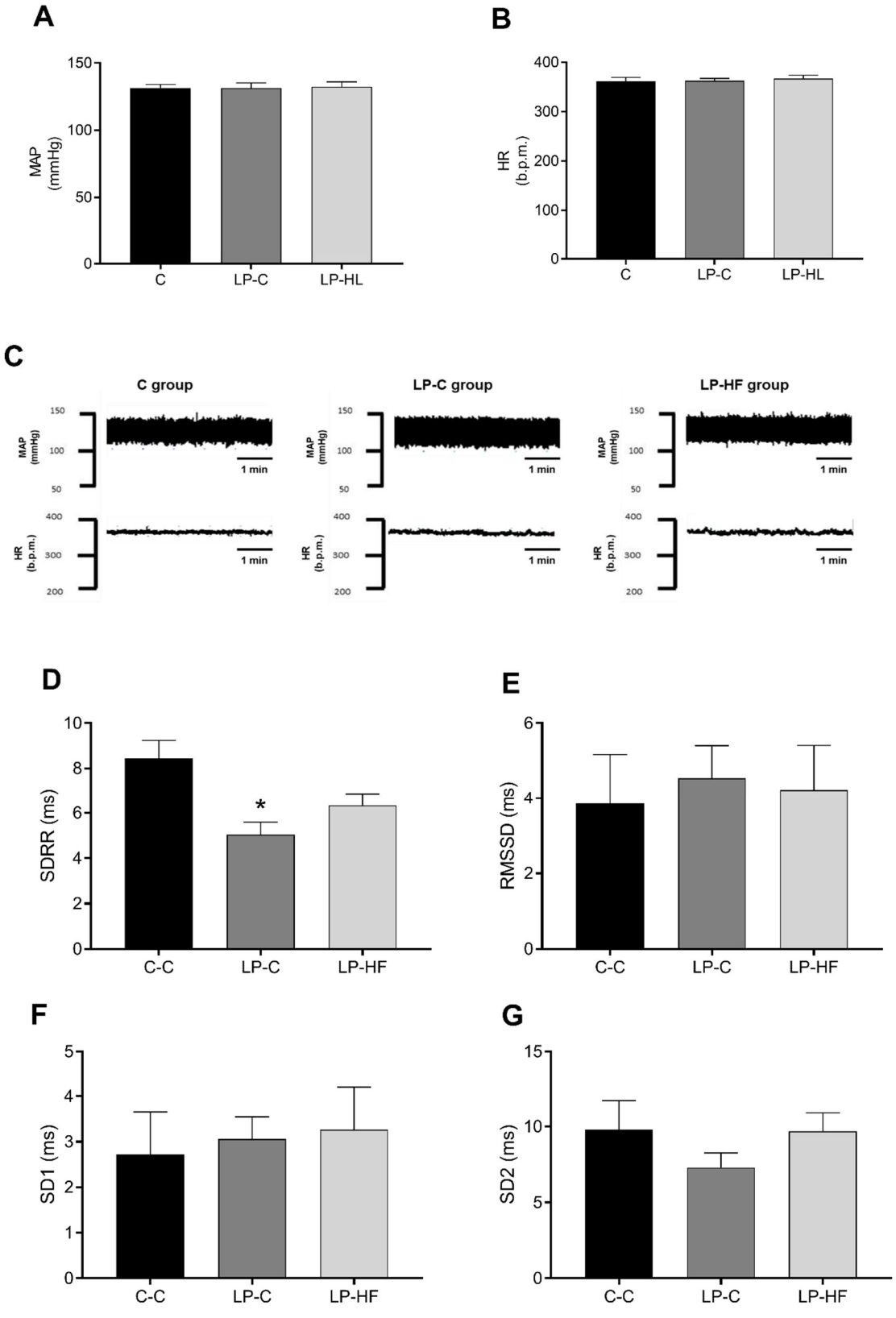
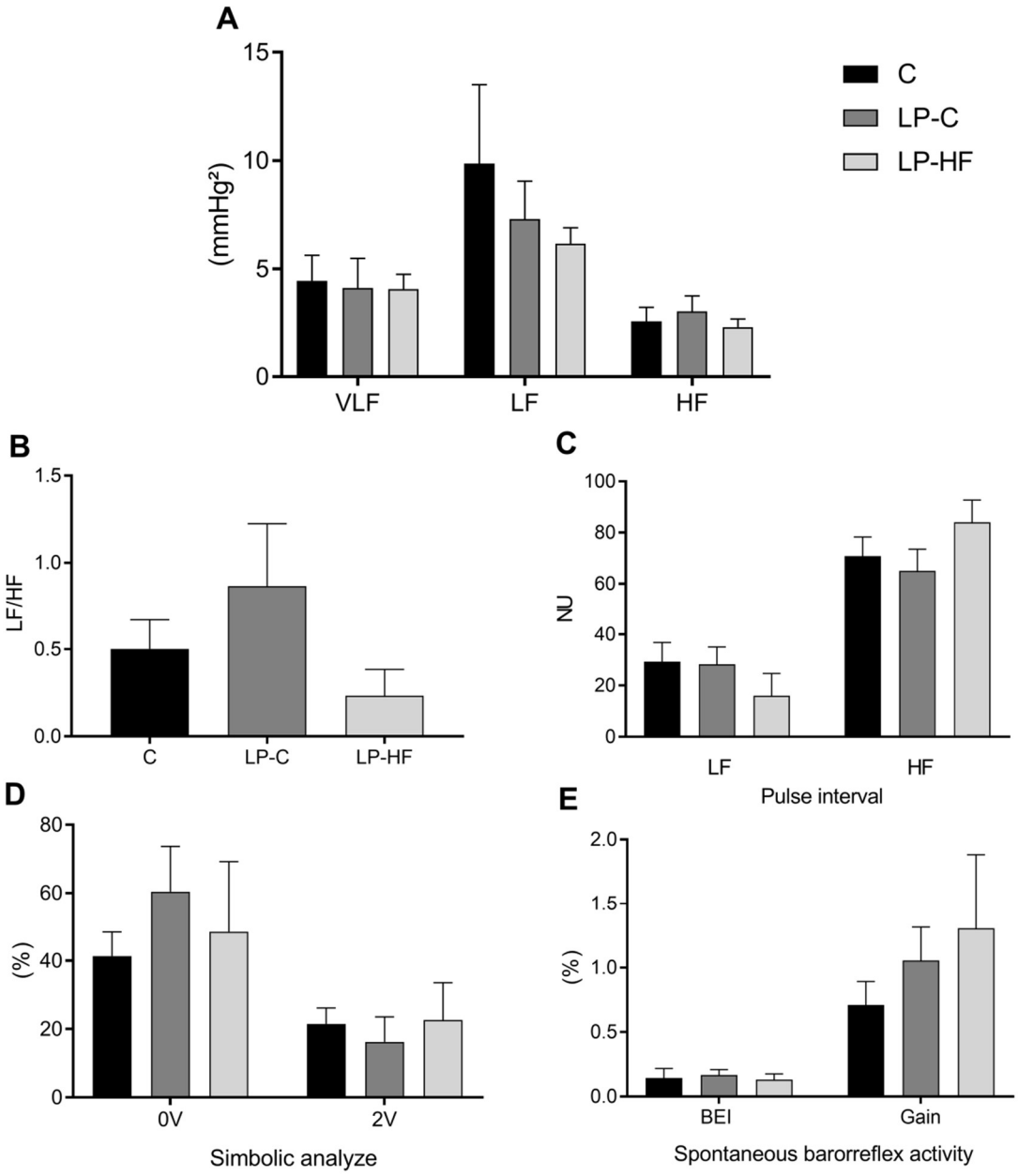

A
MAP (mmHg)
150
100
50
0
C
LP-C
LP-HL
B
HR (b.p.m.)
400
300
200
100
0
C
LP-C
LP-HL
C
C group
LP-C group
LP-HF group
MAP (mmHg)
HR (b.p.m.)
1 min
D
SDRR (ms)
10
8
6
4
2
0
*
C-C
LP-C
LP-HF
E
RMSSD (ms)
6
4
2
0
C-C
LP-C
LP-HF
F
SD1 (ms)
5
4
3
2
1
0
C-C
LP-C
LP-HF
G
SD2 (ms)
15
10
5
0
C-C
LP-C
LP-HF

Fig. 5


A
15
10
5
0
(mmHg²)
VLF
LF
HF
C
LP-C
LP-HF
B
1.5
1.0
0.5
0.0
LF/HF
C
LP-C
LP-HF
C
100
80
60
40
20
0
NU
LF
HF
Pulse interval
D
80
60
40
20
0
(%)
0V
2V
Simbolic analyze
E
2.0
1.5
1.0
0.5
0.0
(%)
BEI
Gain
Spontaneous barorreflex activity

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6 CONSIDERAÇÕES FINAIS

A divergência entre as dietas do ambiente perinatal e pós-natal, inicialmente, parece ter favorecido o funcionamento metabólico e autonômico. Em nível fisiológico, o fígado, como órgão metabólico fundamental, parece ter sido favorecido pela transição nutricional, uma vez que o peso do tecido não foi alterado e não foi observado dano tecidual, quer seja pela análise histopatológica, quer seja pelos níveis de séricos de transferases. Sobre a modulação autonômica, a dieta hiperlipídica demonstrou um efeito protetor proeminente, uma vez que a variabilidade cardíaca dos animais desnutridos que consumiram dieta controle foi reduzida, acompanhada de um aumento no número de células Kupffer, o que sugere implicações iniciais da regulação autonômica com repercussões hepáticas, mas sem repercussão histopatológica. Nossos achados sugerem que o maior consumo de lipídios, relacionado a redução do consumo de carboidratos pode ser mais favorável para reverter a programação simpática induzida pela desnutrição em ratos adultos jovens. Entretanto, em nível molecular, o modelo lipogênico e lipolítico exacerbado, observado no tecido hepático de animais previamente desnutridos e submetidos a uma dieta rica em gorduras (ácidos graxos saturados), indica o declínio da homeostase observada até os 90 dias de vida. Fatores inflamatórios significativamente ativos no adoecimento hepático e relacionados ao desenvolvimento de doenças que compõem o ramo hepático da síndrome metabólica, apresenta hiperexpressão induzida pela dieta hiperlipídica, indicando a necessidade de monitoramento das repercussões a mais longo prazo.

Assim, concluímos que a desnutrição perinatal induz adaptações que mantém a normalidade do metabolismo lipídico e modulação autonômica, e que o consumo de dieta hiperlipídica não representa uma sobrecarga metabólica para animais desnutridos, até a idade observada. No entanto, o aumento da expressão gênica relacionada ao metabolismo lipídico e a fatores inflamatórios, sugere que uma maior ingestão de ácidos graxos saturados por animais previamente desnutridos induz uma resposta molecular inflamatória, que pode desencadear em adoecimento hepático a longo prazo.

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APÊNDICE A – ARTIGO 02

ARTIGO 02 – FETAL PROGRAMMING EFFECTS OF MISMATCHED PERINATAL AND POSTNATAL NUTRITION ON THE DEVELOPMENT OF CARDIOMETABOLIC DISEASES

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Aiany Cibelle Simões-Alves[a1], Ana Paula da Fonseca Arcoverde Cabral de Mello[a2], Luciano Pirola[b3], Beatrice Morio[b4], João Henrique Costa-Silva[a5]

[a]Laboratory of Nutrition, Physical Activity and Phenotypic Plasticity, Universidade Federal de Pernambuco, UFPE, Vitória de Santo Antão – PE, 55608-680, Brazil.

[b]Laboratoire de Recherche en Cardiovasculaire, Métabolisme, Diabétologie et Nutrition (CarMeN), INSERM U1060, INRA U1397, Université Claude Bernard Lyon1, Oullins, Lyon, France

[1] aiany_simoes@hotmail.com; [2] anap.arcoverde@gmail.com; [3] luciano.pirola@univ-lyon1.fr; [4] joaohenriq@hotmail.com.

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Corresponding author:

João Henrique Costa-Silva

Núcleo de Educação Física e Ciências do Esporte – CAV - UFPE

Rua Alta do Reservatório, S/N, Bela Vista, Vitória de Santo Antão, PE.

CEP: 55608-680

Phone/fax: 55 81 31144101

Email: joao.hcsilva@ufpe.br

Abstract: Epidemiological and experimental studies have shown that maternal diet is largely related to morphological, metabolic and physiological changes in offspring. Thus, individuals whose mothers were malnourished during pregnancy and early postnatal may have energy balance dysfunctions in adulthood. This phenomenon can be understood in the context of phenotypic plasticity which, in order to ensure the survival of offspring, promotes metabolic and molecular adjustments, which may occur via epigenetic modulation, altering gene expression without altering DNA sequence. The phenomenon of nutritional transition, characterized mainly by the substitution of low caloric / low protein consumption patterns, followed by increased consumption of high caloric / high fat diets, can induce a metabolic overload with high potential epigenetic modulation, inducing cardiometabolic disorders, especially if nutritional insults occur during the perinatal period. Thus, our main objective was to review the cardiometabolic and epigenetic repercussions of metabolic programming in malnourished organisms during the perinatal period, when submitted to incompatible postnatal diets. The review emphasizes that the incompatibility between the nutritional status of the perinatal and postnatal environment induces accelerated recovery growth and adjustments in autonomic modulation, insulin sensitivity as well as mitochondrial disfunction. A post-weaning high caloric / high fat diets potentiate these adjustments, exacerbating deleterious changes in important metabolic organs: hepatic, adipose and muscular tissue. The epigenetic repercussions of postnatal metabolic overload may be etiological sources of cardiometabolic diseases, which the subjects affected by the nutritional transition.

Keywords: **N**utrition transition; malnutrition; obesity cardiometabolic; dyslipidemia; epigenetic.

Introduction

In the second half of the twentieth century, a change in the dietary habits of the population, mainly in the western part of the world, has been evidenced, which has been called a phenomenon of "nutritional transition" (1, 2). Higher levels of malnutrition have been replaced by higher rates of overweight and obesity related to hyperlipidic and hypercaloric food consumption (3). Epidemiological evidence has shown that nutritional deficiency in the first years of life accompanied by overnutrition, a posteriori, may increase the risk of dyslipidemia, and other cardiometabolic diseases in adulthood, such as hypertension and type II diabetes (4, 5). In this regard, it has been observed that the eating habits and nutritional condition in early phases of life play a key role on the etiology of these diseases by inducing physiological dysfunctions (6-8).

This phenomenon can be understood in the context of phenotypic plasticity. Many papers had suggested that external environmental inputs, such as nutritional, may modify the phenotype, leading to physiological adaptations without genetic changes (9). Recently, it is suggested that mother's nutritional factors play a key role in this context and it has been highlighted since Barker (10-13). In this context, new evidences from epidemiological and clinical studies have showed the association of the maternal under- nutrition with development of cardiometabolic dysfuntions (14-19). Thus, individuals whose mothers were undernutrished early in pregnancy and inappropriate postnatal nutrition, and more specifically rapid catch-up growth, may significantly heighten energy balance dysfunctions in adulthood. In particular, postnatal hypercaloric nutrition is an important accelerator in the etiology of adult-onset disease in human born with low birth weight (20), due to the metabolic load generated by low weight at birth and postnatal hypercaloric nutrition is mismatched with metabolic capacity attained during fetal life. Thus, this review will address the new concepts about the fetal programming effects of mismatched perinatal and postnatal nutrition on the development of cardiometabolic diseases.

Phenotypic plasticity and perinatal origin of cardiometabolic diseases.

Developmental origins of health and diseases proposed by Barker and colleagues in 1986 have been extensively studied as physiological consequences of perinatal nutritional factors (5, 21-23). This field of research proposes that cardiometabolic diseases can be

"programmed" by the "adaptative" effects of both under- and overnutrition during early phases of growth and development on the cell physiology in the phenotype, but without altering the genotype (24-28). In this context, the phenotypic plasticity has as molecular basis the epigenetic alterations, such as DNA methylation, histone acetylation and microRNA expression (29). These modifications "epigenetic" were firstly described by Conrad Waddigton in 1940 and it studies the relationship between cause and effect in the genes to produce a phenotype (30). Nowadays, this concept is employed to describe the process of the gene expression and its linking to modifications in the chromatin structure without altering DNA sequence (31, 32).

The DNA methylation is related to addition of methyl groups on DNA cytosine residues, normally on the cytosine followed by guanine residue (CpG dinucleotides), which can produce inhibition of the gene expression by impairing transcriptional factor binding (33-36). Histone acetylation diminishes the electrostatic affinity between histone proteins and DNA, and thereby promotes a chromatin structure that is more permissive to gene transcription, regulated by the action of histone acetyl transferases (HATs), whereas the removal of acetyl groups is catalysed by histone deacetylase (HDACs) (37). MicroRNAs are small non-coding endogenous RNA molecules involved in gene regulation, and are located at introns of protein-coding genes, introns of non-coding genes, or exons of non-coding genes; they have been implicated in many cellular processes including proliferation, apoptosis, differentiation, senescence, and the responses to stress and immune stimuli (38). In this context, it has been investigated how nutritional aspect may induce these epigenetic modifications.

These epigenetic marks are established early in development and can persist for a lengthy period of time. Epigenetic modifications are widely hypothesized to be an overarching mechanism linking maternal nutrition to metabolic health phenotypes in the offspring (39, 40). Particularly, mice born to mothers supplemented with folic acid, vitamin B12, methionine, zinc, betaine, and choline experienced significantly higher rates of allergic airway inflammation, ensuing from an excessive methylation of the runt-related transcription factor 3 (Runx3), a mediator of T-lymphocyte differentiation predisposing to asthma-like diseases (41). Hence, methylation can act as a double-edged sword, and this finding is consistent with human epidemiologic evidence of a significant association between perinatal folic acid supplementation and increased risk of wheezing at 18 months of age . On the other hand, folic acid deficiency has been associated with an increased expression of inflammatory mediators, such as interleukin (IL)-β, IL-6, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 in the mouse monocyte cell line RAW264.7 (42).

It is considered at least three ways by which nutrients can induce DNA methylation, alter gene expression and modify cellular phenotype: i) by providing methyl group supply for inducing S- adenosyl-L-methionine formation (genomic DNA methylation), modifing the methyltransferase activity or impairing DNA demethylation process; ii) by modifying chromatin remodeling, or lysine and arginine residues in the N-terminal histone tails; and iii) by altering microRNA expression (31, 32, 43, 44). In this context, altered contents of amino acids, such as methionine and cysteine, as well as reduced choline and folate diet amount can modify the process of the DNA methylation leading to both DNA hyper- and hypomethylation (45). In particular, maternal protein restriction reduces the supply of methyl groups from glycine when fetal demand is high. Consistent with this concept, a reduction in overall maternal calories during pregnancy induced hypomethylation of promoters in the liver tissue of the offspring that resulted in the increased expression of genes involved in fatty acid oxidation (e.g., peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ Coactivator (PGC)-1α and reduced expression of genes involved in lipid synthesis (e.g., sterol response element binding protein-1c and diacylglycerol acyltransferase-1) (46). Maternal protein restriction during pregnancy induced hypomethylation of both the glucocorticoid receptor and the PPAR-α promoters in the livers of the offspring that conditioned alterations in the expression of their target genes and metabolic processes under the control of these transcription factors (47). In the islets of the offspring, maternal protein restriction altered histone methylation and increased DNA methylation of the hepatocyte nuclear factor 4α (HNF4α) promoter that ultimately resulted in reduced HNF4α expression (48), besides modifying the chromatin structure, including acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4, was detected at a significantly increased level at the GLUT4 promoter region in female pup muscle following a maternal LP diet (49). Microarray profiling indicated that mmu-miR-615, mmu-miR-124, mmu-miR-376b, and mmu-let-7e were significantly downregulated, while, mmu-miR-708 and mmu-miR-879 were upregulated in maternal low-protein diet offspring; bioinformatic analysis showed target genes were mapped to inflammatory-related pathways. Serum tumor necrosis factor-α (TNF-α) levels were higher and interleukin 6 (IL-6) had a tendency to be elevated (50).

Current Stage of Knowledge

Mismatched nutritional: maternal protein malnutrition and development of cardiometabolic diseases

Maternal malnutrition is associated with the risk of developing cardiovascular disease and co-morbidities in offspring's later life including hypertension, metabolic syndrome and type-II diabetes (51-53). Studies in humans have provided support for the positive association between low birth weight and increased incidence of hypertension (54-57).

Maternal low-protein diet model during both gestation and lactation is one of the most extensively studied animal models of phenotypic plasticity (58-63). Feeding a low-protein diet (8% protein) during gestation and lactation is associated with growth restriction, asymmetric reduction in organ growth, elevated systolic blood pressure, dyslipidaemia and increased fasting plasma insulin concentrations in the most of studies in rodents (58, 60, 62-68). However, it is known that the magnitude of the cardiovascular and metabolic outcomes are dependent on the both time exposure to protein restricted-diet (69, 70) and growth trajectory throughout the postnatal period (29, 71). A rapid and increased catch-up growth and childhood weight gain appear to augment metabolic disruption in end organs, for example liver (72, 73).

Although the relationship between maternal protein restriction, sympathetic overactivity and hypertension have been suggested (61, 74, 75), few studies have described the physiological dysfunctions responsible for producing these effects. Nowadays, it is well accepted that perinatal protein malnutrition raise risks of hypertension by mechanisms that include abnormal vascular function (75-77), altered nephron morphology and function, and stimulation of the renin-angiotensin system (52, 78). Recently, studies have highlighted contribution of the sympathetic overactivity associated to enhanced respiratory rhythm and O2/CO2 sensitivity on the development of the maternal low-protein diet-induced hypertension by mechanisms independent of the baroreflex function (19, 61, 67, 79-81). Offspring from dams subjected to perinatal protein restriction had relevant short-term effects on the carotid body (CB) sensitivity and respiratory control. With enhanced baseline sympathetic activity and amplified ventilatory and sympathetic responses to peripheral chemoreflex activation, prior to the establishment of hypertension (63, 80). The underlying mechanism involved in these effects seems to be linked with up-regulation of hypoxic inducible factor (HIF-1α) in CB peripheral chemoreceptors (80, 82-84). Rangel and colleagues (2014) demonstrated that the mean DNA methylation at individual islet cytosinet-guanine of the DNA (CpG) sites within the angiotensine enzyme converter (ACE) gene promoter was significantly lower in children with low birth weight (LBW) in the 6- to 12-year-old range. This alteration in methylation levels has been associated

to higher ACE activity and AP in LBW children (85). An analysis of fetal brain from rats exposed to protein deprivation during the second half of gestation showed an increase in ACE-1 mRNA associated with reduced methylation at several positions in the ACE-1 promoter region (86). This new evidence suggests how malnutrition may contribute to the high prevalence of hypertension (81).

The central nervous system (CNS) compared to other organ systems has increased vulnerability to reactive oxygen species (ROS). ROS are known to modulate the sympathetic activity and their increased production in key brainstem sites is involved in the etiologic of several cardiovascular diseases, for example, diseases caused by sympathetic overexcitation, such as neurogenic hypertension (Chan et al., 2006; Essick and Sam, 2010). Ferreira and colleagues showed that perinatal protein undernutrition increased lipid peroxidation and decreased the activity of several antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities) as well as elements of the GSH system, in adult brainstem. Dysfunction in the brainstem oxidative metabolism, using the same experimental model, were observed in rats immediately after weaning associated to the increase in ROS production, with a decrease in antioxidant defence and redox status and Mitochondrial dysfunction (87, 88) . Related to the metabolic effects on the heart, it was observed that these animals showed decreased mitochondrial oxidative phosphorylation capacity and increased ROS in the myocardium. In addition, maternal low-protein diet induced a significant decrease in enzymatic antioxidant capacity (superoxide dismutase, catalase, glutathione-S-transferase and glutathione reductase activities) and glutathione level when compared with normal protein group (89).

Maternal protein restriction affects the insulin sensitivity of the offspring. Previous studies found that in rats, maternal protein restriction throughout pregnancy and lactation, induced improved insulin sensitivity in the male offspring (90). iI the same way, Intra-uterine growth restriction (maternal diet, 8% protein) present central insulin resistance in adulthood (91). This central insulin resistance was associated with reduced protein levels of the p110β subunit of phosphoinositide 3-kinase (PI3K) and increased serine phosphorylation of IRS-1 in the arcuate nucleus (ARC) of the hypothalamus. Expression of the gene encoding protein tyrosine phosphatase 1B (PTP1B; Ptpn1) was also increased specifically in region of the hypothalamus (91); the mechanism appeared to be also increased insulin receptor signaling mediated by protein kinase C (PKC)-ζ in skeletal muscle in the offspring of dams that were protein restricted throughout pregnancy and lactation(92).

The ability of skeletal muscle to respond to maternal protein restriction is an

essential evolutionary adaptation to optimize the use of nutrients available during life-span, an important response in this process is the activation of genes that ameliorate or compensate for the protein deficit by stimulating the expression of glucose transporters, glycolytic and lipolytic enzymes that compensate the altered function of the mitochondria (93, 94). The induced persistent reductions in the transcriptional levels of PDK4, in skeletal muscle acute-chronic responses, suggest that persistent epigenetic modifications have occurred during fetal life persisting into adult life (95). Furthermore, these animals show energy wasting and minimal energy expenditure increased, due to both an increased mitochondrial function in skeletal muscle and an increased mitochondrial density in White Adipose Tissue (96).

Regarding hepatic metabolism, studies showed that protein restricted rats had suppressed gluconeogenesis by a mechanism primarily mediated by decrease on the mRNA level of hepatic phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme and enhancement of the insulin signals through the insulin receptor (IR)/IR substrate (IRS)/phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin complex 1 (mTOR) pathway in the liver (Toyoshima et., 2010). In relation to lipid metabolism, there was decreased liver triglyceride content in adult rats exposed to protein restriction during gestation and lactation. It was suggested that this effect could be due to increased fatty-acid transport into the mitochondrial matrix or alterations in triglyceride biosynthesis (Qasem et al., 2015); besides that blood cholesterol and triglycerides were higher in the adult offspring (at 110 days of age) fed a protein-restricted diet and reduced expression of the gene for glucokinase (GCK), the sensor of glucose in the liver. The protein restriction likely leads to inadequate detection of glucose levels (97).

A maternal protein restriction was shown to reduce the lean and increase the fat contents of six-month old offspring with a tendency for reduced number of muscles myofibers associated with reduced expression of mRNA of Insulin-like growth factor 2 gene (IGF2 mRNA) in pigs (Chavatte-Palmer et al., 2016). Consistent with protein restriction having an effect on lipid metabolism, maternal protein restriction resulted in smaller adipocytes in the fat tissues of offspring exposed to protein-restriction during pregnancy and lactation (98). Despite their smaller size, the insulin sensitivity of these adipocytes was impaired in association with reduced protein expression of key insulin signaling molecules (e.g., Akt) (98).

Mismatched nutritional: postnatal overnutrition on the development of cardiometabolic diseases

Nutritional transition is a phenomenon well documented in developing countries in the 20th and 21th centuries, and has induced high incidence of the chronic diseases and high prevalence of the obesity (99-101). It is evident that protein malnutrition was an health problem in the first half of the 20th century. Now, it was replaced by a diet enriched in saturated fat or other high fat diets, predisposing to overweight and obesity (102). Nowadays, it suggested that two billion people in the world are overweight and obese individuals, with major prevalence is related to diet induced-obesity, which have been associated to cardiovascular and endocrine dysfunctions (103-107).

In the late 1980s to 1990s, Barker and colleagues provided epidemiologic evidence of the programming of offspring metabolic syndrome, demonstrating that low birth weight was a significant predictor of adult obesity, diabetes, and cardiovascular disease, for promoting changes in the fetal environment, which can trigger genetic alterations and reflect on the maturation of fetal organs and systems (4, 5). Previous studies have shown that high-fat diet significantly increased weights of malnourished rats in gestation and/or lactation, increased body fat, reduced lean body mass, and accentuated plasma leptin, increase in glucose levels with increased insulin levels and hypertriglyceridemia in male rats (108).

Similarly, study has reported on the interaction between maternal diet and amplification by a post-weaning high-fat diet, and demonstrated that profound adult hyperphagia is a consequence of fetal programming and a key contributing factor in adult pathophysiology (109). Maternal protein restriction (8% casein) during gestation and lactation followed by a postweaning HF diet (41% fat) induced an increased percentage of visceral fat, reduced insulin sensitivity and increased food intake in adult offspring (110). At 60 d of age, the interaction between maternal LP and post-weaning HF diet showed double body weight gain and increased retroperitoneal and epididymal fat depots, even with decrease food intake in pups fed a post-weaning HF diet (111). Continuous intake of a HF diet can also promote hypertrophy and dysfunction of adipocytes, this may induce infiltration of pro-inflammatory macrophages in adipose tissue, enhancing the production of pro-inflammatory cytokines in this tissue (112). Characterizing a physiological state of chronic inflammation, present in obese individuals (104, 113, 114), besides increased NO production by macrophages (111). An elevated NO production has been implicated in macrophage death by apoptosis (115) and in the pathogenesis of inflammatory diseases (116), suggesting that there exists an immunomodulation related to dietary fatty acids after the maternal LP diet-induced metabolic programming.

Within an obesogenic environment, the maternal prenatal undernutrition it seems to modify programs the long-term offspring adipose tissue gene expression profile in a depot-specific manner that may predispose prenatally undernourished individuals to altered lipid metabolism and fat accumulation in adult life. At 120 days, undernourished rats during gestation and lactation submitted to the obesogenic diet showed a catch-up growth to match the body weight of adult control HF animals, suggesting an increase in adiposity while showing hyperleptinemia, the elevated serum leptin concentration suggests an increase of adiposity. Furthermore, unlike hypothalamus, gonadal white adipose tissues under HF diet is a very sensitive target of maternal prenatal undernutrition programming (117). This reinforces the idea of the existence of a brain sparing effect under suboptimal nutritional conditions (118).

In liver, wistar rats maternal protein restriction (8% casein) during gestation and lactation followed by a postweaning HF diet (+59% SFA) in 90 days of life induced reduction in all respiratory states of hepatic mitochondria, presented greater mitochondrial swelling compared to controls, potentiated after Ca2+ addition and prevented in the presence of EGTA (calcium chelator) and cyclosporin A (mitochondrial permeability transition pore inhibitor), increase in liver protein oxidation and lipid peroxidation so with reduction in catalase and glutathione peroxidase activities. Besides that, increase the voltage-dependent anion channel 1 (VDAC1) transcription. This data suggest that adult rats subjected to maternal protein restriction were more susceptible to hepatic mitochondrial damage caused by a diet rich in saturated fatty acids post-weaning (119). In sheep, the HCHF diet post-natal increased hepatic triglyceride content in lambs, associated with down-regulated expressions of energy-metabolism-related genes (GLUT1, PPARα, SREBP1c, PEPCK) (120).

Conclusion

The incompatibility between the nutritional status of the perinatal and postnatal environment induces accelerated recovery growth and adjustments in autonomic modulation, insulin sensitivity as well as mitochondrial disfunction. A post-weaning high caloric / high fat diets potentiate these adjustments, exacerbating deleterious changes in important metabolic organs: hepatic, adipose and muscular tissue. The epigenetic repercussions of postnatal metabolic overload may be etiological sources of cardiometabolic diseases, which affect the subjects affected by the nutritional transition.

Author contributions

ACSA, APFACM, LP and JHCS drafted the work and revised critically for the important intellectual content; wrote the paper; final review of the manuscript.

Conflict of Interest Statement:

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APÊNDICE B – ARTIGO 3

ARTIGO 3 - CONSUMPTION OF A HIGH-FAT DIET DOES NOT POTENTIATE THE DELETERIOUS EFFECTS ON LIPID AND PROTEIN LEVELS AND BODY DEVELOPMENT IN RATS SUBJECTED TO MATERNAL PROTEIN RESTRICTION.

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DR. ALICE VALENÇA ARAÚJO (Orcid ID : 0000-0002-9728-8033)

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Consumption of a high-fat diet does not potentiate the deleterious effects on lipid and protein levels and body development in rats subjected to maternal protein restriction.

Sávio dos Santos Barbosa[1], Ana Paula da Fonseca Arcoverde Cabral de Mello[1], Viviane de Oliveira Nogueira Souza[1], Ially Fabiane da Silva[1], Palloma Emanuelle Dornelas de Melo[1], Carlos Renato dos Santos[2], João Henrique da Costa-Silva[1], Alice Valença Araújo[2*]

[1] Nucleus of Physical Education and Sport Sciences,

[2] Nucleus of Public Health, Centro Acadêmico de Vitória, Universidade Federal de Pernambuco (CAV/UFPE), Vitória de Santo Antão, PE, Brazil.

*** Correspondence:**
Alice Valença Araújo

Address: Universidade Federal de Pernambuco – Centro Acadêmico de Vitória, Rua Alto do Reservatório, s/n, Bela Vista, CEP: 55608-680, Vitória de Santo Antão-PE, Brazil; +55 81 4114 4101; alice.araujo@ufpe.br

Keywords: high-fat diet$_1$, protein undernutrition$_2$, ventilatory parameters$_3$, biochemical parameters$_4$, maternal diet$_5$, body patterning$_6$

Abstract

Maternal undernutrition may cause injuries in several organs of the offspring, as well as lead to diseases in adulthood. Obesity and/or the consumption of a high-fat diet may also induce metabolic and cardiorespiratory diseases. We hypothesized that the consumption of a post-weaning high-fat diet would potentiate the deleterious effects of maternal protein undernutrition. This study evaluated the effects of the association of a low-protein diet during gestation and lactation with a post-weaning high-fat diet on the biochemical and ventilatory parameters of rats. Male Wistar rats from mothers who received a low-protein (9% of protein) or normoprotein diet during pregnancy and lactation received a high-fat (32% of total kilocalories from lipids) or a normal fat diet after weaning. Mass gain and somatic growth of the offspring were monitored. Also examined were biochemical chemical parameters and respiratory frequency, tidal volume (volume of air displaced in each normal respiratory cycle when extra effort is not applied), and pulmonary ventilation. Offspring from undernourished mothers presented lower birth weight ($p=0.0225$), which remained until the end of lactation ($p<0.01$). The rats that consumed high-fat diet and had been submitted to maternal undernutrition presented higher tidal volume when compared to the ones that consumed control diet at the 21st day of life ($p<0.05$). At 30 and 90 days, no further ventilatory changes were observed. Our data show that the consumption of a high-fat diet post weaning did not seem to potentiate the changes induced by maternal undernutrition.

Introduction

The study of undernutrition is important because it is a problem that continues to affect thousands of people worldwide (PATIL, DIVYARANI 2015; DAS, GULSHAN 2017). There is a specific period in which the occurrence of undernutrition leads to more severe damage, known as

the critical period of development. This period includes the intrauterine and lactation phases (MORGANE *et al.* 1993). It is known that the occurrence of maternal undernutrition is related to injuries in some of the organs of their offspring in adulthood (SENE *et al.* 2013; VIEIRA-FILHO *et al.* 2014; TARRY-ADKINS *et al.* 2016). Undernutrition (9%) was observed in a food consumption survey in the State of Pernambuco, in the Northeast Brazil, where the present study was conducted (TEODOSIO *et al.* 1990). The diet consumed by this human population was used as an experimental model of malnutrition (PAIXÃO *et al.* 2001; MAGALHÃES *et al.* 2006).

Obesity is another phenomenon that affects an increasing number of people worldwide. In 2014, it was estimated that around the world more than 600 million adults and about 41 million children under five years old have this condition (WHO 2018). The increase in obesity cases is largely due to changes in eating patterns, known as nutritional transition (BATISTA FILHO, BATISTA 2010). This phenomenon began in Brazil in the early 1970s, when cases of undernutrition began to be progressively reduced and, concomitantly, there were increases in the levels of overweight/obesity (BATISTA FILHO, RISSIN 2003).

Currently, the simultaneous occurrence of undernutrition and obesity, known as the Dual Burden, is a unique and complex challenge for public health. A large recent study has described trends in the national dual burden in children under five years in low- and middle-income countries (TZIOUMIS *et al.*, 2016). Recently, a well-documented study of a phenomenon called "nutrition transition" has been published (POPKIN, ADAIR, NG, 2012). In many countries, protein undernutrition has been replaced by a diet enriched in fat, predisposing children to overweight/obesity and cardiometabolic consequences in adulthood (BATISTA FILHO, RISSIN 2003; BATISTA FILHO, BATISTA 2010; BATISTA, RIBEIRO *et al.*, 2013; RIBEIRO, LIMA *et al.*, 2015). Although the cardiometabolic effects of the different high-fat diets have been well described *in utero*, child- and adulthood, little data is available about the impact of high-fat diets on the lipid and protein blood levels and body development in animals previously subjected to perinatal low protein diet.

Evidence identifying changes in rats that underwent periods of maternal protein undernutrition and consumed a high-fat diet after weaning has been published (SOUZA-MELLO, MANDARIN-DE-LACERDA, AGUILA, 2007; CLAYCOMBE *et al.* 2016; RIZZI *et al.* 2017). Body composition, renal and hepatic morphology and function, blood pressure and blood

biochemistry were evaluated. However, there are no studies showing evidences related to other parameters, like pulmonary ventilation, opening, thus, a large field for new studies. Impairments in lung structure and function have already been identified in models of maternal protein undernutrition and intrauterine growth restriction (KARADAG *et al.,* 2009; DE BRITO ALVES*, et al.,* 2014), but not in association to a high-fat diet. Souza-Mello *et al.* (SOUZA-MELLO, MANDARIN-DE-LACERDA, AGUILA, 2007) were the only studies found that evaluated blood biochemistry; this was in 6-month-old rats. We set out to evaluate some of these parameters in 90-day-old rats. The expectation is that our study, when associated with that of Souza-Mello *et al.*, will help to understand the behavior of blood biochemistry over the life of undernourished animals that have consumed a high-fat diet. Thus, the aim of the present study was to evaluate the effects of the association of a low-protein diet during gestation and lactation with a post-weaning high-fat diet on the biochemical and ventilatory parameters of rats.

Results

During the pregnancy period, the mothers in both groups exhibited similar increase in body mass (**Figure 1A**), with no changes in food or water consumption (**Figure 1C and D**). During the last week of lactation, undernourished mothers lost weight (Normal protein (**NP**): 254.0±9.35 g vs. Low Protein (**LP**): 231.3±5.71 g, p=0.0381, n=6, **Figure 1B**). There was lower food intake in the second week of lactation in the LP group (**NP**: 40.59±1.59 vs. **LP**: 29.9±3,22, p=0.0087, n=6, **Figure 1E**), but no changes in water consumption (**Figure 1F**). The consumption of each macronutrient is shown in Table 2.

The offspring of undernourished mothers presented lower body mass 24 hours after birth when compared to control animals (**NP**: 6.95±0.17 vs. **LP**: 6.47±0.12, p=0.0225, n=22 and 26 respectively, **Figure 2A**). Body mass remained lower in the offspring of undernourished mothers throughout the lactation period (7th day: **NP**: 15.96±0.38 vs. **LP**: 14.04±0.29, p<0.001; 14th day: **NP**: 30.54±0.76 vs. **LP**: 23.27±0.54, p<0.001; 21st day: **NP**: 49.2±0.52 vs. **LP**: 33.29±1.06, p<0.001; n=22 and 26 respectively, **Figure 2A**). The naso-anal length was shorter 24 hours after birth and remained so until the end of lactation, except on the seventh day of life (**Table 3**). The caudal length was shorter in the undernourished animals only in the fourteenth day of life (**Table 3**) and the abdominal circumference was smaller throughout lactation, except on the first day after birth (**Table 3**).

At the end of lactation, the offspring from the control and undernourished groups were divided into normo-fat or high-fat diet, thus forming four groups (NPNF, NPHF, LPNF and LPHF). At this moment (week 3 of life), the groups of undernourished mothers presented lower values of body mass when compared to the animals of control mothers (Normal protein and Normal Fat (**NPNF**): 48.71±0.82 vs. Low Protein and Normal Fat (**LPNF**): 32.58±1.67, $p<0.001$, n=12 and 14 respectively; and Normal Protein and High Fat (**NPHF**): 49.64±0.73 vs. Low Protein and High Fat (**LPHF**): 34.01±1.26, $p<0.001$, n=10 and 12 respectively, **Figure 2B**). However, animals from NPHF and NPNF groups and from LPNF and LPHF groups did not present differences in body mass when compared with each other (**NPHF**: 49.64 ± 0.8 vs. **NPNF**: 48.71 ± 0.8, $p>0.05$, n= 10 and 12 respectively; and **LPNF**: 32.58 ± 1.67 vs. **LPHF**: 34.01 ± 1.26, $p>0.05$, n=14 and 12 respectively, **Figure 2B**). After weaning, the consumption of a control diet by the offspring of the undernourished animals, body mass remained lower until the 10th week of life, except for the 8th and 9rd weeks (**Figure 2B**). When the offspring began to consume high-fat diet, the difference in body mass between the two groups remained until the 6th week of life (**NPHF**: 130.1 ± 3.0 vs. **LPHF**: 109.2 ± 4.1, $p<0.001$, n=10 and 12 respectively, **Figure 2B**). At 11 weeks of age, there were no differences in body mass among the groups (**Figure 2B**). Nor were differences evident judging by their adiposity indices (**NPNF**: 5.95 ± 0.3, **NPHF**: 6.34 ± 0.4, **LPNF**: 5.1 ± 0.6, **LPHF**: 6.5 ± 0.6, $p>0.05$, n= 13, 10, 12 and 11 respectively).

Regarding somatic growth after weaning, the naso-anal length and caudal length of the LPNF group were shorter at week 4 of life and remained so until the 9th and 10th weeks, respectively (**Table 4**) when compared to the NPNF group. Regarding LPHF group, the NPHF group had a longer caudal length from 4th to 10th week, except for the 5th week (**Table 4**). Regarding abdominal circumference, a statistically significant difference was observed only in the 4th week of life, between the NPHF and LPHF groups (**Table 4**).

The food intake after weaning did not differ among groups at any of the evaluated times (**Figure 3A**). There were also no differences in water consumption, except for the first week after weaning, when it was higher in the LPNF group when compared to the LPHF group (**LPNF**: 23.44±3.01 vs. **LPHF**: 12,67±0.32, $p<0.05$, n=6 and 5 respectively, **Figure 3B**). For the analysis, both food and water consumption were registered on a weekly basis.

At the end of lactation (21 days), considering the final formation of the groups (NPNF, NPHF, LPNF and LPHF), the animals from undernourished mothers had lower serum levels of albumin when compared to NPNF (NPNF±0.2 g/dL; NPHF 3.3±0.2 g/dL; LPNF 2.9±0.2 g/dL $p<0.05$; LPHF 2.9±0.1g/dL $p<0.05$). The levels of total protein were lower in LPHF (5.0±0.2 g/dL) when compared to NPHF (5.7±0.3 g/dL, $p<0.05$). Both differences had disappeared by 30 days and after. The levels of triglycerides were also lower in the LPHF on the 30th day (159.2±22.0 mg/dL), when compared to NPHF (30th: 175.27.0), but the difference had disappeared by the 90th day of life. The serum levels of cholesterol, Glutamic-Oxaloacetic Transaminase (GOT), Glutamic Pyruvic transaminase (GPT), as well as fasting blood glucose showed no differences (data not shown).

Taking into account the respiratory parameters evaluated at the end of lactation (21st day), the respiratory frequency was not altered in any group, as well as the respiratpory volume (**Figure 4A and G)**. It was observed that the LPNF group presented higher TV when compared to the NPNF group (**NPNF**: 11.49±0.87 vs. **LPNF**: 15.76±1.08, $p<0.05$, n=13 and 20 respectively, **Figure 4D**). After the consumption of a high-fat or control diet for nine days (from the 21st to the 30th days) or 69 days (from the 21st to the 90^{the} days of life), no difference was observed among all variables analyzed when the groups were compared (**Figure 4B, E and H for 30 days and C, F and I for 90 days**).

There was no statistically significant difference for organ masses (heart, liver, lung, spleen, kidneys, brainstem, hypothalamus and soleus and extensor digitorum longus muscles). When these were adjusted for the animals' weight, the mass of the kidneys was higher in the LPHF, compared to the LPNF (**LPHF**: 0.37±0.02 vs. **LPNF**: 0.31±0.01, $p<0.05$, n=11 and 12 respectively for right kidney; **LPHF**: 0.35±0.02 vs. **LPNF**: 0.28±0.03, $p<0.05$, n=11 and 12 respectively for Left kidney). The adjusted mass for the other organs did not shown difference (data not shown). Moreover, there was no difference among the groups in Fat Percentage, Fat Free Mass, Visceral Adipose Tissue, Subcutaneous Adipose Tissue, Epididymal Adipose Tissue or Retroperitoneal Adipose Tissue (absolute and adjusted masses (data not shown).

Discussion

Understanding the impact of a high-fat diet on the model of maternal protein undernutrition is extremely important for metabolic programming (BARKER 1994). This phenomenon states that individuals who have suffered insults during a critical period of development may have persistent and long lasting effects on the structure and function of their organism (LUCAS 1998), making them more susceptible to damage from insults in the after-weaning period. Although there are many studies showing the effects of a low-protein diet on gestation and lactation or of a high-fat diet during adulthood, our study is the first to show the influence of both types of diets on the respiratory system in a nutritional transition model.

During the pregnancy period, the consumption of food and water and mass gain were not different between the groups. This response has also been observed in other studies (RODFORD *et al.* 2008; TAKEMORI *et al.* 2013) and may be related to the fact that diets are isocaloric. From the second week of lactation, there was a lower consumption of food by LP mothers in our study. It was observed that as the offspring grew larger and older, they started to eat the food, besides take breast milk. Since the offspring of the LP group were smaller, they had more difficulty reaching the food. Therefore, the consumption of the food by the NP may reflect the consumption of the mother and the offspring; and the consumption of the LP reflects mainly the consumption of the mother.

We observed that the offspring of LP presented lower birth weight (24 hours after birth) and this remained true throughout the lactation period. In fact, the low protein diet during gestation has been used as an experimental model of intrauterine growth restriction (MENENDEZ-CASTRO *et al.* 2012; FALCÃO-TEBAS *et al.* 2012; TAKEMORI *et al.* 2013). The diminished somatic growth presented by LP also reflects the change generated by maternal protein undernutrition and agrees with the study of FALCÃO-TEBAS (2012).

All these changes reflect an important phenomenon known as Catch-up Growth, a process that follows a period of growth failure whereby the specific growth rate increases above the rate that would have been expected (GRIFFIN 2015). There are some models to explain this condition but the neuroendocrine hypothesis is probably the most popular and had been tested with rodents, pigs and fish (HEYER, LEBRET 2006; WON, BORSKI 2013; FERNANDEZ-FEIJOO *et al.* 2017). This hypothesis is based on a period of augmented hormone activity (GRIFFIN 2015),

198 leading to a later catch-up growth to compensate for the poor growth period (WON, BORSKI
199 2013).

200 The lower relative renal mass presented by undernourished rats is a response that has been
201 previously observed in a model of protein undernutrition (COSTA-SILVA *et al.* 2009). However,
202 in our study, the NPHF animals maintained renal values similar to the other groups, except when
203 compared to LPNF which had a lower renal weight. Given that some studies that used a low-
204 protein diet (MORGANE *et al.* 1993; COSTA-SILVA *et al.* 2009) or high-fat diet (EBENEZER *et*
205 *al.* 2009; PINHAL *et al.* 2013) showed detrimental alterations in the kidneys, a more detailed
206 evaluation of this organ in the undernourished animals that consumed a high-fat diet is necessary
207 for a better understanding of how the combination of these environmental insults can induce renal
208 modifications.

209 At the end of lactation, NP had a reduction in total protein and albumin values. Castro *et*
210 *al.* (CASTRO *et al.* 2009) also found this response and stated that serum proteins may reflect the
211 level of protein intake. In fact, some authors (GEURSEN, CARNE 1987; PASSOS *et al.* 2000)
212 showed that the consumption of a low protein diet during lactation reduced the synthesis and
213 secretion of α-lactalbumin by the mammary glands, leading to a decrease in protein concentrations
214 in breast milk. In the present study, at 30 days of age, these changes were not observed anymore.
215 Corroborating with what was stated by Castro *et al.* (CASTRO *et al.* 2009), undernourished
216 animals, in our study, since they had been fed a diet containing appropriate levels of protein (for at
217 least nine days), presented normalized total protein and albumin values.

218 At 30 days, the animals of the LPNF group presented increased triglyceride when
219 compared to the LPHF and NPNF groups. Zhu *et al*. (ZHU *et al*. 2016) also identified an increase
220 in the level of hepatic triglyceride in undernourished animals in response to maternal protein
221 undernutrition during pregnancy. In that study, the gene lipase expression of hepatic lipoprotein
222 increased, which could explain the augmented triglycerides, since lipoprotein lipase promotes an
223 increase in the phosphorylation of perilipin in adipose tissue, leading to a greater release of
224 triglycerides into the bloodstream. At 90 days of life, there was no difference in the serum
225 triglyceride between the groups, indicating that, at long term, a normal diet is enough to normalize
226 the serum triglyceride in offspring of undernourished mothers. This benefit may be related to the
227 polyunsaturated and/or monounsaturated fatty acids, which could have a reducing effect on

triglyceride (KRIS-ETHERTON, YU 1997) or triglycerides and HDL cholesterol (SANTOS *et al.* 2013), respectively. Taking into account that this difference among the groups in our study disappeared, the reducing effect of the polyunsaturated and/or monounsaturated fatty acids could have been dependent on a previous alteration in the triglyceride.

Although the diet used in this study was high-fat, its proportion of fatty acids does not seem to have adversely affected serum triglyceride values, nor even cholesterol, after 69 days of consumption.

Despite the difference between NPNF and LPNF in the TV on 21st day of life, no further modification was observed in ventilatory parameters, suggesting that the high-fat diet used in our study was not able to impair ventilation and respiratory rhythm in this experimental model.

Therefore, the findings of the present study confirm the changes observed in a model of low-protein diet during gestation and lactation and show that the consumption of a high-fat diet after the weaning did not further affect the analyzed parameters. Instead, some changes observed during lactation disappeared.

Methods

Animals

The experimental protocols were approved by the Animal Ethics Committee (23076.044287 / 2015-96) of the Federal University of Pernambuco, Brazil.

Wistar rats, provided from the Centro Acadêmico de Vitória (CAV-UFPE), were used. The animals were maintained in a room with controlled temperature (22 ± 1ºC), light-dark cycle (dark: 18:00 - 06:00 hours) and diet (52% carbohydrates, 21% protein and 4% lipids - Labina, Purina Agribands) and water *ad libitum*. Initially, the rats were placed together for up to a week for mating (one male for three nulliparous female rats). Confirmation of pregnancy was made by the presence of spermatozoa in the vaginal smear, which was considered the first day of gestation.

Diets

After the identification of the pregnancy by the vaginal smear, the rats were transferred to individual cages, where they started to receive a normoprotein or a low protein diet that was

255 continued until the end of lactation. The size of the litter and sex of the pups were determined 24
256 hours after birth. Each litter was reduced to eight, in order to reduce the competition for breast
257 milk, so as not to lead to a compromise in the development of offspring. The males were
258 prioritized and, when necessary, females were used to make up the groups. The male offspring
259 were placed in the control (NP-NF and LP-NF) groups or high-fat diet groups (NP-HF and LP-
260 HF) immediately after weaning (22nd day of life) until 90 days of life.

261 The diets were made in the Experimental Nutrition Laboratory - Department of Nutrition,
262 UFPE, according to the American Institute of Nutrition - (AIN-93) (REEVES *et al.* 1993)
263 (normoprotein and low protein) and Family budget survey (IBGE / Brazil, SFB 2002/2003) (high-
264 fat), as previously described (CARVALHO *et al.* 2013) (FERRO CAVALCANTE *et al.* 2013),
265 with minor modifications. Composition of diets is presented in Table 1. Regarding fatty acid
266 composition (saturated, SFA; monounsaturated, MUFA; and polyunsaturated, PUFA), the diets
267 were as follows: normo- and low protein diets (SFA 17.3%, MUFA 29.9% and PUFA 52.8%); and
268 high-fat diet (SFA 29.6%, MUFA 34.8% and PUFA 35.6%). The latter was classified as high-fat
269 because the percentage of lipids was higher than that recommended by the AIN-93 (16.7%)
270 (REEVES *et al.* 1993).

271 **Evaluation of food and water intake by mothers and offspring**

272 Food and water intake were measured during pregnancy and lactation (maternal
273 consumption) and after weaning (offspring consumption). Food consumption was calculated from
274 the difference between the offered diet and what remained after 24 hours, registered in kcal
275 (determined by multiplying the amount of diet ingested by the calories contained in each 100g of
276 diet).

277 The water intake was calculated by the difference between the offered amount of water and
278 what was left after 24 hours.

279 **Evaluation of body mass of mothers and offspring and somatic growth of offspring**

280 The mothers' body mass was evaluated daily after determination of pregnancy and
281 continued to be evaluated daily until the end of lactation. Body mass of the offspring was
282 evaluated daily after birth and continued so until the end of lactation. After this period, body mass

283 was registered weekly and continued weekly until the end of the study (90 days of life). The naso-
284 anal and caudal lengths and abdominal circumference of the offspring were collected weekly,
285 from the first 24 hours of life until the final of the study, by using a 0.1 cm precision tape measure.

286 **Biochemical analyses**

287 At 21, 30 and 90 days of age, animals of all groups were fasted for 12 hours with water *ad*
288 *libitum* and then, blood samples were collected by retro-orbital plexus puncture using a non-
289 heparinized glass capillary tube. After coagulation, the serum was obtained and stored at -20 ° C.
290 The biochemical analyses were carried out by spectrophotometry using the respective reagent and
291 standard kits (Labtest Diagnóstica SA, MG, Brazil). Quantifications of total proteins, albumin,
292 fasting blood glucose, triglycerides and total cholesterol were made.

293 **Determination of organ mass and body adiposity**

294 At 90 days of age, the animals were sacrificed by decapitation and the following organs
295 were removed and weighed: heart, spleen, liver, kidneys (right and left), lung and adipose tissue.
296 Total body fat was measured by the sum of subcutaneous, epididymal, retroperitoneal and
297 mesenteric fats. The adiposity index was calculated according to the following formula:

298 Adiposity index = (total body fat / body mass) x 100 (MEDEI *et al.* 2010).

299 **Measures of pulmonary ventilation**

300 Ventilation measurements were obtained by whole-body plethysmography in a closed
301 system (MALAN 1973) and were made on the 21st, 30th and 90th days of life. First, the animals
302 were adapted to the recording chamber (acrylic chamber with volume of 729 ml for 21st and 5L
303 for 30th and 90th) for a variable period of time (≈30 min). This chamber was ventilated with
304 ambient air during the acclimatization period. During each ventilation measurement, airflow was
305 interrupted and the chamber with the animal remained fully sealed for short periods of time (≈ 2
306 min). The oscillations caused by the ventilation of the animal were captured by a device connected
307 to the chamber containing a pressure differential transducer and a signal amplifier (ML141
308 spirometer, PowerLab, ADInstruments). The signal was then sent to the data acquisition and
309 analysis system (LabChartTM Pro, PowerLab, ADInstruments). The calibration of the volume was
310 obtained during each experiment, injecting a known volume of air (1 mL) into the chamber with

the use of a syringe. Three respiratory variables were measured: respiratory frequency (RF), tidal volume (VT) and pulmonary ventilation (VE).

Data analysis

The results were expressed as mean ± MSE (mean standard error). Normality analysis of the sample was performed using the Shapiro-Wilk test (SHAPIRO, WILK 1965). Group results during gestation and lactation, as well as mass, somatic growth and ventilation of the offspring (during lactation) were compared using the unpaired Student t-test (STUDENT 1908) or the Mann-Whitney test (MANN, WHITNEY 1947) (according to the normality presented by the data). For comparisons of the biochemical and ventilation data (after weaning) and organs weight, One-Way ANOVA were used; this was followed, when necessary, by the Bonferroni posttest or the Kruskal-Wallis test (HECKE 2012); and this was followed, when necessary, by Dunn's posttest (according to the normality presented by the data). Data were analyzed by the statistical program GraphPad Prism (GraphPad Software Corporation, version 5.0, 2007) and R (version 3.3.2, using base and PMCMR packages) (R Core Team, 2017). The level of significance was considered when $p \leq 0.05$.

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TABLE 1. Composition of diets. Adapted from Cavalcante *et. al.* (2013). **Source:** The high-fat diet was adapted from the 2002/2003 Family Budget Survey (POF); The normoprotein diet followed the recommendations of Reeves, Nielsen and Fahey Jr, 1993. The centesimal composition was analyzed in the Laboratory of Bromatology / UFPE.

Ingreditents	**Diets**			**Nutrients**	**Diets**		
	Normoprotein (Control)	**Low Protein**	**High-Fat**		**Normoprotein (Control)**	**Low Protein**	**High-Fat**
	g/100	g/100	g/100		g/100	g/100	g/100
Corn starch	52.9	63.54	15.0	Protein	17.5	9.4	22.8
Wheat flour	-	-	12.0	Animal Protein	17.0	7.0	16.0
Biscuits Cornstarch	-	-	5.0	Vegetable Protein	0.3	0.3	4.8
Soy Flour	-	-	6.0	Lipid	6.5	10.8	15.7
Gum	-	-	0.5	Carbohydrate	59.7	71.1	52.3
Lard	-	-	5.3	Fiber (Cellulose)	5.0	5.0	5.0
Margarine (65 % lipid)	-	-	4.0	Mineral Mix	3.5	3.5	3.5
Cream milk (20 % lipid)	-	-	4.0	Vitamin Mix	1.0	1.0	1.0
Casein protein (85 %)	20.0	9.41	20.0	DL-Methionine	0.3	0.3	0.3
Sucrose	10.0	10.0	18.0	Choline Bitartrate	0.25	0.25	0.25

Soybean oil	7.0	7.0	4.0	BHT	0.0014	0.0014	0.014
Fiber (cellulose)	5.0	5.0	2.0	**Composition according to total energy (%)**			
Mineral mix	3.5	3.5	2.5	Protein	19.1	9.0	20.7
Vitamin Mix	1.0	1.0	0.7	Lipid	15.9	23.1	31.9
DL-Methionine	0.3	0.3	0.25	Carbohydrate	65.0	67.9	47.4
Choline bitartrate	0.25	0.25	0.25				
BHT	0.0014	0.0014	0.014				
Sodium Chloride (NaCl)	-	-	0.3				
Monosodium Glutamate	-	-	0.2				
Total	100.0	100.0	100.0				
Total energy (Kcal/g)	3.6	3.6	4.2				

TABLE 2. Total consumption of each macronutrient. The calculation was made from the sum of all diet consumed during gestation and lactation (mothers' consumption); and from the 22nd day of life (after weaning) until the end of the study (offspring's consumption). The total diet consumed represents the sum of the macro and micronutrients plus the fibers (the composition of the diets is described in table 1).

	Total consumption of each macronutrient (g)			
	Mothers			
	Gestation		**Lactation**	
	NP	**LP**	**NP**	**LP**
Total consumed	348.4 **[1279.6 kcal]**	351.1 **[1431.7 kcal]**	789.5 **[2899.9 kcal]**	629.3 **[2638.1 kcal]**
Protein	61.0 **[243.9 kcal]**	33.0 **[132 kcal]**	138.2 **[552.7 kcal]**	59.2 **[236.6 kcal]**
Carbohydrate	208.0 **[831.9 kcal]**	249.6 **[998.4 kcal]**	471.3 **[1885.4 kcal]**	447.4 **[1789.8 kcal]**
Fat	22.6 **[203.8 kcal]**	37.9 **[341.2 kcal]**	51.3 **[461.9 kcal]**	68.0 **[611.7 kcal]**
	Offspring			
	NPNF	**NPHF**	**LPNF**	**LPHF**
Total consumed	1197.6 **[4398.7 kcal]**	1111.8 **[4911.0 kcal]**	1092.4 **[4012.5 kcal]**	991.6 **[4379.8 kcal]**
Protein	209.6 **[838.3 kcal]**	253.5 **[1014.0 kcal]**	191.2 **[764.7 kcal]**	226.1 **[904.3 kcal]**
Carbohydrate	715.0 **[2859.8 kcal]**	581.5 **[2326.0 kcal]**	652.2 **[2608.7 kcal]**	518.6 **[2074.4 kcal]**
Fat	77.8 **[700.6 kcal]**	174.6 **[1571.0 kcal]**	71.0 **[639.1 kcal]**	155.7 **[1401.1 kcal]**

NP: Normoprotein; **LP**: Low protein; **NPNF**: Normoprotein and normofat; **NPHF**: Normoprotein and high fat; **LPNF**: low protein and normofat; **LPHF**: low protein and high fat.

TABLE 3. Measurements of naso-anal length (NAL), caudal length (CL) and abdominal circumference (AC) during lactation. Somatic growth measurements were performed 24 hours after birth and each week after, in the offspring from mothers that consumed a normoprotein (NP, 17% protein, n =22) or low protein diet (LP, 8% protein, n =26) during gestation and lactation. *Statistical difference ($p<0.05$) when compared to the NP group (control), assessed by Mann-Whitney test.

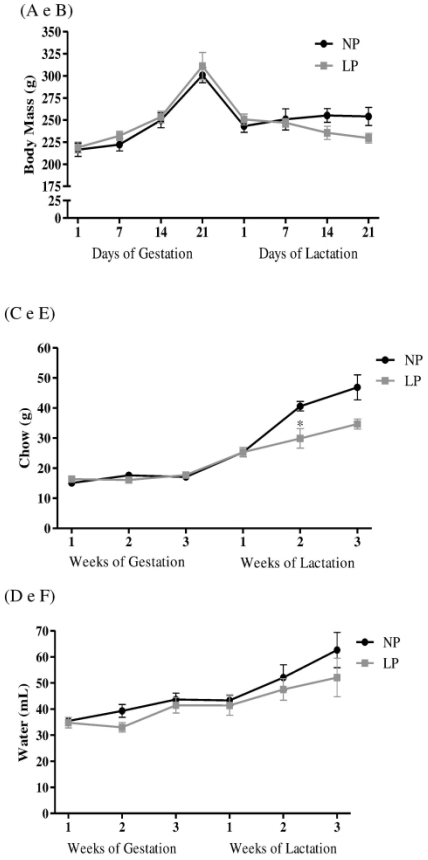
LACTATION

OBSERVED DAY	NAL (cm)		CL (cm)		AC (cm)	
	NP	**LP**	**NP**	**LP**	**NP**	**LP**
1	5.18±0.07	5.06±0.06*	1.72±0.03	1.69±0.03	4.67±0.1	4.63±0.06
7	7.17±0.06	7.07±0.05	3.32±0.06	3.25±0.05	6.13±0.08	5.77±0.12*
14	8.93±0.1	8.48±0.12*	5.41±0.18	4.92±0.07*	7.94±0.19	6.95±0.18*
21	11.08±0.22	10.4±0.1*	7.14±0.22	6.95±0.18	8.77±0.18	8.18±0.13*

TABLE 4. Measurements of naso-anal length (NAL), caudal length (CL) and abdominal circumference (AC) after weaning. Somatic growth measurements in the NPNF, LPNF, NPHF and LPHF offspring **A** - Statistical difference when compared to NPNF group; **B** - Statistical difference when compared to NPHF group. Kruskal-Wallis was used to compare the difference between groups and was followed, when necessary, by the Dunn posttest ($p < 0.05$).

	POST WEANING											
OBSERVED DAY	**NAL (cm)**				**CL (cm)**				**AC (cm)**			
	NPNF	**NPHF**	**LPNF**	**LPHF**	**NPNF**	**NPHF**	**LPNF**	**LPHF**	**NPNF**	**NPHF**	**LPNF**	**LPHF**
Week 3	11.1±0.3	11.0±0.3	10.4±0.2	10.4±0.2	7.1±0.3	7.2±0.4	7.2±0.1	6.8±0.3	8.6±0.3	8.9±0.1	8.1±0.3	8.2±0.2
Week 4	14.0±0.2	14.0±0.2	**12.6±0.4^{A}**	13.3±0.1	10.0±0.2	10.8±0.3	**8.3±0.2^{A}**	**8.5±0.1^{B}**	10.4±0.2	11.5±0.4	9.4±0.2	**9.9±0.2^{B}**
Week 5	16.8±0.2	16.1±0.3	**14.3±0.3^{A}**	14.2±0.5	12.5±0.3	12.7±0.3	**11.0±0.3^{A}**	11.2±0.3	12.3±0.2	12.3±0.1	12.0±0.4	12.5±0.4
Week 6	19.0±0.3	18.2±0.2	**17.2±0.3^{A}**	17.7±0.1	14.9±0.2	14.9±0.2	**13.3±0.3^{A}**	**13.6±0.2^{B}**	13.8±0.1	14.4±0.3	13.5±0.3	14.3±0.5
Week 7	20.5±0.1	20.0±0.3	**19.0±0.1^{A}**	18.8±0.3	16.8±0.2	16.6±0.2	**15.4±0.2^{A}**	**15.1±0.2^{B}**	14.4±0.3	15.1±0.3	14.2±0.2	14.5±0.2
Week 8	21.9±0.4	20.9±0.3	**19.9±0.2^{A}**	20.3±0.4	17.9±0.2	17.8±0.2	**16.6±0.3^{A}**	**16.5±0.2^{B}**	15.8±0.2	15.7±0.3	15.0±0.2	15.5±0.2
Week 9	22.4±0.3	21.9±0.3	**21.2±0.2^{A}**	21.5±0.2	18.3±0.2	18.1±0.2	**17.4±0.2^{A}**	**17.2±0.2^{B}**	16.6±0.1	16.7±0.3	15.6±0.3	16.4±0.3
Week 10	22.6±0.2	22.4±0.2	21.9±0.2	22.4±0.2	18.7±0.3	18.6±0.2	**17.8±0.2^{A}**	**17.7±0.2^{B}**	17.2±0.9	16.6±0.3	16.2±0.3	16.6±0.2
Week 11	23.5±0.2	23.1±0.3	**22.2±0.3^{A}**	22.9±0.4	18.9±0.2	18.8±0.3	18.1±0.2	18.1±0.2	17.3±0.2	17.4±0.2	16.0±0.4	16.8±0.3

Week 12	23.6±0.2	23.3±0.2	22.9±0.1	23.4±0.3	19.4±0.4	18.9±0.2	18.7±0.2	18.6±0.2	16.7±0.2	16.9±0.3	17.3±0.2	16.9±0.4
Week 13	23.9±0.3	24.1±0.3	22.9±0.2	23.4±0.3	19.1±0.1	19.3±0.2	18.9±0.2	18.9±0.3	18.1±0.3	18.4±0.3	17.3±0.4	17.3±0.6



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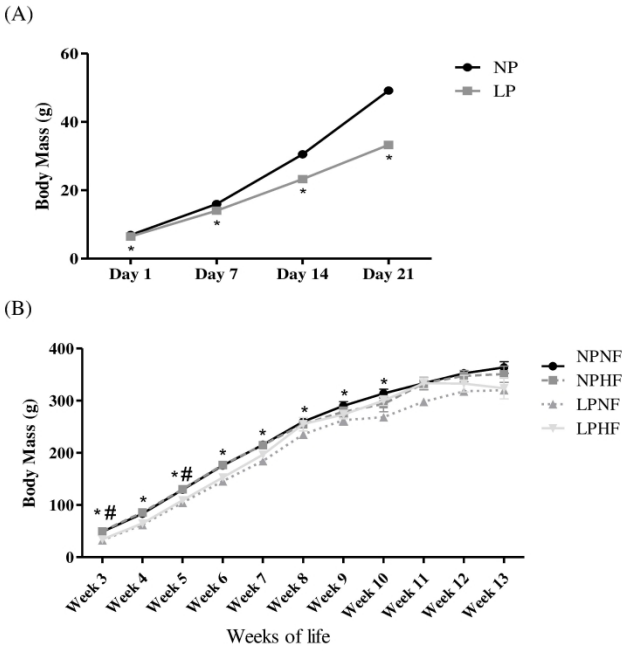

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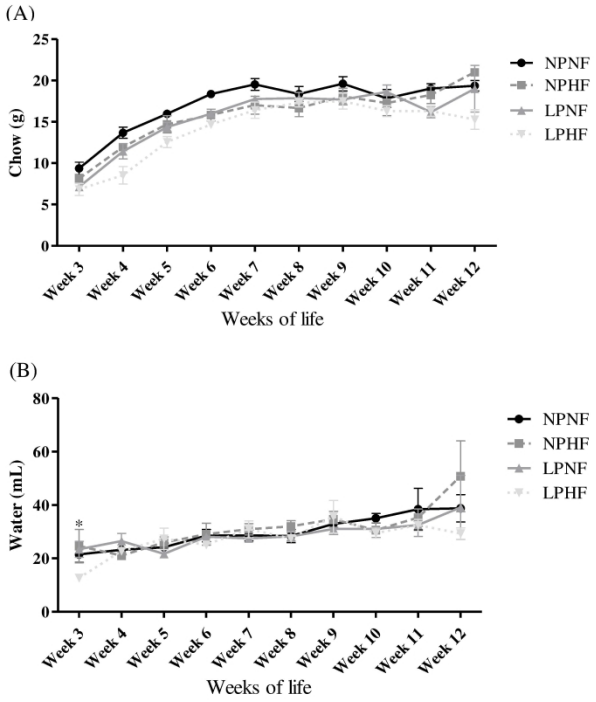

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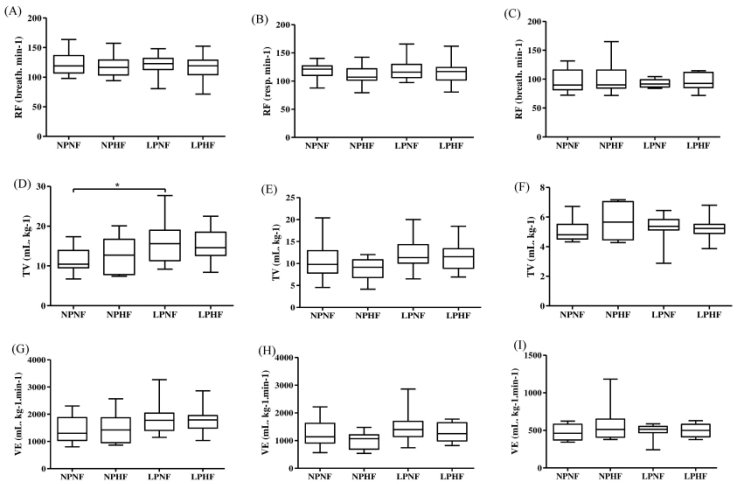

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ANEXO A - DOCUMENTO DE APROVAÇÃO DO COMITÊ DE ÉTICA PARA USO ANIMAL

Universidade Federal de Pernambuco
Centro de Biociências
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife – PE – Brasil
Fones: 2126 8842
ceua@ufpe.br

Recife, 05 de junho de 2019

Ofício nº 30/19

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. João Henrique da Costa Silva**
Centro Acadêmico de Vitória
Universidade Federal de Pernambuco
Processo **nº 23076.046459/2018-17**

Certificamos que a proposta intitulada **"Avaliação dos efeitos de uma dieta em ácidos graxos saturados sobre a composição corporal, o matabolismo lipídio e a pressão arterial de ratos submetidos a desnutrição perinatal."** registrado com o **nº 23076.046459/2018-17** sob a responsabilidade de **Prof. João Henrique da Costa Silva** o 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 DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 28/05/2019.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	28/05/2019 a 01/04/2023
Espécie/linhagem/raça	Rato heterogenico
Nº de animais	68
Peso/Idade	100g/ 30 dias
Sexo	Macho (56) Femea (12)
Origem: Biotério de Criação	Biotério do Departamento de Nutrição
Destino: Biotério de Experimentação	Biotério do CAV

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345691