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RODRIGO VARGAS

EFFECTS OF PROTEIN RESTRICTION DURING LACTATION ON FEMALE RAT METABOLISM AND THEIR OFFSPRING

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

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias

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BANCA EXAMINADORA

_____
Prof. Dr. Paulo Cezar de Freitas Mathias
Universidade Estadual de Maringá

_____
Prof. Dra. Sabrina Grassiolli
Universidade Estadual do Oeste do Paraná

_____
Prof. Dra. Sandra Lucinei Balbo
Universidade Estadual do Oeste do Paraná

_____
Prof. Dr. Egberto Gaspar de Moura
Universidade do Estado do Rio de Janeiro

_____
Profa. Dra. Ananda Malta
Instituto de Pesquisa Aplicada em Medicina

Biografia

Rodrigo Vargas nasceu em 29/01/1991 em Foz do Iguaçu/PR. Possui graduação em ciências biológicas pela Universidade Estadual do Oeste do Paraná (UNIOESTE) (2013). Conclui o mestrado em Biociências e Saúde no ano de 2018, pela mesma instituição, com a dissertação intitulada “A exposição ao glifosato, durante a prenhez e lactação, altera o metabolismo lipídico hepático de camundongos alimentados com dieta hiperlipídica”. Atualmente é doutorando no Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Maringá e professor de biologia celular, bioquímica e fisiologia na Universidade Cesumar (UNICESUMAR). Tem experiência na área de biologia celular e fisiologia, atuando principalmente nos seguintes temas: desnutrição, metabolismo hepático, secreção de insulina e homeostase da glicose.

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Apresentação

Esta tese é composta de dois artigos científicos experimentais, sendo o primeiro intitulado *“Low-protein diet during lactation programs lean phenotype and improve antioxidative system in adult female rat offspring”,* o qual foi redigido e submetido de acordo com as normas da revista *Journal of Developmental Origins of Health and Disease*, com atual fator de impacto 3,034. O segundo artigo foi intitulado *“Protein-restriction during lactation causes transgenerational metabolic dysfunction in adult rat offspring”,* o qual foi redigido e submetido de acordo com as normas da revista *Frontier in Nutrition*, com atual fator de impacto de 6.790. Ambos os trabalhos tratam sobre a plasticidade fisiológica causada pela oferta da dieta hipoproteica durante a lactação no metabolismo das fêmeas e seus descendentes.

Resumo geral:

Introdução: Diversas evidências apontam que injúrias em fases críticas do desenvolvimento do organismo, como o período intrauterino e o neonatal, elevam a probabilidade do desenvolvimento de síndrome metabólica na vida adulta, devido ao desenvolvimento do sistema regulador do balanço energético ocorrer neste período. Este processo, intitulado programação metabólica, pode ser explicado pelo conceito das Origens Desenvolvimentistas de Saúde e Doença (DOHaD), o qual, através de diversas evidências científicas, explica como os fatores ambientais primordiais podem induzir a alterações fisiológicas no organismo fetal, neonatal e de primeira infância e programar estas consequências ao longo da vida; e pela hipótese do fenótipo econômico. A hipótese do "fenótipo econômico" sugere que o desenvolvimento do feto é sensível ao ambiente nutricional, aumentando assim a chance de sobrevivência do indivíduo em condições nutricionais precárias e intermitentes, resultando num metabolismo pós-natal alterado. Porém, se houver grande disponibilidade de nutrientes após o período lactacional, o indivíduo torna-se susceptível ao surgimento de doenças metabólicas e cardiovasculares na idade adulta. Assim, a presente tese buscou avaliar o impacto da restrição proteica durante a lactação no metabolismo de ratas fêmeas adultas e seus efeitos na prole F2.

Metodologia: A fim de entender os efeitos da restrição proteica no metabolismo de fêmeas adultas e seus descentes, ratas Wistar lactentes receberam dieta hipoproteica (4% de proteína) nos primeiros 14 dias da lactação, constituindo o grupo LP; enquanto o grupo controle recebeu dieta normoproteica (23% de proteína), compondo o grupo NP. Durante a idade adulta, um grupo de fêmeas sofreu eutanásia e os parâmetros metabólicos foram analisados. Outro grupo foi

induzido ao cruzamento, com ratos controle externos ao experimento, e o metabolismo de sua prole macho foi analisado. Na vida adulta, as proles foram expostas a dieta normolipídica ou hiperlipídica, compondo quatro grupos: NPNF-F2, NPHF-F2, LPNF-F2 e LPHF-F2.

Resultados: As fêmeas LP adultas apresentaram um fenótipo magro, com redução de peso corporal e estoques de gordura na vida adulta. Na prole F2, foi possível identificar um efeito transgeracional da dieta LP. Contudo, a programação metabólica foi eficiente em minimizar os impactos da dieta hiperlipídica no metabolismo da prole, com compensação hepática.

Conclusão: A prole fêmea apresentou fenótipo magro enquanto a prole F2 apresentou efeito transgeracional da dieta no metabolismo hepático e na homeostase da glicose.

Palavras-chave: desnutrição, metabolismo hepático, homeostase da glicose.

General abstract

Introduction: Several evidence indicate that injuries in critical phases of the organism's development, such as the intrauterine and neonatal period, increase the probability of developing metabolic syndrome in adult life, due to the development of the energy balance regulatory system occurring in this period. This process, called metabolic programming, can be explained by the concept of the Developmental Origins of Health and Disease (DOHaD), which, through diverse scientific evidence, explains how primordial environmental factors can induce physiological changes in the fetal, neonatal and early childhood and program these consequences throughout life; and by the economic phenotype hypothesis. The “economic phenotype” hypothesis suggests that fetal development is sensitive to the nutritional environment, thus increasing the individual's chance of survival in poor and intermittent nutritional conditions, resulting in altered postnatal metabolism. However, if there is great availability of nutrients after the lactation period, the individual becomes susceptible to the emergence of metabolic and cardiovascular diseases in adulthood. Thus, the present thesis sought to evaluate the impact of protein restriction during lactation on the metabolism of adult female rats and its effects on the F2 offspring.

Methodology: To understand the effects of protein restriction on the metabolism of adult females and their offspring, infant Wistar rats received a low-protein diet (4% protein) in the first 14 days of lactation, constituting the LP group; while the control group received normoproteic diet (23% of protein), composing the NP group. During adulthood, a group of females was euthanized and metabolic parameters were analyzed. Another group was induced to cross, with control rats external to the experiment, and the metabolism of their male offspring was

analyzed. In adulthood, the offspring were exposed to a normolipid or high fat diet, comprising four groups: NPNF-F2, NPHF-F2, LPNF-F2 and LPHF-F2.

Results: Adult LP females showed a lean phenotype, with reduced body weight and fat stores in adulthood. In the F2 offspring, it was possible to identify a transgenerational effect of the LP diet. However, metabolic programming was efficient in minimizing the impacts of the high-fat diet on offspring metabolism, with hepatic overload.

Conclusion: The female offspring showed a lean phenotype while the F2 offspring showed a transgenerational effect of the diet on hepatic metabolism and glucose homeostasis.

Keywords: malnutrition, hepatic metabolism, glucose homeostasis.

Artigo 1

Low-protein diet during lactation programs lean phenotype and improve antioxidative system in adult female rat offspring

Rodrigo Vargas[1,2], Isabela Peixoto Martins[1,2,3], Anna Carolina Huppes de Souza[1], Julia Berno Oliveira[2], Ariadny Martins de Almeira[2], Camila Cristina Ianoni Matiusso[1,3], Camila Benan Zara[1], Audrei Pavanello[2], Sarah Rickli[1], Camila Quaglio Neves[3], Jean Carlos Fernando Besson[2], Tânia Cristina Alexandrino Becker[4], Ananda Malta[1], Paulo Cezar de Freitas Mathias[1].

[1]Department of Biotechnology, Genetics, and Cellular Biology, State University of Maringa, Maringa, PR, Brazil.

[2]Health Sciences Center, Unicesumar, Maringa, PR, Brazil.

[3]Departament of Morphological Sciences, State University of Maringa, Maringa, PR, Brazil.

[4]Department of Basic Health Sciences, State University of Maringa, Maringa, PR, Brazil.

Correspondence to: Professor Rodrigo Vargas; Department of Biotechnology, Genetic and Cell Biology; Laboratory of Secretion Cell Biology – Block H67, room 19, State University of Maringa/UEM - Phone/Fax + 55 (44) 3011 4892. Colombo Avenue 5790, 87020-900, Maringá/PR – Brazil. E-mail address: rodrigokockvargas@gmail.com

Abstract

Nutritional insults early in life have been associated to phenotype alterations and promote adverse permanent effects that impaired capacity of maintaining energy balance in adulthood. We aimed to evaluate the long-term effects of a low-protein diet (LP) during lactation on the metabolism and antioxidant system of adult female rat offspring. Adult female Wistar rats were fed either a low-protein diet (LP; 4% protein) during the first two weeks of lactation or a normal-protein diet (NP; 23% protein). Their female offspring received a standard diet throughout the experiment. At 90 days old, LP female offspring decreased body weight and fat pad stores, even feeding efficiency was higher. LP adult female offspring displayed brown adipose tissue hypertrophy, without alterations in glucose homeostasis. LP diet decrease liver triglycerides content and improve antioxidant system. We concluded that LP diet during suckling phase promotes a lean phenotype and improve hepatocyte antioxidant system in adult female offspring.
Keywords: metabolic programming, protein restriction, suckling phase

1. Introduction

A recent report from the United Nations presents updates on the state of food and nutrition security around the world. It includes the latest estimates of the cost and affordability of healthy food, evidencing that the number of people affected by hunger globally has risen to around 828 million in 2021 [1]. In the poorest populations, it was possible to observe a transition from energy and protein malnutrition to a gradual increase of dietary lipid content [2]. Protein is the most expensive food component of the human diet. These data become more worrisome due to COVID-19 pandemic [1]. Studies in Brazil and around the world showed that the human population was looking for high caloric and ultra-processed food due to high prices of healthy food in this period, such as those rich in protein [3–5]. The harmful impact of these choices on the human population is incalculable. Nevertheless, epidemiological and experimental studies displayed impairment on metabolism and increased in non-communicable disease when this dietary mismatch is applied.

The Development Origins in Health and Disease (DOHaD) concept suggests that maternal malnutrition in developmental vulnerability windows, such as suckling phase, programs offspring metabolism, resulting in different phenotypes and promoting adverse permanent effects that impaired capacity of maintaining energy balance in adulthood [6–9]. The low-protein diet (LP) is an experimental model widely used to investigate maternal malnutrition early in life [10–14] due to replacing protein content with carbohydrate [15]. Even low-protein diet effects on offspring can be beneficial in some metabolic parameters like

obesity [13], thrifty phenotype hypothesis alerts to changes in food habits throughout life.

In male offspring rat, our research group and others showed that maternal LP during lactation provoke lower body weight, food intake and fat pad stores, hypoinsulinemia, glucose intolerance, higher insulin sensitivity, reduced insulin secretion [12,13] and beta-cell mass, down-regulation of key genes regulating beta-cell development [16], altered autonomic nervous system function [13], impaired hypothalamic development [9] and hepatic damage [17] in adulthood. Those metabolic dysfunctions can be transmitted to next generations [18,19].

While several reports show the LP effects in male metabolism, very few studies have explored the metabolic programmed by this diet during lactation in female rats. Moreover, understanding scientific findings in the context of sex is very important for correctly applying research-derived knowledge [20].

In the present study, we evaluated the long-term effects of a LP during lactation on the body composition, lipid profile, glucose homeostasis, hepatic metabolism, and antioxidant system of adult female rat offspring.

2. Methods

2.1 Ethical approval

All experiments were conducted according to the ARRIVE guidelines [21] and with the Brazilian Association for Animal Experimentation (COBEA) standards. Protocols were approved by the Ethics Committee in Animal Research of the State University of Maringa (protocol number 8625310521) and performed in the sectorial animal facility of the Secretion Cell Biology Laboratory.

2.2 Nutritional insult and animal groups

After 1 week of acclimatization, female and male Wistar rats (70 and 80 days old, respectively) were mated in a ratio of three females to each male, and the pregnant females were transferred to individual cages and fed a standard diet. At birth, the litter were standardized to eight pups per dam, in 1:1 sex ratio, preferentially, and dams were fed either a normal-protein diet (NP; 23% protein; Nuvital; Curitiba/PR, Brazil; n=6) or a low-protein diet [22] (LP; 4% protein; n=6) during the first 14 days of lactation. At postnatal day 21, female offspring were weaned, housed four per cage and fed a standard diet throughout all experimental periods. The male offspring were previously evaluated by our research group [13]. The experimental procedures were conducted at 90 days of age. Throughout the experimental period, the animals were kept under controlled temperature (23 C ± 2 C) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle) conditions. The animals received water and food *ad libitum*.

2.3 Biometric parameters and food intake

Body weight (BW) and food intake (FI) were assessed weekly (n=5-10 rats from 4-6 litter per group) from weaning until 90 days of age to area under curve (AUC) measurement. The FI of rats was calculated by the formula [FI(g) = (Df – Di)/7] which is the difference between the amount of diet remaining (Df) and the amount presented previously (Di) divided by the number of days. The feeding efficiency [food consumption (g)/body weight (g)] was calculated. At 90 days rats were anaesthetized (thiopental, 45 mg/kg of bw), decapitated and laparotomized to remove their liver, pancreas, retroperitoneal, mesenteric, uterine, ovarian, and

brown fat pads stores**.** The weight of the tissues collected were expressed in relation to BW (g/100 g of BW).

2.4 Glucose metabolism assessment

At 90 days, a batch of female rats (n=5-10 rats from 4-6 litter per group) were submitted to the fasting of a 6-h to perform intraperitoneal insulin tolerance test (ipITT). Glycemia was determined from the collection of blood from the caudal vein and was measured using a glucometer at five different times (0, 15, 30, 45 and 60 minutes), once before and four after the application of intraperitoneal injection of insulin (1 U/kg of BW). Then the rate constant for plasma glucose disappearance (K_{itt}) was calculated [23]. Additionally, after two days, a surgery was performed for implantation of a cannula to execute an intravenous glucose tolerance test (ivGTT), as previously described [22]. After a 12-h fast, blood samples were removed before the injection of glucose (1g/kg of BW) (0 min) and 5, 15, 30 and 45 min afterward. The glucose response during the test was calculated by AUC.

2.5 Biochemical parameters

Glucose concentrations were measured by the glucose oxidase method using a commercial kit (GoldAnalisa®; Belo Horizonte, MG, Brazil) (n=5-10 rats from 4-6 litter per group). Fasting insulin was measured by ELISA. Total cholesterol, HDL cholesterol, triglycerides, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes were measured in plasma samples by colorimetric method using commercial kits (Gold Analisa®; Belo Horizonte, MG, Brazil). The absorbance reading was performed in a spectrophotometer

(Bioplus®, Barueri/SP, Brazil). The VLDL cholesterol and LDL cholesterol was calculated by Friedewald formula [24]

2.6 Oxidative stress assessment

Right-lobe hepatic samples (approximately 100mg, n=7-12 rats from 4-6 litter per group) were weighed and homogenized in a potassium phosphate buffer (200 mM, pH 6.5). A total of 60 µL of the homogenate was transferred to polypropylene tubes to determine the reduced glutathione (GSH) levels [25,26]. The other part of the homogenate was centrifuged for (20 min at 9000 ×g), and part of the supernatant was used to determine the lipid hydroperoxide (LOOH) levels according to the methodology previously described [27]. The supernatant was collected for biochemical assays to determine the enzymatic activity of superoxide dismutase (SOD) and glutathione S-transferase (GST).

The GSH levels were measured according to the methodology previously described [28]. For the GSH reaction, 25 µL of the sample was mixed with 5 µL of 5,5′-dithiobis-2-nitrobenzoic acid and 280 µL of tris (hydroxymethyl) aminomethane hydrochloride 1 (Tris-HCl) buffer (0.4 M; pH 7.0) and read at 412 nm. Individual values were interpolated based on the GSH standard curve and expressed as µg of GSH/g of liver.

The total LOOH was determined according to the methodology previously described [27]. The centrifuged supernatant (200 µL) was used for the LOOH analyses. LOOH concentrations were determined using an extinction coefficient of 4.3 mmolar 1/cm, and the results are expressed as mmol/mg of tissue. Readings were performed at 560 nm using a spectrophotometer.

The enzymatic SOD assay is based on the ability of SOD to inhibit the autoxidation of pyrogallol [29]. Readings were performed at 405 nm using a spectrophotometer. The results are expressed as U of SOD/mg of protein.

The homogenate was used to measure the GST enzymatic activity. GST enzyme activity was measured based on the capacity of the former conjugate of glutathione and 1-chloro-2, 4-dinitrobenzene, which was read at 340 nm using a spectrophotometer, according to the method previously described [30]. An extinction coefficient of 9.6 mmolar 1/cm was used, and the results were expressed as µmol/min/mg of protein.

2.7 Hepatic levels of cholesterol and triglycerides

Left-lobe hepatic samples of approximately 100 mg were removed (n=5-10 rats from 4-6 litter per group) to determine total lipids using the Folch method [31]. The extract was evaporated and then diluted in isopropanol. The cholesterol and triglycerides contents were measured using a commercial kit in accordance with the manufacturer's instructions (GoldAnalisa; Belo Horizonte, MG, Brazil).

2.8 Pancreas, brown and white fat histology

At 90 days, fat pad stores were collected for histological analysis. Retroperitoneal white adipose tissue and interscapular brown adipose tissue samples were removed (n= 6 rats from 6 litter per group), fixed in 4% paraformaldehyde for 24 hours, then dehydrated in alcohol of increasing concentration, and, after diaphanization in xylene, was embedded in histologically paraffin. Slices of 5-µm thickness were prepared for staining with

hematoxylin and eosin (H&E). The histological sections had followed the interval of 30 μm between the slices. After processing and fixation on the histology slide, the sections were examined using capture light microscopy (20 optic zones per animal in 40x). ImageJ for Windows (Open Source) was used in the analysis.

2.9 Statistical analysis

Data are presented as the mean ± the SEM and were analyzed by GraphPad Prism, version 8.0, for iOS (GraphPad Software, Inc. San Diego, CA, USA). The statistical test used was Student's t-test and values $p<0.05$ are considered statistically significant.

3. Results

3.1 Biometric parameters, food intake and morphometric analysis

Protein restriction caused lower body weight gain by 22% in the LP group (Figure 1A; $P<0.0001$) compared to the NP group, as shown by AUC. The LP group had increased food intake (+16.35%; $P<0.001$; Figure 1B) and feeding efficiency (+20.72%; $P<0.0001$; Figure 1C) when compared with NP group.

At 90 days old, metabolic programming by protein restriction decreased body weight (-15.81%; $P<0.0001$; Figure 1D), liver (-14.73%; $P<0.01$; Figure 1E), ovarian (-31.2%; $P<0.001$; Figure 1F), mesenteric (-16.6%; $P<0.01$; Figure 1G), retroperitoneal (-36.1%; $P<0.0001$; Figure 1H), and uterine (-44.4%; $P<0.0001$; Figure 1I) fat pad compared to NP group.

According to Figure 1L-M, LP rats had higher number (+35.45%; $P<0.01$) and area (+2%; $P<0.05$) of brown adipocytes compared to NP rats (Figure 1P-

Q). No difference was observed in number (Figure 1J) and area (Figure 1K) of white adipocytes (Figure 2N-O) between groups.

3.2 Glucose metabolism

At adulthood, LP group demonstrated normal glucose levels at ivGTT (Figure 2A) and no difference in insulin sensitivity, as demonstrated by K_{itt} (Figure 2B), even fasting glycemia were higher (+13%; $P<0.05$; Figure 2C) when compared to NP group. Protein restriction did not change fasting insulinemia (Figure 2D), pancreas weight (Figure 2E) and islet area (Figure 2F-H).

3.3 Biochemical parameters

Regarding lipid profile, the LP group displayed lower serum total cholesterol (-16.6%; $P<0.05$; Figure 3A) and HDL-C (-23.6%; $P<0.01$; Figure 3C) levels when compared to the NP group. No difference was observed in serum triglycerides (Figure 3B), LDL-C (Figure 3D), VLDL-C (Figure 3E), AST (Figure 3F) and ALT (Figure 3G) levels between groups.

In liver tissue, LP increases SOD (+42%; $P<0.01$; Figure 3H) and GST (20,43%; $P<0.05$; Figure 3K) activity in female offspring. No difference was demonstrated in liver CAT (Figure 3I) activity and GSH (Figure 3J) content. Liver triglycerides content is lower (-26.6%; $P<0.05$; Figure 3N) in LP group. Protein restriction increased liver LOOH (+20.29%; $P<0.05$; Figure 3L) and CHOL (+11.63%; $P<0.05$; Figure 3M) content compared to NP group.

4. Discussion

In the present study, we evaluated the metabolic programming by protein-restriction during the first 14 days of lactation in biometric and biochemistry parameters of adult female rat offspring. At 90 days old, LP diet decreased body weight and fat pad stores, even food intake and brown adipose tissue area was higher. Besides, these results suggested a lean phenotype without expressive alterations in glucose homeostasis. Metabolic alterations provoked by diet decreased serum cholesterol and increased their deposition within hepatocytes, improving liver antioxidant system.

Feeding behavior is regulated by hypothalamus through orexigenic and anorexigenic neuropeptides [32]. The brain has a full development during the suckling phase in rats [33]. LP diet offer during lactation decreased pup milk intake and protein content in breast milk [11], suggesting lower production of these neuropeptides and provoking malformation of hypothalamus [34]. Moreover, poor maternal nutrition can impair behavioral outcomes as anxiety and reward by damage in neuron development of the frontal cortex and limbic system [35]. In the present study, we show for the first time that LP diet increases food intake and feeding efficiency in adult female offspring while male offspring had normophagia at the same age, as we previously showed [13]. However, as observed in males [13], female offspring had lower body weight and fat pad stores, as also shown by Bertasso *et al* (8% protein content in diet) [17]. In LP high-carbohydrate murine model, lower body weight in male rats is associated with increase in brown adipose tissue thermogenesis due to higher expression of mitochondrial electron transport chain uncoupling protein 1 (UCP1) [36]. Here, we show for the first time that LP diet provokes BAT hypertrophy in female

offspring, suggesting higher activity of the sympathetic nervous system, as we previously showed in LP male offspring [13].

The hypermetabolic status can alter glucose homeostasis due to hormonal influences in key-enzymes. Glucokinase (GCK) is the first and rate-limiting step of glycolysis in the liver and pancreas [37]. Phosphoenolpyruvate carboxykinase (PEPCK-1) is the main enzyme which catalyzes the first committed step in hepatic gluconeogenesis [38]. In adult LP female offspring (8% protein content), GCK and PEPCK-1 expression was higher [17]. Here, we show that female offspring had normal glucose tolerance during GTT. The higher levels of PEPCK-1 can reflect the formation of new glucose and increase fasting glucose, as we show.

Maternal malnutrition by protein-restriction alters lipid profile in male and female offspring [13,17]. The diet used in this study has very low protein content (4%) [22]. The replace from protein to carbohydrate in this diet leads to change in lipid profile with lower HDL-C levels in both sex [15,39] and in the next generation (unpublished data). Here, we show that female offspring displayed decreased CHOL levels in plasma and increased in liver. Decrease in plasma CHOL levels can suggest an increase in tissue uptake to steroid hormone synthesis. Also, CHOL levels in plasma and liver, shown in this study, suggest an increased hepatic LDL-C uptake. However, we do not observe a decrease in plasma LDL-C levels.

In male offspring, LP diet promotes fatty free acid (FFA) uptake in hepatocytes by increasing white adipose tissue lipolysis [17]. Studies has been shown that estrogen appears protects against the development of hepatic steatosis [40]. In fact, we not observed steatosis in female rats. Interestingly, in ovariectomized rats, estrogen replacement reversed hepatic steatosis and

reduced hepatic lipogenic protein expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in high-fat high-fructose model [41]. Moreover, we show that TG liver content was lower in female offspring. Reduction in liver TG content can occur due to an increase in TG utilization, suggesting increase in sympathetic nervous system, as observed in adult male offspring [13]. In addition, female offspring show increased β-oxidative status by higher carnitine palmitoyl-transferase 1α (CPT-1α) content [17], suggesting a hepatic compensatory mechanism. In ovariectomized rats, fatty acid oxidation is decreased, suggesting an estrogen dependent pathway [42]. Indeed, liver lipid metabolism is essential to neutralize the huge impact of FFA in hepatocyte membrane integrity. Here, we show for the first time that liver lipid hydroperoxide (LOOH) content, lipid peroxidation hallmark, increased in LP female offspring.

The increase in liver glutathione-S-transferase (GST) levels, responsible for eliminating lipid peroxidation products [43], suggest an effective antioxidant system. Superoxide dismutase (SOD) converts superoxide anion to hydrogen peroxide (H_2O_2), that can be converted, by catalase (CAT) action, to water and oxygen [44]. Here, we show that SOD activity is higher in LP female offspring, but not CAT. It's important to remember that mitochondria are the main organelle producers of reactive oxygen species and mitochondrial genome is inherent from maternal lineage [45]. Thus, our data suggest that LP during lactation can positively modulate the antioxidant system and avoid damage in mitochondrial and nuclear DNA.

In conclusion, LP diet during lactation promotes a lean phenotype, improving antioxidant system in adult female offspring. These outcomes are

essential to understand the impact of these metabolic alterations in the next generation. Other studies were required to understand mechanisms involved.

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6. Statement of authors' contributions to manuscript

R. V.: researched data, study design, manuscript writing; I. P. M. and C. C. I. M.: researched data and study design; A. C. H. S., J. B. O., A. M. A., C. B. Z., A. P., S. R. and C. Q. N.: researched data; J. C. F. B. and T. C. A. B.: contributed to manuscript discussion; A. M. and P. C. F. M.: contributed to discussion and reviewed manuscript.

7. Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Biometric parameters and fat pad morphometry. Body weight gain (A), food intake (B), feeding efficiency (C), body weight at 90 days old (D), liver (E), ovarian (F), mesenteric (G), retroperitoneal (H) and uterine fat pad store (I), number of white adipocyte (J), retroperitoneal adipocyte area (K), number of brown adipocyte (L), brown adipocyte area (M), NP WAT (N), LP WAT (O), NP BAT (P) and LP BAT (Q). Hematoxylin & Eosin-stained sections. Magnification: 200x. Scale bar = 50μm. The data are expressed as the mean ± S.E.M. and were obtained from 8-12 rats of each group (from 3-6 different litter). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 2. Glucose homeostasis. Plasma glucose during ivGTT (A), insulin tolerance test and K_{itt} (B), fasting glycemia (C), fasting insulin concentration (D), pancreas weight (E), islet area (F), NP islet (G) and LP islet (H). Hematoxylin & Eosin-stained sections. Magnification: 400x. Scale bar = 50μm. The data are expressed as the mean ± S.E.M. and were obtained from 6-12 rats of each group (from 3-6 different litter). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 3. Biochemistry parameters. Total cholesterol (A), triglycerides (B), HDL-C (C), LDL-C (D), VLDL-C (E), AST (F), ALT (G), liver SOD activity (H), liver CAT activity (I), liver GSH content (J), liver GST activity (K), liver LOOH content (L), liver cholesterol content (M) and liver triglycerides content (N). The data are expressed as the mean ± S.E.M. and were obtained from 8-12 rats of each group (from 3-4 different litter). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figures

Figure 1

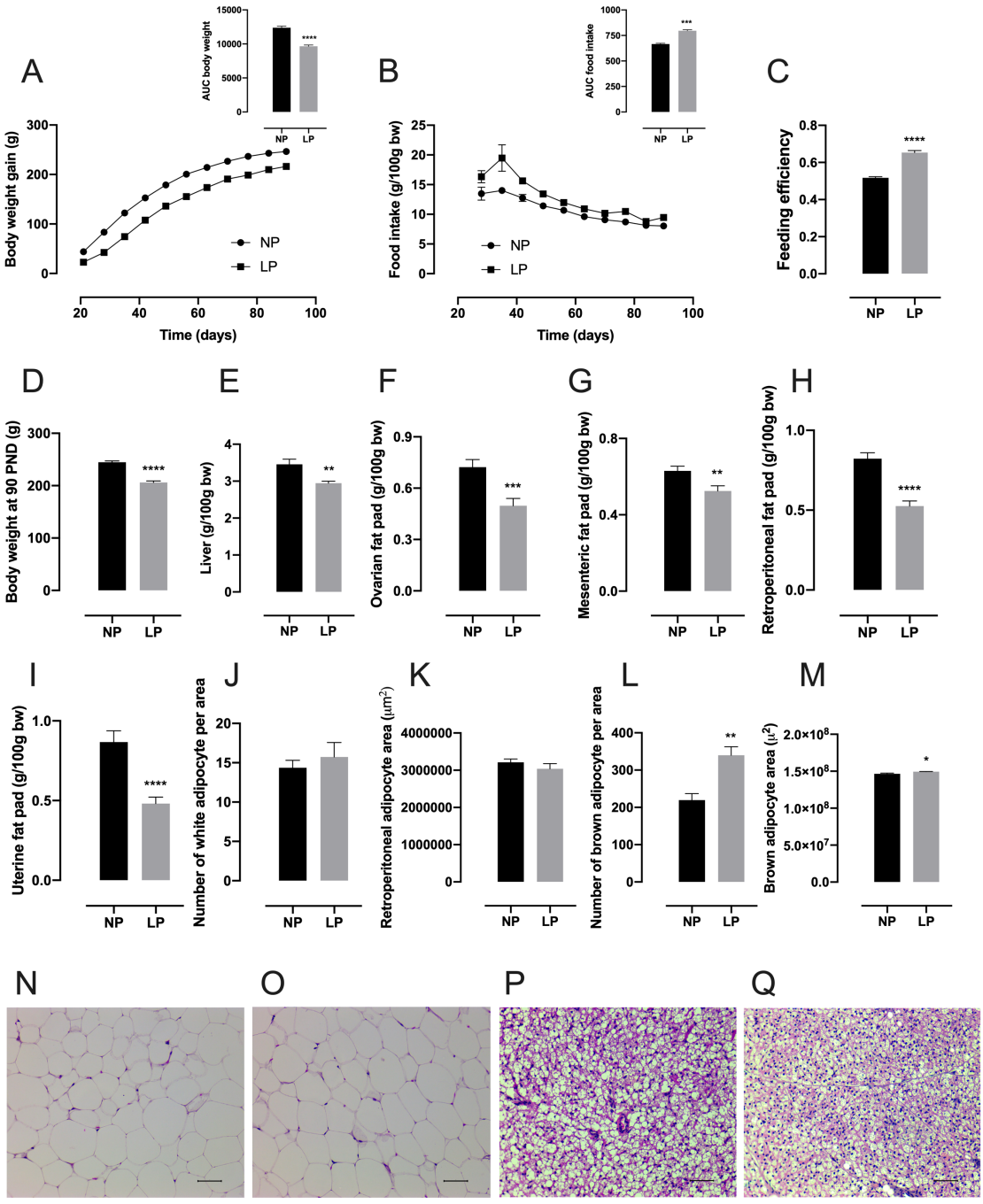


Figure 2

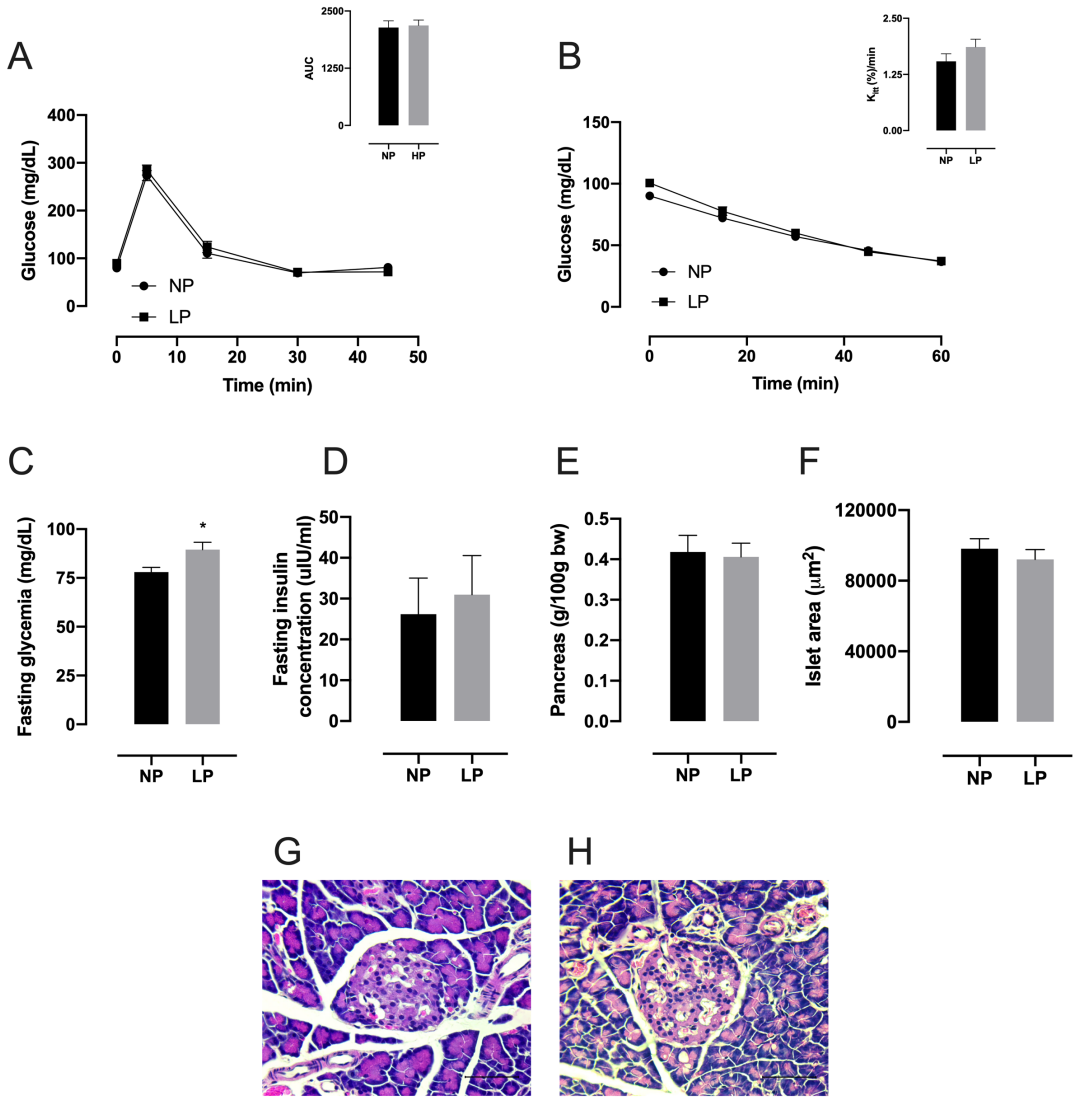
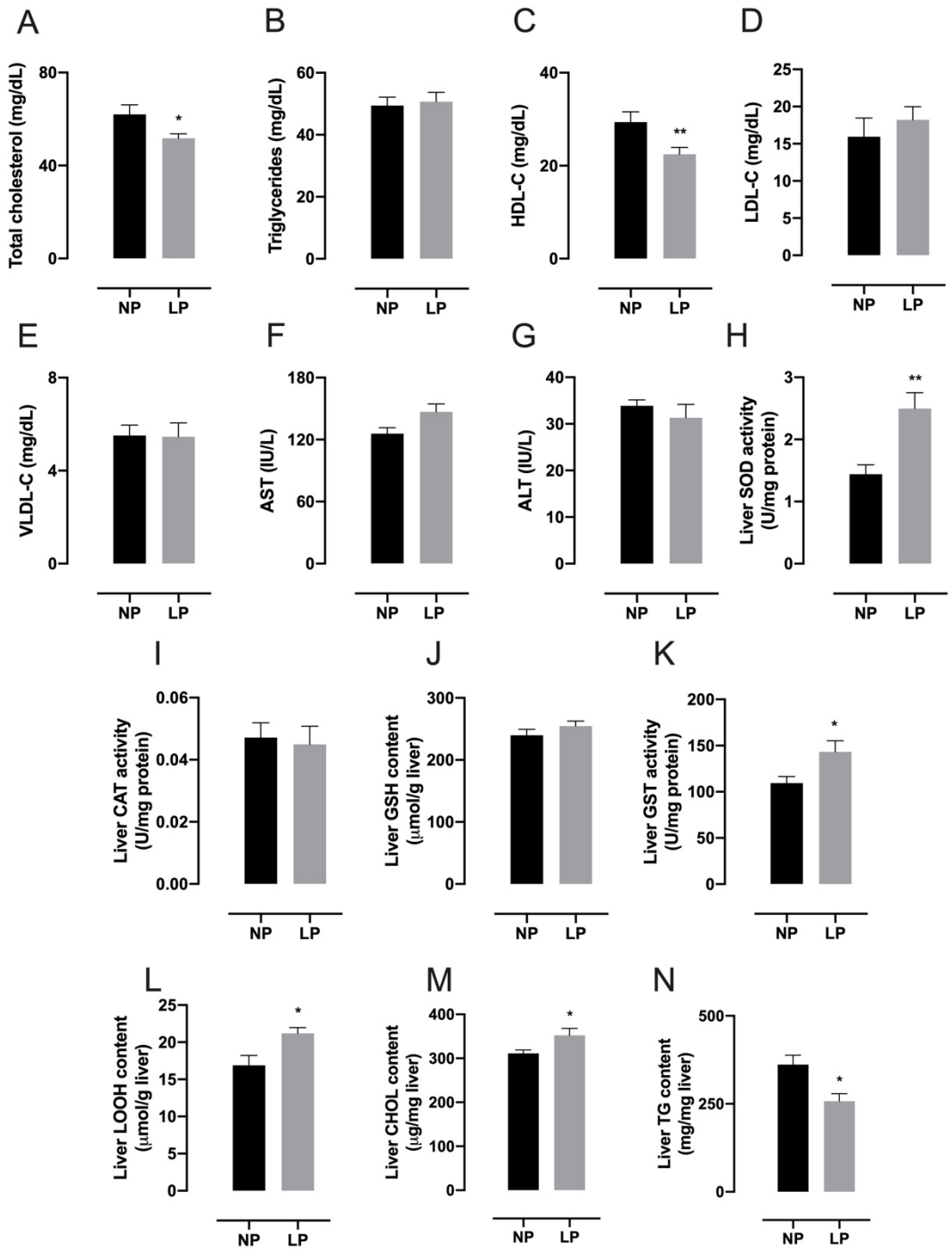

A
Glucose (mg/dL)
Time (min)
NP
LP
AUC
NP
HP
B
Glucose (mg/dL)
Time (min)
NP
LP
NP
LP
C
Fasting glycemia (mg/dL)
NP
LP
D
Fasting insulin concentration (uIU/ml)
NP
LP
E
Pancreas (g/100g bw)
NP
LP
F
Islet area (μm²)
NP
LP
G
H

Figure 3


A
Total cholesterol (mg/dL)
80
40
0
*
NP
LP
B
Triglycerides (mg/dL)
60
40
20
0
NP
LP
C
HDL-C (mg/dL)
40
20
0
**
NP
LP
D
LDL-C (mg/dL)
25
20
15
10
5
0
NP
LP
E
VLDL-C (mg/dL)
8
4
0
NP
LP
F
AST (IU/L)
180
120
60
0
NP
LP
G
ALT (IU/L)
40
30
20
10
0
NP
LP
H
Liver SOD activity (U/mg protein)
3
2
1
0
**
NP
LP
I
Liver CAT activity (U/mg protein)
0.06
0.04
0.02
0.00
NP
LP
J
Liver GSH content (μmol/g liver)
300
200
100
0
NP
LP
K
Liver GST activity (U/mg protein)
200
150
100
50
0
*
NP
LP
L
Liver LOOH content (μmol/g liver)
25
20
15
10
5
0
*
NP
LP
M
Liver CHOL content (μg/mg liver)
400
300
200
100
0
*
NP
LP
N
Liver TG content (mg/mg liver)
500
250
0
*
NP
LP

Artigo 2

Protein-restriction during lactation causes transgenerational metabolic dysfunction in adult rat offspring

Rodrigo Vargas[1,3], Isabela Peixoto Martins[1,2,3], Camila Cristina Ianoni Matiusso[1,3], Raiana Aparecida Casagrande[3], Camila Benan Zara[1], Anna Carolina Huppes de Souza[1], William Pereira Horst[3], Taina Cristine Sieklicki[3], Tania Cristina Alexandrino Becker[4], Naiara Cristina Lucredi[5], Jurandir Fernando Comar[5], Ananda Malta[1], Paulo Cezar de Freitas Mathias[1].

[1]Department of Biotechnology, Genetics, and Cellular Biology, State University of Maringa, Maringa, PR, Brazil.

[2]Departament of Morphological Sciences, State University of Maringa, Maringa, PR, Brazil.

[3]Health Sciences Center, Unicesumar, Maringa, PR, Brazil.

[4] Department of Basic Health Sciences, State University of Maringa, Maringa, PR, Brazil.

[5] Department of Biochemistry, State University of Maringa, Maringa, PR, Brazil.

Correspondence to: Rodrigo Vargas; Department of Biotechnology, Genetic and Cell Biology; Laboratory of Secretion Cell Biology – Block H67, room 19, State University of Maringa/UEM - Phone/Fax + 55 (44) 3011 4892. Colombo Avenue 5790, 87020-900, Maringá/PR – Brazil. E-mail address: rodrigokockvargas@gmail.com

Abstract

Protein restriction during lactation can induce metabolic dysfunctions and has a huge impact on the offspring's phenotype in the later in life. We tested whether the effects of a maternal low-protein diet (LP) in rats can be transmitted to the F2 generation and increase their vulnerability to dietary insults in adulthood. Female Wistar rats (F0) were fed either a low-protein diet (LP; 4% protein) during the first two weeks of lactation or a normal-protein diet (NP; 23% protein). The female offspring (F1 generation) were maintained on a standard diet throughout the experiment. Once adulthood, female F1 offspring from both groups (i.e., NP-F1 and LP-F1) were bred to proven males, outside the experiment, to produce the F2 generation. Male F2 offspring from both groups (NP-F2 and LP-F2 groups) received a standard diet until 60 days old, at which point they received either a normal fat (NF; 4.5% fat) or a high fat diet (HF; 35% fat) for 30 days. At 90 days old, LPNF-F2 offspring had increased lipogenesis and fasting insulinemia compared to NPNF-F2, without alteration in insulin sensitivity. HF diet caused increased gluconeogenesis and displayed glucose intolerance in LPHF-F2 offspring compared to LPNF-F2 offspring. Additionally, the HF diet led to damage to lipid metabolism (such as steatosis grade 3), and higher body weight, fat pad stores, and hepatic lipid content. We concluded that an F0 maternal protein-restricted diet during lactation can induce a transgenerational effect on glucose and liver metabolism in the F2 generation, making offspring's liver more vulnerable to nutritional injury later in life.

Keywords: metabolic programming, maternal malnutrition, steatosis, thrifty phenotype hypothesis, metabolism.

1. Introduction

A relationship between malnutrition and chronic diseases has been observed worldwide (1). Exponential evidence indicates that perinatal environmental factors, such as maternal malnutrition status, promote long-term effects on the metabolic phenotype of offspring (2,3); this process is known as the metabolic programming. Epidemiological and experimental studies suggest that early life nutritional programming is associated with a higher risk for the development of cardiometabolic syndrome (4–6); an impaired capacity to maintain energy balance is not only limited to exposed individuals, but also to subsequent generations, even though nutritional conditions are favorable (7).

According to the thrifty phenotype hypothesis, maternal malnutrition provokes metabolic adaptations in offspring that support further development and survival by altered intrauterine growth and an adjusted metabolic phenotype, with a reduced energy demand appropriate for poor nutritional conditions (8–11). However, even an adequate, or excessive postnatal food supply in later life may have negative consequences, mainly on glucose homeostasis (12) and hepatic lipid metabolism (13). This notion is supported by studies on perinatal nutrient restriction during critical periods of development.

Several reports have also focused on the effects of maternal malnutrition. For example, a low-protein diet suggests that the programmed metabolic dysregulation observed in different vulnerability windows may be associated with different biological mechanisms and have a great impact on the phenotype induced in the offspring (7,14).

Lactation is a window of susceptibility due to the development and maturation of major organs and tissues, which determine the offspring's metabolic phenotype (2,15). Thus, exposure to undernutrition during this period can affect metabolism and pancreatic function. Our research group showed that a low-protein diet during lactation increased glycemia, even though offspring displayed higher peripheral insulin sensitivity and lower fasting insulinemia (15). Insulin is a major anabolic hormone involved in hepatic metabolism and dietary protein malnutrition can induce hepatic fat accumulation (16). Maternal low-protein diet effects have also been observed in several studies (17,18) in the F2 (19,20) and F3 generations (21,22), contributing to the early life origin of the risk of chronic diseases.

While the investigation of the transgenerational effects of developmentally programmed traits is widening, very few studies have explored the potential for these traits to be transmitted with post-weaning diets other than adequate controls. In the present study, we assessed the transgenerational transmission of programmed phenotype outcomes on glucose homeostasis and lipidic hepatic metabolism through the maternal lineage and offspring vulnerability to a food insult later in life with a high-fat diet.

2. Material and methods

2.1 Ethical approval

All experiments were conducted according to ARRIVE guidelines (23) and Brazilian Association for Animal Experimentation (COBEA) standards. The

protocols were approved by the Ethics Committee in Animal Research of the State University of Maringa (n. 5409020520) and performed in the sectional animal facility of the Secretion Cell Biology Laboratory.

2.2 Dams

2.2.1 Experimental design and diets

After 1 week of acclimatization, female and male Wistar rats (70 and 80 days old, respectively) were mated at a ratio of three females to one male. The pregnant females were transferred to individual cages and fed a standard diet. Pregnant F0 females were used to compose the F1 group (Figure 1). At birth, the litter was standardized to eight pups per dam, with a 1:1 sex ratio, and F0 dams were fed either a normal-protein diet (NP; 23% protein; Nuvital; Curitiba/PR, Brazil; n=12) or a low-protein diet (24) (LP; 4% protein; n=12) during the first 14 days of lactation. At postnatal day 21, the F1 female offspring were weaned, housed in groups of four per cage, and fed a standard diet. The F1 male offspring were not evaluated in the present study.

F1 female offspring (n=1 per litter) were kept until 70 days old and mated to proven male rats outside the experiment to compose the NP-F2 and LP-F2 groups. F1 females were fed a standard diet throughout the experimental period. After birth, the litter was standardized to eight pups per dam, in a 1:1 sex ratio. After weaning, only male offspring (NP-F2 and LP-F2) were used in the experiments to avoid estrogen influences.

The experimental procedures were conducted at 90 days old. Throughout the experimental period, the animals were kept under controlled temperature (23

C± 2°C) and photoperiod (7:00 a.m. to 7:00 p.m., daylight cycle) conditions. Animals received water and food *ad libitum*.

2.2.2 Fasting glycemia and oral glucose tolerance test (oGTT) during pregnancy

Glucose concentration was measured via the glucose oxidase method using a commercial kit (GoldAnalisa; Belo Horizonte, MG, Brazil) (25). At the 18th day of pregnancy (26), after a 6 h fast, blood samples were collected before gavage administration of glucose (1 g/kg of body weight, 0 min, n=6-7) and 15, 30, 45, and 60 min afterward. Glucose response during the test was calculated using the area under the curve (AUC).

2.2.3 Intraperitoneal insulin tolerance test (ipITT)

On the 20th day of pregnancy, an ipITT was performed after a 6 h fast (n=6-7). Dams received an injection of insulin (1U/kg of body weight) and blood samples were collected as previously reported (27). Subsequently, the rate of glucose tissue uptake or rate constant for plasma glucose disappearance (K_{itt}) was calculated (28).

2.2.4 Biometric parameters and caloric intake during lactation.

Body weight (BW) and food intake were measured daily during the suckling phase. Food intake (FI) was calculated as the difference between the amount of remaining diet (D_f) and the amount presented previously (D_i): [FI (g) =

($D_f - D_i$)]. Even though the energy values of the diets were the same, food intake was presented in calories (kcal/100 g of body weight). The AUC for food intake and feeding efficiency [food consumption (g)/body weight (g)] was calculated.

2.3 Offspring

2.3.1 Experimental design and diet

At 60 days old, a subset of male offspring from the NP-F2 and LP-F2 groups were fed a normal-fat diet (NF; 7% fat; Nuvital; Curitiba/PR, Brazil) or a high-fat diet (HF; 35% fat) [26] until they were 90 days old. The four experimental groups used were as follows: NPNF-F2, control offspring that were fed an NF diet; LPNF-F2, low-protein offspring that were fed an NF diet; NPHF-F2, control offspring that were fed a HF diet; and LPHF-F2, low-protein offspring that were fed an HF diet (n=8 litter per group).

2.3.2 Body weight gain, caloric intake, feed efficiency, liver weight and fat pad store measurements

Body weight (BW) and food intake were determined daily from birth to weaning. They were then examined weekly until they reached 90 days old. Food intake was calculated weekly. Considering that the energetic values of the diets were different, food intake was presented in calories. The AUC for food intake and feeding efficiency were calculated. At 90 days old, the rats were anesthetized with thiopental (45 mg/kg of body weight), weighed, decapitated, and laparotomized to remove their liver and retroperitoneal, perigonadal, and

mesenteric fat pad stores. The weights of the fat pads and liver were expressed in relation to the BW of each animal (g/100 g of BW).

2.3.3 Intravenous glucose tolerance test (ivGTT)

At 90 days old, a batch of animals (n=10-12 rats from 3-4 litters per group) was subjected to a surgical procedure to perform ivGTT, as previously described (24). After a 12 h fast, blood samples were collected before the injection of glucose (1 g/kg of body weight, 0 min) and 5, 15, 30, and 45 min afterward. Glucose response during the test was calculated using the AUC.

2.3.4 Intraperitoneal insulin tolerance test (ipITT)

Another batch of animals (n=10-12 rats from 3-4 litters per group) was cannulated, and ipITT was performed after a 6h fast. They received an injection of insulin (1U/kg of body weight), and blood samples were collected, as previously reported (29). Subsequently, the rate of glucose tissue uptake or the rate constant for plasma glucose disappearance (K_{itt}) was calculated (28).

2.3.5 Blood glucose levels and lipid profile

Glucose concentration was measured by the glucose oxidase method using a commercial kit (GoldAnalisa; Belo Horizonte, MG, Brazil) (25). Triglycerides (TG), total cholesterol, and high-density lipoprotein cholesterol (HDL-C) levels were measured in plasma samples using a colorimetric method

and commercial kits (GoldAnalisa; Belo Horizonte, MG, Brazil). Low-density lipoprotein cholesterol (LDL-C) and very-low-density LDL cholesterol (VLDL-C) values were calculated using the Friedewald formula (30).

2.3.6 Hepatic levels of cholesterol and triglycerides

Left-lobe hepatic samples of approximately 100 mg were removed (n=5-10 rats from 5-10 litters per group) to determine total lipids using the Folch method (31). The extract was evaporated and then diluted in isopropanol. Cholesterol and TG contents were measured using a commercial kit, in accordance with the manufacturer's instructions (GoldAnalisa; Belo Horizonte, MG, Brazil).

2.3.7 Pancreas, liver, and retroperitoneal fat histology

Pancreas, liver, and retroperitoneal fat samples (n=5-6 rats from 5-6 litters per group) were removed and fixed in 4% paraformaldehyde for 24h. Subsequently, the samples were dehydrated in an alcohol increasing series of concentrations. After diaphanization in xylene, the samples were embedded in histological paraffin. Slices of 5-μm thickness were prepared for staining with hematoxylin and eosin (H&E). In the pancreas and fat slices, islets (40 per animal, 40x magnification) and retroperitoneal fat (20 per animal, 20x magnification) photomicrographs were randomly acquired using an Olympus DP71 camera coupled to an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan). ImageJ for Windows (Open Source) was used for analysis. Liver slices

(30 fields per animal, 20x magnification) were examined under a light microscope. These values were classified as previously described for the magnitude of steatosis (32). Thus, steatosis was graded as follows: 0 (none to 5% of hepatocytes affected), 1 (>5%-33% affected), 2 (>33%-66% affected), and 3 (>66% affected). The predominant distribution pattern of steatosis was graded as follows: 0 (zone 3), 1 (zone 1), 2 (azonal), or 3 (panacinar).

2.3.8 RNA isolation and real-time quantitative RT-qPCR

Liver samples were collected and stored in liquid nitrogen at -80 ºC pending total RNA extraction. RNA was isolated from 100 mg frozen tissue using TrizolTM reagent (Thermo Fisher Scientific, Waltham, MA). The RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm (NanoDrop ND 1000 NanoDrop Technologies, Wilmington, DE). cDNA was synthetized using Platus Transcriber RNaseH cDNA First Strand kit (Sinapse inc, BR) and quantitation of the tissue expression of selected genes was done by quantitative PCR in the Rotor-Gene® Q (Qiagen) with “HOT FirePol® EvaGreen® qPCR Supermix” (Solis BioDyne, EE). The glyceraldehyde 3 phosphate dehydrogenase (GADPH) gene was utilized as a reference gene. The 2-ΔCT method (33) was used for the relative quantification analysis, and data were expressed as an arbitrary unit (AU). Primers for phosphoenolpyruvate carboxykinase (PEPCK) and fatty acid synthase (FASN) are listed in the Supplementary Material.

2.4 Statistical analysis

The results were normalized and presented as the mean ± standard error (S.E.M.). Statistical analysis was performed using Student's t-test or two-way analysis of variance, followed by Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant for the effects of low-protein diet-fed dams (LP), a high-fat diet-fed offspring (HF), or interaction (I) of factors. Analyses were conducted using GraphPad Prism version 6.01 for iOS (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1 Dams

3.1.1 Body weight, food intake and glucose homeostasis during pregnancy

During the oGTT, as observed by the AUC, LP-F1 dams showed mild glucose intolerance (Figure 2D; +10.21%; $P < 0.05$) and fasting hyperglycemia (Figure 2F; +17.02%; $P < 0.01$), without differences in insulin sensitivity, as demonstrated by K_{itt} (Figure 2E).

As shown in Figure 2, protein restriction during lactation did not modify BW gain or food intake in dams (Figure 2A-B). However, LP-F1 dams showed a 13.24% decrease in feed efficiency (Figure 2C) during lactation ($P < 0.01$).

3.2 Offspring

3.2.1 Biometric parameters and food intake

Protein restriction caused pup weight at birth to be lower by 23.7% in LP-F2 offspring (Figure 3A; $P < 0.0001$) compared to the NP-F2 offspring. Metabolic programming showed no change in BW gain between offspring groups during the suckling phase (Figure 3B) or until postnatal day 60 (PND) (Figure 3C). However, LP-F2 offspring had increased food intake (Figure 3E; +24.5%; $P < 0.05$) and feed efficiency (Figure 3D; +14.54%; $P < 0.0001$) when compared with the control offspring.

Figure 3F shows that there was no difference in BW gain between LPNF-F2 and NPNF-F2 offspring in adulthood. However, exposure to an HF diet resulted in an increased of 33.68% ($P < 0.0001$) and 36.18% ($P < 0.001$) in body weight gain in NPHF-F2 and LPHF-F2 offspring, respectively. No other differences were observed between the NPHF-F2 and LPHF-F2 offspring. Food intake did not differ between groups (Figure 3H). Additionally, feeding efficiency did not differ between the NPNF-F2 and LPNF-F2 offspring. However, the LPHF-F2 group showed an increase of 31.83% ($P < 0.0001$) and 13.07% ($P < 0.05$) in feeding efficiency compared to the LPNF-F2 and NPHF-F2 groups, respectively (Figure 3G), with interactions between factors.

As shown in Figure 3I, at PND90, there was no difference bin BW etween the NPNF-F2 and LPNF-F2 offspring. The NPHF-F2 group had 23.14% increase in BW compared with the NPNF-F2 group ($P < 0.0001$). Additionally, the LPHF-F2 group has an 11.58% increase in BW compared to the LPNF-F2 group ($P < 0.0001$). Among the groups that received the HF diet, LPHF-F2 group had a 7.7% decrease in body weight ($P < 0.05$), with interactions between factors.

3.2.2 Biochemical parameters and lipid profile

As shown in Table 1, fasting glycemia was 15.39% lower in LPNF-F2 offspring than in NPNF-F2 offspring ($P < 0.05$). Offering an HF diet, the NPHF-F2 offspring showed a 13.27% increase in fasting glycemia ($P < 0.05$). However, no difference was observed between LPHF-F2 compared with LPNF-F2 offspring. Nevertheless, the LPHF-F2 offspring showed lower fasting glucose (16.89%) than the NPHF-F2 offspring ($P < 0.01$), with interactions between factors.

Fasting insulinemia was 34.6% higher in the LPNF-F2 offspring compared with NPNF-F2 ($P < 0.001$). The NPHF-F2 and LPHF-F2 offspring showed no differences compared to their counterparts. However, LPHF-F2 displayed an increased of 36.75% in fasting insulinemia compared with NPHF-F2 ($P < 0.001$).

Regarding total cholesterol levels, there was no difference between the LPNF-F2 compared with NPNF-F2 offspring. However, as expected, NPHF-F2 offspring had significantly higher total cholesterol levels than NPNF-F2 offspring (+28%; $P < 0.001$). In contrast, HF diet intake showed no statistical difference in total cholesterol between the LPHF-F2 and LPNF-F2 offspring. Interestingly, LPHF-F2 showed lower cholesterol levels than NPHF-F2 (-17.5%; $P < 0.01$), with interactions between factors.

TG levels did not differ between the LPNF-F2 and NPNF-F2 groups. Additionally, a HF diet did not increase TG levels in NPHF-F2 offspring compared to NPNF-F2 or LPHF-F2 offspring. However, the LPHF-F2 offspring showed an increase of 21.07% in plasma TG levels compared to the LPNF-F2 offspring ($P < 0.05$), with interactions between factors.

The LPNF-F2 group showed a 48.61% decrease in HDL-C levels compared to the NPNF-F2 group ($P < 0.0001$). As expected, HDL-C levels in the LPHF-F2 group were decreased by 43.29% compared to the NPHF-F2 group ($P < 0.0001$). In addition, a HF diet increased HDL-C levels by 24.41% and 31.50% in the NPHF-F2 and LPHF-F2 groups ($P < 0.05$), respectively, compared to their counterparts, with interactions between factors.

In plasma LDL-C levels, the LPNF-F2 group had levels increased by 44.81% compared with the NPNF-F2 group ($P < 0.001$). Moreover, NPHF-F2 offspring showed a tendency to have increased LDL-C levels compared to NPNF-F2 offspring ($P = 0.0944$). The LPHF-F2 offspring showed an increase of 27.93% and 42.52% compared with LPNF-F2 ($P < 0.05$) and NPHF-F2 offspring ($P < 0.0001$), respectively, with interactions between factors.

VLDL-C levels did not differ between the LPNF-F2 and NPNF-F2 offspring. Curiously, the NPHF-F1 offspring were not significantly different from the NPNF-F2 or LPHF-F2 offspring in terms of VLDL-C levels. However, the LPHF-F2 group showed an increase of 21.07% in VLDL-C levels compared to the LPNF-F2 group ($P < 0.05$), with interactions between factors.

3.2.3 Glucose homeostasis during the glucose and insulin tolerance test

During the ivGTT, as observed by the AUC, the LPNF-F2 group showed no difference in glycemia or peripheral insulin sensitivity compared with the NPNF-F2 group (Figure 4A), as demonstrated by K_{itt} (Figure 4B). As expected, the HF diet resulted in glucose intolerance (Figure 4A; +28.44%; $P < 0.001$) and insulin resistance (Figure 4B; -40.48%; $P < 0.05$) in NPHF-F2 offspring compared

with NPNF-F2 offspring. However, no differences were observed between the LP-HF-F2 and LP-NF-F2 offspring in glucose levels and kITT. Although the LPHF-F2 offspring showed significantly lower glucose concentrations during the test then the NPHF-F2 offspring (Figure 4A; -30.36%; $P < 0.01$), no difference was observed in insulin sensitivity (Figure 4B). Glucose tolerance tests showed interactions between the factors.

3.2.4 Pancreatic islet morphometry

Optical analysis showed that pancreatic islet architecture was not altered in the offspring. However, the islet area was lower in the LPNF-F2 offspring than in the NPNF-F2 offspring (Figure 4C; -29.75%; $P < 0.01$). The LPHF-F2 and NPHF-F2 offspring showed no difference in islet area compared to their counterparts. However, this parameter was decreased by 40.38% in the LPHF-F2 group compared to that in the NPHF-F2 group (Figure 4C; $P < 0.0001$).

3.5 Fat pad store composition and morphometry

As shown in Figure 5A-C, no difference was observed in fat pad stores between the LPNF-F2 and NPNF-F2 offspring. As expected, the NPHF-F2 offspring had a higher retroperitoneal (Figure 5A; +63.30%; $P < 0.001$), perigonadal (Figure 5B; +63.19%; $P < 0.0001$), and mesenteric fat pad (Figure 5C; +61.98%; $P < 0.0001$) than the NPNF-F2 offspring. Similarly, the white adipose tissue (WAT) mass was higher in the LPHF-F2 offspring than in the LPNF-F2 offspring, with an increase of 48.84% ($P < 0.0001$), 47.63% ($P <$

0.0001), and 52.64% (P < 0.0001) for retroperitoneal, perigonadal, and mesenteric fat pad, respectively. While retroperitoneal fat stores were not different, the LPHF-F2 offspring had lower perigonadal (Figure 5B; -18.48%; P < 0.01) and mesenteric fat stores (Figure 5C; 25.68%; P < 0.001) compared with the NPHF-F2 offspring, with interactions between factors in all fat pad stores.

Regarding the morphometric analysis of retroperitoneal fat, no differences were observed between the LPNF-F2 and NPNF-F2 offspring. However, the NPHF-F2 and LPHF-F2 groups showed increases of 24.67% and 25.74%, respectively, in the retroperitoneal adipocyte area, compared to their counterparts (Figure 5D; P < 0.05). No differences were observed between the LPHF-F2 and NPHF-F2 groups.

3.6 Hepatic morphofunction and lipid profile

As shown in Figure 5E, the LPNF-F2 group did not show a difference in liver weight compared with the NPNF-F2 group. Thus, an HF diet did not induce any difference in liver weight in the NP-F2 offspring. However, the LPHF-F2 group showed an increase of 8.18% in liver weight compared with the LPNF-F2 group. No difference was observed in liver weight between the LPHF-F2 and NPHF-F2 offspring.

Regarding total liver fat content, no difference was observed between the NPNF-F2 and LPNF-F2 offspring. However, the HF diet increased liver fat content by 36.11% and 29.41% in the NPHF-F2 and LPHF-F2 offspring, respectively, compared with their counterparts (Figure 5F; P < 0.001). No

difference was observed in the LPHF-F2 offspring compared to the NPHF-F2 offspring.

Liver TG content was similar between the LPNF-F2 and NPNF-F2 offspring. Curiously, an HF diet did not induce a difference in liver TG content in the NPHF-F2 offspring compared with the NPNF-F2 offspring (Figure 5G). However, liver TG content in the LPHF-F2 offspring increased by 138.17% ($P < 0.0001$) and 158.40% ($P < 0.01$) compared with the LPNF-F2 and NPHF-F2 offspring, respectively, with interactions between factors.

The same pattern of results as for total liver fat content was observed for liver cholesterol content (Figure 5H). No statistical difference was observed between the LPNF-F2 and NPNF-F2 offspring or between the LPHF-F2 and NPHF-F2 offspring. However, a HF diet increased liver cholesterol content by 38.72% and 29.08% ($P < 0.01$) in the NPHF-F2 and LPHF-F2 groups, respectively, compared with their counterparts.

The livers of the LPNF-F2 and NPNF-F2 offspring exhibited a brown-reddish color with no optical evidence of hepatic lipid alteration. Microscopical observation of the hepatocytes showed that they were arranged in rows, delimited by connective tissue containing sinusoids capillaries. As shown in Table 2, NPNF-F2 hepatocytes had a homogeneous cytoplasm without fat vacuoles (score 0; Figure 6A), while the nucleus displayed a central position. LPNF-F2 histopathological analyses showed that 80% of the samples displayed mild steatosis (score 1; Figure 6B) in zone 1.

The livers of the HF-fed offspring presented a yellowish aspect, which macroscopically characterizes hepatic steatosis (Table 2). This was confirmed via histopathological analyses, which showed that the hepatocytes of the NPHF-

F2 and LPHF-F2 offspring exhibited displacement of the nucleus to the cell periphery and the presence of large cytosolic fat vacuoles. These analyses demonstrated that 66.67% of the NPHF-F2 offspring displayed severe macrovesicular steatosis (score 3) distributed in zone 1 (50%) or without a distribution pattern (azonal; 50%). In the LPHF-F2 offspring, a HF diet increased severe macrovesicular steatosis (80%; score 3; Figure 6D) evenly distributed throughout the hepatic tissue (panacinar; 60%).

3.7 Liver PEPCK and FASN expression

As shown in Figure 5I, phosphoenolpyruvate carboxykinase (PEPCK) displayed no difference between the LPNF-F2 and NPNF-F2 offspring. Additionally, a HF diet did not induce a difference in PEPCK expression between the NPHF-F2 and NPNF-F2 offspring. However, the LPHF-F2 group showed an increase of 139.10% and 132.17% in PEPCK expression compared with the NPHF-F2 ($P < 0.05$) and LPNF-F2 ($P < 0.01$) groups, respectively, with interactions between factors.

Fatty acid synthase (FASN) was increased by 49.79% in the LPNF-F2 group compared to the NPNF-F2 group ($P < 0.05$) (Figure 5J). Moreover, the NPNF-F2 and NPHF-F2 groups showed no difference in this parameter. FASN expression in the LPHF-F2 group was decreased by 58.75% compared with the LPNF-F2 group ($P < 0.05$). No statistical difference was observed between the LPHF-F2 and NPHF-F2 offspring, with interactions between the factors.

4. Discussion

In this study, we evaluated the transgenerational transmission of the programmed phenotype by LP in the first 14 days of lactation through the maternal lineage to adult male F2 offspring, and their susceptibility to damage induced by an HF diet later in life. First, we observed that LP alters glucose homeostasis in LPNF-F2 (second generation) offspring, resulting in lower fasting glycemia and islet area. Insulin sensitivity was not altered. However, insulin levels were higher. In addition, we showed lipid hepatic alterations with increased lipogenesis and grade 1 steatosis, verifying the transgenerational effects of grandmother low-protein diet-fed. After a HF diet was offered during adulthood, we show for the first time that the LPHF-F2 offspring are more susceptible to hepatic damage that the NPHF-F2 offspring. They also showed decreased total cholesterol, HDL-C, and fat pad stores. Furthermore, the hepatic tissue is completely compromised by intracellular fat vesicles. These outcomes corroborates Barker's hypothesis that metabolic programming during critical developmental periods results in altered postnatal metabolism, leaving future generations (such as the F2 generation) more susceptible to diseases (9–11).

The hypothalamus is a well-regulated brain center and an important structure in the control of energy balance (34). The expression of neuropeptides involved in the control of eating behavior is altered in male rats with undernutrition through an increase in the expression of orexigenic hypothalamic peptides, with a concomitant decreased in anorexigenic peptides(35). This results in an increase in caloric intake and a decrease in fat pad stores in adult life (15). In female adult rats, exposure to a low-protein diet during critical periods of development can affect feeding behaviors (36) by causing malformation of the

hypothalamus, which remains in adult life (37). However, body weight gain and caloric intake were not altered in F1 dams that were fed a low-protein diet during the suckling phase. In the LP, the removed protein is replaced with carbohydrates to maintain the energy content of the diet. Consumption of these diets can reduce food intake by increasing serotonin production (38).

Several studies have shown that the nutritional status of the dams during critical developmental periods is essential for pups' normal growth and development (26,38,39). Maternal milk is considered a better feeding source for newborns (40), and malnutrition during the suckling phase can negatively affect offspring growth, metabolism, and organ development (15,26). In addition, offspring health is directly influenced by intrauterine milieu (41). Here, we show for the first time that LP female offspring F1 displayed fasting hyperglycemia and glucose intolerance during pregnancy. Pregnancy is considered a diabetogenic situation *per se*. Maternal hyperglycemia and diabetes can compromise the food supply and induce adaptations in pancreatic fetal development due to glucose transportation through the placenta (41). Pregnant dams with gestational diabetes mellitus produce offspring with normal or low birth weights (39), which show impaired glucose tolerance during adulthood. This diabetogenic effect can be transmitted to the next generation of individuals (41).

Nevertheless, perinatal undernutrition determines a preference for a HF diet and increases dopaminergic action (42), which indicates the vulnerability of pathways that regulate food intake. However, an increase in HF intake was previously not observed in male offspring LP-F1 (15) and LP-F2 programmed during the suckling phase. A HF diet increased feeding efficiency in the LPHF-F2

offspring compared to the NPHF-F2 offspring, demonstrating catch-up growth. This could indicate a higher risk of obesity and related disorders (43).

A HF diet is directly associated with obesity, dyslipidemia, insulin resistance, and glucose intolerance (15,38). Indeed, our research group previously showed that these parameters were higher in NPHF-F1 adult rats than in LPHF-F1 rats (15). Here, we show for the first time that LPHF-F2 offspring displayed similar results compared to LPHF-F1 male offspring; thus showing non-genomic phenotype transmission by epigenetic mechanisms from the maternal lineage.

Maternal glucose intolerance can harm lipid metabolism and promote fat accumulation in offspring due to the upregulation of *Insr, Lpl, Pparg* and *Adipoq* mRNA. Adipocyte hypertrophy is associated with an increase in IL-6 levels, which disrupts insulin signaling (39). The LPHF-F2 group showed high-fat pad gain and an altered lipid profile with a smaller magnitude than the NPHF-F2 group. This transgenerational transmission can contribute to the worldwide pandemic of obesity and type 2 diabetes.

Alterations in lipid profiles have been observed in children with Kwashiorkor syndrome, a deficit in calories and protein. Patients with Kwashiorkor show lower serum TG, and TG accumulation on hepatocytes, due to decreased VLDL-C synthesis. Lower VLDL-C secretion occurs because of a severe protein deficiency. A block in the release of hepatic triglycerides is the major mechanism of fatty liver disease in Kwashiorkor syndrome (44). The LP used in this study had a very low protein content (4%). The restriction of some nutrients in the maternal diet leads to changes in the lipid profile of the offspring

(45), such as HDL-C and LDL-C. This is significant as increased LDL-C levels are an important hallmark of cardiovascular disease (46).

A high carbohydrate content in the diet can be associated with decreased HDL-C levels (47). Similarly, LPNF-F2 and LPHF-F2 adult offspring also had lower HDL-C levels, although LP-F1 dams were fed a normal protein diet throughout the experimental period. This profile modification may be due to changes in the expression of the transcription factors that regulates lipolysis and lipogenesis. Suppression of these transcription factors has been shown in rats fed protein restriction during the perinatal period (48), which presented with increased serum TG levels. Triglycerides are the main storage form of energy in adipocytes and hepatocytes in humans and rats. Their release from their stores must be regulated to avoid their toxic potential (49). The liver is an important site for storing excess free fatty acids and is released to control energy homeostasis. TG release occurs in the VLDL-C form by re-esterification at the endoplasmic reticulum with a requirement for apoB and microsomal triglyceride transfer protein (MTP) in the hepatic acinus pericentral zone. Lower VLDL-C secretion leads to the accumulation of TG in hepatocytes, resulting in hepatic steatosis (48).

Hepatic steatosis is characterized by lipid accumulation in hepatocytes, leading to inflammation and the potential progression to liver failure and cirrhosis (50). Even LPHF-F2 offspring had an increase in VLDL-C compared to LPNF-F2 offspring, which is not enough for TG transportation from the liver to the peripheral tissues. Hormonal and dietary factors also affect VLDL-C levels (51). For the first time, we show that protein restriction during the suckling phase had a harmful transgenerational effect on liver tissue when a HF diet was offered. In this study,

the LPHF-F2 group displayed higher hepatic TG levels, liver weight, and steatosis grade (score 3) than the NPHF-F2 group.

FASN is one of the major genes responsible for lipid homeostasis and *de novo* lipogenesis, and is controlled by hormones and nutritional status. After a meal, blood glucose and insulin levels increase and stimulate *de novo* lipogenesis (52). FASN mRNA expression was higher in the LPNF-F2 offspring, suggesting a compensatory mechanism for energy homeostasis maintenance with low-grade steatosis (score 1). Similarly, a restricted diet during pregnancy increased hepatic FASN mRNA expression in the F0 generation, with a trend in the F1 generation. The increase in FASN mRNA levels correlated with lower HDL-C content in the female progeny (45). Transgenerational studies have shown that LP can affect the hepatic transcriptional profile of thousands of genes until the F3 generation (53). In addition, restricted calorie intake during the preconception or gestational period impairs lipid metabolism, altering FASN mRNA levels in the adult offspring (54). Diets with long-chain fatty acids significantly inhibited *de novo* lipogenesis by decreasing AKT2 phosphorylation (55), which could explain low FASN expression in the LPHF-F2 and NPHF-F2 groups.

The liver consists of distinct zones with phenotypic heterogeneity, depending on their acinus or lobular localization. Enzymes involved in fatty acid metabolism can exhibit flexibility according to their physiological needs. TG accumulates in hepatocytes starting in the pericentral areas, advancing to the intermediate and periportal areas (56). For the first time, we demonstrated that LPNF-F2 shows steatosis grade 1, with a zone 1 predominant distribution. However, the HF diet increased steatosis to grade 3, with full distribution of lipids in the liver tissue (panacinar distribution). These results imply that F0 maternal

LP could induce a transgenerational effect on liver metabolism and be susceptible to non-alcoholic fatty liver disease in the F2 generation. Liver lipid metabolism is essential to neutralize the impact of FFA-mediated lipotoxicity in peripheral tissues and pancreatic beta cells (56).

The pancreas is the major organ involved in the maintenance of glucose metabolism. Early LP during pregnancy impairs pancreatic beta cell development due to glucose dependence for beta cell maturation, resulting in altered structure and function (14). At the end of gestation, the fetus can control its own glucose levels owing to the adaptation of insulin production and insulin action (26,41). Studies have shown that poor carbohydrate supply during lactation can change islet structure (37) and stimulate involution of the pancreas, which is more pronounced in litters from diabetic dams (41). The offspring of hyperglycemic dams display glucose intolerance in adulthood (26). In adulthood, pancreatic mass is normalized (41). In this study, we demonstrated the transgenerational effect of a low-protein diet on glucose homeostasis. The LPNF-F2 group was associated with hypoglycemia and hyperinsulinemia compared to the NPNF-F2 group. Additionally, the LPHF-F2 group showed lower glucose intolerance and hyperinsulinemia than the NPHF-F2 group, insulin sensitivity was not altered, and islet area was lower. These structural alterations may be compensatory mechanisms to maintain glucose homeostasis early in life (57).

The liver is the only organ that produces and exports glucose. Liver glucose production is dependent on the PEPCK enzyme, one of the main regulators of the gluconeogenic pathway. PEPCK is activated only after birth and catalyzes oxaloacetate decarboxylation to produce phosphoenolpyruvate in the presence of GTP, which is essential for gluconeogenesis pathway (58).

Nutritional status is the primary target of PEPCK regulation. Lipid accumulation is highly associated with higher PEPCK transcription in Zucker genetically fatty rats (59). In LPHF-F2, PEPCK expression increased. This induction may contribute to gluconeogenesis, which can be associated with glucose intolerance and hyperinsulinemia observed in the LPHF-F2 offspring.

In conclusion, F0 maternal protein-restricted diet during lactation could induce a transgenerational effect on glucose and hepatic metabolism in the F2 generation, making liver offspring more vulnerable to nutritional injury later in life. Further studies are required to understand the transgenerational mechanisms of low-protein diet.

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6. Statement of authors' contributions to manuscript

Rodrigo Vargas: Conceptualization, methodology, formal analysis, investigation, writing – original draft; Isabela Peixoto Martins and Camila Cristina Ianoni Matiusso, Raiana Aparecida Casagrande, Camila Benan Zara, Anna Carolina

Huppes de Souza, William Pereira Horst, Taina Cristine Sieklicki and Naiara Cristina Lucredi: investigation; Tania Cristina Alexandrino Becker, Jurandir Fernando Comar: Resources and supervision; Ananda Malta, Paulo Cezar de Freitas Mathias: Resources, supervision and writing – review and editing.

7. Conflict of interest

The authors declare that they have no conflict of interest.

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Tables + legends

Table 1 – Effect of HF consumption on biochemical parameters at PND90 of adult F2 offspring from F1 dams programmed by protein restriction on lactation

Parameters	NPNF-F2	NPHF-F2	LPNF-F2	LPHF-F2	LP	HF	I
Fasting glycemia (mg/dl)	94.7±3.0	109.2±5.5$^{\phi}$	80.2±2.7$^{\#}$	90.8±3.4$^{\#\#}$	ns	****	**
Fasting insulinemia (⌈U/ml)	153.6±18	160.6±5.5	234.9±7.2$^{\#\#\#}$	253.9±8.8$^{\#\#\#}$	ns	****	ns
Total cholesterol (mg/dl)	87.6±3.8	123.1±4.0$^{\phi\phi\phi}$	88.7±1.4	101.5±3.9$^{\#\#}$	**	*	****
Triglycerides (mg/dl)	66.1±2.4	82.8±6.4	71.6±4.4	90.7±5.5$^{\phi}$	ns	ns	**
HDL-C (mg/dl)	51.9±5.1	68.6±2.4$^{\phi}$	26.7±2.4$^{\#\#\#\#}$	38.9±2.6$^{\#\#\#\#\phi}$	ns	****	***
LDL-C (mg/dl)	21.2±1.5	30.7±1.6	38.5±1.6$^{\#\#\#}$	53.4±3.1$^{\#\#\#\#\phi}$	ns	****	****
VLDL-C (mg/dl)	13.9±0.6	18.1±1.2	14.3±0.9	18.1±1.1$^{\phi}$	ns	ns	***

All data are expressed as the mean ± S.E.M. and were obtained from 10-12 rats of each group (from 10-12 different litter). NPNF-F2: offspring of dam (F1) born from NP dam (F0) than received NF during adulthood; NPHF-F2: offspring of dam (F1) born from NP dam (F0) than received HF during adulthood; LPNF-F2: offspring of dam (F1) born from LP dam (F0) than received NF during adulthood; LPHF-F2: offspring of dam (F1) born from LP dam (F0) than received HF during adulthood. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 to NP-F2 vs. LP-F2 in the same conditions; $^{\phi}$P<0.05, $^{\phi\phi}$P<0.01, $^{\phi\phi\phi}$P<0.001, $^{\phi\phi\phi\phi}$P<0.0001 to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor; and I: interaction between LP and HF factors. *P<0.05, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on two-way analysis of variance.

Table 2 – Effect of HF consumption on hepatic steatosis of adult F2 offspring from dams programmed by protein restriction during lactation

		% Responses in category				
Item		Score	NPNF-F2 (n=5)	NPHF-F2 (n=6)	LPNF-F2 (n=5)	LPHF-F2 (n=5)
Steatosis						
	<5%	0	100%	0%	20%	0%
	5% - 33%	1	0%	0%	80%	0%
	>33% - 66%	2	0%	33,33%	0%	20%
	>66%	3	0%	66,67%	0%	80%
Location						
	Zone 3	0	0%	0%	0%	0%
	Zone 1	1	0%	50%	100%	0%
	Azonal	2	0%	50%	0%	40%
	Panacinar	3	0%	0%	0%	60%

All data were obtained from 5-6 rats of each group (from 5-6 different litter). NPNF-F2: offspring of dam (F1) born from NP dam (F0) than received NF during adulthood; NPHF-F2: offspring of dam (F1) born from NP dam (F0) than received HF during adulthood; LPNF-F2: offspring of dam (F1) born from LP dam (F0) than received NF during adulthood; LPHF-F2: offspring of dam (F1) born from LP dam (F0) than received HF during adulthood.

Figure legends

Figure 1. Experimental design. NP: normal-protein diet; LP: low-protein diet; NF: normal-fat diet; HF: high-fat diet; PND: post-natal day.

Figure 2. Dams' biometric parameters and glucose homeostasis analysis. Plasma glucose during oGTT (A), insulin tolerance test and K_{itt} (B), and fasting glycemia (C) during pregnancy; body weight gain (D), food intake (E), and feeding efficiency (F) during lactation. The data are expressed as the means ± S.E.M. and were obtained from 6-12 dams (from 6-12 different litter). The inset represents the AUC. *P<0.05, **P<0.01 for Student's *t* test. NP-F1: female rats of dams fed a normal-protein diet during lactation; LP-F1: female rats of dams fed a low-protein diet during lactation.

Figure 3. Offspring's biometric parameters, caloric intake and feeding efficiency. Litter weight at born (A), body weight gain during lactation (B), body weight gain from 21 to 60 PND (C), feeding efficiency from 21 to 60 PND (D), food intake from 21 to 60 PND (E), body weight gain from 60 to 90 PND (F), feeding efficiency from 60 to 90 PND (G), food intake from 60 to 90 PND (H), and body weight at 90 PND (I). The data are expressed as the means ± S.E.M. and were obtained from 12-15 rats of each group (from 4-5 different litter). The inset represents the AUC. *P<0.05, ****P<0.0001 for Student's *t* test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 to NP-F2 vs. LP-F2 in the same conditions; $^{\phi}$P<0.05, $^{\phi\phi}$P<0.01, $^{\phi\phi\phi}$P<0.001, $^{\phi\phi\phi\phi}$P<0.0001 to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet

factor; and I: interaction between LP and HF factors. *P<0.05, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on two-way analysis of variance.

Figure 4. Glucose homeostasis and pancreas morphometry at 90 days old. Plasma glucose during ivGTT (A), insulin tolerance test and K_{itt} (B), islet area (C), NPNF-F2 islet (D), NPHF-F2 islet (E), LPNF-F2 islet (F) and LPHF-F2 islet (G). The data are expressed as the means ± S.E.M. and were obtained from 6-12 rats of each group (from 3-6 different litters). $^{\#\#}$P<0.01, $^{\#\#\#\#}$P<0.0001 to NP-F2 vs. LP-F2 in the same conditions; $^{\phi}$P<0.05, $^{\phi\phi\phi}$P<0.001 to NF vs. HF in the same group for the probability based on Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor; and I: interaction between LP and HF factors. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on two-way analysis of variance.

Figure 5. Fat pad store and hepatic profile at 90 days old. Retroperitoneal (A), perigonadal (B), mesenteric fat pad store (C), retroperitoneal adipocyte area (D), liver weight (E), total liver fat content (F), liver triglycerides content (G), liver cholesterol content (H), PEPCK (I) and FASN expression (J). The data are expressed as the mean ± S.E.M. and were obtained from 8-12 rats of each group (from 3-4 different litter). $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 to NP-F2 vs. LP-F2 in the same conditions; $^{\phi}$P<0.05, $^{\phi\phi}$P<0.01, $^{\phi\phi\phi}$P<0.001, $^{\phi\phi\phi\phi}$P<0.0001 to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP: low-protein diet factor; HF: high-fat diet factor; and I: interaction

between LP and HF factors. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ and ns, no significant difference, based on two-way analysis of variance.

Figure 6. Hepatic steatosis. Representative images of microscopical analyses of steatosis grade 0 (none to 5% of hepatocytes affected) (A; NPNF-F2), grade 1 (>5% - 33% affected) (B; LPNF-F2), grade 2 (>33% - 66% affected) (C; NPHF-F2) and grade 3 (> 66% affected) (D; LPHF-F2). Hematoxylin & Eosin-stained sections. Hash: examples of macrovesicular steatosis, in all hepatocytes, fat inclusions displaced nucleus to the periphery; Arrow: cluster of inflammatory cells. Magnification: 200x. Scale bar = 50μm.

Figures

Figure 1.

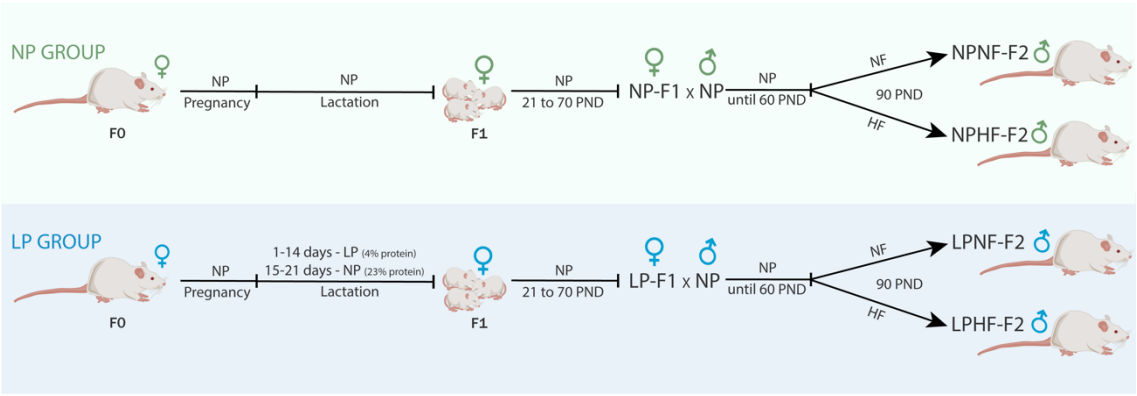


Figure 2.

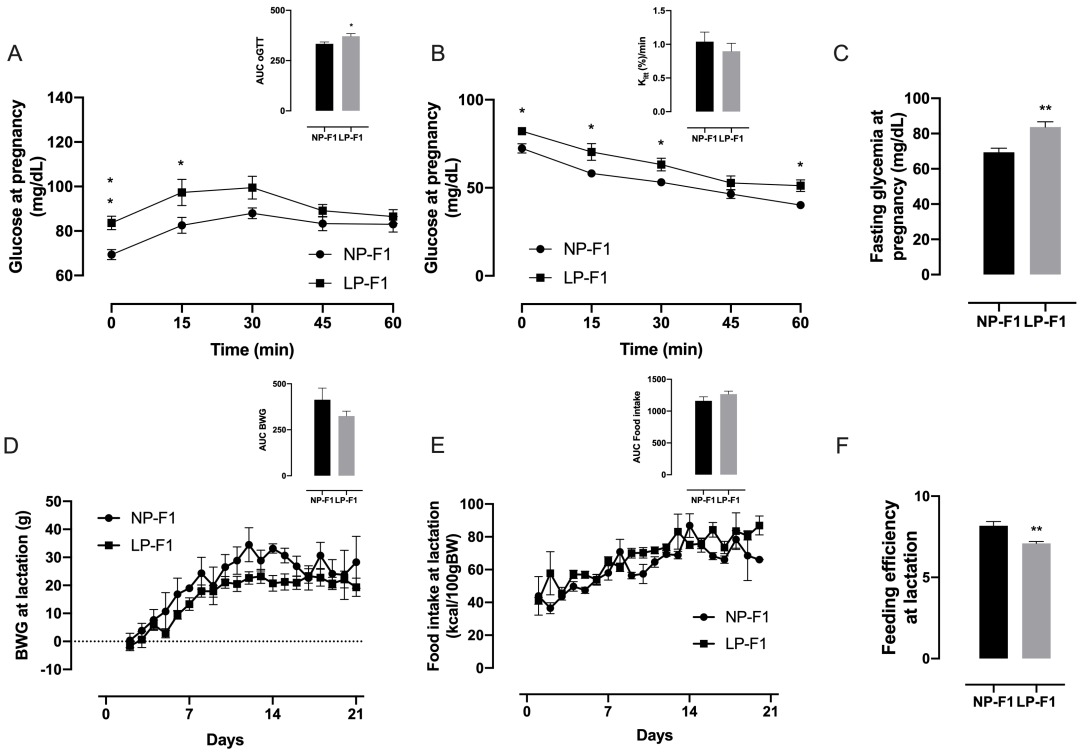


Figure 3.

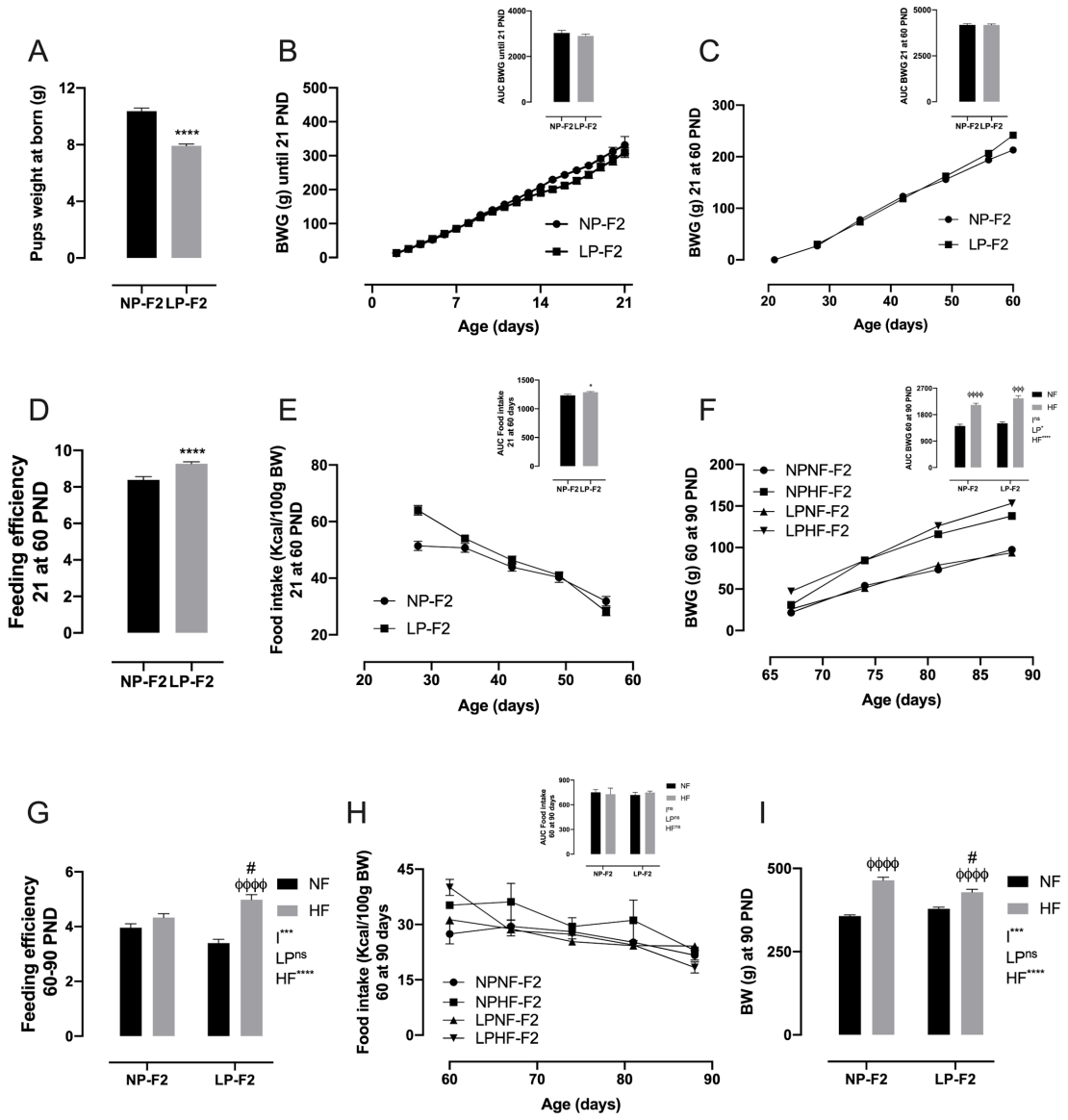

A
Pups weight at born (g)
****
NP-F2 LP-F2
B
BWG (g) until 21 PND
AUC BWG until 21 PND
NP-F2
LP-F2
Age (days)
C
BWG (g) 21 at 60 PND
AUC BWG 21 at 60 PND
NP-F2
LP-F2
Age (days)
D
Feeding efficiency 21 at 60 PND
****
NP-F2 LP-F2
E
Food intake (Kcal/100g BW) 21 at 60 PND
AUC Food intake 21 at 60 days
NP-F2
LP-F2
Age (days)
F
BWG (g) 60 at 90 PND
AUC BWG 60 at 90 PND
NPNF-F2
NPHF-F2
LPNF-F2
LPHF-F2
Age (days)
G
Feeding efficiency 60-90 PND
NF
HF
I***
LPns
HF****
NP-F2
LP-F2
H
Food intake (Kcal/100g BW) 60 at 90 days
AUC Food intake 60 at 90 days
NPNF-F2
NPHF-F2
LPNF-F2
LPHF-F2
Age (days)
I
BW (g) at 90 PND
NF
HF
I***
LPns
HF****
NP-F2
LP-F2

Figure 4.

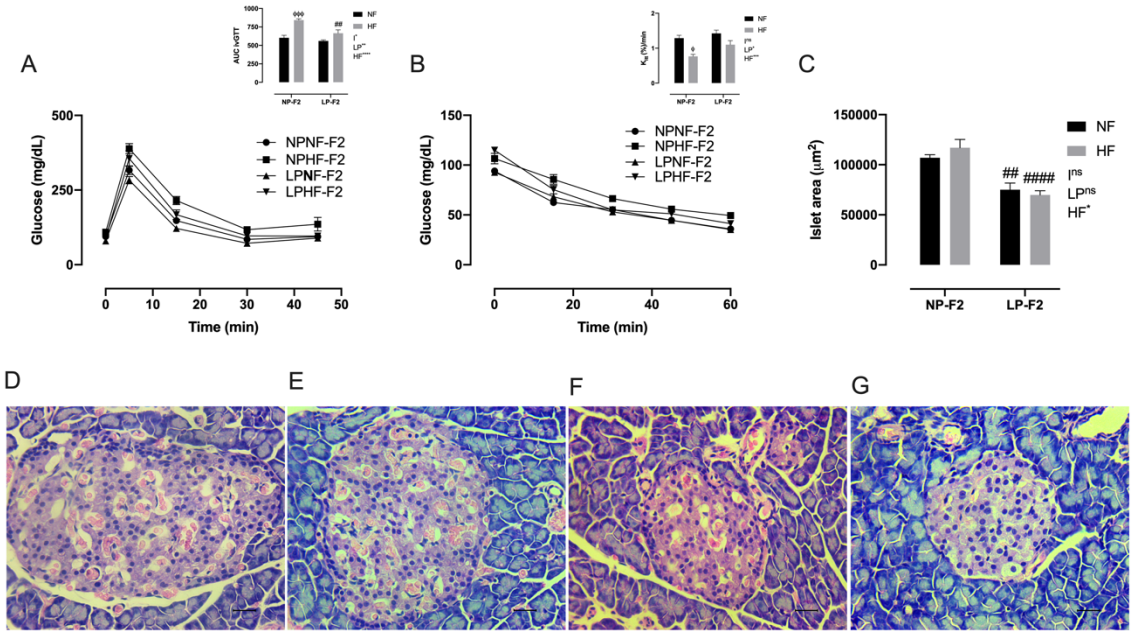

A
B
C
Glucose (mg/dL)
Time (min)
NPNF-F2
NPHF-F2
LPNF-F2
LPHF-F2
Islet area (µm²)
NF
HF
NP-F2
LP-F2
D
E
F
G

Figure 5.

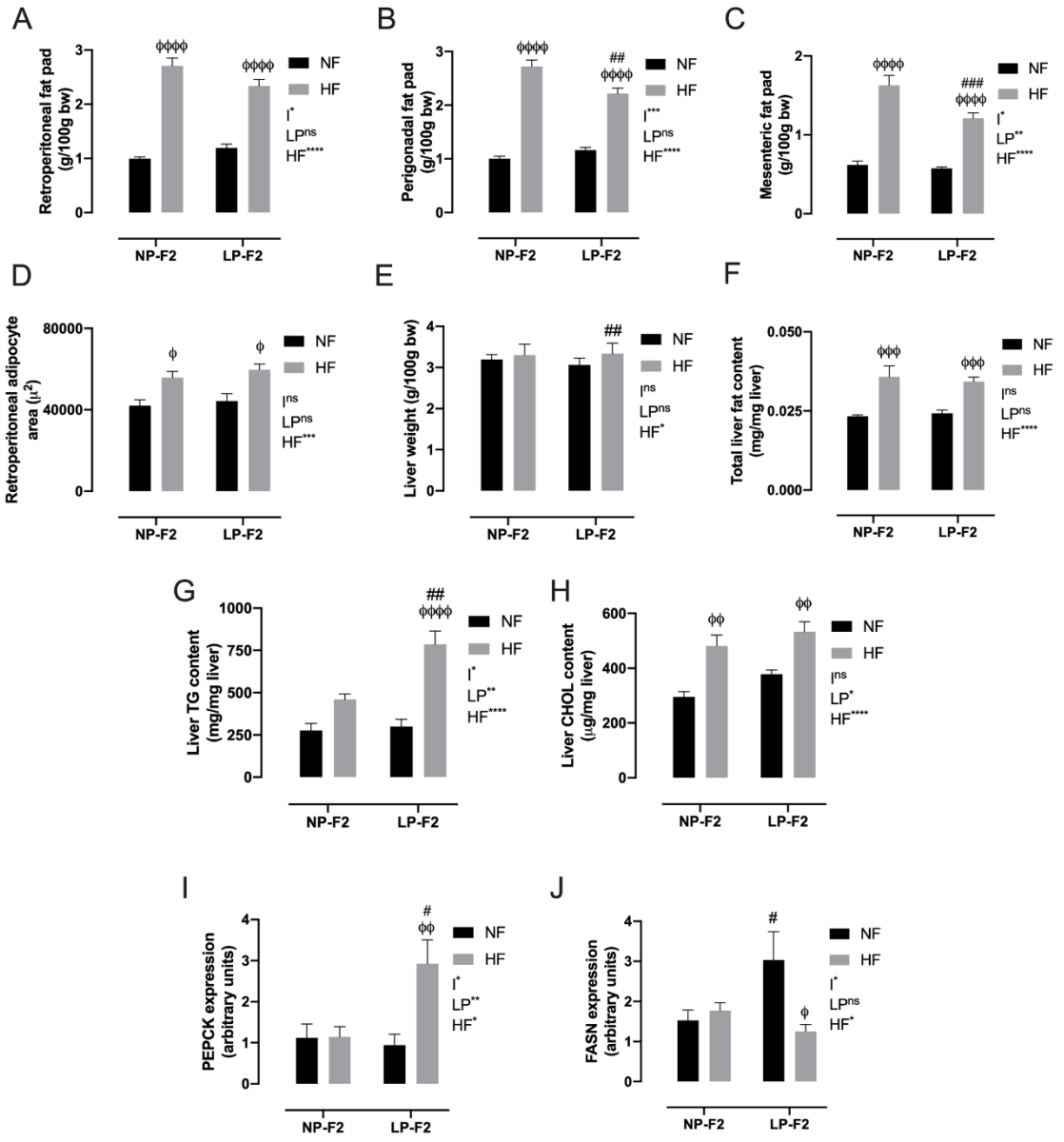
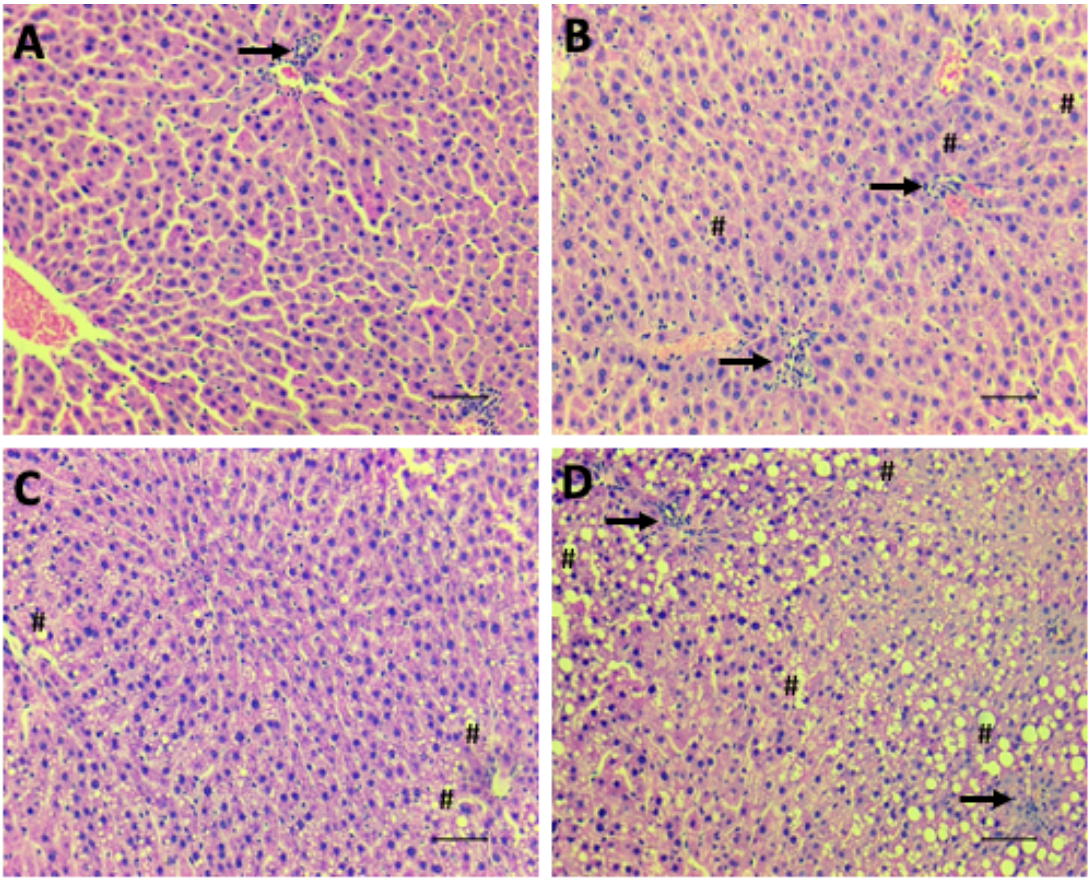

A
Retroperitoneal fat pad (g/100g bw)
NF
HF
I*
LPns
HF****
NP-F2
LP-F2
B
Perigonadal fat pad (g/100g bw)
I***
LPns
HF****
C
Mesenteric fat pad (g/100g bw)
I*
LP**
HF****
D
Retroperitoneal adipocyte area (μ²)
Ins
LPns
HF***
E
Liver weight (g/100g bw)
Ins
LPns
HF*
F
Total liver fat content (mg/mg liver)
Ins
LPns
HF****
G
Liver TG content (mg/mg liver)
I*
LP**
HF****
H
Liver CHOL content (μg/mg liver)
Ins
LP*
HF****
I
PEPCK expression (arbitrary units)
I*
LP**
HF*
J
FASN expression (arbitrary units)
I*
LPns
HF*

Figure 6.



Supplementary material

Sequences of the primers used in PCR

Gene	Forward	Reverse
GADPH	TTGTGCAGTGCCAGCCTC	GAGAAGGCAGCCCTGGTAAC
PEPCK	GGGGGTGTTTACTGGGAAGG	CGGTTCCTCATCCTGTGGTC
FAS	ACCTGTGGAATTCCCGGTTC	ACTCGGAACTGGCGTCAATG