Journal of Developmental Origins of Health and Disease

www.cambridge.org/doh

Puberty as a DOHaD programming window: high-fat diet induces long-term hepatic dysfunction in male rats

Beatriz Gonçalves dos Santos[1], Rosiane Aparecida Miranda[2], Lucas Paulo Jacinto Saavedra[3], Flávio Andrade Francisco[3], Maiara Vanusa Guedes Ribeiro[3], Anna Rebeka Oliveira Ferreira[3], Marcos Divino Ferreira-Junior[1], Keilah Valéria Naves Cavalcante[1], Carlos Henrique Xavier[4], Egberto Gaspar de Moura[2], Patrícia Cristina Lisboa[2], Ariel Penha Carvalho da Mota[1], Gustavo Rodrigues Pedrino[4], James Andrew Armitage[5], Paulo Cezar de Freitas Mathias[3], Kesia Palma-Rigo[3] and Rodrigo Mello Gomes[1]

[1]Department of Physiological Sciences, Laboratory of Endocrine Physiology and Metabolism, Federal University of Goiás, Goiânia, GO, Brazil; [2]Department of Physiological Sciences, Laboratory of Endocrine Physiology, Institute of Biology Roberto Alcantara Gomes, Rio de Janeiro State University, Rio de Janeiro, Brazil; [3]Department of Biotechnology, Genetics and Cell Biology, Laboratory of Secretion Cell Biology, State University of Maringá, Maringá, Brazil; [4]Department of Physiological Sciences, Federal University of Goiás, Goiânia, GO, Brazil and [5]School of Medicine and IMPACT Institute, Deakin University, Waurn Ponds, Australia

Original Article

Cite this article: dos Santos BG, Miranda RA, Saavedra LPJ, Francisco FA, Ribeiro MVG, Oliveira Ferreira AR, Ferreira-Junior MD, Cavalcante KVN, Xavier CH, de Moura EG, Lisboa PC, Mota APC, Pedrino GR, Armitage JA, Mathias PCF, Palma-Rigo K, and Gomes RM. (2023) Puberty as a DOHaD programming window: high-fat diet induces long-term hepatic dysfunction in male rats. *Journal of Developmental Origins of Health and Disease* **14**: 614–622. doi: 10.1017/S2040174423000272

Received: 29 March 2023
Revised: 10 August 2023
Accepted: 11 September 2023
First published online: 13 November 2023

Keywords:
High-fat diet; puberty; obesity; DOHaD; NAFLD

Corresponding author:
R. M. Gomes; Email: gomesrm@ufg.br

Abstract

The aim of this study was to evaluate whether high-fat (HF) diet intake during puberty can program obesity as well as generate glucose imbalance and hepatic metabolic dysfunctions in adult life. Male Wistar rats were randomly assigned into two groups: rats fed standard chow (NF) and rats fed a HF from postnatal 30-day-old (PND30) until PND60. Then, both groups were fed a standard chow from PND60 until PND120. Euthanasia and samples collections occurred at PND120. HF animals were overweight (+11%) and had increased adiposity, hyperphagia (+12%), hyperglycaemia (+13%), hyperinsulinemia (+69%), and hypertriglyceridemia (+34%). Plasma glucose levels during intravenous glucose tolerance test (ivGTT) and intraperitoneal insulin tolerance test (ipITT) were also higher in the HF group, whereas K_{itt} was significantly lower (–34%), suggesting reduced insulin sensitivity. In the same sense, HF animals present pancreatic islets hypertrophy and high β-cell mass. HF animals also had a significant increase in blood glucose levels during pyruvate tolerance test, indicating increased gluconeogenesis. Hepatic morphology analyses showed an increase in lipid inclusion in the HF group. Moreover, PEPCK and FAS protein expression were higher in the livers of the HF animals (+79% and + 37%, respectively). In conclusion, HF during puberty causes obese phenotype leading to glucose dyshomeostasis and nonalcoholic fatty liver disease, which can be related to the overexpression of proteins PEPCK and FAS.

Introduction

Programming periods, such as gestation, lactation, and puberty, are markedly known by the phenotypic plasticity, increasing the risk of cardiometabolic diseases late in life.[1] The "Developmental Origins of Health and Disease" (DOHaD) concept predicts that adaptations during critical developmental periods determine health or illness in adulthood.[2]

Puberty is physiologically characterized by sexual and physical maturation, and an adequate nutritional support is necessary for a normal development.[3] Nutritional disorders, such as overnutrition or undernutrition during phenotypic plasticity periods program to obesity, diabetes, and cardiovascular disorders.[4–6] Epidemiological and experimental studies have demonstrated that a high-fat (HF) diet induces overweight/obesity, hyperphagia, insulin resistance, glucose intolerance, hepatic dysfunctions, and other disturbances.[7–10] Specifically, HF intake during the puberty induces abnormalities in reproductive function, adiposity, and impairment of hippocampal-dependent memory associated with enhanced hippocampal inflammation later in life in male rats.[5,11] Moreover, prepubertal HF female rats had polycystic ovary syndrome.[12] In humans, eating behavior among adolescents contribute to obesity onset and related disorders.[13,14]

Eating habits and obesity are important risk factors for liver diseases, such as nonalcoholic fatty liver disease (NAFLD), characterized by fat accumulation in the liver accompanied by different degrees of inflammation and fibrosis.[15] Among adolescents, the prevalence of NAFLD

is high and increases the risk of glucose dyshomeostasis.[16,17] In addition, NAFLD is more common in men than in women.[18] Several molecular mechanisms have been proposed to explain the development of NAFLD, although it is not fully understood. Studies suggest that the capacity of the liver is overwhelmed by excess substrates, leading to the accumulation of lipotoxic lipids and consequent hepatocellular injury.[19] Liver lipotoxicity may be related to failure in gluconeogenesis and/or lipogenesis processes, impacting hepatic lipid uptake, synthesis, oxidation, and export of fat. HFD fed animals increased the expression of PEPCK, that contributes with high blood glucose in this animal model.[20,21] On the other hand, in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase (PEPCK), an enzyme responsible for converting cytoplasmic oxaloacetate into phosphoenolpyruvate (PEP), intermediates of the tricarboxylic acid (TCA) cycle accumulate, and hepatic steatosis is observed.[22] Increased hepatic *de novo* fatty acid synthesis and upregulation of key lipogenic enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), are also observed in clinical and experimental models of NAFLD.[23] Maternal HF diet induces molecular alterations in pathways of lipid metabolism in the liver in offspring rats during the puberty.[24] When these same animal models are exposed to high-fructose diet during puberty, liver injury is intensified, demonstrating that this period of life is extremely susceptible to the metabolic programming.[25] Regarding exposure to HF, some aspects of its effects remain unclear, especially in relation to puberty as a window of programming. We hypothesized that the exposure to HF during puberty can induce liver dysfunction at adulthood, promoting fat accumulation. Thus, we investigated the long-term repercussions of HF during puberty on (1) body weight, food intake and adiposity, (2) hepatic morphology and functions, (3) glucose homeostasis, and (4) protein content of PEPCK, ACC, and FAS. In addition, insulin sensitivity in adult rats were also investigated.

Materials and methods

Ethical approval

The handling of animals and experimental procedures were performed according to the rules of the National Council of Animal Experiments Control (CONCEA) and the Brazilian Society of Science in Laboratory Animals (SBCAL) and approved by the Ethics Committee on Animal Use of State University of Maringá - CEUA/UEM (protocol number 1527130815).

Experimental design and diets

Twenty-five-day-old male Wistar rats were housed in the Animal Facility of the Laboratory of Secretion Cell Biology, Department of Biotechnology, Genetic and Cell Biology of State University of Maringa, in polypropylene cages (45 × 30 × 15 cm) maintained on a 12 h light–dark cycle (07:00 lights on) and controlled temperature (22.0 ± 2°C). At the postnatal day 30 (PND30), after 5 days of environmental adaptation, the animals were divided into two groups: control group fed a standard chow, normal fat diet (NF; 15.66 kJ/g; n = 40), and rats fed a HF (HF; 22.46 kJ/g; n = 40). Table 1 shows the composition of both diets. Diets were manufactured as previously described[26] and were given *ad libitum* during 30 days, from PND30 until PND60. Subsequently, from PND60 to PND120 both NF and HF groups were fed with standard chow. Fig. 1a summarizes the experimental protocol. Animals had unlimited access to food and water. The animals were maintained in cages with 3 to 4 animals. Body weight and food intake were evaluated weekly throughout the experimental period.

Table 1. Composition of diets

	Normal fat diet (NF)		High-fat diet (HF)	
Ingredients	g/100g	kJ/100g	g/100g	kJ/100g
Casein	20.00	294.00	20.00	294.00
Sucrose	10.00	161.90	10.00	161.90
Cornstarch	42.75	681.44	11.55	184.11
Dextrinized starch	13.20	230.74	13.20	230.74
Lard	–	–	31.20	1177.49
Soybean oil	4.00	153.48	4.00	153.48
Cellulose	5.00	40.00	5.00	40.00
Mineral mix (AIN–93)	3.50	–	3.50	–
Vitamin mix (AIN–93)	1.00	–	1.00	–
L–cystine	0.30	4.72	0.30	4.72
Choline bitartrate	0.25	–	0.25	–
TOTAL	100 g	1566.27 kJ	100 g	2246.43 kJ

Intravenous glucose tolerance test

At PND120, a cohort of adult offspring (n = 10 per group) from both experimental groups were anesthetized (ketamine 75 mg + xylazine 15 mg/kg of BW, i.m.) and a silicone cannula (Silastic, Dow Corning, Midland, MI, USA) was implanted in the right jugular vein. After overnight fasting, an intravenous glucose tolerance test (ivGTT) was performed in conscious rats, as previously described.[27] Blood samples were collected and centrifuged at 10,000 r.p.m. for 5 min for plasma collection, and then the plasma was stored at −20 °C for subsequent measurements of glucose and insulin. Animals used for the ivGTT were not used in any other experimental procedures.

Intraperitoneal insulin tolerance test

At PND120, another cohort of adult offspring (n = 10 / group) from both experimental groups received an intraperitoneal injection of human recombinant insulin (2 U/kg of BW, Eli Lilly, São Paulo, Brazil). The Intraperitoneal insulin tolerance test (ipITT) was performed in fed and conscious rats. Blood samples were collected through a small cut at the tip of the tail, and blood glucose was measured using a glucometer (ACCU-CHEK Advantage, Roche Diagnostics, Mannheim, Germany). Blood samples were collected at 0 (prior to insulin injection) and 5, 10, 15, 20, and 25 min after injection, and glycemia was measured at each corresponding time. Thereafter, the constant rate for glucose decay (K_{itt}) was calculated by the formula K_{itt} (%/min) = $0.693/t_{1/2}$. The plasma glucose $t_{1/2}$ was calculated from the slope of the least-squares analyses of plasma glucose concentrations during the linear decay phase.[28] Animals used in this test were not used in any other experimental procedures.

Intraperitoneal pyruvate tolerance test

Another cohort of adult offspring (PND120, n = 10 / group) was fasted for 16 hours and submitted to an intraperitoneal pyruvate

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

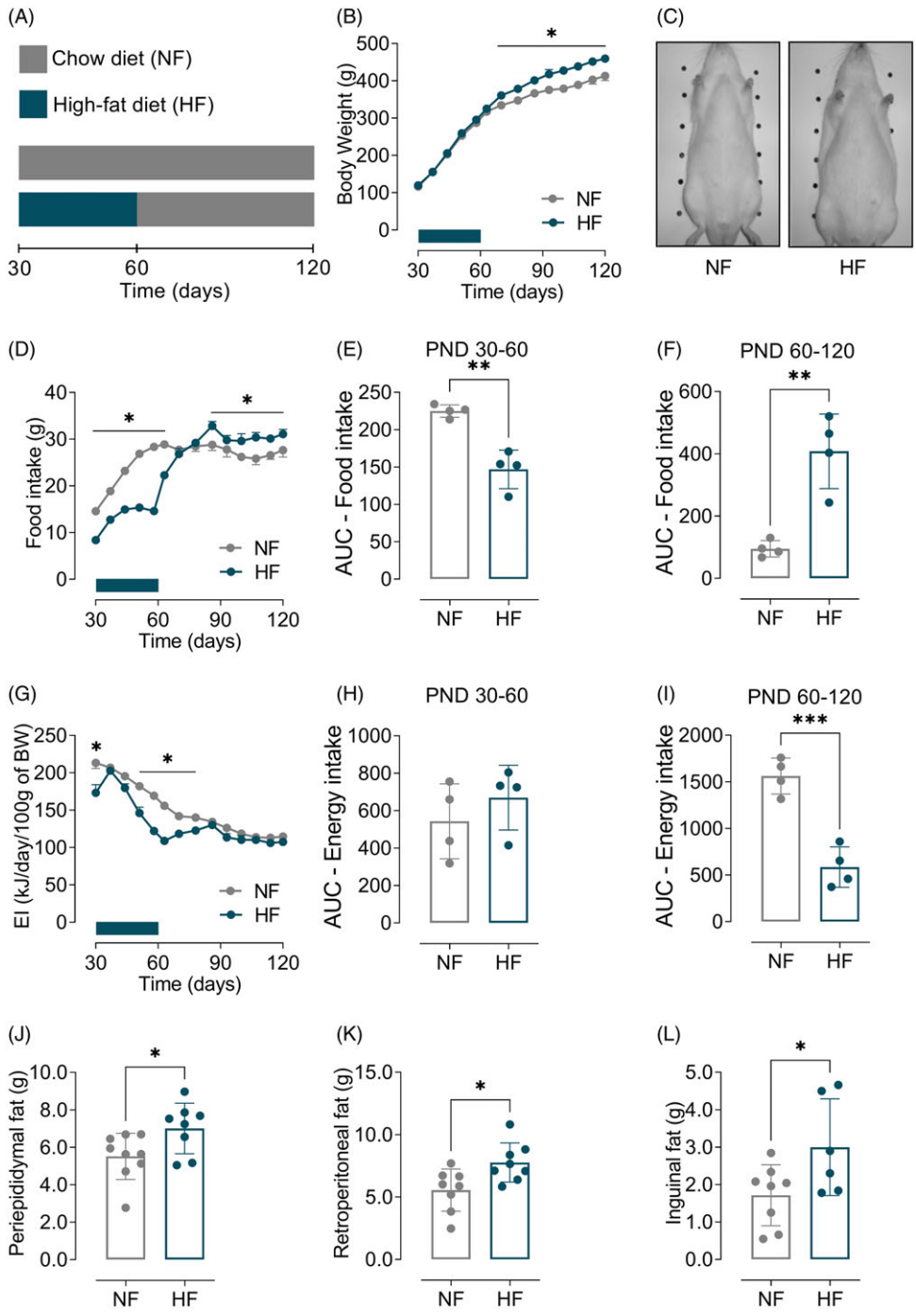

Figure 1. Long-term effects of high-fat diet during puberty on food intake and body composition of adult rats. Experimental protocol (A), curve of body weight throughout experimental period (B), representative images showing the obese phenotype of the HF rat (C), food intake throughout experimental period (D), area under curve of food intake from PND30 until PND60 (E), area under curve of food intake from PND60 until PND120 (F), energy intake throughout experimental period (G), area under curve of energy intake from PND30 until PND60 (H), area under curve of energy intake from PND60 until PND120 (I), periepididymal fat (J), retroperitoneal fat (K), and inguinal fat (L) at PN120. Data are presented as the mean ± SEM. Two-way ANOVA (B, D, and G) *$p < 0.05$; Student's *t*-test (bar graphs) *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

tolerance test (ipPTT). Conscious rats received an intraperitoneal injection of 25% (w/v) sodium pyruvate solution diluted in normal saline (2 g / kg of BW; Sigma-Aldrich, St Louis, MO, USA). Blood samples were collected at 0 min (before pyruvate injection) and 15, 30, 60, 90, and 120 min after injection. Blood samples were collected through a small tail tip cut, and blood glucose was measured using a blood glucose meter (ACCU-CHEK Advantage, Roche Diagnostics, Mannheim, Germany).[29] Animals used for the ipPTT were not used in any other experimental procedures.

Euthanasia and sample collection

At PND120, another cohort of fasted animals (n = 10 / group) was anesthetized with sodium thiopental (40 mg/kg of BW, i.p.;

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

Thiopentax, Cristália, Brazil) and euthanized by exsanguination for blood, liver, and white adipose tissue (WAT) sample collection.

Liver morphological analyses

Liver samples were removed, frozen in liquid nitrogen, and stored in a -80 °C freezer. Subsequently, these samples were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA, USA). Nonserial histological sections (10 µm thick) were obtained using a Leica CM1850 cryostat (Leica Biosystems, Wetzlar, Germany) and stained with Oil Red O. The percentage of lipid inclusion was determined by the ratio between the red oil marked area and total area of the images (% = marked area / total area × 100). Morphological analyses were performed using digital images (TIFF 24-bit color, 2560 × 1920 pixels) obtained with a light microscope (Olympus BX41, Tokyo, Japan) and a camera QColor 3 Olympus (×400 magnification). The images were acquired randomly from 5 rats per group (20 images per rat). The analyses were performed using the software ICY (Institut Pasteur, Paris, France).

Immunohistochemistry

Pancreas samples were fixed in 10% buffered formalin, dehydrated, embedded in histological paraffin, and sectioned (5 µm) in nonserial cuts. Sections were incubated for 60 min at room temperature with a specific mouse monoclonal primary antibody diluted to 1:500 (anti-insulin - catalogue # I2018 from Sigma-Aldrich Inc., St Louis, MO, USA). The immunohistochemistry assay was performed using a commercial kit (Histostain-Plus, Invitrogen, Carlsbad, CA, USA), as previously described.[27] Finally, sections were counterstained with hematoxylin. The morphometric analyses were performed using digital images (TIFF 24-bit color, 2560 × 1920 pixels). Quantitative analyses of the pancreatic islets number were performed using digital images (×20 magnification) from 6 different fields from each animal. Analyses of the pancreatic islet area and pancreatic β-cell mass were performed using 40 digital images (×400 magnification) from each animal. Analyses were performed using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Biochemical and hormonal analyses

Blood samples were centrifuged (10,000 r.p.m. for 5 min), and plasma used to determinate blood glucose, triglycerides, and total and HDL cholesterol by the enzymatic-colorimetric method with a commercial kit (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil) according to the manufacturer's instructions. Blood insulin was measured by radioimmunoassay in a gamma counter (Wizard2 Automatic Gamma Counter, TM-2470, PerkinElmer, Shelton, CT, USA). It was used as standards human insulin and anti-insulin antibody (catalogue # I9278 and 4200691 Sigma-Aldrich, St Louis, MO, USA) and recombinant human insulin ^{125}I-labeled (catalogue # NEX420 PerkinElmer, Shelton, CT, USA). The intra-assay coefficients of variation were in the range of 8–10%. The limit of detection was 0.006 ng/ml. The measurements were taken in a single assay.

Western blotting

Liver samples (n = 4 / group) were homogenized in lysis buffer (PBS [137 mM NaCl, 2.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4], 8.8 mM IGEPAL CA-630, 12 mM sodium deoxycholate, 3.47 mM SDS, 2 mM Na_3VO_4, 1 mM PMSF, 2.34 µM leupeptin, 0.154 µM aprotinin, 1.45 µM pepstatin) in a glass homogenizer at 4°C and centrifuged at 10,000 r.p.m. at 4°C for 20 minutes. After, total protein content was quantified by the bicinchoninic acid method (Sigma-Aldrich, Missouri, EUA) according to the manufacturer's instructions. Samples were denatured in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.001% bromophenol blue). Aliquots of 30 µg of protein from each sample were subjected to separation by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Amersham Protran, GE Healthcare, Little Chalfont, BUX, UK), The membranes were incubated with a blocking solution (5% skim powdered milk, 10 mM Tris, 150 mM NaCl, 0.02% Tween 20) under mild agitation for 90 min at room temperature. Subsequently, the membranes were washed with TBS and then incubated overnight at 4°C with the following specific primary antibodies: PEPCK (at 1:1000 dilutions; catalogue # sc-32879 from Santa Cruz Biotechnology, CA, USA), ACC (at 1:1000 dilutions; catalogue # 4190 from Cell Signalling Technology MA, USA), FAS (at 1:1000 dilutions; catalogue # 3180 from Cell Signalling Technology MA, USA), β-actin (at 1:1000 dilution; catalogue # A2228 from Sigma-Aldrich MO, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH at 1:1000 dilution; catalog # SC-25778 from Santa Cruz Biotechnology, CA, USA). Then, the membranes were gently washed (3x 5 min; 10 mM Tris, 150 mM NaCl, 0.02% Tween 20) and incubated with the manufacturer-specific HRP-conjugated secondary antibody (anti-rabbit at 1:10,000 dilution; catalogue # B7389 from Sigma-Aldrich MO, USA) or (anti-mouse at 1:10,000 dilution; catalogue # B8520 from Sigma-Aldrich MO, USA) for 90 min. The protein bands were visualized with chemiluminescence (ECL kit; Amersham Biosciences, London, UK) and exposed to ImageQuant LAS (GE Healthcare, Buckinghamshire, UK). The area and density of the bands were quantified by ImageJ software (NIH, MA, USA). β-Actin and GAPDH were used as a load control.

Statistical analysis

Data are expressed as Mean ± standard error of the mean (SEM). To compare differences between groups unpaired Student's *t*-test was used. To compare the differences in body weight, food intake, ivGTT, ipITT, and ipPTT curves, two-way ANOVA was used followed by the Sidak post hoc test. GraphPad Prism 9 software (v9.01, GraphPad, San Diego, CA, USA) was used for data analyses and build graphics. Differences were considered significant at $p < 0.05$.

Results

Long-term effects of HF diet during puberty on food intake and body composition

As shown in Fig. 1b, during HF intake period (PND30 – PND60) animals from both groups had no change in the body weight. However, at PND70 we observed a clear increase on the body weight of HF animals compared to NF animals (NF 334.70 ± 5.39 g *vs* HF 360.70 ± 5.60 g; $p < 0.05$), which gradually increased throughout experimental period, with a difference lasting up to PN120 (NF 413.10 ± 12.30 g *vs* HF 459.60 ± 10.00 g; $p < 0.05$).

As depicted in Fig. 1d–f, during the treatment (PND30 – PND60), HF animals had low food intake compared to NF animals (AUC food intake NF 224.90 ± 4.22 g *vs* HF 146.90 ± 12,89 g; $p < 0.0001$). However, from PND60 until PND120, HF animals

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

had higher food intake (AUC food intake NF 94.68 ± 13.16 g vs HF 408.00 ± 59.79 g n = 4; $p < 0.05$).

In the same sense, the energy intake of HF animals was similar than that of their counterparts during the dietary intervention (AUC of energy intake 30–60 days NF 543.2 ± 100.1 *vs* HF 669.3 ± 86.5; Fig. 1h), however after the dietary intervention the energy intake of HF group was lower than NF group (AUC of energy intake 60-120 days NF 1562.0 ± 96.7 *vs* HF 586.1 ± 108.4; $p < 0.001$; Fig. 1i).

For WAT mass, we observed an effect of programming as shown in representative Fig. 1c, and it was confirmed by quantitative measurements. HF animals had higher periepididymal fat (NF 5.52 ± 0.41 g vs HF 7.01 ± 0.47 g; $p < 0.05$; Fig. 1j), retroperitoneal fat (NF 5.56 ± 0.59 g vs HF 7.76 ± 0.55 g; $p < 0.01$; Fig. 1k), and inguinal fat stores (NF 1.71 ± 0.28 g vs HF 2.99 ± 0.52 g; $p < 0.05$; Fig. 1l).

Long-term effects of HF diet during puberty on glucose homeostasis and pancreatic islets morphology

HF animals showed increased (+13%) fasting blood glucose (NF 71.78 ± 4.05 mg/dL *vs* HF 80.70 ± 1.50 mg/dL; $p < 0.05$; Fig. 2a) and had higher (+69%) fasting blood insulin (NF 0.48 ± 0.06 ng/mL *vs* HF 0.81 ± 0.12 ng/mL; $p < 0.05$; Fig. 2d) compared to NF animals.

As depicted in Fig. 2b, HF animals had higher blood glucose levels during ivGTT compared to NF animals, leading to increased (+56%) area under the curve of the test (NF 1408 ± 196.8 *vs* HF 2200 ± 283.2 $p < 0.05$; Fig. 2c). In addition, HF animals had higher blood glucose levels during ipITT (Fig. 2e). On the other hand, K_{itt} was significantly lower (−34%) in HF animals than in your counterparts (NF 2.26 ± 0.11 %/min *vs* HF 1.49 ± 0.28 %/min; $p < 0.05$; Fig. 2f).

The pancreas mass of the HF animals was heavier than the pancreas mass of NF group (Fig. 2g; NF 0.76 ± 0.02 g vs HF 0.99 ± 0.09 g; $p < 0.05$). Also, HF animals showed high number (NF 2.68 ± 0.12 islets/mm^2 *vs* HF 4.34 ± 0.16 islets/mm^2; Fig. 2i; $p < 0.0001$) and hypertrophied pancreatic islets (NF 85.80 ± 12.61 x10^3 μm^2 *vs* HF 177.4 ± 13.02 x10^3 μm^2; Fig. 2j; $p < 0.001$), with high beta cell mass (NF 1.41 ± 0.08 % *vs* HF 2.21 ± 0.12 %; Fig. 2k; $p < 0.0001$).

Long-term effects of HF diet during puberty on gluconeogenesis, lipid profile, liver morphology, and key-enzymes expression in the liver of adult rats

As depicted in Fig. 3a, HF animals had higher (+18%) liver mass compared to NF animals (NF 14.26 ± 0.48 g *vs* HF 16.85 ± 0.44 g; $p < 0.01$). Moreover, HF animals showed increased blood glucose during ipPTT (Fig. 3b), leading to higher (+55%) AUC compared to NF animals (NF 3247 ± 390.20 *vs* HF 5040 ± 534.00; $p < 0.05$; Fig. 3c), suggesting an increase in the hepatic gluconeogenesis.

HF animals also presented higher (+34%) blood triglycerides (NF 155.90 ± 6.74 mg/dL *vs* HF 209.60 ± 19.41 mg/dL; $p < 0.05$; Fig. 3d), with no changes in total (NF 90.22 ± 4.17 mg/dL *vs* HF 95.00 ± 6.15 mg/dL; $p < 0.05$; Fig. 3e) or HDL cholesterol (NF 52.78 ± 3.04 mg/dL *vs* HF 56.00 ± 2.94 mg/dL; $p < 0.05$; Fig. 3f) when compared to NF group. Besides, HF animals also showed an increase of 10-fold in lipid inclusion in the liver compared with the NF animals (NF 1.13 ± 0.23 % vs HF 11.18 ± 0.81 %; $p < 0.01$; Fig. 3g–h).

HF animals also showed 79% higher protein content of PEPCK (NF 100.00 ± 10.47 % of control *vs* HF 178.70 ± 46.07 % of control; $p < 0.05$; Fig. 3i). Regarding hepatic biomarkers of lipogenesis at PND120, HF animals had no change in protein content of ACC compared to NF animals (NF 100.00 ± 18.81 % of control *vs* HF 107.30 ± 13.24 % of control; $p = 0.761$; Fig. 3j). However, HF animals showed 37% higher protein content of FAS compared to NF animals (NF 100.00 ± 10.43 % of control *vs* HF 137.20 ± 8.85 % of control; $p < 0.05$; Fig. 3k).

Discussion

Traditionally, there is a strong emphasis on the role of the fetal period in metabolic disease programming; however, there is a growing interest in understanding other programming windows. As already mentioned, puberty is also a period of intense neuroendocrine and reproductive changes[30,31] and could be linked to disorders later in life. Studies have shown that eating patterns during adolescence have changed in the last two to three decades and have affected body weight gain, which can contribute to overweight and obesity from this period of life.[13] In the current study, we demonstrated that HF consumption during puberty can induce metabolic dysfunctions in rats at adulthood. HF animals showed obese phenotype, hyperphagia, hypertriglyceridemia, higher liver mass, hepatic steatosis, hepatic insulin resistance and PEPCK and FAS overexpression. Fig. 4 summarizes the main findings of our study and the proposed mechanism for the observed changes. To our knowledge, our results are the first to demonstrate metabolic programming of hepatic dysfunction in this experimental model induced by HF during puberty.

It is also important to highlight that the decreased food intake observed during HF challenge (PND30-60) is associated with the high caloric content of this diet and the lower palatability, which induces satiety faster than a standard diet. In fact, we prove that HF-fed animals ingested lower amounts of energy at the end of dietary intervention. However, the energy intake after dietary intervention was similar, when corrected to body weight, in both groups. In addition, the hyperphagia observed in HF group later in life could be an effect of hypothalamic inflammation, as previously observed in other animal models and in humans, that appears prior to the weight gain,[32–34] demonstrating the presence of a programming effect. However, it is a limitation of this study do not assess hypothalamic inflammation, that should be assessed by other studies. Reinforcing that, in a previous study, we showed that exposure to HF during puberty induces long-term obesity.[5] Other studies have also shown these effects in similar HF models.[35,36]

We also observed hyperglycaemia, hyperinsulinemia, and insulin resistance, as suggested by the K_{itt} results. Other study showed that maternal HF intake during gestational and lactational periods induces hyperinsulinemia and hepatic insulin resistance in offspring.[37] Our group also showed hyperinsulinemia and insulin resistance in HF-fed male Wistar rats.[26] Although those studies differ from our experimental model, it has been well established by the literature that an HF is able to dysregulate glucose and insulin metabolism and could induce the development of diabetes mellitus.[38,39] In this sense, we also showed that HF animals presented increased and hypertrophied islets, and higher β-cell mass, that is well described as a signal of systemic insulin resistance.

We hypothesized that rats fed a HF during puberty develop obesity, insulin resistance, and that liver metabolism could be affected. Here, we evaluated hepatic glucose production by intraperitoneal pyruvate administration and, as expected, HF animals had a significant increase in blood glucose levels after

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

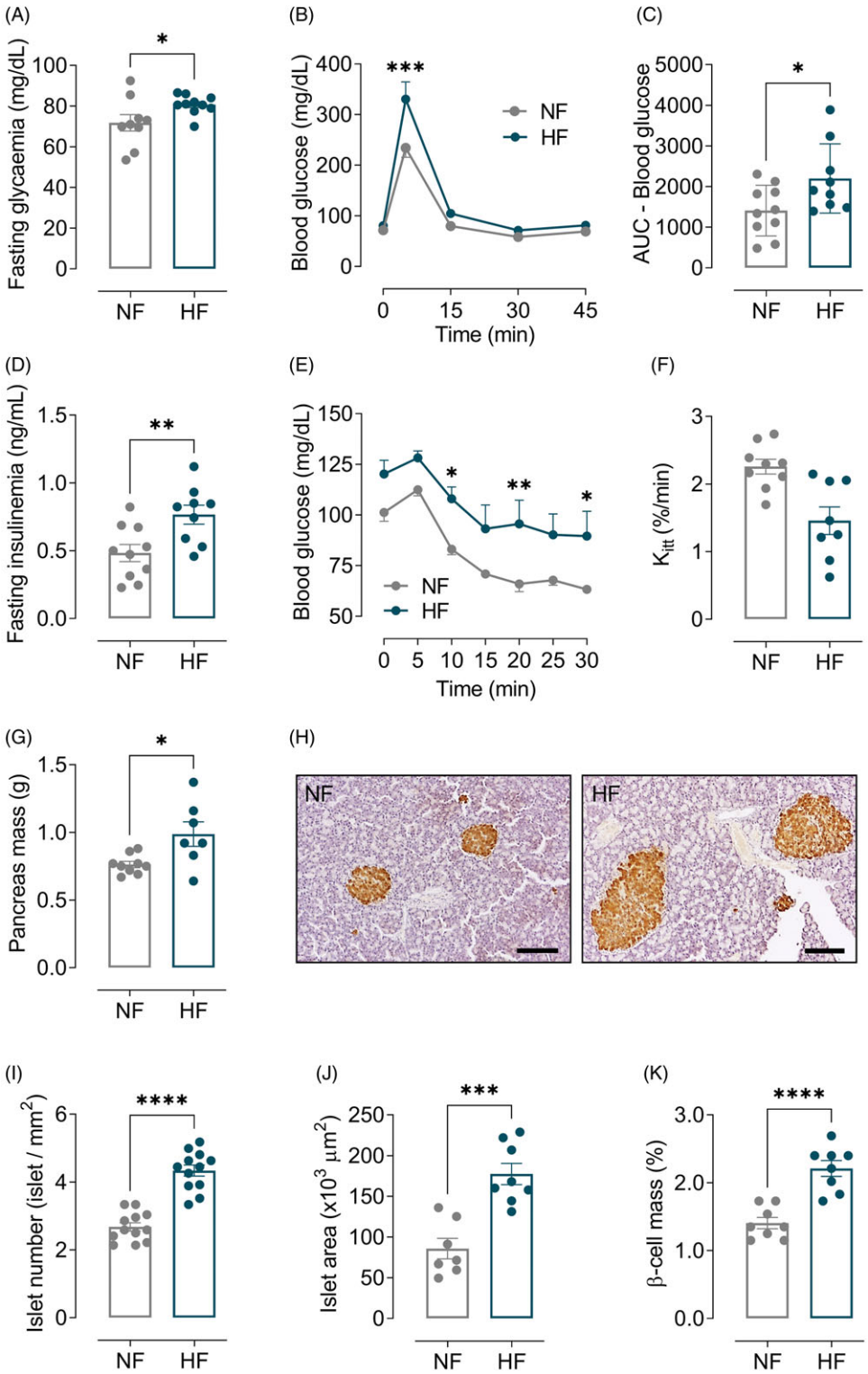

Figure 2. Long-term effects of high-fat diet during puberty on glucose homeostasis of adult rats. Fasting blood glucose (A), curve of blood glucose during ivGTT (B), AUC of blood glucose (C), fasting blood insulin (D), curve of blood glucose during ipITT (E), K_{itt} (F), pancreas mass (G), representative photomicrographs (×100 magnification, scale bars = 200 µm) shows pancreas sections immunostained with anti-insulin antibody (H), quantitative analyses of the islet number (I), islet area (J), and β-cell mass (K). Data are presented as the mean ± SEM. Two-way ANOVA (B and E) *$p < 0.05$; Student's *t*-test (bar graphs) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

pyruvate injection. These increased of glucose levels indicates an increase in hepatic gluconeogenesis and insulin resistance. Corroborating this, PEPCK, an enzyme responsible for converting cytoplasmic oxaloacetate into PEP during gluconeogenesis, was overexpressed in the liver form HF animals. Thus, these results suggest that these animals develop hepatic insulin resistance as well

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

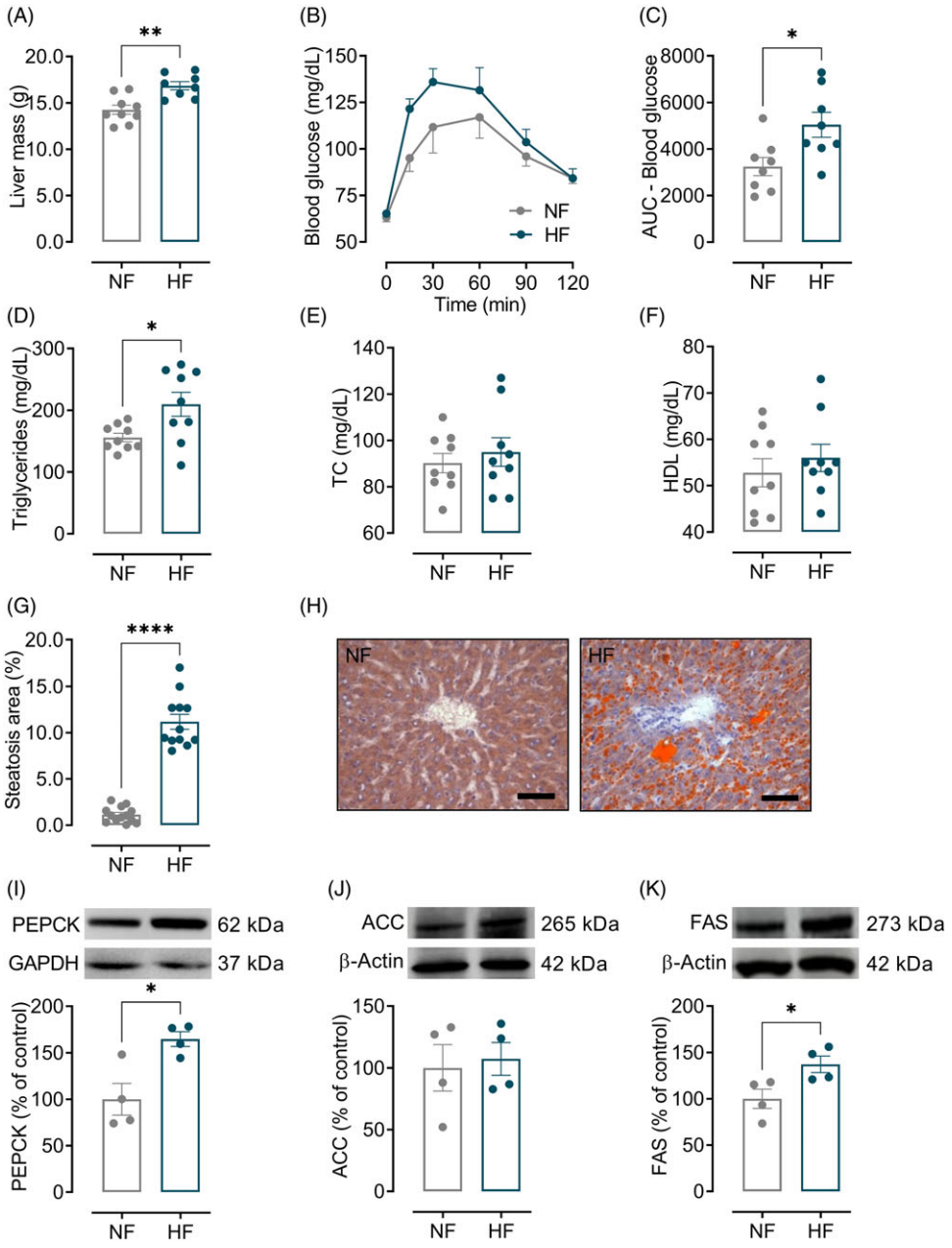

Figure 3. Long-term effects of high-fat diet during puberty on gluconeogenesis, lipid profile, liver morphology, and key-enzymes expression in the liver of adult rats. Liver mass (A), curve of blood glucose during ipPTT (B), AUC of blood glucose (C), blood triglycerides levels (D), total cholesterol (E), HDL cholesterol (F), steatosis area in the liver (G), representative photomicrographs (×400 magnification, scale bars = 50 μm) showing steatosis area in the liver stained with red oil (H). Western blot analysis of PEPCK (I), ACC (J), and FAS (K) and in the liver of the NF and HF rats. Representative immunoblots of proteins are show above the graphs. β-actin and GAPDH were used as a loading control. Data are presented as the mean ± SEM. Two-way ANOVA followed by Tukey's post hoc test (B) *$p < 0.05$; Student's *t*-test (bar graphs) *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

as lower peripheral insulin sensitivity. Some studies in different models have demonstrated that HF alters gluconeogenesis in the liver.[37,40] As a limitation of our study, we did not perform any analyses in other peripheral tissues; however, we suggest that HF animals may develop peripheral insulin resistance, reducing glucose uptake by muscle and adipose tissue.

HF animals had higher triglycerides levels and presented increased lipid inclusion in the liver, which characterizes hepatic steatosis. This condition seems to be related to hepatic insulin resistance, which could be suggested by FAS overexpression. The enzymes ACC and FAS are involved in the steps of *de novo* lipogenesis, and both activities are insulin-regulated. FAS overexpression is known in livers from HF animals.[41] As ACC and FAS levels were highly sensitive to nutritional and hormonal changes, we hypothesized that disruptions in the plasticity of these enzyme mechanisms observed in this work, confirming the puberty as a programming window. It is possible that ACC expression during HF intake was affected; however, in the long-term, this change disappeared, which did not occur to FAS, demonstrating a plastic effect of the expression of this enzyme, i.e., in the adult HF group. Unfortunately, we did not perform any analyses immediately after HF challenge.

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

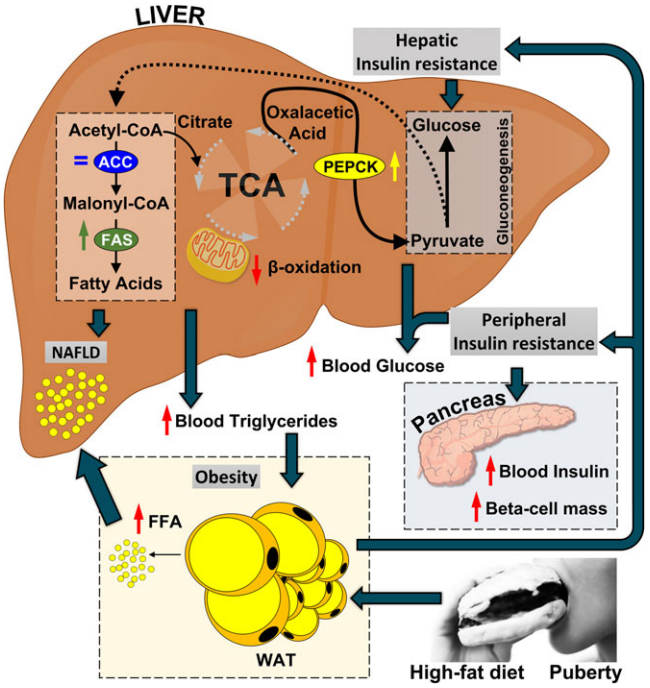

Figure 4. Summary of key findings about hepatic metabolic long-term programming in adult male Wistar rats in response to HF intake during puberty. High-fat diet during puberty led to an increase in white adipose tissue. Increased WAT led to increased circulating free fatty acids, and insulin resistance. Peripheral insulin resistance favored increased insulinemia and hypertrophy of pancreatic β-cells. In the liver, insulin resistance led to greater activation of the gluconeogenic pathway, which provides substrates for lipogenic pathway activity (ACC/FAS). Lipogenesis, combined with a higher amount of free fatty acids and insulin resistance, led to hepatic steatosis. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; FFA, free-fat acids; HFD, high-fat diet; NAFLD, nonalcoholic fatty liver disease; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; TCA, tricarboxylic acid; WAT, white adipose tissue.

In fact, fat accumulation in the liver is highly associated with insulin resistance and NAFLD progression. Free fatty acids (FFAs) from WAT as well as from *de novo* lipogenesis and from the diet are responsible for the increase of ectopic fat in the liver. Moreover, hepatic steatosis is also associated with liver inflammation caused by increased FFA. Furthermore, β-oxidation which occurs in liver mitochondria, may also be impaired by the increased FFA load in NAFLD.[42] Some of these interactions were demonstrated in mice fed an HF and indicate that lipid accumulation in the liver leads to subacute hepatic inflammation, causing insulin resistance in the liver and also in other insulin-dependent tissues.[43] Considering that obesity is a public health problem and is a potential condition for the development of NAFLD,[44,45] our findings in this study provide evidence of how HF intake during puberty affects metabolic biomarkers and morphology in the long-term, even after the HF intake was interrupted.

Conclusion

In summary, our results demonstrated that HF consumption during puberty increased the susceptibility to obesity and impaired glucose metabolism and liver injury later in life. The exact mechanisms that lead to these dysfunctions still need further investigation. However, hepatic insulin resistance and lipid accumulation directly affect some important proteins involved in gluconeogenesis (PEPCK) and lipogenesis (FAS) and seem to be important contributing factors. Furthermore, considering future clinical investigations, we must consider the poor eating habits of adolescents as an important risk factor for developing obesity and NAFLD later in life.

Acknowledgments. All authors are grateful to Maroly Valentim Alves Pinto for technical assistance.

Financial support. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) – Finance Code 001.

Competing interests. None.

References

1. Fernandez-Twinn DS, Ozanne SE. Early life nutrition and metabolic programming: early life programming. *Ann Ny Acad Sci*. 2010; 1212(1), 78–96.
2. Barker D. The developmental origins of chronic adult disease. *Acta Paediatr*. 2007; 93(s446), 26–33.
3. Das JK, Salam RA, Thornburg KL, *et al.* Nutrition in adolescents: physiology, metabolism, and nutritional needs: adolescents: physiology, metabolism, and nutrition. *Ann NY Acad Sci*. 2017; 1393(1), 21–33.
4. de Oliveira JC, de Moura EG, Miranda RA, *et al.* Low-protein diet in puberty impairs testosterone output and energy metabolism in male rats. *J Endocrinol*. 2018; 237(3), 243–254.
5. Ibáñez CA, Erthal RP, Ogo FM, *et al.* A high fat diet during adolescence in male rats negatively programs reproductive and metabolic function which is partially ameliorated by exercise. *Front Physiol*. 2017; 8, 807.
6. Chavaglia Cavalet L, dos Santos Ribeiro LC, Rosa GB, *et al.* Long-term effects of early overfeeding and food restriction during puberty on cardiac remodeling in adult rats. *J Dev Orig Health Dis*. 2020; 11(5), 492–498.
7. Seong J, Kang JY, Sun JS, Kim KW. Hypothalamic inflammation and obesity: a mechanistic review. *Arch Pharm Res*. 2019; 42(5), 383–392.
8. Cerf ME. High fat programming and cardiovascular disease. *Medicina*. 2018; 54(5), 86.
9. Billingsley H, Carbone S, Lavie C. Dietary fats and chronic noncommunicable diseases. *Nutrients*. 2018; 10(10), 1385.
10. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: modeling the metabolic disorders of human obesity in rodents*. *Obesity*. 2007; 15(4), 798–808.
11. Boitard C, Cavaroc A, Sauvant J, *et al.* Impairment of hippocampal-dependent memory induced by juvenile high-fat diet intake is associated with enhanced hippocampal inflammation in rats. *Brain Behav Immun*. 2014; 40, 9–17.
12. Patel R, Shah G. High-fat diet exposure from pre-pubertal age induces polycystic ovary syndrome (PCOS) in rats. *Reproduction*. 2018; 155(2), 139–149.
13. Moreno LA, Rodríguez G, Fleta J, *et al.* Trends of dietary habits in adolescents. *Crit Rev Food Sci*. 2010; 50(2), 106–112.
14. Simmonds M, Burch J, Llewellyn A, *et al.* The use of measures of obesity in childhood for predicting obesity and the development of obesity-related diseases in adulthood: a systematic review and meta-analysis. *Health Technol Assess*. 2015; 19(43), 1–336.
15. Wesolowski SR, Kasmi KCE, Jonscher KR, Friedman JE. Developmental origins of NAFLD: a womb with a clue. *Nat Rev Gastroenterol Hepatol*. 2017; 14(2), 81–96.
16. Welsh JA, Karpen S, Vos MB. Increasing prevalence of nonalcoholic fatty liver disease among united states adolescents, 1988-1994 to 2007-2010. *J Pediatr*. 2013; 162(3), 496–500.e1.
17. Armstrong MJ, Adams LA, Canbay A, Syn W-K. Extrahepatic complications of nonalcoholic fatty liver disease: hepatology. *Hepatology*. 2014; 59, 1174–1197.
18. Tsuneto A, Hida A, Sera N, *et al.* Fatty liver incidence and predictive variables. *Hypertens Res*. 2010; 33(6), 638–643.

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press

19. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med*. 2018; 24(7), 908–922.
20. Arçari DP, Santos JC, Gambero A, Ferraz LFC, Ribeiro ML. Modulatory effects of yerba maté (Ilex paraguariensis) on the PI3K-AKT signaling pathway. *Mol Nutr Food Res*. 2013; 57, 1882–1885.
21. Kim SJ, Quan HY, Jeong KJ, *et al.* Beneficial effect of betulinic acid on hyperglycemia via suppression of hepatic glucose production. *J Agric Food Chem*. 2014; 62(2), 434–442.
22. Burgess SC, Hausler N, Merritt M, *et al.* Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. *J Biol Chem*. 2004; 279(47), 48941–48949.
23. Geisler CE, Renquist BJ. Hepatic lipid accumulation: cause and consequence of dysregulated glucoregulatory hormones. *J Endocrinol*. 2017; 234(1), R1–R21.
24. Oliveira LS, Souza LL, Souza AFP, *et al.* Perinatal maternal high-fat diet promotes alterations in hepatic lipid metabolism and resistance to the hypolipidemic effect of fish oil in adolescent rat offspring. *Mol Nutr Food Res*. 2016; 60(11), 2493–2504.
25. Oliveira LS, Caetano B, Miranda RA, *et al.* Differentiated hepatic response to fructose intake during adolescence reveals the increased susceptibility to non-alcoholic fatty liver disease of maternal high-fat diet male rat offspring. *Mol Nutr Food Res*. 2020; 64(3), 1900838.
26. Gomes RM, Tófolo LP, Rinaldi W, *et al.* Moderate exercise restores pancreatic beta-cell function and autonomic nervous system activity in obese rats induced by high-fat diet. *Cell Physiol Biochem*. 2013; 32(2), 310–321.
27. Gomes RM, Bueno FG, Schamber CR, *et al.* Maternal diet-induced obesity during suckling period programs offspring obese phenotype and hypothalamic leptin/insulin resistance. *J Nutr Biochem*. 2018; 61, 24–32.
28. Miranda RA, da Silva Franco CC, Previate C, *et al.* Particulate matter exposure during perinatal life results in impaired glucose metabolism in adult male rat offspring. *Cell Physiol Biochem*. 2018; 49(1), 395–405.
29. Milanski M, Arruda AP, Coope A, *et al.* Inhibition of hypothalamic inflammation reverses diet-induced insulin resistance in the liver. *Diabetes*. 2012; 61(6), 1455–1462.
30. Ebling FJP. The neuroendocrine timing of puberty. *Reproduction*. 2005; 129(6), 675–683.
31. Salam RA, Bhutta ZA. 2.7 adolescent nutrition. In *World Review of Nutrition and Dietetics* (eds. Koletzko B, Bhatia J, Bhutta ZA, *et al.*), 2015; pp. 122–126. Karger AG, Switzerland.
32. Morselli E, Criollo A, Rodriguez-Navas C, Clegg DJ. Chronic high fat diet consumption impairs metabolic health of male mice. *Inflamm Cell Signal*. 2014; 1(6), e561.
33. Thaler JP, Yi C-X, Schur EA, *et al.* Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest*. 2012; 122(1), 153–162.
34. del Fernández-Arjona MM, León-Rodríguez A, Grondona JM, López-Ávalos MD. Long-term priming of hypothalamic microglia is associated with energy balance disturbances under diet-induced obesity. *Glia*. 2022; 70(9), 1734–1761.
35. Ullah R, Su Y, Shen Y, *et al.* Postnatal feeding with high-fat diet induces obesity and precocious puberty in C57BL/6J mouse pups: a novel model of obesity and puberty. *Front Med*. 2017; 11(2), 266–276.
36. Holtrup B, Church CD, Berry R, *et al.* Puberty is an important developmental period for the establishment of adipose tissue mass and metabolic homeostasis. *Adipocyte*. 2017; 6(3), 224–233.
37. Ashino NG, Saito KN, Souza FD, *et al.* Maternal high-fat feeding through pregnancy and lactation predisposes mouse offspring to molecular insulin resistance and fatty liver. *J Nutr Biochem*. 2012; 23(4), 341–348.
38. Holemans K, Caluwaerts S, Poston L, Van Assche FA. Diet-induced obesity in the rat: a model for gestational diabetes mellitus. *Am J Obstet Gynecol*. 2004; 190(3), 858–865.
39. Moreno-Fernández S, Garcés-Rimón M, Vera G, *et al.* High fat/High glucose diet induces metabolic syndrome in an experimental rat model. *Nutrients*. 2018; 10(10), 1502.
40. Maeda Júnior A, Constantin J, Utsunomiya K, *et al.* Cafeteria diet feeding in young rats leads to hepatic steatosis and increased gluconeogenesis under fatty acids and glucagon influence. *Nutrients*. 2018; 10(11), 1571.
41. Dorn C, Riener M-O, Kirovski G, *et al.* Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol*. 2010; 3(5), 505–514.
42. Sanyal AJ, Campbell-Sargent C, Mirshahi F, *et al.* Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology*. 2001; 120(5), 1183–1192.
43. Cai D, Yuan M, Frantz DF, *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. *Nat Med*. 2005; 11(2), 183–190.
44. Milić S, Lulić D, Štimac D. Non-alcoholic fatty liver disease and obesity: biochemical, metabolic and clinical presentations. *World J Gastroenterol*. 2014; 20, 9330–9337.
45. Browning JD, Szczepaniak LS, Dobbins R, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*. 2004; 40, 1387–1395.

https://doi.org/10.1017/S2040174423000272 Published online by Cambridge University Press