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Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring

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ABSTRACT

Background: Maternal high-caloric nutrition and related gestational diabetes mellitus (GDM) are associated with a high-risk for developing metabolic complications later in life and in their offspring. In contrast, exercise is recognized as a non-pharmacological strategy against metabolic dysfunctions associated to lifestyle disorders. Therefore, we investigated whether gestational exercise delays the development of metabolic alterations in GDM mothers later in life, but also protects 6-week-old male offspring from adverse effects of maternal diet.
Methods: Female Sprague-Dawley rats were fed with either control (C) or high-fat high-sucrose (HFHS) diet to induce GDM and submitted to gestational exercise during the 3 weeks of pregnancy. Male offspring were sedentary and fed with C-diet.
Results: Sedentary HFHS-fed dams exhibited increased gestational body weight gain ($p < 0.01$) and glucose intolerance ($p < 0.01$), characteristic of GDM. Their offspring had normal glucose metabolism, but increased early-age body weight, which was reverted by gestational exercise. Gestational exercise also reduced offspring hepatic triglycerides accumulation ($p < 0.05$) and improved liver mitochondrial respiration capacity ($p < 0.05$), contributing to the recovery of liver bioenergetics compromised by maternal HFHS diet. Interestingly, liver mitochondrial respiration remained increased by gestational exercise in HFHS-fed dams despite prolonged HFHS consumption and exercise cessation.
Conclusions: Gestational exercise can result in liver mitochondrial adaptations in GDM animals, which can be preserved even after the exercise program cessation. Exposure to maternal GDM programs liver metabolic setting of male offspring, whereas gestational exercise appears as an important preventive tool against maternal diet-induced metabolic alterations.

Abbreviations: ALT, alanine-aminotransferase;; AST, aspartate-aminotransferase; AUC, area under curve; C, control diet; ETC, electron transport chain; FA, fatty acids;; FCCP, carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone; G/M, glutamate/malate; GDM, gestational diabetes mellitus; OGTT, oral glucose tolerance test; HDL, high-density lipoproteins; HFD, high-fat diet; HFHS, high-fat high-sucrose diet; IR, insulin resistance; LDL, low-density lipoproteins; NAFLD, non-alcoholic fatty liver disease; OXPHOS, oxidative phosphorilation; RCR, respiratory control ratio; S/R, succinate/rotenone; T2DM, type 2 diabetes mellitus; TG, triglycerides; UCP2, uncoupling protein-2.

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1. Introduction

Increasing rates of metabolic diseases due to high-caloric intake and sedentary lifestyle are reaching epidemic magnitudes [1,2]. Along with several other prevalent metabolic complications, unhealthy lifestyles and excessive weight gain during pregnancy may provoke hyperglycaemia and insulin resistance (IR), characteristic of gestational diabetes mellitus (GDM) [3]. While in most women GDM-related metabolic alterations usually disappear after delivery, an increased risk for impaired glucose tolerance and fatty liver later in life may exist [4–6]. With an organism already pressured by anatomical, physiological, and metabolic adaptations during pregnancy to answer to fetal metabolic demands and endure the demands of pregnancy, exposure to additional environmental factors may exacerbate these adaptations and consequently alter fetal development [7]. In fact, in-utero environment during the critical periods of fetal programming can influence the predisposition of offspring for chronic disease development through physiological and/or epigenetic changes [8]. Offspring of GDM mothers also have affected insulin signalling, disrupted liver metabolome, higher risk for type 2 diabetes (T2DM) and hepatic steatosis [9,10].

Considering the potential severity of maternal unhealthy lifestyles, the implications of GDM and particularly the transgenerational burden, it is important to adequately manage eventual complications and protect both mothers and offspring. Anti-diabetic drugs are currently available to lower blood glucose levels. Still, the effectiveness of these therapies on mothers and offspring is not completely known [11]. Additionally, liver-related drug metabolism and clearance may be affected even in an uncomplicated pregnancy due to distinct pregnancy-related physiological changes [12]. Therefore, non-pharmacological interventions, such as maternal lifestyle changes, should emerge as first-line strategies to implement during pregnancy and, eventually, counteract GDM and its adverse transgenerational impact [13]. Physical exercise has already been recognized as an effective protective and/or therapeutic non-pharmacological strategy against liver-related disorders, with consistent beneficial metabolic outcomes on high-fat diet (HFD)-induced mitochondrial dysfunction [14–17]. Moreover, maternal exercise before and/or during pregnancy has also been reported as beneficial in protecting the offspring from diet-induced obesity, hepatic steatosis, and alterations in glucose metabolism [17–19].

The important role of liver mitochondria in metabolism, as well as their particular sensitivity to liver-related disorders, such as non-alcoholic fatty liver disease (NAFLD) or T2DM [20,21], suggest that liver mitochondrial dysfunction may be a sensitive and early marker that can precede a clear clinical manifestation of the disease. However, studies regarding the GDM impact on liver status of mothers later in life, as well as on their offspring at early age, with the focus on liver mitochondrial alterations are very scarce. Considering the hepatic protective effects of exercise in liver-related disorders, the novel and clinically relevant hypothesis of the present study is that gestational exercise may alleviate GDM-related liver dysfunction and contribute to a more resistant and "fitness" mitochondrial phenotype in the post-partum period of GDM mothers, but even more, that gestational exercise may counteract the deleterious impact of unhealthy maternal diet, ultimately decreasing GDM-imposed liver malfunction in the offspring.

2. Material and methods

2.1. Animals

Seven-week-old female Sprague-Dawley rats (150–200 g) (Charles River, L'Arbresele, France), were randomly divided into 2 diet groups: control (C) and high-fat high-sucrose (HFHS) diet. After 7-weeks on respective diets, dams were mated with sedentary C-fed male rats (Charles River, L'Arbresele, France). Pregnant HFHS-fed dams were divided into two subgroups: sedentary animals and animals submitted to gestational exercise protocol during pregnancy. This resulted in 3 experimental groups ($n = 6$ per group): P-C-S - sedentary C-fed dams; P-HFHS-S - sedentary HFHS-fed dams; and P-HFHS-E – exercised HFHS-fed dams. Dams delivered naturally and litter size was reduced to 3 male and 3 female pups to avoid food competition and effects on pups' body mass. The offspring were weaned after 3-weeks of nursing. Male offspring were assigned to 3 experimental groups ($n = 6$ per group): C—S offspring of sedentary C-fed dams; HFHS-S offspring of sedentary HFHS-fed dams; and HFHS-E offspring of exercised HFHS-fed dams. The offspring from all experimental groups were sedentary and fed with C-diet. Dams were euthanised 8-weeks post-partum and the offspring at 6-weeks of age (Fig. 1A).

All animals were housed in polyethylene type III-H cages in a normal environment (21–22 °C; 50–60% humidity, 12 h light/dark cycles), receiving food and water ad libitum, in a specific-pathogen free animal facility at the Institute for Research and Innovation in Health – i3S. The animals during mating and those later submitted to the exercise protocol during pregnancy were housed in polyethylene type IV cages equipped with a running wheel (Type 304 Stainless steel, Tecniplast, Casale Litta, Italy). To facilitate the expression of species-typical behaviour and promote psychological wellbeing, the cages were filled with corn cob bedding and environmental enrichment (shelter and nesting materials) was provided. During the experimental protocol, the allocation sequence of the animals of the different experimental groups was blindly performed through allocation concealment by independent animal facility technicians. The Ethical Committee of the Institute for Research and Innovation in Health – i3S, University of Porto, and National Government Authority (*Direção Geral de Alimentação e Veterinánia* – No.0421/000/000/2018) approved the experimental protocol, which was in compliance with the *Guidelines for Care and Use of Laboratory Animals in Research* advised by the Federation of European Laboratory Animal Science Associations (FELASA). All the authors are accredited by FELASA to perform animal experiments.

2.2. Diets and exercise protocol

Animals from maternal generation consumed C or HFHS-diet (E157452–047 or D12451(II)mod., Ssniff, Soest, Germany, respectively) throughout 18-weeks of the experimental protocol. HFHS previously used to induce GDM in animal models [10], contained 42% metabolizable energy from fat (vs. 10% in C), 27% from proteins (vs. 20% in C), and 31% from carbohydrates (mainly sucrose, vs. 70% in C with 1% sucrose), with crude fat of 23.1% (vs. 4.1% in C), high cholesterol content and increased proportion of long-chain fatty acids (FA) (S1).

Regarding exercise protocol, dams from P-HFHS-E group were exercised on a motor-driven treadmill (LE8700, Panlab Harvard Apparatus, MA, USA) during the 3-weeks of pregnancy, 6 days/week, 20–60 min/day during the dark cycle (Fig. 1B). One week before the exercise protocol, P-HFHS-E animals were adapted to the treadmill. In the first week of the protocol, the training duration was gradually increased from 20 min/day to 60 min/day until the speed of 18 m/min was reached. In the following 2-weeks, the duration was kept at 60 min, but speed was gradually increased until 21 m/min to raise exercise intensity (Fig. 1B). With a pup delivery approaching, the intensity and duration of exercise were adjusted for each animal in the last few days of pregnancy. Additionally, during pregnancy, the exercising dams were housed in cages equipped with running-wheels to stimulate voluntary physical activity. To avoid confounding factors and favour similar handling and environmental conditions, the sedentary animals were kept in cages with blocked wheels and placed on a static treadmill during the same period as the exercised counterparts.

2.3. Glucose homeostasis

Oral glucose tolerance test (OGTT) was performed in dams 1-week before mating and on the embryonic day E15.5 to confirm GDM-

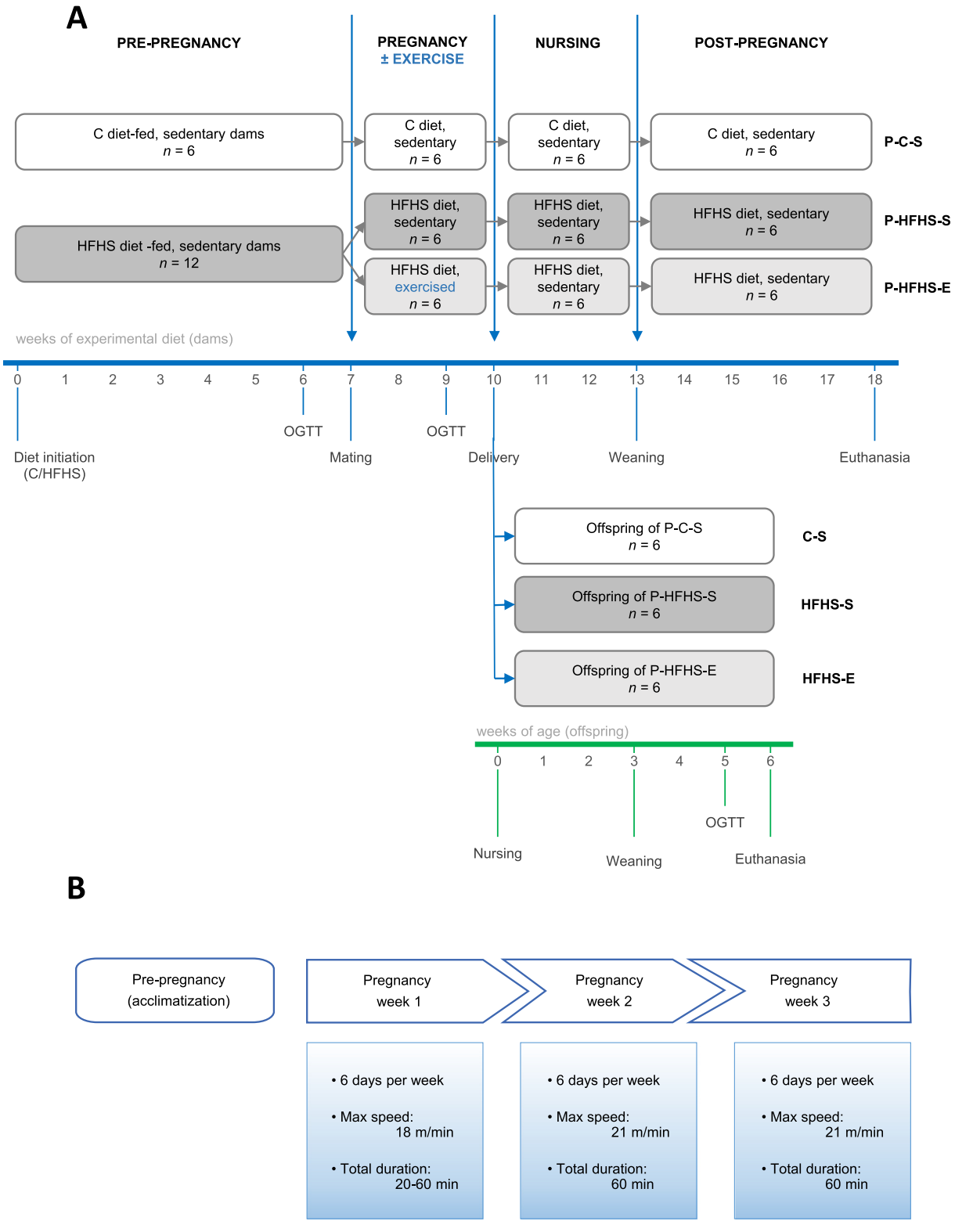

Fig. 1. Experimental (A) and exercise (B) protocol. C – maternal control diet; HFHF – maternal high-fat high-sucrose diet; P – pregnant animals in maternal generation; S – sedentary animals in maternal generation; E – exercising animals in maternal generation. OGTT – oral glucose tolerance test.

related glucose intolerance during pregnancy. In the offspring, OGTT was performed 1-week before euthanasia. Glucose was administered to the rats at a dose of 2 g/kg body weight. To avoid potential harm induced by intraperitoneal injection or oral gavage to (pregnant) mothers during OGTT, glucose was orally administered in the form of non-flavoured jelly (Globo Gelatina Neutra, A Colmeia do Minho, Portugal) [22]. To avoid novel objects avoidance, rats were trained to eat jelly without glucose. Moreover, since prolonged hypoglycaemia in pregnancy could influence the fetus development, animals were submitted to a 6-h fasting period prior to OGTT. The same fasting period was followed in pre-mating OGTT and in offspring OGTT.

2.4. Animal euthanasia and tissue sampling

Animals were anaesthetized (induction: 5% isoflurane, 1 L/m O_2; maintenance: 2.5% isoflurane, 0.4 L/m O_2) after overnight fasting. The liver was rapidly excised, rinsed with ice-cold PBS, weighed, and separated for light microscopy, mitochondria isolation, and other analysis. The blood was collected from inferior vena cava and centrifuged (3000 ×*g*, 10 min, 4 °C). Collected plasma was stored at −80 °C until later use for biochemical and metabolomics analysis. In order to reduce performance and detection bias in the following techniques and to avoid researchers from knowing the animals' treatment groups, the animals were labelled with a blinded coding system during data acquisition until the statistical analysis.

2.5. Light microscopy and liver triglyceride content

Liver tissues were fixed in 4% paraformaldehyde (pH 7.4), dehydrated in a graded series of alcohol (70–100%), and embedded in paraffin. Tissue sections were made at 4 μm in adhesive slides and stained with hematoxylin/eosin. Stained slides were blindly evaluated by two veterinary pathologists (TBG and FG) using the Carl Zeiss Axio Observer light microscope equipped with Zen2 software.

Histologic evaluation took into account the main histologic features of NAFLD in rodent models [23]. The following parameters were considered: (1) microvesicular steatosis, (2) macrovesicular steatosis, (3) hypertrophy, (4) lobular inflammatory foci (all lymphocytic foci except for portal location), (5) portal inflammation. Microgranuloma was evaluated as well, but not used for the final score due to high similarities between groups. All parameters were evaluated in a four-level scoring system, in which 0 corresponds to absence of the alteration (< 5%), 1 to low-grade lesion (5–33% altered), 2 to moderate-grade lesion (33–66%), and 3 to high-grade lesion (> 66% altered).

Triglyceride (TG) content in the liver was determined with Triglyceride Quantification Kit (BioVision, Milpitas, USA) following the manufacturer instructions.

2.6. Analysis of acylcarnitine levels in plasma and liver homogenates

Plasma spots were collected on Whatman 903 paper (Guthrie cards) and acylcarnitines detected and quantified by tandem mass spectrometry (MS/MS), using standard protocols [24]. Acylcarnitine quantification in liver was performed using the protocol adapted from Petucci et al. [25]. Liver tissue was homogenised in ice-cold 80% methanol in ratio 1:10, vortexed and centrifuged at 14000 rpm (10 °C, 5 min). Afterwards, 140 μL of supernatant were collected to a 96-well plate and 100 μL of methanol containing deuterated acylcarnitine internal standard solutions (Cambridge Isotope labs, USA) was added to each well. The mixture was subsequently dried using nitrogen flow and shortly thereafter derivatised to the corresponding buthyl esters by incubation with 60 μL of 3 N buthanolic HCl at 70 °C for 15 min. Samples were dried once again using nitrogen reconstituted with 200 μL of 80% methanol for flow injection MS/MS in an API 4000 QTRAP (Sciex, Washington, D.C., USA).

2.7. Isolation of liver mitochondria and mitochondrial respiratory assays

Mitochondria were isolated from the median liver lobule by a conventional differential centrifugation method [15]. An aliquot of fresh mitochondrial fraction was used for in vitro oxygen consumption assays, while rest was stored at -80 °C for later Western blotting semi-quantification. Mitochondrial respiratory function was measured using Biological Oxygen Monitor System and a Clark-Type oxygen electrode (Hansatech Instruments, England). Reactions were performed at 30 °C in a magnetically-stirred glass chamber containing 1 mg/mL of

Table 1
Animal data and plasma biochemical analyses.

	Maternal generation			Non-pregnant (NP) counterparts of maternal generation		
	P-C-S	P-HFHS-S	P-HFHS-E	NP-C-S	NP-HFHS-S	NP-HFHS-E
Body weight (g)	323.43 ± 10.97	323.50 ± 10.98	339.33 ± 9.84	338.27 ± 10.04	328.91 ± 13.11	334.00 ± 13.49
Liver weight (g)	9.44 ± 0.38	9.52 ± 0.46	10.53 ± 0.35	9.50 ± 0.26	9.06 ± 0.33	9.44 ± 0.25
Liver weight/body weight (mg•g^{-1})	29.22 ± 0.73	29.42 ± 1.04	31.04 ± 0.62	28.22 ± 1.09	27.80 ± 1.12	28.41 ± 1.00
Glucose (mg/dL)	191.29 ± 9.35	232.40 ± 6.04*	205.67 ± 5.78#	226.00 ± 10.17	201.91 ± 5.11	201.00 ± 6.28
Triglycerides (mg/dL)	73.40 ± 5.98	82.80 ± 3.52	74.8 ± 7.88	82.20 ± 8.11	77.60 ± 6.59	61.40 ± 7.08
Cholesterol (mg/dL)	85.86 ± 7.07	80.33 ± 6.22	81.00 ± 3.62	82.25 ± 2.32	75.20 ± 2.90	73.50 ± 2.67
HDL (mg/dL)	56.71 ± 4.02	54.67 ± 4.65	54.17 ± 2.26	60.67 ± 3.64	56.00 ± 1.77	50.67 ± 2.63
LDL (mg/dL)	21.14 ± 1.93	20.33 ± 2.09	22.17 ± 1.66	24.33 ± 2.33	21.45 ± 1.52	18.67 ± 1.28
AST (U/L)	78.14 ± 6.31	80.83 ± 8.18	81.25 ± 8.70	72.20 ± 5.67	96.90 ± 9.52	78.60 ± 5.25
ALT (U/L)	23.40 ± 1.32	29.33 ± 1.05*	28.50 ± 1.38	22.83 ± 0.91	34.82 ± 2.24*	30.25 ± 2.17
		Offspring generation				
		C-S		HFHS-S		HFHS-E
Body weight (g)		212.40 ± 8.74		221.20 ± 10.02		199.30 ± 10.23
Liver weight (g)		8.03 ± 0.56		7.53 ± 0.43		7.47 ± 0.59
Liver weight/body weight (mg•g^{-1})		36.06 ± 1.29		33.91 ± 1.58		33.99 ± 1.61
Glucose (mg/dL)		193.43 ± 11.60		180.20 ± 3.44		211.83 ± 5.78#
Triglycerides (mg/dL)		64.00 ± 4.78		79.00 ± 0.63*		51.50 ± 2.14#
Cholesterol (mg/dL)		98.83 ± 2.65		103.40 ± 1.50		87.25 ± 1.01#
HDL (mg/dL)		65.86 ± 2.47		65.60 ± 1.21		64.33 ± 3.96
LDL (mg/dL)		33.14 ± 2.18		30.00 ± 0.84		29.67 ± 2.44
AST (U/L)		101.50 ± 5.65		109.60 ± 12.58		91.80 ± 3.49
ALT (U/L)		46.29 ± 4.42		44.80 ± 1.53		47.00 ± 3.25

Values are means ± SEM ($n = 6$).
P – pregnant animals (maternal generation); NP – non-pregnant counterparts of maternal generation; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers;
ALT – alanine aminotransferase; AST – aspartate aminotransferase; HDL – high-density lipoproteins; LDL – low-density lipoproteins.
* vs. P-C-S ($p < 0.05$) or vs. C—S ($p < 0.05$) in maternal or offspring generation, respectively.
# vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

mitochondrial protein in respiration buffer (130 mM sucrose, 10 mM HEPES, 2.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM KH2PO4, 0.1 mM EGTA, pH 7.4). Mitochondrial respiration through electron transport chain (ETC) complex I or complex II was initiated by adding glutamate/malate (G/M, 10 mM and 5 mM, respectively) or succinate (10 Mm) plus rotenone (1.5 μM), respectively. State 3 respiration and phosphorylation cycle were obtained by adding ADP (150 nmol) and the rate of oxygen consumption after ADP phosphorylation represented state 4. The state 3/state 4 ratio used to calculate the respiratory control ratio (RCR). ADP/O ratio represented the ratio between the number of nmol ADP phosphorylated by nmol O_2 consumed during state 3. Additionally, the state 4 was measured in the presence of oligomycin (2 μg) in order to inhibit proton influx through the ATP synthase.

2.8. Western blotting

Equivalent amounts (20 μg) of liver mitochondria were denatured in sample loading buffer and separated by SDS/PAGE, followed by transfer to PVDF membranes. Membranes were blocked with non-fat dry milk and afterwards incubated with primary antibodies: anti-OXPHOS cocktail (1:1000; Abcam, ab110413) and anti-UCP2 (1:500; Sigma Aldrich, SAB2501087), and subsequently, with secondary antibodies: horseradish peroxidase-conjugated anti-goat (1:10000; Santa Cruz, sc-2354) or anti-mouse (1:10000; Santa Cruz, sc-2005). Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, #1705061) and acquired by ChemiDoc-XRS Image System (Bio-Rad). Density was determined by using the Image Lab software 6.0.1 (Bio-Rad). Ponceau S staining was used for normalization of protein loading or transfer differences [26]. The final data were expressed as percentage variation of control values (%P-C-S or %C-S, in maternal and offspring generation, respectively).

2.9. Statistical analysis

Sample size was estimated assuming $\alpha = 5\%$ and a power of 0.90 (G*Power, Düsseldorf) from previous study. Results are expressed as the mean ± SEM. Statistical analysis was performed in GraphPad-Prism 8.0 (GraphPad software, USA), using one-way ANOVA test followed by Holm-Sidak *post-hoc* test, with the significance level at 5%.

3. Results

3.1. Maternal and offspring characteristics and glucose homeostasis

After 18-weeks on respective diets, no differences were found in body weight between C and HFHS dams (Table 1). However, HFHS induced a noteworthy 1.4-fold gestational body weight gain in sedentary dams compared to C-diet-fed or exercised HFHS-fed dams (Fig. 2A). Maternal HFHS-diet also increased litter size and offspring body mass by 20% (Fig. 2B-D). The impact of maternal HFHS-diet and the protective

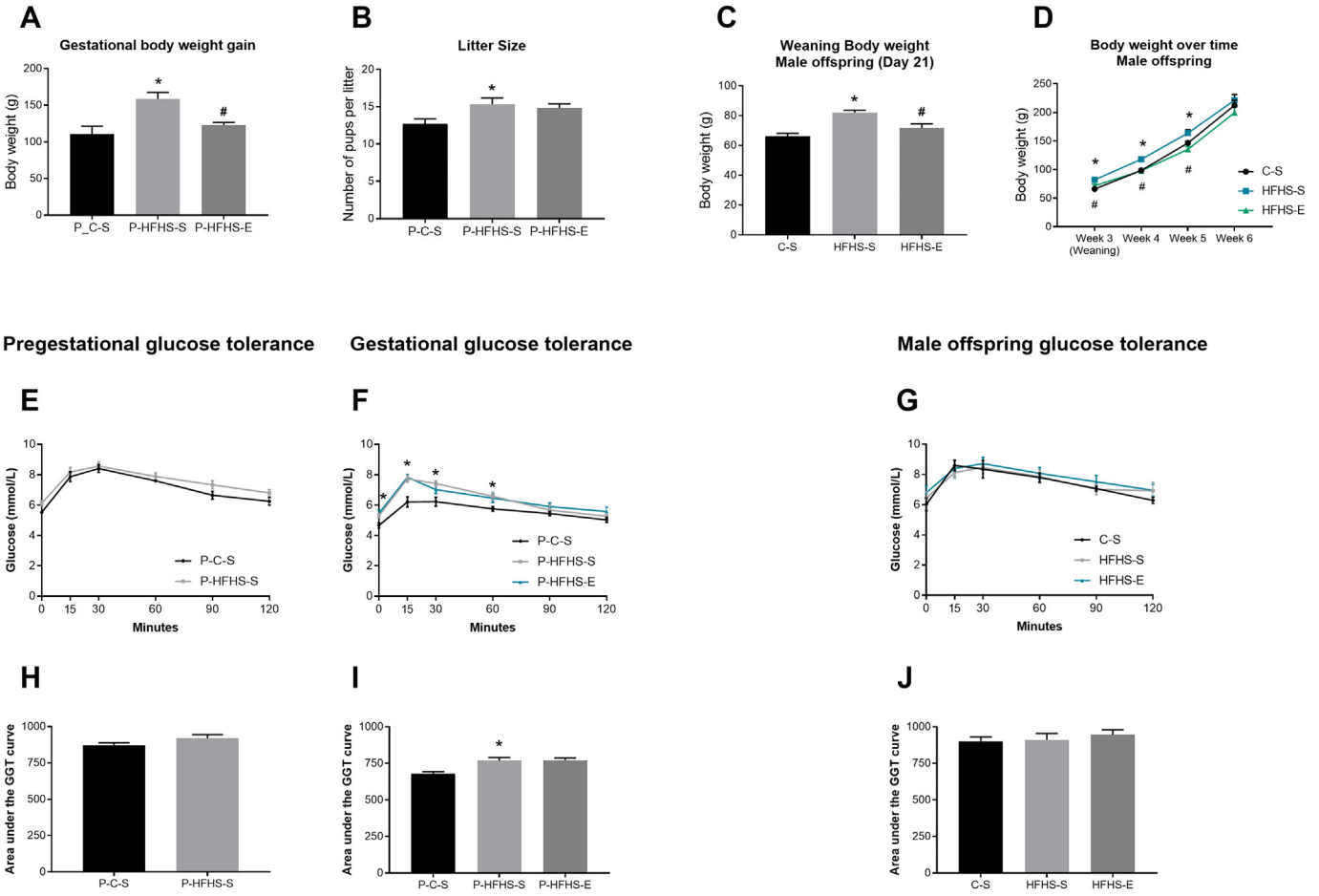

Fig. 2. Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal body weight gain during gestation period (A), litter size (B) and offspring body weight changes from weaning (C) to the euthanasia point at 6 weeks of age (D), maternal glucose tolerance before pregnancy (E, H) and gestational glucose tolerance (F, I) and offspring glucose tolerance at 5 weeks of age (G, J) OGTT – glucose tolerance test. P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM ($n = 6$). * vs. P-C-S ($p < 0.05$) or vs. C—S ($p < 0.05$) in maternal or offspring generation, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

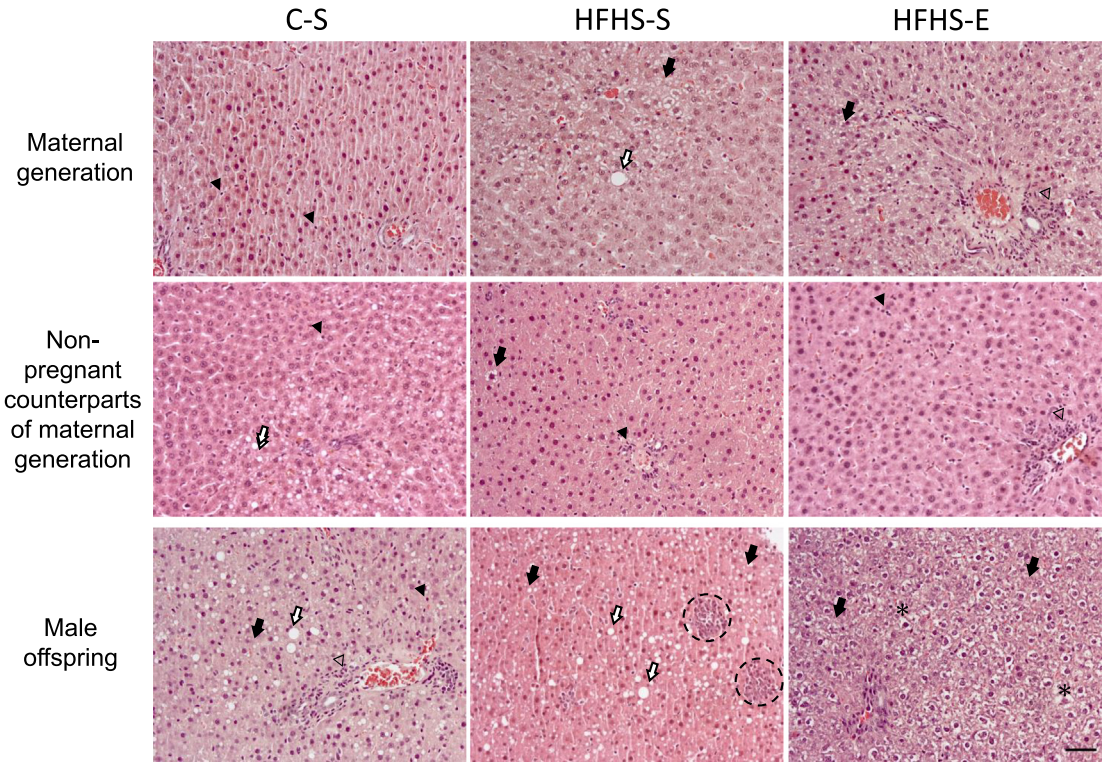

Fig. 3. Representative light micrographs (×200) of liver tissue from all groups and generations: C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. The presence of microvesicular steatosis (black arrow), macrovesicular steatosis (white arrow), lobular inflammation (black arrow head), portal inflammation (white arrow head), hypertrophy (asterisk).

effect of gestational exercise on offspring body weight was prolonged until the 6th weeks of age, when only similar trends were observed (Fig. 2D).

Dams' plasma glucose and alanine-aminotransaminase (ALT) levels were elevated by HFHS-diet, whereas gestational exercise prevented this effect, pointing out the diet impact on glucose metabolism during the gestational period and reinforcing a potential priming condition for GDM. In the offspring, maternal exercise increases glucose level ($p < 0.05$), but lessens the effects of HFHS-diet on plasma TG ($p < 0.001$) and cholesterol levels ($p < 0.001$) (Table 1).

Prior to mating, glucose tolerance was not different between C-fed and HFHS-fed animals (Fig. 2E, H). However, at mid-gestation, an increased glucose intolerance and glucose AUC ($p < 0.01$) were noticed in both HFHS groups (Fig. 2F, I), confirming the presence of gestational glucose intolerance characteristic for GDM. No alterations in glucose tolerance were observed in their offspring (Figs. 2G, 3J).

3.2. Histological analysis and liver triglyceride content

We examined the liver for histological evidence of steatosis using hematoxylin/eosin-staining and calculated the NAFLD score (Table 2 and Fig. 3). Although without statistical meaning, the GDM mothers, sedentary or exercised, showed lower NAFLD score than C-fed mothers ($p = 0.56$ vs. P-C-S or $p = 0.84$ vs. P-HFHS-S, respectively). To assess whether pregnancy per se affected this score, we also examined the liver of their non-pregnant counterparts. Surprisingly, non-pregnant sedentary HFHS-fed dams have tendency for higher NAFLD score ($p = 0.15$, vs. NP-C-S), while 3-weeks of exercise (corresponding to gestational exercise period of the pregnant dams) decreased the NAFLD score ($p = 0.37$, vs. NP-HFHS-S). In contrast, maternal HFHS-diet increased offspring NAFLD score, while gestational exercise lessened the adverse effect of maternal HFHS-diet (Fig. 3).

Accordingly, animals from maternal generation did not differ in hepatic TG content, despite a slight decrease in HFHS groups (Fig. 4A). In

Table 2
NAFLD score recorded from mothers' and offspring livers of all experimental groups, blindly observed under light microscope.

	Maternal generation			Non-pregnant (NP) counterparts of maternal generation		
NAFLD score	P-C-S	2.5 ± 0.5		NP-C-S	2.5 ± 0.4	
	P-HFHS-S	1.8 ± 0.4	$P = 0.56$ vs. P-C-S	NP-HFHS-S	3.6 ± 0.4	$P = 0.15$ vs. NP-C-S
	P-HFHS-E	1.7 ± 0.5	$P = 0.84$ vs. P-HFHS-S	NP-HFHS-E	3.0 ± 0.4	$P = 0.37$ vs. NP-HFHS-S
		Offspring generation				
NAFLD score		C-S	4.7 ± 1.0			
		HFHS-S	5.6 ± 0.7			$P = 0.72$ vs. C-S
		HFHS-E	5.2 ± 1.0			$P = 0.77$ vs. HFHS-S

Values are means ± SEM (n = 6).
P – maternal generation; NP – non-pregnant counterparts of maternal generation; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers.

contrast, offspring of HFHS sedentary mothers showed tendency towards an increase ($p = 0.26$) in liver TG content compared to their C—S offspring, whereas maternal exercise remarkably reduced ($p = 0.04$) hepatic TG levels (Fig. 4B), which are in line with the tendency observed in the NAFLD score.

3.3. Plasma and liver acylcarnitine levels

The levels of plasma (Fig. 5A) and hepatic (Fig. 5B) acylcarnitines were not influenced by HFHS and/or gestational exercise in dams, except for C12. However, in the offspring, the increase in the levels of some long-chain acylcarnitines in plasma (C16, C18, C18:1) and liver (C16, C18) due to maternal HFHS-diet was significantly restored to control levels by gestational exercise (Fig. 5C, D).

3.4. Liver mitochondrial bioenergetics

After 18-weeks of HFHS-diet, sedentary dams did not show any changes in the RCR with G/M- or S/R-related substrates; however gestational exercise increased the RCR ($p < 0.05$, vs. P_HFHS_S), when G/M were used as substrates, which was maintained even after exercise cessation (Fig. 6A-C). The same effect of gestational exercise (P_HFHS_S vs. P_HFHS_E) was noticed in respiratory rates with oligomycin (G/M and S/R) (Fig. 7E). In contrast, maternal HFHS-diet and sedentarism reduced offspring RCR, an effect which was nor observed in the offspring of exercised HFHS-fed mothers ($p < 0.05$, vs. P_HFHS_S) (Fig. 6E-G). Respiration with oligomycin (G/M) was greatly increased in offspring of sedentary HFHS mothers when compared to sedentary C mothers, whereas maternal gestational exercise diminished the impact of maternal HFHS (Fig. 8F). The ADP/O ratio was not affected by maternal diet and exercise in both generations (Fig. 7D, H).

3.5. Oxidative phosphorylation system (OXPHOS) subunits and UCP2 protein expression

Even though HFHS-diet per se did not affect protein expression of OXPHOS subunits (Fig. 7A), consistently with unchanged hepatic mitochondrial respiration, gestational exercise increased the protein expression of CIII, CIV, and CV subunits in dams (Fig. 7A). In the offspring, gestational exercise prevented maternal HFHS-induced decrease in CI protein expression and prompted an increase of CIV protein expression in HFHS-E group compared to HFHS-S (Fig. 7B).

More reduced levels of UCP2 in liver mitochondria fractions were observed in sedentary HFHS dams compared to sedentary C counterparts, while gestational exercise restored UCP2 levels (Fig. 7C). However, a completely opposite effect of maternal diet was observed in the offspring, where gestational exercise prevented an increase in UCP2 expression due to maternal HFHS (Fig. 7D).

4. Discussion

Modern lifestyle contributes to the development and increasing prevalence of numerous metabolic disorders, such as GDM and NAFLD. Maternal metabolic inability to cope with high-caloric intake because of sedentary behaviour can prime offspring for later liver dysfunction since the fetus' metabolism cannot buffer the excess of nutrients transmitted through the placenta [21]. Our study explores the impact of GDM-related metabolic alterations on both mothers and their 6-week-old male offspring and intends to clarify the role of exercise performed during the critical and sensitive moment of pregnancy and in utero development against metabolic deleterious consequences. To our knowledge, this is the first study to analyse effects of gestational moderate exercise combined with voluntary physical activity on HFHS-diet-induced liver mitochondrial bioenergetics dysfunction in both generations. Data showed that gestational exercise prevented some of the deleterious mitochondrial alterations induced by HFHS and specifically related to GDM in mothers, but also had a strong impact in reverting maternal GDM-induced mitochondrial bioenergetics impairments and hepatic lipid accumulation in the offspring.

4.1. Animal characteristics and model of GDM

In our study, female rats were fed with HFHS prior to mating to encourage a specific metabolic milieu before pregnancy that would, in the particular conditions of pregnancy, enable the development of metabolic remodelling, characteristic of GDM. However, the HFHS feeding, did not affect dams' body weight. Still, the metabolizable energy of C and HFHS-diets is similar (15.1 MJ/kg and 19.1 MJ/kg, respectively). Actually, isocaloric intake of HFD may not induce body weight gain, but may provoke alterations in adiposity, which rather depends on diet composition, particularly dietary FA [27]. Nevertheless, HFHS-diet and sedentary lifestyle resulted in high gestational weight gain, a risk factor for GDM. However, this negative effect was reverted by gestational exercise, which is in accordance with meta-analysis studies suggesting that gestational exercise interventions can reduce body weight gain in

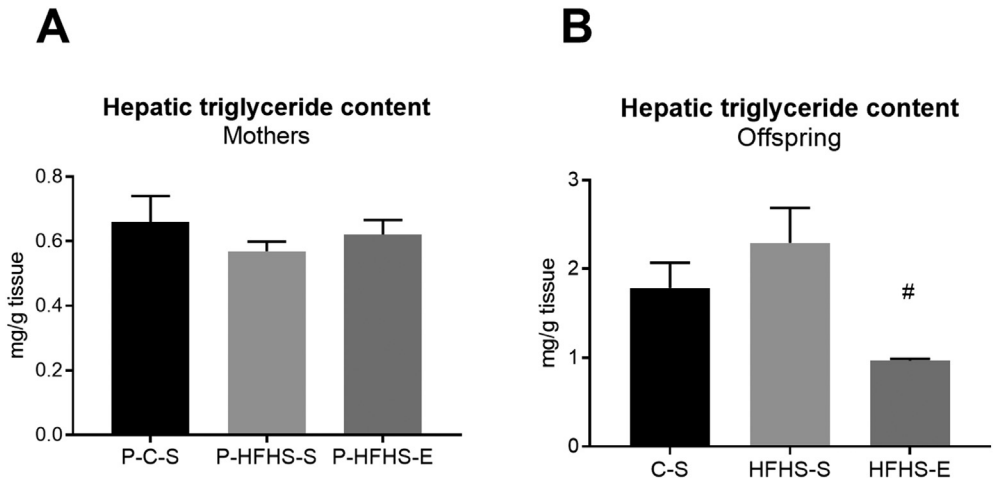

Fig. 4. Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal (A) and offspring (B) liver triglyceride content; P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM ($n = 6$). # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

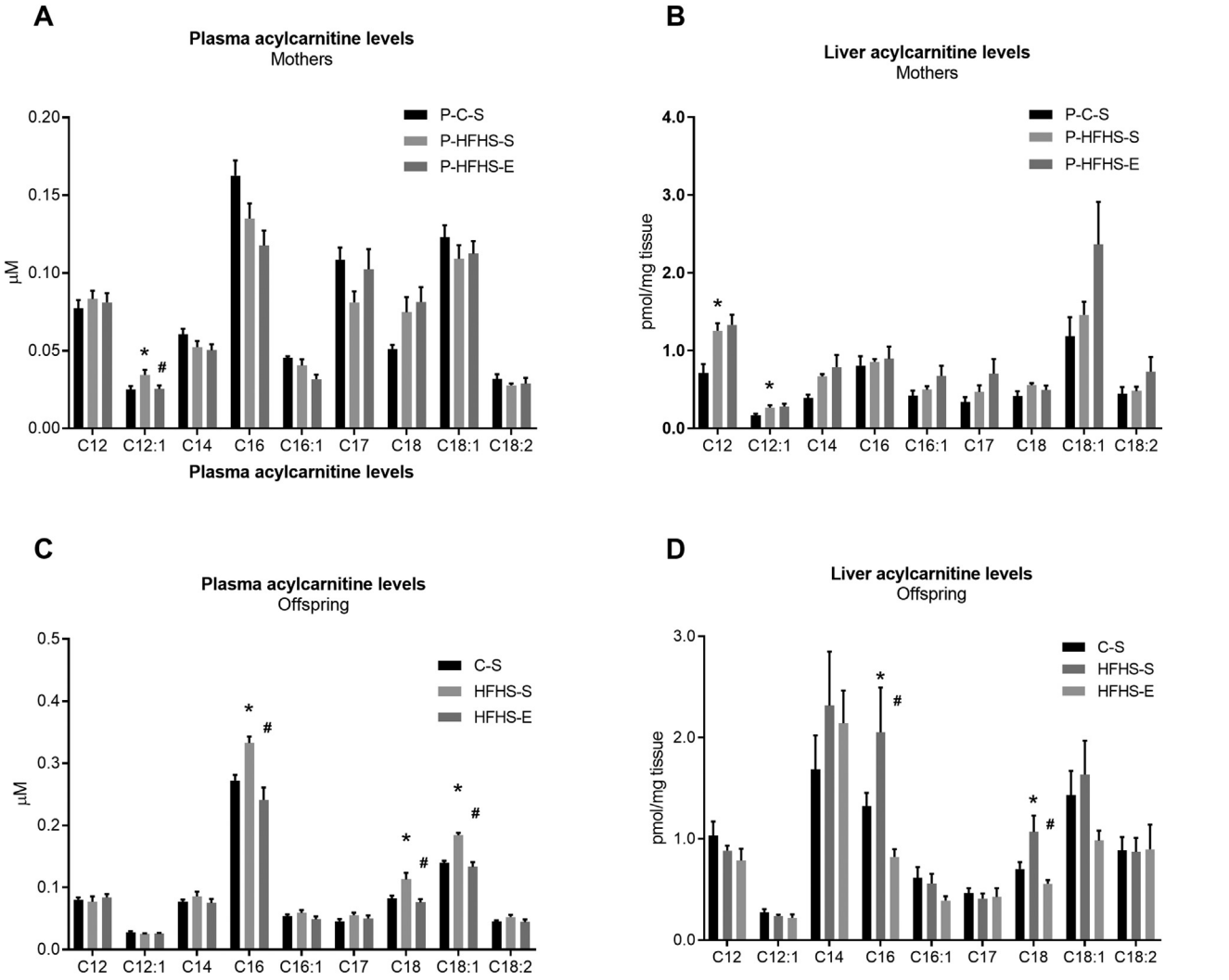

Fig. 5. Effect of maternal high-fat high-sucrose diet and gestational exercise on acylcarnitine levels in (A) plasma and (B) liver in the maternal generation on acylcarnitine levels in plasma (C) and liver (D) in the offspring generation. C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM ($n = 6$). * vs. P-C-S (0.05) or vs. C—S ($p < 0.05$) in maternal or offspring generation, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

pregnant women [28,29]. Interestingly, HFHS feeding increased litter size regardless of physical activity levels. The data on the influence of maternal HFD and/or exercise on litter size are, however, inconsistent [10,30–32]. Since in favourable conditions an increase in litter size may be favoured [33], it could be speculated that HFHS animals allocated more energy to increase litter and offspring size. The offspring of sedentary HFHS mothers had also higher body weight at weaning compared to the offspring of sedentary C mothers, which can be explained by maternal GDM-associated hyperglycaemia, hyperinsulinemia, and hyperlipidemia, and consequent increase in fetus store of protein and fats, and macrosomia [34,35]. This strong impact of maternal lifestyle on offspring body weight increase was maintained until the 6th week of age suggesting that post-weaning nutrition option (C-diet) could have prevented obesity development later in life. Interestingly, gestational exercise also appears to be a very strong stimulus against obesity development at early age, as the body weight of the offspring from exercised HFHS mothers was maintained at the level of their sedentary C counterparts.

Glucose intolerance during pregnancy is another important marker of GDM. To confirm that GDM-related glucose intolerance was developed only at the onset of the pregnancy and not before, we performed OGTT before and during pregnancy. The HFHS-diet consumption before pregnancy did not alter basal glucose levels or glucose tolerance between C-fed and HFHS-fed animals. However, during pregnancy, basal glucose level and glucose AUC were higher in HFHS animals compared to C animals, and this difference in glucose levels remained for 60 min after glucose ingestion. Similar results were reported by Pereira et al. [10] in HFHS-diet-induced GDM. However, 2-weeks of exercise in HFHS-fed animals did not improve glucose tolerance during pregnancy, although a slight decrease in 30 and 60 min after the glucose ingestion was noticed compared to sedentary HFHS group. This suggests that the 2-weeks of exercise during pregnancy were insufficient to revert the glucose metabolism impairments but were able, at least, to alleviate HFHS-diet impact. On the opposite, gestational exercise restored a HFD-induced increase in glucose AUC in mice [30]. Moreover, 4-weeks of exercise protocol in rats lowered glucose AUC only in the control diet group but not in the HFD group suggesting that some advantageous effects of exercise can be impaired by HFD and thus restrict exercise from reversing HFD-induced metabolic alterations [36].

4.2. Hepatic lipid accumulation

Women with GDM seem to have higher risk for hepatic steatosis, although this was only observed as early as 5–16 years post-partum [5,6].

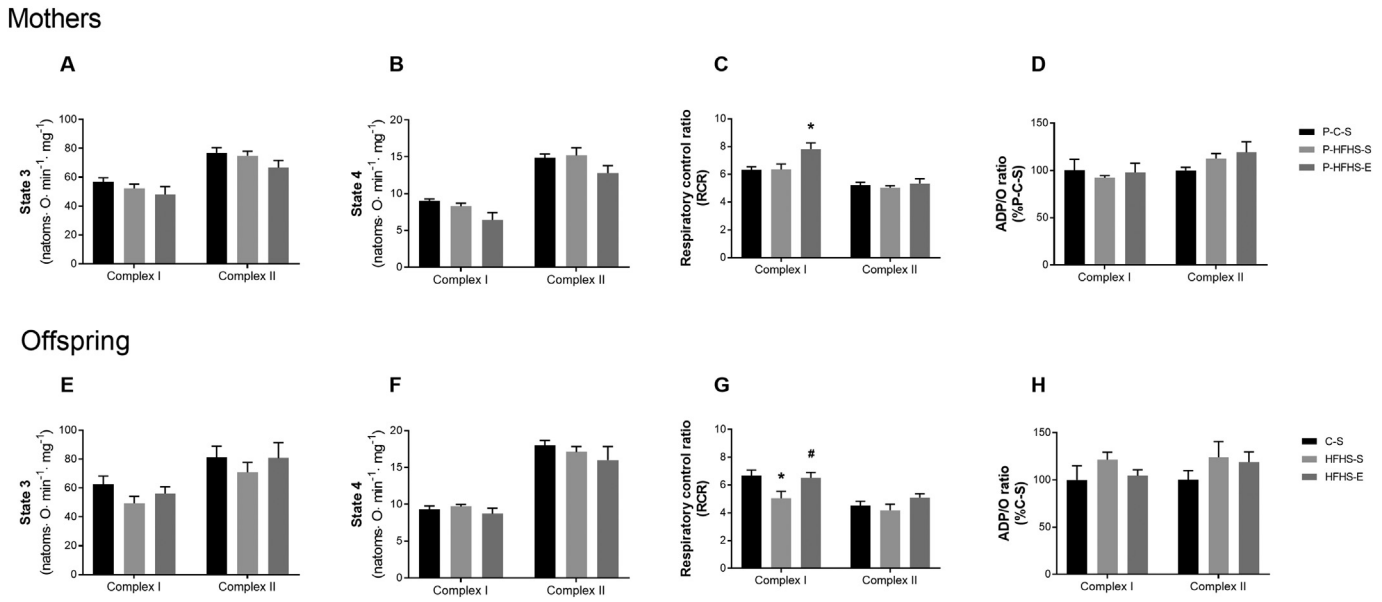

Fig. 6. Effect of maternal high-fat high-sucrose diet and gestational exercise on liver mitochondria (A) state 3 respiration, (B) state 4 respiration, (C), respiratory control ratio (RCR), and (D) ADP/O ratio in the maternal generation; and (E) state 3 respiration, (F) state 4 respiration, (G), respiratory control ratio (RCR), and (H) ADP/O ratio in the offsrpring generation. P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM (n = 6). * vs. P-C-S ($p < 0.05$) or vs. C—S ($p < 0.05$) in maternal or offspring generation, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

Surprisingly, in the present study, HFHS-diet predisposed towards a reduction of NAFLD score in the maternal generation regardless of exercise, which was supported by hepatic TG content. Interestingly, in non-pregnant counterparts, HFHS-diet increased NAFLD score, while 3-week of exercise lessen this effect, suggesting that gestational exercise per se promotes some protective mechanisms, which can be additionally triggered by some environmental stimuli, such as nutrition, to protect mother and fetus from detrimental consequences. We can also speculate that lower β-oxidation of FA due to GDM-related hyperglycaemia [37], may allow greater availability of maternal dietary FA to the fetus, which is actually supported by offspring acylcarnitine profile (Fig. 4).

The effects of maternal HFHS-diet, and especially gestational exercise, highlight the relevance of physical exercise against early-stage NAFLD. Gestational exercise prevented maternal diet-induced deleterious consequence in NAFLD score, and importantly in hepatic TG content, especially because hepatic TGs seems to be a better marker of steatosis in rodent studies [23]. Maternal high-caloric diet, related or not with GDM, can induce NAFLD development in their offspring, especially if followed by energy-rich diet consumption by offspring themselves [9,10,38,39]. Although hepatic steatosis in the offspring is not as obvious at the statistical level in our study, the observed trends can suggest that offspring of sedentary HFHS mothers are at the threshold of the development of hepatic steatosis, which can be successfully prevented by gestational exercise.

4.3. Liver mitochondrial function

Acylcarnitine profiling is used to describe alterations in mitochondrial β-oxidation in animal models of obesity and IR [40]. In the dams of the present study, HFHS feeding did not alter long-chain acylcarnitine levels. Eventually, the 8 or 12-weeks of HFD did not influence hepatic acylcarnitines due to liver capacity to export the excess of FA to circulation [40,41]. Conversely, offspring have increased exactly those plasma and liver acylcarnitines (C16, C18, and C18:1), that correspond to different FA composition between C and HFHS-diets consumed by mothers. Considering that fetus receives nutrient supplies, especially glucose and FA, from maternal diet through the placenta [42], these data suggest that increased accumulation of those acylcarnitine in the offspring plasma and liver are a consequence of maternal diet. Furthermore, reduced placental β-oxidation in GDM encourages the accumulation of triglycerides in placenta [37], which may result in an increased transfer of placental FA to the fetus liver FA pool. Fortunately, it seems that maternal gestational exercise emerged as a very effective tool against excessive energy originated from mothers' HFHS-diet. Accordingly, less FA seem to be transferred through placenta to the fetus, resulting in the control levels of acylcarnitines in plasma and liver of the offspring from exercised HFHS mothers. Even though liver and muscle contribute differently to the plasma acylcarnitine pool during exercise, i.e. muscle mainly contribute to plasma medium and long-chain acylcarnitines and liver only for short-chain acylcarnitines [43], our data showed that maternal exercise promoted a decrease in hepatic and plasma long-chain acylcarnitines in the offspring when compared to their sedentary counterparts. Nevertheless, Xu and collaborators provided evidence that upon exercise, skeletal muscle can release long-chain acylcarnitines to plasma, that are further taken by liver [43]. Indeed, plasma levels of acylcarnitine above a certain threshold seem to represent a stimulus for their uptake by the liver [44]. In the liver, however, due to the increased fitness mitochondrial phenotype stimulated by exercise (Fig. 6), long-chain acylcarnitines are efficiently shortened and exported to circulation as short-chain acylcarnitines resulting in the decrease of the hepatic long-chain acylcarnitines levels [43]. Another possible interesting mechanism that could explain this role of exercise includes muscle-secreted myokines [45]. The secretion of myokines such as interleukins (IL)-6, fibrobrast growth factor 21 (FGF21), and fibronectin type III-domain containing 5 (FNDC5), later cleaved to irisin, are found to be increased upon skeletal muscle contraction [46]. Irisin may further modulate PPARalpha signalling pathways, which consequently regulates lipid metabolism through thermogenesis mechanisms or upregulation of FGF21, which can eventually improve liver insulin sensitivity [47]. Furthermore, considering that PPARalpha is also involved in hepatic (acyl)carnitine metabolism [48], this irisin-

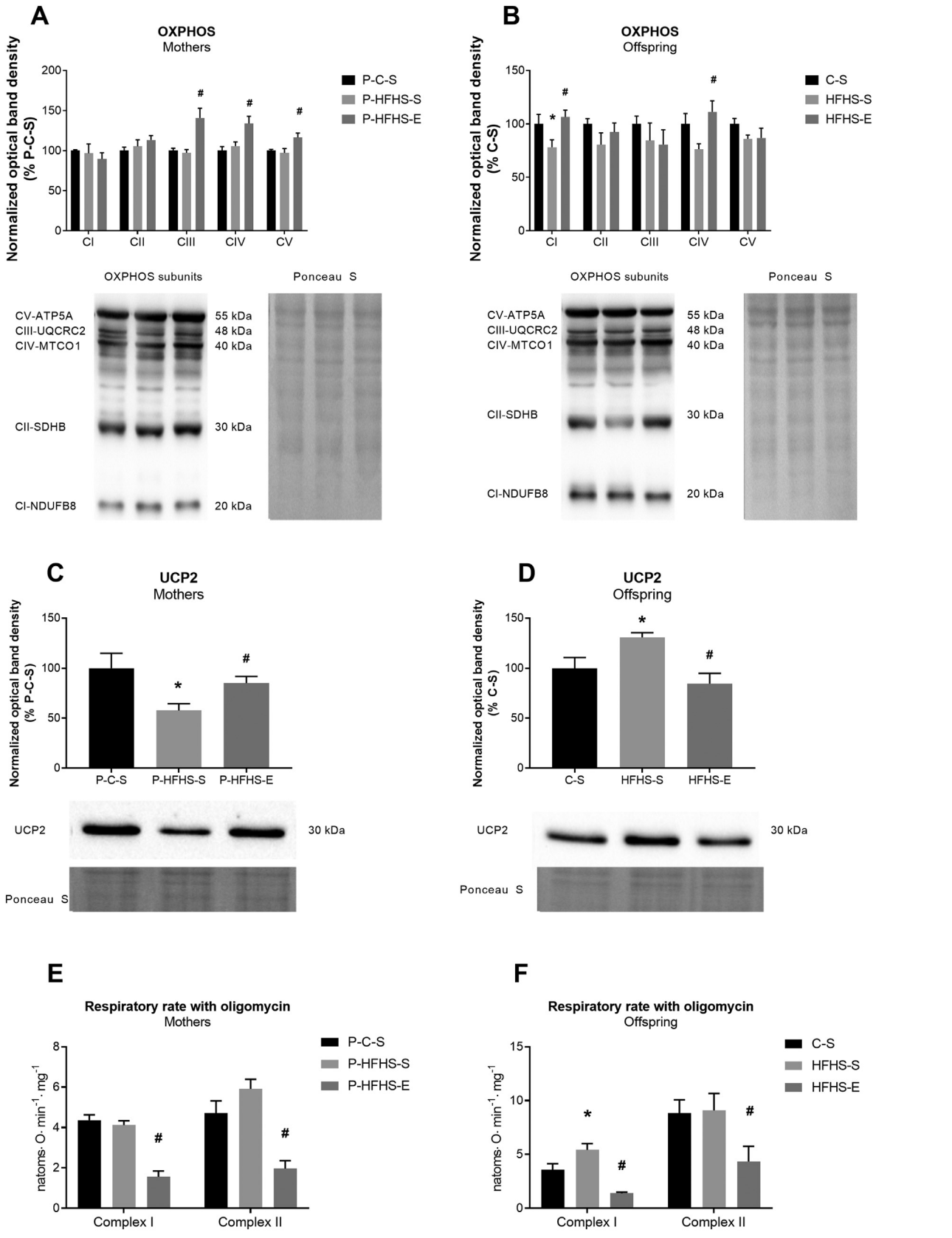

Fig. 7. Effect of maternal high-fat high-sucrose diet and gestational exercise on OXPHOS subunits in (A) maternal generation and (B) offspring generation and UCP2 content in (C) maternal generation and (D) offspring generation; and respiratory rate with oligomycin in (E) maternal generation and (F) offspring generation. CI – complex I CI-NDUFB8 subunit; CII – complex II CII-SDHB subunit; CIII – complex III CIII-UQCRC2 subunit; CIV – complex IV CIV-MTCO1 subunit; CV – complex V CV-ATP5A subunit. UCP2 – Uncoupling protein 2. P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM (n = 6). * vs. P-C-S ($p < 0.05$) or vs. C—S ($p < 0.05$) in maternal or offspring generation, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in maternal or offspring generation, respectively.

PPARalpha-acylcarnitine axis could indirectly explain, at least in part, a possible role of exercise in the regulation of hepatic mitochondrial function.

Moreover, under sedentary conditions, HFD may induce the initial increase in β-oxidation, but without a concomitant increase in TCA cycle activity. Therefore, β-oxidation flux may be disrupted and promote acylcarnitine accumulation in mitochondria, possibly contributing to mitochondrial failure in physically inactive animals [40]. In line with the alterations in offspring acylcarnitines levels that result from maternal unhealthy lifestyle, we observed alterations in offspring liver mitochondrial function. Maternal HFHS-diet and sedentary lifestyle compromised RCR (when using complex I-linked substrates) of their offspring, and reduced complex I protein expression. Reduced expression or activity of OXPHOS has been detected in liver mitochondria of offspring of obese [49] or HFD-fed mothers [38] in rodent studies. The offspring of sedentary HFHS mothers also showed a declining tendency in the expression of other respiratory complexes, suggesting slightly reduced liver oxidative capacity. However, this bioenergetic failure was remarkably attenuated by gestational exercise even in maternal HFHS-fed conditions. This increased bioenergetic efficiency boosted by gestational exercise may be, at least in part, supported by regulation of ETC complex I protein expression, as well as, complex IV expression. A similar increase in complex IV activity or expression due to maternal exercise has been reported in offspring muscle and fetal heart [50–52]. Being a rate-limiting enzymatic complex in OXPHOS [53], complex IV is importantly stimulated by maternal exercise, therefore rescuing the compromised liver mitochondrial ETC activity in adverse conditions. Overall, this suggests that gestational exercise can contribute to the recovery of the offspring OXPHOS system compromised by maternal HFHS-diet. Surprisingly, a study by Siti et al. [52] suggested that pre-gestational and gestational exercise (combined with regular diet) did not alter offspring liver complex I-related RCR values, but reduced complex-II related RCR, followed by a decrease in complex II activity and an increase in complex IV activity. This discrepancy in RCR values might be a consequence of different exercise protocols, as well as animal models and diets. Actually, in our model, we combined endurance training with the free-running wheel, which has been suggested to prompt rats' oxidative capacity [54].

Additionally, UCP2 upregulation, associated with increased proton leakage, was observed in the offspring of sedentary HFHS mothers. In another study, the same impact of maternal energy-rich diet on offspring UCP2 levels was observed [55]. The impairment of respiratory chain due to maternal HFHS diet was also reflected in the increased rate of proton leak (in the presence of oligomycin), which was, in contrast, remarkably reduced by maternal gestational exercise. Upregulation of UCP2 and related mitochondrial proton leakage are proposed as underlying events in NAFLD development. With an increase in energy supplies over energy requirements, liver mitochondria can activate an alternative pathway of substrate oxidation not coupled to ATP synthesis, but involving UCP2 [56]. Additionally, hepatic UCP2 expression can be stimulated by fatty acids [57]. Therefore, increased accumulation of liver acylcarnitines in the offspring of sedentary HFHS mothers may have represented an increase in energy supplies, justifying the consequent UCP2 upregulation and related uncoupling. Once again, maternal exercise during GDM pregnancy prevented the adverse effect of HFHS-diet, and considerably reduced UCP2 expression in the offspring liver mitochondria. These data are consistent with reduced hepatic TG levels, promoting gestational exercise as an important preventive agent against the development of GDM-related liver pathology in offspring.

To our knowledge, there are no data regarding the effect of exercise cessation on liver mitochondrial function of GDM animals. In the present study, HFHS-diet per se did not affect liver mitochondrial function or OXPHOS expression in mothers. Indeed, in other studies, HFD did not alter liver mitochondria respiratory rates in female rodents [58,59]. Interestingly, we observed that even 8-weeks after exercise cessation, mitochondrial adaptations were maintained, as reflected in increased RCR values when using complex I-related substrates and increased protein expression of complex III, complex IV, and ATP synthase. Even though increased oxygen consumption was not followed by upregulation of complex I, the increase of complexes III and IV expression may suggest that gestational exercise promoted the formation of ETC supercomplex. In fact, enhanced supercomplex formation was observed in human skeletal muscle upon exercise [60]. This dynamic supercomplex, consisting of complexes I, III, and IV, can represent a mechanism through which mitochondrial ETC might adapt to cell requirements [61]. Within supercomplexes, the stability of complex I seems to be maintained by complex III [62] and complex IV [63], which may explain the improvement of complex I-related RCR due to exercise without any alterations in complex I protein expression.

Despite not affecting mitochondrial respiration, HFHS feeding reduced maternal UCP2 levels even though it would be expected from HFHS to prompt an opposite effect, as previously discussed. However, UCP2 mRNA regulations depends on ATP pool regulation. Reduced intracellular ATP levels are correlated with reduced UCP2 levels, which could prevent UCP2-uncoupling and impending ATP depletion [64]. Actually, women with GDM are at higher risk for acute ischemia [65] and could suffer from liver ischemia [66] during complicated pregnancy, promoting anaerobic metabolism and consequent reduction in ATP levels, which can explain reduced UCP2 expression in our data and no alterations in oxygen consumption with oligomycin. This UCP2 reduction could probably be induced at the onset of the pregnancy and related to GDM pathology, considering that UCP2 content between exercised GDM mothers and sedentary C mothers do not differ. These results argue that gestational exercise was able to reduce liver alterations that are associated with GDM and develop at the onset of the pregnancy.

5. Conclusions

The results of this innovative study demonstrated for the first time that gestational exercise was able to boost liver mitochondrial respiratory capacity in a diet-induced GDM model preserved even 8-weeks after delivery and exercise cessation. However, both maternal diet and gestational exercise seem to have a high impact on the next generation, with maternal gestational exercise emerging as a more potent factor. Hepatic TG accumulation and impaired liver mitochondrial capacity, also associated with NAFLD development, suggest that these offspring are at the very early stage of liver disease before a clinical image can be histologically observed. Nevertheless, gestational exercise was undoubtedly effective in preventing these metabolic deleterious consequences from detrimental maternal HFHS-diet. Therefore, this work highlights the role of exercise as a first-line non-pharmacological strategy, able to prevent liver metabolic disorders in the context of GDM. In addition, considering the positive impact it can have on mothers, but also in children health, it advocates the social and economic importance of exercise in pregnancy clinical counselling.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2021.154704.

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CRediT authorship contribution statement

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Visualization, Project administration. **Jorge Beleza:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Pedro Coxito:** Investigation, Writing – review & editing. **Susana Pereira:** Conceptualization, Investigation, Writing – review & editing, Project administration. **Hugo Rocha:** Investigation, Writing – review & editing. **Tiago Bordeira Gaspar:** Investigation, Writing – review & editing. **Fátima Gärtner:** Investigation, Writing – review & editing. **Rossana Correia:** Investigation, Writing – review & editing. **Maria João Martins:** Investigation, Writing – review & editing. **Tiago Guimarães:** Investigation, Writing – review & editing. **Sandra Martins:** Investigation, Writing – review & editing. **Paulo Oliveira:** Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **António Ascensão:** Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **José Magalhães:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

No conflict of interest.

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