

University of Porto
Faculty of Sport
Research Center in Physical Activity, Health and Leisure
Laboratory for Integrative and Translational Research in Population Health
Laboratory of Metabolism and Exercise

**Breaking the intergenerational cycle of chronic liver disease
with maternal exercise:**

Effects of gestational physical exercise on the modulation
of liver mitochondrial function in mothers and offspring

The present dissertation was submitted to obtain the Ph.D. degree included in the doctoral course of Physical Activity and Health designed the Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, according to the Law 74/2006 from March 24th.

Supervisor: Professor José Fernando Magalhães Pinto Pereira
Co-supervisor: Professor António Alexandre Moreira Ribeiro de Ascensão

Jelena Stevanović Silva
Porto, 2022

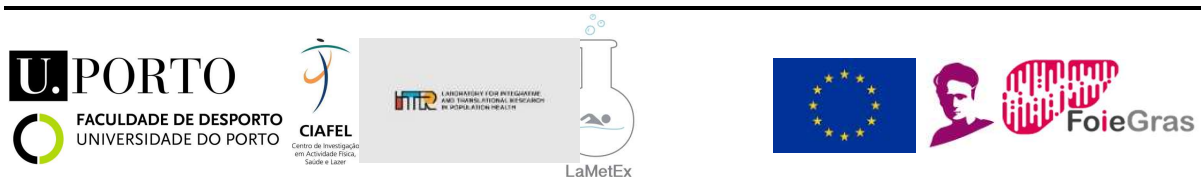
Stevanović Silva, J. (2022). *Breaking the intergenerational cycle of chronic liver disease with maternal exercise: Effects of gestational physical exercise on the modulation of liver mitochondrial function in mothers and offspring*. Porto: J. Stevanović Silva. Dissertação de Doutoramento em Atividade Física e Saúde, apresentada à Faculdade de Desporto da Universidade do Porto.

KEYWORDS: GESTATIONAL EXERCISE; GESTATIONAL DIABETES; FETAL PROGRAMMING; OFFSPRING HEALTH; LIVER MITOCHONDRIA.

Funding

This doctoral thesis is part of a project that has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722619.

The present work was conducted at the Faculty of Sport of University of Porto, Research Center in Physical Activity, Health and Leisure; Laboratory for Integrative and Translational Research in Population Health; at the Laboratory of Metabolism and Exercise.



Acknowledgements

Approaching the end of this doctoral journey, I look back and realize that throughout all these years I have met many different people to whom I feel deeply indebted and whose support and help have been indispensable in the completion of this dissertation. Therefore, I cannot but express my sincere gratitude to all of them.

To my supervisors, Professor José Magalhães and Professor António Ascensão. I owe them very special thanks for giving me the opportunity to be part of the “Foie Gras” project, but above all, to be a member of their team and introduce me to the world of Exercise Physiology.

To Professor José, for all his help from the moment I moved to Portugal and started working on this dissertation. He has been a supervisor, a teacher, but most of all, a friend. I am very grateful for his boundless patience, willingness to exchange ideas, and selfless support. I am deeply appreciative for his trust in me, his optimism, and for challenging me and pushing me forward when everything seemed to be grey. Everything he taught me, his inspiring enthusiasm and knowledge, his advice and criticism, all of this have contributed to my professional development. Without him, I would never be a researcher as I am today. It has been a privilege and an honour being guided by him.

To Professor António, for supporting me in the critical and important moments of my dissertation, for his scientific rigor and unconditional support. His insightful feedback pushed me to sharpen my academic thinking and brought this dissertation to a higher level.

To my laboratory colleagues, for their support in the experimental part of this dissertation, for all the hard-working moments, all the laughs and tears. This has been a long journey and without you it would have never reached the end.

To Jorge Beleza, for being a friend more than anything, for helping me to adapt to Portuguese society and teaching me how to deal with Portuguese bureaucracy. I am thankful for all the help and countless hours spent with me in

the laboratory, learning and adjusting techniques, discussing, planning and changing plans, complaining, but in the end enjoying the work. Without his help I would have never been able to complete this doctoral journey, therefore, I feel that this dissertation is his as much as it is mine.

To Pedro Coxito, I am more than a grateful for his expertise in laboratory techniques and detail-oriented skills, which immensely improved my research, for all his kind words and understanding, for his friendship.

To Rita Pereira, for her kindness, friendship, and for her unselfish help during the critical moments of our research.

To all the members of the “Foie Gras” team: to principal investigators for all their advice, criticism, and scientific debates, and to research colleagues for their friendship and optimism.

To senior researchers and collaborators from other research institutions, for their indispensable support, help, and advice.

To Dr. Sofia Lamas from the Animal facility of the i3S, for all the patience, enthusiasm, useful advice, and selfless support in working with experimental animals. Without her great expertise and willingness to help, it would be impossible to deal with such an experimental protocol.

To Professor Rita Ferreira from the University of Aveiro, for her kindness to share her knowledge and for always being available to resolve any doubt with Western Blots and biochemical proceedings.

To Professor Alexandra Gouveia and Professor Adriana Rodrigues from the Faculty of Medicine, for welcoming me in their laboratory and for their generosity to share their knowledge and teach me procedures in optical microscopy and PCR.

To Professor Paulo Oliveira from the University of Coimbra, for scientific contribution that was encouraging and inspiring and for his generous comments and suggestions for each manuscript writing.

To all my friends from Serbia, who remained part of my life despite the distance, and to those from Portugal, who made this country feel like home to me.

To my brother and my parents, for everything. Without their support, faith in me, and, most importantly, their love, I would not be what I am now. I am eternally grateful to my parents for being wind in my sails as well as my safe harbour when needed. I dedicate my dissertation to them. Мама и тата, хвала на свему!

To André and Maria, for their love. Without them, nothing of this would be worthy.

*Нека би Господ дао да резултати ове дисертације буду
искоришћени за добробит других и никако другачије.*

Table of Contents

Acknowledgements	v
List of Figures	xi
List of Tables	xv
Resumo	xvii
Abstract	xix
List of Abbreviations	xxi
CHAPTER I	
General Introduction	25
CHAPTER I	
Aims	19
CHAPTER II	
Theoretical Background	25
Review Article I	
Fit mothers for a healthy future: Breaking the intergenerational cycle of NAFLD with maternal exercise	27
Review Article II	
Physical exercise and liver “fitness”: Role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease	47
CHAPTER III	
Experimental Work	63
Original Article I	
Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring	65
Original Article II	
Gestational exercise antagonizes the impact of maternal high-fat high-sucrose diet on liver mitochondrial alterations and quality control signalling in male offspring	81

Original Article III	
Exercise performed during pregnancy positively modulates liver metabolism and promotes mitochondrial biogenesis of female offspring in an animal model of diet-induced gestational diabetes	119
CHAPTER IV	
General Discussion	157
CHAPTER V	
General Conclusions	173
REFERENCES	179

List of Figures

CHAPTER II

Review Article I

- Figure 1.** Potential mechanisms behind the development of non-alcoholic fatty liver disease (NAFLD) in children 31
- Figure 2.** Liver-muscle crosstalk..... 34
- Figure 3.** Breaking the intergenerational cycle of NAFLD with maternal exercise 35

CHAPTER III

Original Article I

- Figure 1.** Experimental and exercise protocol. 69
- Figure 2.** Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal body weight gain during gestation period 71
- Figure 3.** Representative light micrographs (×200) of liver tissue from all groups and generations..... 72
- Figure 4.** Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal and offspring liver triglyceride content. 73
- Figure 5.** Effect of maternal high-fat high-sucrose diet and gestational exercise on acylcarnitine levels in plasma and liver in the maternal and offspring generation..... 74
- Figure 6.** Effect of maternal high-fat high-sucrose diet and gestational exercise on liver mitochondria state 3 respiration, state 4 respiration, respiratory control ratio (RCR), and ADP/O ratio in the maternal and offspring generation..... 75
- Figure 7.** Effect of maternal high-fat high-sucrose diet and gestational exercise on OXPHOS subunits, UCP2, and respiratory rate with oligomycin in maternal and offspring generation..... 76
- Supplementary data 1 (S1).** The difference in fatty acid composition between Control diet and High-fat high-sucrose diet 80

Original Article II

- Figure 1.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of regulators of liver glucose and lipid metabolism in mothers and offspring and representative transmission electron microscopy images of liver parenchyma of male offspring..... 93
- Figure 2.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of different markers of liver mitochondrial biogenesis in mothers and offspring..... 95
- Figure 3.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of different markers of liver mitochondrial dynamics in mothers and offspring..... 96
- Figure 4.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of ER α in the liver tissue and mitochondria of maternal and offspring generation 97
- Figure 5.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of different markers of auto(mito)phagy signalling in liver and liver mitochondria in mothers and offspring..... 98
- Figure 6.** Effects of maternal high-fat high sucrose diet and gestational exercise on the protein expression of different markers of apoptotic signalling in mothers and offspring 99
- Figure 7.** Effects of maternal high-fat high sucrose diet and gestational exercise on the micro-RNA expression in the liver in mothers and offspring 100

Original Article III

- Figure 1.** Effects of maternal high-fat high-sucrose diet and gestational exercise on female offspring body weight changes from weaning period to the sacrifice point at 6 weeks of age; and offspring glucose tolerance at 5 weeks of age..... 132

Figure 2. Effects of maternal high-fat high-sucrose diet and gestational exercise on representative light micrographs and transmission electron micrographs of liver parenchyma.....	134
Figure 3. Effects of maternal high-fat high-sucrose diet and gestational exercise on acylcarnitine levels in plasma and liver in the female offspring	135
Figure 4. Effects of maternal high-fat high-sucrose diet and gestational exercise on liver OXPHOS subunits and UCP2	135
Figure 5. Effects of maternal high-fat high-sucrose diet and gestational exercise on the protein expression of different markers of liver glucose and lipid metabolism	136
Figure 6. Effects of maternal high-fat high-sucrose diet and gestational exercise on the protein expression of different markers of liver mitochondrial biogenesis	138
Figure 7. Effects of maternal high-fat high-sucrose diet and gestational exercise on the protein expression of different markers of liver mitochondrial dynamics	139
Figure 8. Effect of maternal high-fat high-sucrose diet and gestational exercise on the hepatic protein expression of different markers of mitophagy signalling	141
Figure 9. Effects of maternal high-fat high-sucrose diet and gestational exercise on the micro-RNA expression in the liver	142

CHAPTER V

General Conclusions

Figure 1. Principal component analysis of all parameters studied in this dissertation in mothers, male offspring, and female offspring.....	177
--	-----

List of Tables

CHAPTER II

Review Article I

Table 1. Alterations in offspring hepatic metabolism in response to maternal exercise performed before and/or during pregnancy.....	37
---	----

CHAPTER III

Original Article I

Table 1. Animal data and plasma biochemical analyses	70
Table 2. NAFLD score recorded from mothers' and offspring livers of all experimental groups, blindly observed under light microscope.	72

Original Article II

Table 1. Semiquantitative analysis of ultrastructural examination of offspring liver tissue.	92
---	----

Original Article III

Table 1. Animal data and plasma biochemical analyses	131
Table 2. NAFLD score recorded from offspring livers of all experimental groups, observed blindly under light microscope, hepatic triglyceride content, and semiquantitative analysis of ultrastructural examination of offspring liver tissue.....	133

Resumo

O estilo de vida materno e potenciais distúrbios metabólicos relacionados com o mesmo, como a diabetes mellitus gestacional (DMG), têm sido associados a alterações no ambiente intrauterino e são fortes moduladores da programação fetal, originando eventualmente condições metabólicas deletérias e stressantes para o feto. O exercício físico assume-se como uma estratégia essencial na prevenção/terapia de doenças hepáticas, mas pouco se conhece sobre o impacto do mesmo quando realizado durante a gravidez e os seus efeitos no desenvolvimento dos descendentes. Esta dissertação, composta por dois estudos de revisão e três estudos experimentais, pretendeu analisar o papel do exercício gestacional na função hepática dos descendentes quando comprometida pela DMG materna (estudos I, II e III), mas também na condição hepática pós-DMG materna (estudos I e II). Neste modelo animal de DMG induzida por dieta, avaliámos a função respiratória mitocondrial hepática das mães e dos descendentes masculinos (estudo I), bem como a histopatologia hepática, a acumulação de triglicerídeos e de acilcarnitinas (estudos I e III), marcadores de biogénese mitocondrial e de sinalização de controlo de qualidade, e a regulação epigenética via mecanismos de microRNA (estudos II e III) em mães e descendentes masculinos e femininos. A bioenergética mitocondrial hepática foi estimulada através de exercício gestacional, prevenindo os problemas infligidos pela dieta materna nos descendentes masculinos e proporcionando adaptações maternas preservadas até 8 semanas após o parto. O estilo de vida materno afetou notavelmente o metabolismo lipídico dos descendentes masculinos, motivando alterações em fatores transcripcionais associados ao metabolismo glicolítico, tendo-se verificado um menor impacto nos descendentes femininos. Adicionalmente, o exercício gestacional revelou-se como um poderoso regulador da biogénese e dinâmica mitocondrial, prevenindo ou revertendo a tendência induzida pela dieta materna no sentido da fissão mitocondrial e não da fusão, e demonstrou ser um modulador mais potente de mecanismos epigenéticos associados à expressão de microRNA do que a dieta materna. As vias de sinalização apoptótica e auto(mito)fágica, exclusivamente em mitocôndrias, foram alteradas apenas nas mães. Em termos gerais, os

nossos dados sugerem que o exercício gestacional pode prevenir consequências metabólicas relacionadas com a DMG nos descendentes, devendo, por isso, ser considerado como um modulador muito poderoso e favorável do ambiente intrauterino e como uma importante estratégia contra a DMG.

PALAVRAS-CHAVE: Exercício gestacional; Diabetes gestacional; Programação fetal; Saúde dos descendentes; Mitocôndrias hepáticas.

Abstract

Maternal lifestyle and potential related metabolic disorders, such as gestational diabetes mellitus (GDM), have been associated with alterations in *in utero* environment and are strong modulators of fetal programming, eventually leading to aggravated and stressful metabolic conditions for the fetus. Physical exercise has been proved as the cornerstone in the prevention/therapy of liver diseases, yet less is known about physical exercise performed during pregnancy and its effects on offspring development. This dissertation, comprised of two review and three experimental studies, intended to analyze the role of gestational exercise on offspring liver health jeopardized by maternal GDM (studies I, II, and III), but also on maternal post-GDM liver condition (studies I and II). In this diet-induced GDM animal model, we assessed maternal and male offspring hepatic mitochondrial respiratory function (study I), as well as hepatic histopathology, triglyceride and acylcarnitine accumulations (studies I and III), markers of mitochondrial biogenesis and quality control signaling, and epigenetic regulation via microRNA mechanisms (studies II and III) in mothers and male and female offspring. Hepatic mitochondrial bioenergetics was stimulated by gestational exercise, thus preventing impairments imposed by maternal diet in male offspring, but also providing adaptations in mothers preserved even 8 weeks post-partum. Maternal lifestyle remarkably affected lipid metabolism in male offspring, with less impact on female offspring, that rather expressed changes in glucose-related transcriptional factors. Also, gestational exercise was a powerful regulator of mitochondrial biogenesis and dynamics, preventing or reverting maternal diet-induced shift towards mitochondrial fission rather than fusion, and it was a more potent modulator of microRNA mechanisms than the maternal diet itself. Apoptotic and auto(mito)phagy signaling pathways, exclusively in mitochondria, were altered only in mothers. In general, our data suggest that gestational exercise can prevent GDM-related metabolic consequences in the offspring, proposing it as a very powerful modulator of *in utero* environment and important strategy against GDM.

KEYWORDS: Gestational exercise; Gestational diabetes; Fetal programming; Offspring health; Liver mitochondria.

List of Abbreviations

ADA - American Diabetes Association
ALT - Alanine aminotransferase
AST - Aspartate aminotransferase
ATP - Adenosine-triphosphate
AUC - Area under the curve
ChREBP - Carbohydrate response element binding protein
DGS - General Directorate of Health
DRP1 - Dynamin-related protein 1
ER - Estrogen receptor
ERE - Estrogen response elements
ET - Endurance training
ETC - Electron transport chain
FA - Fatty acids
FCCP - Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FFA - Free fatty acids
FGF - Fibroblast growth factor
Fis1 - Mitochondrial fission 1
G/M - Glutamate/malate
GCKR - Glucokinase regulatory
GDM - Gestational diabetes mellitus
GE - Gestational exercise
HDL - High-density lipoproteins
HFD - High-fat diet
IADPSG - International Association of Diabetes and Pregnancy Study Groups
IMM - Inner mitochondrial membranes
IR - Insulin resistance
LC3 - Microtubule-associated protein-1 light chain 3
LDL - Low-density lipoproteins
LYPLAL1 - Lysophospholipase-like-1
MBOAT7 - Membrane bound O-acyltransferase domain-containing-7

MFN1 - Mitofusin1
MFN2 - Mitofusin2
miRNA - microRNA
mPTP - Mitochondrial permeability transition pore
mtDNA - Mitochondrial DNA
NADH - Nicotinamide adenine dinucleotide
NAFLD - Non-alcoholic fatty liver disease
NASH - Non-alcoholic steatohepatitis
NICE - National Institute for Health and Care Excellence
OGTT - Oral glucose tolerance test
OMM - Outer mitochondrial membranes
OPA1 - Optic atrophy 1
OXPHOS - Oxidative phosphorylation
PCA - Principal component analysis
PE - Physical exercise
PEPCK - Phosphoenolpyruvate carboxykinase
PGC-1 α - Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
PINK1 - PTEN Induced Kinase 1
PNPLA3 - Patatin-like phospholipase domain-containing 3
PPAR - Peroxisome proliferators-activated receptor
RCR - Respiratory control ratio
ROS - Reactive oxygen species
S/R - Succinate/rotenone
SIRT1 - Sirtuin 1
SM - Skeletal muscle
SREBP1 - Sterol regulatory element binding 1
T2DM - Type 2 diabetes mellitus
TFAM - Mitochondrial transcription factor A
TG - Triglycerides
TM6SF2 - Transmembrane 6 superfamily member-2
TOM20 - Translocase of outer membrane 20
UCP - Uncoupling protein

VLDL - Very-low-density lipoprotein

VPA - Voluntary physical activity

CHAPTER I

General Introduction

Developmental Origins of Health and Disease

The etiology of human diseases includes genetic and environmental factors that rarely act independently (Liu et al., 2009). Without neglecting the genetic component, the interaction of numerous environmental factors, biological and/or socio-economic, may ultimately affect developmental processes and human health (Hoffman et al., 2017). Recent scientific evidence and emerging progress in the field of epigenetics bring a new concept that explains disease etiology, the so-called developmental origins of health and disease (Heindel & Vandenberg, 2015). This concept, explaining the link between early-life exposure to environmental factors and later-life disease development, was developed by David Baker in the 1980s (Barker & Osmond, 1986). According to this paradigm, alterations in *in utero* environment, due to maternal stress, (mal)nutrition, infections, drugs, and other environmental chemicals, may be reflected in the fetal cell differentiation processes and thus in developmental plasticity. However, some effects of environmental influences might not be phenotypically as apparent at birth, but rather promote delicate alterations in gene expression, cell numbers, and even metabolism. Therefore, an adverse *in utero* environment may trigger fetal adaptive responses, and consequently increase the risk for later-life development of metabolic dysfunction or disease, or even an earlier onset and/or an increased severity of disease (Gluckman et al., 2008; Heindel & Vandenberg, 2015).

In fact, the growing scientific evidence support this hypothesis. By intaking of excessive calories and not having a way to “spend” them due to low levels or total absence of physical exercise, pregnant women with this kind of lifestyle usually transmit this “high-caloric burden” through placenta, making *in utero* environment more cytotoxic. Recent studies have shown that the consumption of high-caloric diet by the maternal generation can remarkably affect offspring metabolism, particularly in the liver, adipose tissue, and skeletal muscle (Mouralidarane et al., 2013; Saben et al., 2013). Maternal “junk” food diet during pregnancy and lactation induces molecular metabolic adaptations in the offspring liver regardless of offspring post-weaning diet. Altered *in utero* environment, due to maternal diet, induces irreversible liver damage in the offspring. In fact, these offspring still

develop fatty liver even if fed with a balanced diet after weaning, although the condition is not as exacerbated as in the offspring fed with “junk” food (Bayol et al., 2010). Similar studies also reported that maternal high-fat diet (HFD) predisposes offspring to non-alcoholic fatty liver disease (NAFLD) with observed hyper-responsiveness if the offspring also consumes HFD (Wankhade et al., 2018; Wankhade et al., 2017) and with male offspring being more prone to NAFLD phenotype (Wankhade et al., 2018). Furthermore, exposure to intrauterine hyperglycemia or insulin resistance (IR), which are common in gestational diabetes mellitus (GDM), has the potential to program liver metabolic capacity of the fetus. Maternal GDM-related alterations of *in utero* environment resulted in offspring IR (Li et al., 2017; Yamashita et al., 2003), impaired glucose (Strakovsky et al., 2011) and lipid (Bush et al., 2011; Chang et al., 2019; Li et al., 2017; Talton et al., 2019; Zhang et al., 2017) metabolism, and even increased intrahepatic lipid accumulation and consequent NAFLD (Brumbaugh et al., 2013; Pereira et al., 2015). Bearing in mind that pregnancy is a particular state with many physiological and metabolic alterations to respond to the needs of the growing fetus, it is not a surprise that any external insult, such as maternal lifestyle alterations towards improper dietary habits and sedentarism, may modify the very precisely coordinated and regulated pregnancy metabolism, resulting in adverse *in utero* environment and impaired fetal development and growth.

Gestational diabetes mellitus (GDM)

Even during healthy pregnancies, glucose and lipid metabolism undergo some significant alterations. Pregnancy is a dynamic state with numerous anatomic, physiological, and biochemical adaptations in order to meet essential metabolic demands of a developing and growing fetus. Over the course of pregnancy, the metabolic state changes from an anabolic to a catabolic phase. In the first two trimesters, the anabolic state involves maternal increase in insulin sensitivity and in lipid deposition, resulting from hyperphagia and enhanced *de novo* lipogenesis. This nutrient storage is needed for maternal and fetal demands in later gestation and lactation. In the last trimester, the switch to a catabolic state occurs resulting in an accelerated lipid breakdown as well as increased IR. Consequently,

maternal glucose and free fatty acid (FFA) concentrations rise to respond to the increasing nutritional demands (Lain & Catalano, 2007; Zeng et al., 2017). Even though this shift from early-gestation insulin sensitivity to late-gestation IR represents a logical sequence of events in a healthy pregnancy, in women with a history of diabetes, obese women, or those with hyperglycemia, these metabolic alterations are more aggravated during pregnancy. Therefore, with further enhanced gestational IR, the risk for GDM increases (Lain & Catalano, 2007).

This GDM represents a condition of glucose intolerance and resulting hyperglycemia with the first onset occurring during pregnancy (American Diabetes Association, 2003; Buckley et al., 2012). However, there is no consensus regarding the diagnostic criteria. In fact, the recommended cut-off value for fasting glucose levels is 92 mg/dL (5.1 mmol/L) according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) (International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010) and the American Diabetes Association (ADA) (American Diabetes Association, 2016), or 100 mg/dL (5.6 mmol/L) according to the United Kingdom National Institute for Health and Care Excellence (NICE) (Webber et al., 2015). Generally, the oral glucose tolerance test (OGTT) is performed at 24-28 weeks of gestation, using the previously mentioned fasting glucose cut-off values and post-prandial 180 mg/dL (10 mmol/L) 1-hour after glucose intake and 153 mg/dL (8.5 mmol/L) (IADPSG and ADA) (American Diabetes Association, 2016; International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010) or 140 mg/dL (7.8 mmol/L) (NICE) (Webber et al., 2015) 2-hour after glucose intake. In Portugal, following the ADPSG recommendations, the General Directorate of Health (DGS) recommends that GDM may be diagnosed in the first trimester if the blood glucose values ranged between 92 mg/dL and 126 mg/dL, avoiding the OGTT of the second trimester. Therefore, for pregnant women with fasting glucose values lower than 92 mg/dL, the second trial to GDM is performed regularly at 24-28 weeks of gestations using OGTT (Almeida et al., 2017). Yet, the fasting blood glucose screening at initial medical visits is still not recommended by any of these three associations (American Diabetes Association, 2016; International Association of Diabetes and Pregnancy Study

Groups Consensus Panel, 2010; Webber et al., 2015). Interestingly, although the comparison of the early screening and detection of GDM with more convenient guidelines showed no apparent neonatal benefit, it was associated with an increased risk for small-for-gestational-age infants, as well as with higher healthcare costs due to more frequent medical visits (Ferreira et al., 2018). Furthermore, ADA defines GDM as diabetes diagnosed in the second or third trimester (American Diabetes Association, 2016) considering that an increase in IR, possibly related to GDM, usually begins at 20-24 weeks of gestation due to the rising levels of pregnancy-associated hormones (Kamana et al., 2015). It is assumed that about 7% of all pregnancies are related to GDM, with reported range of 1-14% in the United States (American Diabetes Association, 2003). In Europe, the prevalence ranges 2-6% of pregnancies (Buckley et al., 2012), but it seems that the global prevalence can vary between 9% and 45% (Agarwal et al., 2015).

Besides prevalent metabolic complications and possible genetic predisposition, an excessive weight gain during pregnancy and some lifestyle factors, such as malnutrition and sedentary behavior, can also be important risk factors for GDM as maternal dietary intake rich in fat and/or carbohydrates might influence glucose tolerance during pregnancy and the risk of developing GDM. It is also speculated that nutritional habits established years before pregnancy may trigger IR in pregnancy, whereas diet during pregnancy has low additional effect on this factor (Radesky et al., 2008). Furthermore, low levels of physical activity and sedentary behavior were associated with a higher risk of developing GDM (Leng et al., 2016; Pons et al., 2015; Zhang et al., 2006).

Also, women with GDM and suffering from hyperglycemia, hyperlipidemia, and hyperinsulinemia are at higher risk for pregnancy and delivery complications, including cesarean delivery and neonatal macrosomia and hypoglycemia (DeSisto et al., 2014; Kc et al., 2015). Moreover, even though GDM signs usually disappear soon after delivery, metabolic alterations that took place during the pregnancy period could have already provoked some metabolic stress in mothers and fetus, which would be expressed only later in life (Coustan, 2013). In fact, women with history of GDM are vulnerable to develop diabetes mellitus 5-10

years post-partum (Auvinen et al., 2020; Feig et al., 2008; Noctor & Dunne, 2015), but also NAFLD, although much later in life (5-16 years after delivery) (Ajmera et al., 2016; Foghsgaard et al., 2017; Forbes et al., 2011; Lavrentaki et al., 2019). Moreover, children from GDM mothers are more prone to impaired glucose tolerance (Dabelea et al., 2008) and especially to liver diseases (Brumbaugh et al., 2013; Patel et al., 2016), as their fetal metabolism is unable to buffer the excess of placental lipid transport (Brumbaugh & Friedman, 2014). In fact, recent research emphasized that the exposure to maternal GDM can cause an increased lipid accumulation in the offspring liver, which also increases the susceptibility to hepatic steatosis (Bush et al., 2011; Li et al., 2017; Pereira et al., 2015; Talton et al., 2019).

Fetal liver metabolism in normal and GDM-related pregnancies

Mechanisms of embryology, development and function of fetal liver could explain the association between maternal GDM and increased susceptibility to offspring hepatic impairment and lipid accumulation. Fetal liver has two important functions – hemopoietic and cardiovascular, the latter serving as a vascular link between the placenta and the heart (Giancotti et al., 2019). During fetal development, about half of the blood from the placenta enters the portal vein, supplying liver with oxygenated and nutrient-rich blood, whereas the rest of the blood goes directly to the fetal heart and brain (Tan & Lewandowski, 2020). Changes in placental nutrient supply, i.e., due to maternal malnutrition and increased body fat stores, may affect fetal liver perfusion and hepatic development, therefore, increasing the risk for metabolic and cardiovascular diseases later in life (Haugen et al., 2005). As an adaptive mechanism to diminish nutrition or oxygenation, the fetus redistributes its blood flow towards more essential organs, such as brain, at the expense of liver, muscle, and gut (Giancotti et al., 2019; Haugen et al., 2005). However, this nutrient allocation depends on the (im)balance between fetal nutrient demand and placental nutrient supply (Godfrey et al., 2012). In a condition of nutrients (glucose and fatty acids (FA)) excess, when mothers are obese, or with impaired glucose tolerance characteristic of GDM, placental nutrient transport is increased and can lead to prenatal lipid deposition (Godfrey

et al., 2012) and consequent risk of macrosomia (Herrera & Desoye, 2016). Due to increased placental nutrient transport, the blood flow to the fetal liver increases (Delhaes et al., 2018). Actually, in normal pregnancies, the increase of placental lipid flux and transport matches the development of adipose tissue (Stewart et al., 2013), which appears at around mid-gestation (Symonds et al., 2012), much later than the liver that appears at about the third week of gestation and rapidly grows until the 10th week (Giancotti et al., 2019). However, in pregnancies associated to maternal obesity, IR, and/or GDM, an early catabolic switch might occur resulting in augmented placental lipid transport, especially in early-to-mid pregnancy before the development of adipose tissue, leading to an ectopic lipid accumulation in liver and other developing tissue (Stewart et al., 2013). This condition establishes a lipotoxic *in utero* environment and eventually triggers the development of metabolic disturbances reflected as IR, metabolic syndrome, diabetes, and/or NAFLD in adulthood (Giancotti et al., 2019; Parretti et al., 2020; Stewart et al., 2013). Therefore, if already primed during the fetal development, the liver of the children whose mothers were diagnosed with pregnancy hyperglycemia and GDM more vulnerable to any possible insult during childhood and adulthood.

Liver metabolism

When the liver is metabolically compromised, liver homeostasis is harmed, which can further worsen any existing liver dysfunction. As a high-metabolic tissue, liver coordinates numerous processes, including glucose, lipid, and protein metabolism, xenobiotic detoxification, bioenergetics, urea metabolism, and secretion of some plasma proteins. Also, being one of the largest glands of the body, liver expresses its exocrine (through the production of bile) and endocrine functions (through the secretion of hormones, including angiotensinogen, thrombopoietin, and insulin-like growth factors) (Si-Tayeb et al., 2010) with crucial impact in body metabolism and role in the interplay between various organs, such as skeletal muscle and adipose tissue (Rui, 2014). As nutrients from diet, i.e., glucose, FA, and amino acids are absorbed through the hepatic portal vein, liver has first access to them when compared to the peripheral tissues (Moore et al.,

2012). However, depending on the physiological conditions and bodies' nutritional needs, glucose and triglycerides (TG) can be mobilized from the liver and secreted into the circulation to be metabolized by other tissues, including muscle and adipose tissue (Rui, 2014). Therefore, based on the glycemic state, two metabolic scenarios can be ascribed for liver glucose: 1) liver can release glucose, originated from glycogenolysis or gluconeogenesis, to the circulation, or 2) after entering the hepatocytes, glucose can be used for glycogen synthesis, whereas the excess of glucose can be converted to FA (Adeva-Andany et al., 2016; Rui, 2014). The FA influx from diet, *de novo* lipogenesis in liver, or lipolysis in adipose tissue can contribute to the hepatic lipid input. Thus, to prevent eventual lipid excess, FFA undergo β -oxidation in liver mitochondria, or esterification into TG in cytoplasm, which are either stored or packed and secreted into the circulation as very-low-density lipoprotein (VLDL) (Nassir et al., 2015). Eventually, FFA availability in the liver can be also regulated by lipophagy, a selective form of autophagy with an important function in the regulation of lipid metabolism in the liver (Martinez-Lopez & Singh, 2015). Dynamic regulation of hepatic glucose and lipid metabolic pathways and tight interaction of liver with other tissues has a crucial part in meeting the systemic metabolic demands. Therefore, a well and precise coordination of hepatic metabolism is needed, and any eventual dysregulation of liver metabolism signaling and subsequent liver failure, contribute to the development of chronic liver disorders, including NAFLD (Jiang et al., 2020).

Mitochondrial dysfunction in metabolic diseases

In metabolically disturbed conditions, liver mitochondria are one of the first sensors of hepatic cytotoxicity. As liver is very rich in mitochondria (~18% of mitochondria *per* hepatocyte volume), mitochondria have a vital role in its metabolism (Wei et al., 2008). In fact, besides their role in Ca^{2+} and redox homeostasis, pH regulation, and cellular remodeling and adaptation, mitochondria are the primary site of β -oxidation and oxidative phosphorylation (OXPHOS). As cellular powerhouses, mitochondria produce adenosine-triphosphate (ATP) through OXPHOS using substrates obtained from glucose,

lipids as well as proteins. Therefore, they can easily sense any alteration in glucose and lipid metabolism and their failure to manage subcellular metabolic processes may result in the impaired regulation of hepatic metabolism (Wei et al., 2008).

Mitochondrial respiration involves metabolic reactions and processes that accomplish the oxidation of nutrients-derived substrates, converting the macronutrient energy to ATP, the universal cellular energy donor (Hoeks et al., 2012). Under physiological conditions, more than 80% of the demanded ATP is produced through OXPHOS, whereas the remaining is mainly provided through glycolysis (Papa et al., 2012). According to the Mitchell's chemiosmotic theory from 1961, the transfer of electrons derived from substrate oxidation is coupled with ATP synthesis in the mitochondria (Mitchell, 1961). The electron transport chain (ETC), consisting of four enzyme complexes (CI, CII, CIII, and CIV) and two mobile carriers (coenzyme Q and cytochrome c), receives electrons derived from oxidizable substrates at CI and CII that are then passed throughout the remaining ETC and transferred to molecular oxygen (O_2). Matched with this electron carrier transport, an electrochemical proton gradient is generated and subsequently used by ATP synthase to phosphorylate ADP to ATP (Benard et al., 2006; Mitchell, 1961; Zhao et al., 2019). These mitochondrial respiration-related processes need to be precisely orchestrated and controlled, with continuous import of necessary substrates and ADP and export of ATP, as an impairment in mitochondrial respiration can result in metabolic dysfunction (Papa et al., 2012). Nevertheless, the OXPHOS is not 100% efficient, even in physiological conditions, due to the proton and electron leak. Hence, neither all the electrons are transported to their final electron acceptor O_2 , resulting in reactive oxygen species (ROS) production, nor the proton motive force is completely coupled with ATP synthesis. Yet, proton leak regulation by uncoupling proteins (UCPs) and ROS generation have an important role in physiology of the cell (Zhao et al., 2019). In chronic liver diseases, such as non-alcoholic steatohepatitis, an increase in FFA availability over stimulates β -oxidation and the generation of nicotinamide adenine dinucleotide (NADH) leading to the increased electron flux through the ETC (Gonçalves et al., 2013). Ultimately, this

stressful condition is associated to enhanced electron ETC leakage and correspondent increase in ROS generation (Koliaki et al., 2015; Patterson et al., 2016). Therefore, under such prolonged state of excessive hepatic lipid accumulation, mitochondrial bioenergetics will become compromised resulting in reduced rates of mitochondrial respiration and ATP synthesis. Concomitantly, lower hepatic ATP content (Szendroedi et al., 2009) and ATP synthase flux (Schmid et al., 2011) were related to IR and increased lipid accumulation in type 2 diabetes patients, and diminished mitochondrial respiration was observed in NAFLD patients (Koliaki et al., 2015). Moreover, maternal HFD during gestation may reduce the expression or activity of ETC complexes even in the offspring regardless of them post-weaning diet (Borengasser et al., 2011; Bruce et al., 2009).

Fine coordination between generation of new mitochondria and removal of the old and damage ones is important for the maintenance of mitochondrial homeostasis and pool of healthy mitochondrial network. Mitochondrial biogenesis is closely coupled with mechanisms of mitochondrial dynamics, fission and fusion, as well as mitophagy (Popov, 2020). In this fine-tuning process, the main regulator of biogenesis - peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 α) attracts a lot of attention in the research of mitochondriopathies. In fact, as a co-transcriptional regulation factor, PGC-1 α activates transcription factors and thus promotes the expression of mitochondrial transcription factor A (TFAM), involved in transcription and replication of mitochondrial DNA (mtDNA) (Jornayvaz & Shulman, 2010). Reduced PGC-1 α expression or activity are associated with increased lipid accumulation in the liver (Aharoni-Simon et al., 2011; Morris et al., 2013; Morris et al., 2012), IR, and diabetes (Patti et al., 2003). In the condition of maternal diabetes, abnormal placental function can occur leading to diminished mitochondrial biogenesis in the placenta and reduction in PGC-1 α and TFAM expression, and mtDNA copy number in a sex-specific manner (S. Jiang et al., 2017). Similarly, maternal HFD intake during pregnancy reduced the activity of PGC-1 α in the liver of male but not female offspring (Borengasser et al., 2011), suggesting sex-specific adaptations during *in utero* development under adverse conditions.

As referred above, the mitochondrial dynamics is closely related to the processes of mitochondrial biogenesis, allowing mitochondrial genetic and functional complementation (Seo et al., 2010). Nutrient availability and supply, as well as stress levels can influence the dynamics of the mitochondrial network – fusion or fission, toward a condition that is more prone to maintain mitochondrial homeostasis and bioenergetics. Under the lack of nutrients, mitochondria undergo fusion (regulated by mitofusin 1 (MFN1) and mitofusin 2 (MFN2) in the outer mitochondrial membrane, and optic atrophy 1 (OPA1) in the inner mitochondrial membrane) and elongate to enhance its bioenergetics efficiency (Yu & Pekkurnaz, 2018). In contrast, in a nutrient-rich environment, mitochondrial network tends to fragment to avoid energy losses, which also decreases bioenergetic efficiency, in a process mediated by dynamin-related protein 1 (DRP1) and mitochondrial fission 1 (Fis1) (Schrepfer & Scorrano, 2016). Additionally, mitochondrial damage or presence of high cellular stress stimulate fission to separate the healthy mitochondrial network from the damaged sections that will further undergo degradation through mitophagy (Nasrallah & Horvath, 2014). Aberrant mitochondrial dynamics has been observed in pathological conditions, such as obesity, IR, diabetes, and NAFLD (Bach et al., 2003; Bonnard et al., 2008; Longo et al., 2021; Mansouri et al., 2018; Patti et al., 2003). Also, it is suggested that mtDNA quality can control mitochondrial dynamics. During fusion, the contents between mitochondria exchanges, yet the redistribution of ETC might enhance ROS production and mtDNA content. The consequent increase in mtDNA would then provide sufficient copies of mtDNA for continual cycles of fusion and fission (Sadakierska-Chudy et al., 2017). In contrast, it is suggested that hyperglycemia and hyperlipidemia favor mitochondrial fragmentation, thus altering mitochondrial bioenergetics (Bonnard et al., 2008), which actually matches with mtDNA copy numbers depletion in such conditions (Kamfar et al., 2016; Litvinova et al., 2019; Pinti et al., 2019). Additionally, mtDNA, as a circular double-stranded molecule in the mitochondrial matrix is very sensitive to oxidative damage due to the lack of histones and DNA repair mechanisms, which means that mitochondrial integrity and function can easily be

jeopardized under several stressful stimuli or pathological conditions (Wei et al., 2008).

MicroRNAs in liver health and disease

The evidence that *in utero* stress may modulate fetal epigenome are emerging. Alterations in maternal metabolism homeostasis may provoke differential fetal adaptation responses, such as increased energy store capacity or alterations in metabolic pathways, ultimately leading to phenotypic changes and metabolic disorders in adult offspring (Goyal et al., 2019). The GDM may be one of the insults resulting in alterations of fetal epigenome and developmental origin of offspring metabolic diseases. In fact, upon exposure to altered maternal metabolism, i.e., hyperglycemia, hyperlipidemia and related alterations in placental transport of glucose, lipids, and amino acids, fetus gets conditioned by adaptive processes at cellular, tissue or organ levels that might involve epigenetic mechanisms. These prolonged epigenetic processes, additionally pressured by adult offspring lifestyle, may contribute to the onset of metabolic disorders even in young adulthood (Agarwal et al., 2018).

Considered as the link between the internal (genetic) and external (environmental) factors underlying the expression of the phenotype, the epigenetic events behind the epigenetic landscape involve DNA methylation, histone modifications, and micro-RNA (miRNAs) regulation (Wei et al., 2008). MiRNAs are non-coding RNAs involved in post-transcriptional gene expression regulation. In fact, controlling gene regulation through a dynamic process that maintains a steady state of gene expression (Fu et al., 2013; Iliopoulos et al., 2010; O'Brien et al., 2018; Tüfekci et al., 2014), miRNAs play an important role in fetal development and various biological processes, such as cellular proliferation, metabolism or cell death. Subcellular localization, cell type, cell state, abundance of target mRNA, and the affinity of miRNA-mRNA regulation are some of the factors modulating miRNAs activity and efficiency in gene regulation (O'Brien et al., 2018). Thus, aberrant miRNA expression has been associated to human diseases, including pregnancy complications (Fu et al., 2013).

Regarding hepatocytes, one of the most abundant miRNAs is miR-122-5p that is involved in lipid metabolism (Ceccarelli et al., 2013). In this particular case, the expression of a variety of genes involved in lipid metabolism is downregulated by this miRNA, including fatty acid synthesis, peroxisome proliferator-activated receptor α , and sterol regulatory element binding 1 (SREBP1), which regulates hepatic TG synthesis (Kalaki-Jouybari et al., 2020). It is also involved in mitochondrial metabolic gene regulation, being the overexpression of miR-122 in human hepatoma cell associated with the increase of PGC-1 α expression (Burchard et al., 2010). In contrast, reduced levels of miR-122 in the liver were observed in NAFLD patients (Miyaaki et al., 2014), diet-induced diabetic rats (Kalaki-Jouybari et al., 2020) and in the liver of male, but not female, fetuses of GDM mothers, suggesting sex-specific epigenetic *in utero* regulation (Fornes et al., 2020; Fornes et al., 2018). Moreover, the inhibition of mir-122 has been associated with increased hepatic lipid accumulation, reinforcing its protective role against NAFLD (Hu et al., 2019). In fact, being also an important regulator of cell cycle, miR-122 in cooperation with anti-apoptotic miR-34a balances cell survival and proliferation in normal liver (McDaniel et al., 2014). Aberrant expression of these two miRNAs (miR-34a upregulation and miR-122 downregulation) is present in NAFLD and hyperglycemic conditions, suggesting their important role in hepatic energy metabolism (Cheung et al., 2008; Li et al., 2009). Furthermore, miR-34a overexpression seems to induce SREBP1 overactivation through Sirtuin 1 (SIRT1) inhibition, shifting the lipid metabolism towards more lipogenic (Wu et al., 2020). Aberrantly elevated mir-34a in HFD-fed mice with fatty livers can attenuate fibroblast growth factor (FGF) 19 signaling (Fu et al., 2012), and thus hepatic glycogen synthesis (Kir et al., 2011). In a similar study, miR-34a overexpression downregulated SIRT1, subsequently decreasing transcriptional activity of PGC-1 α through SIRT1-induced PGC-1 α acetylation, but also diminished FGF21 signaling (Fu et al., 2014), which is important in repressing the transcription of SREBP1 (Zhang et al., 2011). This clearly suggests a role of miR-34a in promoting, although indirectly, hepatic lipid accumulation. Also, PGC-1 α and SREBP1 expression can be dependent on miR-130b. It has been reported that its overexpression is related with a slight

upregulation of SREBP1, as well as with downregulation of PGC-1 α and TFAM and consequent impaired mitochondrial biogenesis (Shaoning Jiang et al., 2017; Zhang et al., 2020). On the contrary, miR-494 downregulation has been associated with an increase of mitochondrial content and TFAM expression (Yamamoto et al., 2012). However, considering the complexity of miRNAs' mechanisms of action, its abundance and variety, the specific role of each miRNA in complex diseases, especially in those with increased intergenerational transmission risk, is yet to be elucidated.

Preventive and therapeutic potential of physical exercise against chronic liver diseases

The inability of maternal organism to overcome the condition of IR that emerges during pregnancy increases the risk of both mothers, fetus, and neonate to develop GDM-related complications. Hence, the proper management and treatment of GDM is an extremely important clinical and health-related concern. As previously referred above, maternal GDM is associated with the risk of pediatric fatty liver disease. However, as no medications for the NAFLD treatment of adults and children are yet approved, pharmacological therapies targeting some of the associated comorbidities (obesity, IR, diabetes) are recommended. Some of these have been studied in children, such as vitamin E, which may be used in patients with non-alcoholic steatohepatitis as an antioxidant, although its long-term safety is not known, whereas probiotics, omega-3 fatty acids, metformin, and ursodeoxycholic acid are still not advised as more research is needed. Experimental therapies in children, including leptin analogs, anti-lipopolysaccharide antibodies, or some antioxidants are so far in early phases of clinical trials. Among some of the non-pharmacological therapies proposed to children, physical exercise and diet are used in a clinical practice, while bariatric surgery is only considered for adolescents with severe obesity or related severe comorbidities. Therefore, lifestyle interventions among children against NAFLD are highly recommended (Draijer et al., 2019).

In fact, nowadays, physical exercise has been widely recognized as the most important and potential non-pharmacological therapeutic strategy to provide

systemic (Bhat et al., 2012; Fiuza-Luces et al., 2018) and mitochondrial benefits against chronic liver diseases (Fletcher et al., 2014; Gonçalves, Maciel, et al., 2014; Gonçalves, Passos, et al., 2014; Linden et al., 2015; Santos-Alves et al., 2014; Santos-Alves et al., 2015). Moreover, studies have shown that physical exercise is an effective therapy for IR and type 2 diabetes (Marson et al., 2016; Motahari-Tabari et al., 2014; Sampath Kumar et al., 2019), as well as for ectopic lipid accumulation and NAFLD (Lee et al., 2019; Zhang et al., 2016) in adults or adolescents. Enhanced hepatic mitochondrial respiration followed by increase in PGC-1 α expression and improved mitochondrial metabolism are only some of the physical exercise-related mitochondrial adaptations (Fletcher et al., 2014). Similarly, physical exercise promoted mitochondrial adaptive remodelling through improved mitochondrial biogenesis, dynamics, and autophagy in the liver (Santos-Alves et al., 2015).

In addition, there is also increasing evidence that early-life physical exercise has an important role in the modulation of children metabolic phenotype and later-life metabolic health (Beleza et al., 2021). In line, early-life physical activity has been associated with a lower prevalence of chronic endocrine and cardiometabolic diseases later in life (Fernandes & Zanesco, 2010).

Interestingly, recent works also have shown that early-life exercise practice can rescue increased adiposity and mitigate IR susceptibility in the offspring of obese mothers. However, the extent of metabolic profile improvements was dependent on the offspring post-weaning diet (Bahari et al., 2013; Caruso et al., 2013; Rajia et al., 2013).

Considering that preventive strategies are always more advisable and beneficial than therapeutic approaches from a clinical, social, and economic point of view, the scientific interest and enthusiasm to uncover and develop preventive tools that are efficient even in the earlier development stage, such as during *in utero* life is emerging. In fact, as maternal GDM-related metabolic burden can affect the *in utero* fetus development, several preventive strategies are proposed to protect both mother and fetus being lifestyle changes, such as in diet and physical exercise habits primary goals (Garrison, 2015). Still, an analysis of 19 trials was not able to associate different dietary advices for women with GDM and related

perinatal outcomes (Han et al., 2017). Oral medications, such as those commonly used in the treatment of type 2 diabetes mellitus – metformin, glyburide, and insulin therapy are also prescribed to prevent GDM-characteristic hyperglycemia (Garrison, 2015). Nevertheless, precaution is needed as during pregnancy regular changes in, among others, maternal cardiovascular, respiratory, gastrointestinal, and renal homeostasis might occur together with alterations in pharmacodynamics and pharmacokinetics. Pregnancy-related changes in maternal physiology and metabolism may also affect drug distribution, bioavailability, and clearance in unpredictable manners, although conclusive research data is still lacking (Ansari et al., 2016; Feghali et al., 2015). Furthermore, the placenta is as semipermeable drug barrier as the blood-brain barrier and capable of dealing with drug metabolism. Thus, drugs transferred through or metabolized by placenta might also affect drug pharmacokinetics itself and raises concerns about fetal safety (Ansari et al., 2016).

Considering all the above mentioned, lifestyle interventions, particularly the promotion of physical exercise, emerge as the most efficient, safest, and beneficial approach in this context (American College of Obstetricians and Gynecologists, 2020; American College of Sports Medicine, 2020), although the mechanisms related to the modulation of several metabolic processes are very complex and some of them are yet to be uncovered by science. Nevertheless, the interplay between epigenetic, endocrine, and metabolic-related adaptations associated to physical exercise, highlights the role of maternal gestational exercise (GE) as a promising non-pharmacological preventive and therapeutic approach to mitigate potential deleterious conditions associated to pregnancy both for mothers and offspring.

CHAPTER I

Aims

The metabolic impact of maternal diet and GE and potentially related development of gestational diabetes mellitus on the offspring health, particularly on the hepatic mitochondrial modulation, due to an adverse *in utero* environment, raised a lot of attention in the recent research. Considering the putative physical exercise protective mechanisms against metabolic disorders, including insulin resistance, diabetes, and fatty liver diseases, the main purposes of this doctoral dissertation were to analyze: (1) the role of GE against maternal diet-induced *in utero* “unhealthy” environment and consequent liver mitochondrial deleterious alterations in 6-week-old offspring, and (2) alterations in maternal metabolism 8-weeks post-partum and GE cessation, particularly focusing on liver mitochondrial function and structure and the epigenetic alterations involved in the intergenerational transmission of maternal metabolic burden.

Therefore, the general hypothesis of this dissertation is that physical exercise performed during pregnancy is an important preventive strategy to reduce the risk of developing liver disorder later in life, as well as to prevent transmitting the impaired hepatic metabolic burden to offspring.

Through three original experimental articles, that constitute an integral part of this dissertation, several objectives were designed in order to answer to the general hypothesis and contribute to the main purpose.

Original article I

The general aim was to analyze the effects of GE against deleterious impact of maternal diet and gestational diabetes mellitus on liver mitochondrial function and hepatic fat accumulation in the mothers and 6-week-old male offspring.

The specific objectives concern the effects of GE on:

- (i) gestational diabetes characteristics in the mothers;
- (ii) liver structural changes and fat accumulation in mothers 8-week post-partum and in the male offspring;
- (iii) systemic and hepatic acylcarnitine profile in both generations;
- (iv) liver mitochondrial function in both generations;

- (v) liver mitochondrial expression of OXPHOS subunits and UCP2 in both generations.

Original article II

The general aim was to analyze the effects of GE against deleterious impact of maternal diet and gestational diabetes mellitus on liver mitochondrial quality control signaling in the mothers and 6-week-old male offspring.

The specific objectives concern the effects of GE on:

- (i) liver and mitochondrial structural alterations in both generations;
- (ii) liver expression of mitochondrial biogenesis markers (PGC-1 α , TFAM, TOM20, and mtDNA copy number) in both generations;
- (iii) liver expression of estrogen receptor alpha in both generations;
- (iv) liver expression of mitochondrial dynamics markers (MFN1, MFN2, OPA1, and DRP1) in both generations;
- (v) liver expression of auto(mito)phagy signaling markers (Beclin-1, Bcl2, LC3-II, Parkin, and PINK1) in both generations;
- (vi) liver expression of apoptosis signaling markers (Bax, Bcl2, cytochrome c) in both generations;
- (vii) liver expression of microRNAs modulating lipid metabolism (miR-122, miR-34a, miR-370, miR-130b, and miR-494) in both generations.

Original article III

The general aim was to analyze the effects of GE against deleterious impact of maternal diet and gestational diabetes mellitus on liver hepatic fat accumulation and mitochondrial quality control signaling in the 6-week-old female offspring.

The specific objectives concern the effects of GE on:

- (i) liver structural changes and fat accumulation;
- (ii) systemic and hepatic acylcarnitine profile;
- (iii) liver and mitochondrial structural alterations;
- (iv) liver expression of mitochondrial biogenesis markers (PGC-1 α , TFAM, TOM20, and mtDNA copy number);

- (v) liver expression of estrogen receptor alpha in both generations;
- (vi) liver expression of mitochondrial dynamics markers (MFN1, MFN2, OPA1, and DRP1);
- (vii) liver expression of auto(mito)phagy signaling markers (Beclin-1, Bcl2, LC3-II, Parkin, and PINK1);
- (viii) liver expression of apoptosis signaling markers (Bax, Bcl2, cytochrome c);
- (ix) liver expression of microRNAs modulating lipid metabolism (miR-122, miR-34a, miR-370, miR-130b, and miR-494) in both generations.

CHAPTER II

Theoretical Background

Fit mothers for a healthy future: Breaking the intergenerational cycle of NAFLD with maternal exercise

Jelena Stevanović-Silva¹, Jorge Beleza², Pedro Coxito¹, Rui Carlos Costa³, António Ascensão¹, José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

³ Department of Communication and Art, Research Institute for Design, Media and Culture (ID+), Aveiro University, Aveiro, Portugal.

Published on: *European Journal of Clinical Investigation*,
2021 Jun; e13596.

doi: 10.1111/eci.13596

Received: 30 March 2021 | Revised: 1 May 2021 | Accepted: 6 May 2021
DOI: 10.1111/eci.13596

ORIGINAL ARTICLE

WILEY

Fit mothers for a healthy future

Breaking the intergenerational cycle of non-alcoholic fatty liver disease with maternal exercise

Jelena Stevanović-Silva¹  | Jorge Beleza²  | Pedro Coxito¹  | Rui Carlos Costa³  |
António Ascensão¹  | José Magalhães¹ 

¹Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal

²Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

³Department of Communication and Art, Research Institute for Design, Media and Culture (ID+), Aveiro University, Aveiro, Portugal

Correspondence

Jelena Stevanović-Silva, Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, R. Dr. Plácido da Costa 91, 4200-450 Porto, Portugal.
Email: jela.stevanov@gmail.com

Funding information

Fundação para a Ciência e a Tecnologia, Grant/Award Number: POCI-01-0145-FEDER-016657-PTDC/DTPDES/1082/2014, POCI-01-0145-FEDER-016690-PTDC/DTPDES/7087/2014, SFRH/BD/129645/2017 and UIDB/00617/2020-base; EU's Horizon 2020 Research and Innovation programme under the Marie Skłodowska-Curie Actions, Grant/Award Number: 722619 and 734719

Abstract

Special issue: 'FOIEGRAS—Bioenergetic Remodelling in the Pathophysiology and Treatment of Non-Alcoholic Fatty Liver Disease'.

Background: Non-alcoholic fatty liver disease (NAFLD) emerges as significant health burden worldwide. Lifestyle changes, unhealthy dietary habits and physical inactivity, can trigger NAFLD development. Persisting on these habits during pregnancy affects in utero environment and prompts a specific metabolic response in foetus resulting in offspring metabolic maladjustments potentially critical for developing NAFLD later in life. The increasing prevalence of NAFLD, particularly in children, has shifted the research focus towards preventive and therapeutic strategies. Yet, designing effective approaches that can break the NAFLD intergenerational cycle becomes even more complicated. Regular physical exercise (PE) is a powerful non-pharmacological strategy known to counteract deleterious metabolic outcomes. In this narrative review, we aimed to briefly describe NAFLD pathogenesis focusing on maternal nutritional challenge and foetal programming, and to provide potential mechanisms behind the putative intergenerational effect of PE against metabolic diseases, including liver diseases.

Methods: Following detailed electronic database search, recent existing evidence about NAFLD development, intergenerational programming and gestational exercise effects was critically analysed and discussed.

Results: PE during pregnancy could have a great potential to counteract intergenerational transmission of metabolic burden. The interplay between different PE roles—metabolic, endocrine and epigenetic—could offer a more stable in utero environment to the foetus, thus rescuing offspring vulnerability to metabolic disturbances.

Conclusions: The better understanding of maternal PE beneficial consequences on offspring metabolism could reinforce the importance of PE during pregnancy as an indispensable strategy in improving offspring health.

KEYWORDS

foetal programming, maternal exercise, NAFLD, offspring health, physical exercise

© 2021 Stichting European Society for Clinical Investigation Journal Foundation. Published by John Wiley & Sons Ltd.

Eur J Clin Invest. 2021;00:e13596.
<https://doi.org/10.1111/eci.13596>

wileyonlinelibrary.com/journal/eci | 1 of 17

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is considered the most common worldwide cause of chronic liver diseases.¹ Described as the hepatic manifestation of metabolic syndrome, it includes a range of conditions characterized by excessive hepatic lipid accumulation. Hepatic steatosis reflects “simple” fat accumulation in the liver; however, if additionally pressured by the activation of inflammatory and fibrotic signalling pathway activation, steatosis can progress to non-alcoholic steatohepatitis, or even cirrhosis and hepatocellular carcinoma. As a complex disease, NAFLD results from the interaction of genetic and environmental factors, particularly unhealthy dietary patterns and physical inactivity.^{2,3}

About 25% of the world's population has NAFLD, with very high prevalence even in children—3%–17%,¹ and up to 70%–80% among obese children.⁴ NAFLD in children may occur as a consequence of obesity and/or intrauterine environment.^{5,6} Environmental factors to which mothers are exposed during pregnancy (diet, stress, smoking, alcohol consumption and/or gestational diabetes mellitus (GDM)) are important determinants of foetal programming.⁶ Given that maternal lifestyle can seriously affect the metabolic status of future generations,^{7–11} it is urgent to ‘design’ efficient preventive tools against NAFLD. Since there are no approved medications for NAFLD treatment, lifestyle changes, mainly the engagement in physical exercise (PE) programmes, are recommended.^{12–14} Being already evidenced as effective in the prevention and treatment of numerous metabolic, neurological, oncological, cardiovascular, musculoskeletal, pulmonary and psychiatric diseases,¹⁴ PE has a great potential to prevent intergenerational transmission of metabolic impairments via metabolic, endocrine and/or epigenetic mechanisms if implemented during critical moments of pregnancy. The promotion of PE during pregnancy should be emphasized to break the intergenerational cycle of NAFLD and provide healthy early-life environment. Thus, in this narrative review, we aim to critically analyse both the pathophysiology of NAFLD and countermeasures to mitigate this disease, as well as how PE acts through distinct pathways and at several levels of systemic and cellular organization in order to counteract NAFLD. Insights into how skeletal muscle (SM) behaves as an endocrine organ, the metabolic action of PE through mitochondrial-related adaptations and PE-induced epigenetic adaptations against NAFLD are provided. Also, recommendations regarding maternal PE and its beneficial consequences on offspring metabolism are discussed.

2 | NAFLD AS A MULTIORGAN DISEASE

Although NAFLD is characterised by excessive hepatic lipid accumulation, these complications are not exclusive to the

liver tissue.^{15,16} NAFLD is closely related to other metabolic disorders, such as insulin resistance (IR), diabetes mellitus, obesity, cardiovascular diseases, and GDM.^{16–18} Extra-hepatic complications also involve chronic kidney disease, colorectal cancer, atherosclerosis, osteoporosis, thyroid dysfunction, obstructive sleep apnoea and polycystic ovarian syndrome.^{16,18} Indeed, cardiovascular diseases are the most common cause of death in NAFLD patients.¹⁸ Thus, NAFLD can be considered a multiorgan disease, manifesting in several organs, with a very complex interplay between different tissues.¹⁵ Yet, NAFLD can occur even in non-obese subjects, where genetic and lifestyle factors play a part in the development of the so-called lean NAFLD.¹⁹ The imbalance between energy intake and expenditure, due to high-caloric intake and low levels of physical activity, may result in ectopic lipid accumulation. In such conditions, lipid storage capacity of the adipose tissue gets saturated, and excess lipids are forwarded to liver, muscle and pancreas. Once these organs also become saturated, excess lipids can trigger the production of toxic reactive lipid species and lipotoxicity.²⁰ A significant role in the pathogenesis of metabolic diseases belongs to IR in SM^{21,22} since SM acts as a sensor of low expenditure of stored energy and signal for events that will ultimately result in IR.²¹ The subsequent hyperinsulinaemia can trigger a sequence of metabolic pathways that favour the development of metabolic disorders, among which hepatic steatosis is of high concern.²¹

2.1 | The role of mitochondria in NAFLD

As a high-metabolic rate tissue with numerous metabolic functions, the liver is highly susceptible to disturbances due to excessive lipid accumulation that may lead to several metabolic impairments. In this context, the role of mitochondria in cellular energy production through oxidative phosphorylation seems to be crucial.²³ Additionally, liver mitochondrial dysfunction has been related to cellular death and inflammation, and thus, it is proposed as one of the first events during steatohepatitis development.¹²

Due to the imbalance between caloric intake and energy expenditure, perturbed regulation of glucose and lipid metabolism results in an excessive hepatic accumulation of fatty acids and triglycerides and consequently in steatosis. To recover from this lipid burden, hepatic β -oxidation rate increases in the beginning, as well as the electron flux through the electron transport chain. However, enhanced reductive supply to the electron transport chain, when coupled with membrane permeabilization and consequent cytochrome c release, through permeability transition pore opening, leads to an electron transport chain over-reductive state. In these circumstances, increased electron leakage promotes inefficiency of mitochondrial ATP synthesis, alongside augmented reactive oxygen

species production and oxidative stress.^{3,12,24} Moreover, inflammatory and fibrogenic responses can aggravate steatosis and 'push' its progression towards steatohepatitis.^{12,25}

The regulation and promotion of β -oxidation in liver mitochondria are dependent on key molecular players, such as peroxisome proliferator-activated receptors (PPARs) and their coactivator PPAR γ -coactivator-1 α (PGC-1 α).²⁶ Yet, PGC-1 α and its downstream target mitochondrial transcription factor A (TFAM) are reported to be decreased in hepatic steatosis.²⁷ The consequent change in mitochondrial homeostasis and efficiency, together with structural alterations in mitochondrial network morphology, can be dangerous to cell viability. Therefore, the dynamic and inter-related adaptive processes involved in mitochondrial plasticity and turnover occur through an interplay of biogenesis, fusion, fission and auto(mito)phagy activation, contributing to the maintenance of mitochondrial network. However, in NAFLD conditions, the function, structure and dynamic processes of liver mitochondria are considerably altered. Disturbance in mitochondrial quality control contributes to hepatic lipid accumulation and subsequent pathogenesis of NAFLD.²³

2.2 | Intergenerational cycle of NAFLD

The attempts to describe pathogenesis of such a complex disorder can be summarized in a 'multiple hit hypothesis' considering that multiple insults (IR, release of adipokines and proinflammatory cytokines, over-nutrition, altered gut flora and (epi)genetic challenges) work together to prompt NAFLD development.² Among these intertwined environmental and genetic factors in NAFLD pathogenesis, in utero environment emerges as the new 'first' hit.²⁸ In fact, children of steatosis-diagnosed parents are at increased risk for NAFLD development even if they do not have other metabolic-related risk factors.²⁸ Likewise, numerous animal studies stressed that maternal obesity or improper dietary habits during pregnancy and/or lactation are important factors that promote metabolic and mitochondrial dysfunction and eventually hepatic steatosis.^{7-11,29} Intrauterine exposure to hyperglycaemia, typical of GDM, may increase the risk of later-life metabolic disorders, being *Pgc-1 α* epigenetic alteration one of the potential mechanisms triggering this metabolic malprogramming.³⁰

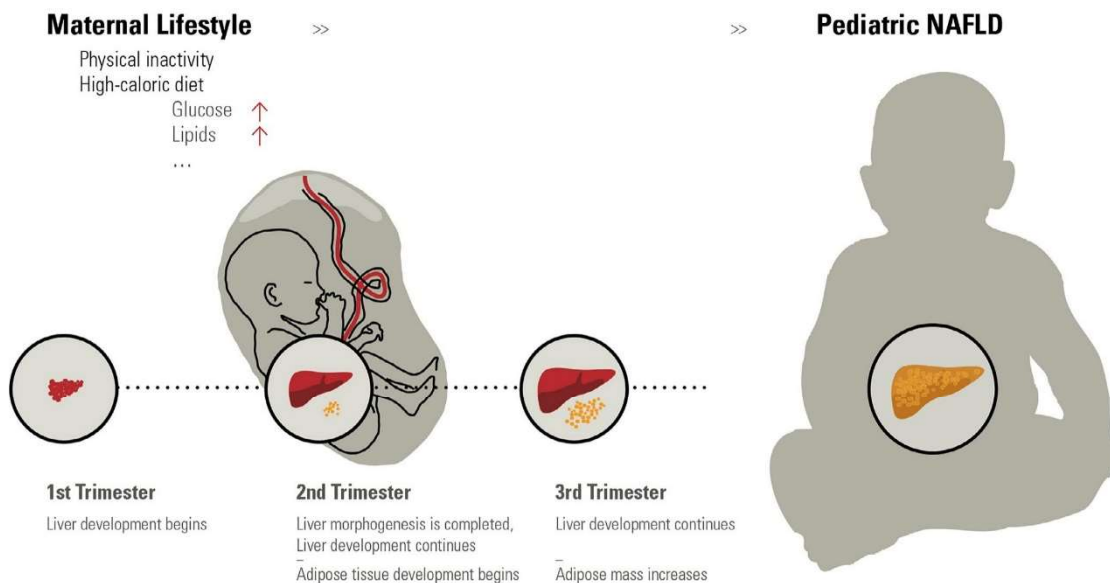


FIGURE 1 Potential mechanisms behind the development of non-alcoholic fatty liver disease (NAFLD) in children. In utero environment has been recognized as a critical factor in foetal development as it can affect a foetus' risk of developing metabolic disorders, such as NAFLD, later in life. In uncomplicated pregnancies, placental glucose and lipid transport are well regulated and matches with the development of foetal liver and adipose tissue. However, inappropriate maternal lifestyle, namely physical inactivity and high-caloric nutrition, results in the maternal nutrient overload that may cause adverse alterations to the in utero environment in early pregnancy. In such circumstances, the foetus is exposed to lipid excess that needs to be accumulated in not yet competent or developed organs. As the adipose tissue begins to develop during mid-pregnancy, a need for other lipid accumulation sites such as liver emerges. Foetal liver, still not completely developed, but already pressured by lipid excess, will become susceptible to metabolic disturbances, not only throughout intrauterine developmental period but also later in life, especially if additionally 'stressed' by an inadequate lifestyle during childhood. The likely end result is a health frailty childhood condition characterized by features of paediatric NAFLD

Accordingly, maternal nutrient overload and lipid excess may result in foetal lipotoxicity. In early gestation, *de novo* lipogenesis in foetal liver is limited and β -oxidation exists only at basal levels, making the liver inefficient when dealing with lipid surplus. Therefore, excessive lipid influx to foetal liver during early-to-mid gestation, before foetal adipose tissue development, results in ectopic lipid accumulation in liver making it susceptible to later-in-life NAFLD development (Figure 1).²⁸ This altered in utero environment that foetus is exposed to, specifically maternal high-fat diet (HFD), may provoke offspring metabolic disturbances, which extent depends on postnatal offspring diet. Actually, offspring from HFD-fed mice were vulnerable to develop steatosis even if fed with standard chow diet (SCD) in their early-life, whereas early-life HFD feeding aggravated their liver metabolism to progressive steatohepatitis.⁸ Similarly, despite 23-week-long post-weaning SCD consumption, re-exposure to HFD in adult mice resulted in steatosis and inflammation, and higher susceptibility to steatohepatitis development.³¹ In rats, even the prolonged SCD consumption by the offspring of HFD-fed mothers was not effective in reducing hepatic steatosis, suggesting that the metabolic intrauterine deleterious consequences might be irreversible at the post-weaning period.⁷ Instead, HFD-fed offspring still developed steatosis, despite maternal SCD consumption during pregnancy and lactation, whereas offspring vulnerability to develop steatohepatitis increases when mothers are fed with HFD during pregnancy.^{7,31} Furthermore, HFD feeding for three consecutive generations resulted in increasingly severe NAFLD manifestations at each generation, which can be explained, at least partly, as a consequence of transgenerational accumulation of epigenetic alterations.³² In contrast, changing maternal diet from HFD to a low-fat diet in the subsequent pregnancy improved triglyceride levels in foetal livers,³³ suggesting that pregnancy can be proposed as a critical window for the implementation of intervention strategies. Taken together, these studies highlight that pregnancy is a critical period for programming offspring predisposition to NAFLD development and progression.

2.3 | Prevention and treatment of NAFLD

Given the social, economic and healthcare burden of NAFLD, a need for effective preventive and/or therapeutic approaches is emerging. However, clinical management against such a complex multiorgan disease is a complicated process. As there are still no approved medications for NAFLD treatment, recommended medications usually target associated metabolic disorders—obesity, IR, diabetes or lipid disorders. Some of the pharmacological treatments include antioxidant vitamin supplementation, PPAR agonists, or anti-diabetic,

anti-obesity, anti-apoptotic, anti-inflammatory and anti-fibrotic agents.³⁴ As NAFLD prevalence is remarkably high in (morbidly) obese individuals,¹⁷ bariatric surgery and surgery-related weight loss are proposed as another strategy against NAFLD. Still, this approach cannot be implemented in patients who are not either obese or have a high risk for surgical complications due to other metabolic disorders.³⁵ Thus, lifestyle interventions (diet and PE) appear as the first-line approach in the management of NAFLD, a disease that is, in fact, mainly triggered by lifestyle modifications. Numerous studies have proven that PE is efficient at reducing hepatic lipids even without dietary changes or weight loss.^{13,36–39} A meta-analysis study also recommended PE as a therapeutic strategy against NAFLD, despite differences in PE type, duration and/or intensity.¹³ Accordingly, data from animal NAFLD models also showed that PE is able to ameliorate NAFLD-related metabolic and mitochondria-related impairments.^{40,41}

With intergenerational transmission of metabolic impairments coming to the fore, efficient therapeutic strategies against developmental programming of NAFLD become a prime concern. Early-life intervention strategies, such as early and prolonged breastfeeding and dietary supplements with anti-inflammatory and antioxidant properties, are suggested as beneficial.^{5,42} Moreover, some animal studies highlight the importance of offspring PE to ameliorate the detrimental metabolic influence of maternal obesity.^{43–45} Yet, these approaches are focused on therapeutic levels and not on breaking the intergenerational inheritance of NAFLD. Therefore, the critical timeframe of in utero development could be the right moment to implement potential preventive programmes in pregnant mothers and avert children's metabolic impairment. Nevertheless, choosing the precise pharmacological therapy is not an easy task as pharmacokinetics and pharmacodynamics may vary even in normal pregnancies due to complex changes in maternal physiology, which can eventually affect foetus safety.⁴⁶ Thus, there is a need to encourage healthy lifestyle during pregnancy for the benefit of future generation health.⁴⁷ Since PE is proposed as the cornerstone in NAFLD prevention, PE during pregnancy may also have benefits in preventing the intergenerational inheritance of NAFLD.

3 | 'EXERCISE IS MEDICINE'

Considering the role of PE as a stimulus to mitochondrial biogenesis and function, PE can be an effective preventive or therapeutic approach to metabolic disorders in which mitochondrial failure is an essential cornerstone, such as NAFLD. Despite PE characteristics, including type, duration, intensity, volume and/or frequency can differently contribute to the eventual beneficial short- or long-term adaptations in

muscle and other tissues, it is unanimously considered a powerful non-pharmacological tool against deleterious effects of modern lifestyles. However, the underlying mechanisms associated with these PE-related beneficial effects in the intergenerational context are still not clearly understood. Thus, in the following paragraphs, we aimed to briefly review putative mechanisms of PE that could counteract deleterious metabolic outcomes.

3.1 | Metabolic action of exercise

Physical inactivity has been associated with the development of numerous chronic metabolic disorders. As discussed before, IR in SM due to physical inactivity can contribute to the development of hepatic steatosis. A decrease in energy storages turnover is one of the primary events associated with physical inactivity, eventually leading to decreased glycogen and lipid turnover in SM.²¹ Actually, whereas SM insulin-sensitive individuals store most of ingested energy as glycogen in both liver and muscle, in SM insulin-resistant individuals, the storage of ingested energy is shifted towards liver over SM. This can initiate hepatic de novo lipogenesis and consequent increase in hepatic triglyceride synthesis and plasma hypertriglyceridaemia.²² Therefore, targeting SM-related IR might prevent the development of NAFLD and associated diseases. Indeed, resistance and aerobic training can improve insulin sensitivity in obese,⁴⁸ diabetes³⁷ or NAFLD^{36,39} patients. Interestingly, PE improves insulin action more strongly in the offspring of diabetic patients than in individuals with no family history of diabetes.^{49,50}

Besides IR regulation, PE modulates multiple processes that may protect liver from NAFLD. Aerobic exercise can mitigate NAFLD through regulation of hepatic lipid metabolism by increasing β -oxidation-related cellular machinery and reducing lipogenesis, as well as through amelioration of redox-, inflammation- and apoptosis-driven status. Moreover, PE also activates hepatic protective pathways through autophagy-related mechanisms.⁵¹ Among PE-induced adaptations, regulation of mitochondrial remodeling and quality control is of great importance in preventing pathological outcomes. PE can stimulate mitochondrial biogenesis, dynamics and auto(mito)phagy to promote the generation of new mitochondria, replacing, at the same time, the old and less-functional network fragments, thus maintaining the pool of healthy mitochondria and improving metabolic function. At molecular level, particular relevance has been attributed to PGC-1 α and its regulatory role in mitochondrial biogenesis in the context of PE.⁵² In fact, data suggest that PE can improve mitochondrial function and bioenergetics which are compromised in various liver diseases, including NAFLD.^{37,39-41,53}

3.2 | Skeletal muscle as an endocrine organ

Besides its metabolic effects, SM also functions as an endocrine organ. Metabolic and mechanical stimuli promoted by PE's contractile activity can express pleiotropic effects on non-contractile tissues. During the exercise and post-exercise recovery periods, muscle cells release molecules with paracrine or endocrine effects, known as myokines.^{54,55} Once released into the bloodstream and recognized by specific receptors on different target organs, myokines activate various signalling pathways and modulate metabolic processes. The first identified myokine—interleukin-6—is stimulated by PE in an intensity- and duration-dependent manner. It promotes glucose uptake by SM, thus contributing to the maintenance of SM glucose homeostasis and lipolysis.⁵⁵ PE-dependent interleukin-6 stimulation might be important in the management of NAFLD, considering that interleukin-6 can protect liver from steatosis and ischaemia/reperfusion injury.⁵⁶ Interleukin-6 and fibronectin type-III domain-containing protein-5, cleaved as irisin, are recognized by specific receptors on liver and other tissues, initiating signalling pathways and ultimately activating mitochondrial master regulator PGC-1 α .^{54,57} PGC-1 α activation further stimulates mitochondrial biogenesis, dynamics and quality control processes, as well as electron transport chain complex activities,^{54,55,57,58} which is an important countermeasure to NAFLD pathology. Considering that SM contributes to insulin-mediated glucose uptake and β -oxidation, myokines are likely to affect the whole-body metabolism in a beneficial manner. The myokine-induced positive modulation of liver mitochondrial remodelling can prepare liver to resist to adverse stimuli that are involved in development and pathogenesis of metabolic diseases, including NAFLD.

3.3 | Epigenetic adaptations to exercise

Among numerous lifestyle factors that modulate human health via epigenetic alterations in a harmful or beneficial fashion, PE represents a beneficial epigenetic influence. It is known that PE-induced adaptive response also involves alterations in epigenetic landscape that includes DNA methylation, histone modifications and microRNA regulation.⁵⁹ Barrès and co-workers showed that acute PE caused hypomethylation of metabolic genes, such as *Pgc-1 α* , *TFAM*, *PPAR β* and consequent increase in gene expression in SM of sedentary people, in a PE intensity-dependent manner.⁶⁰ Moreover, PE prevented liver DNA hypermethylation and epigenome susceptibility to HFD-induced metabolic disturbance in mice.⁶¹ Such PE effect could have clinical implications. In fact, hepatic *Pgc-1 α* methylation seems to be highly correlated with peripheral IR and reduced mitochondrial biogenesis in NAFLD patients.⁶²

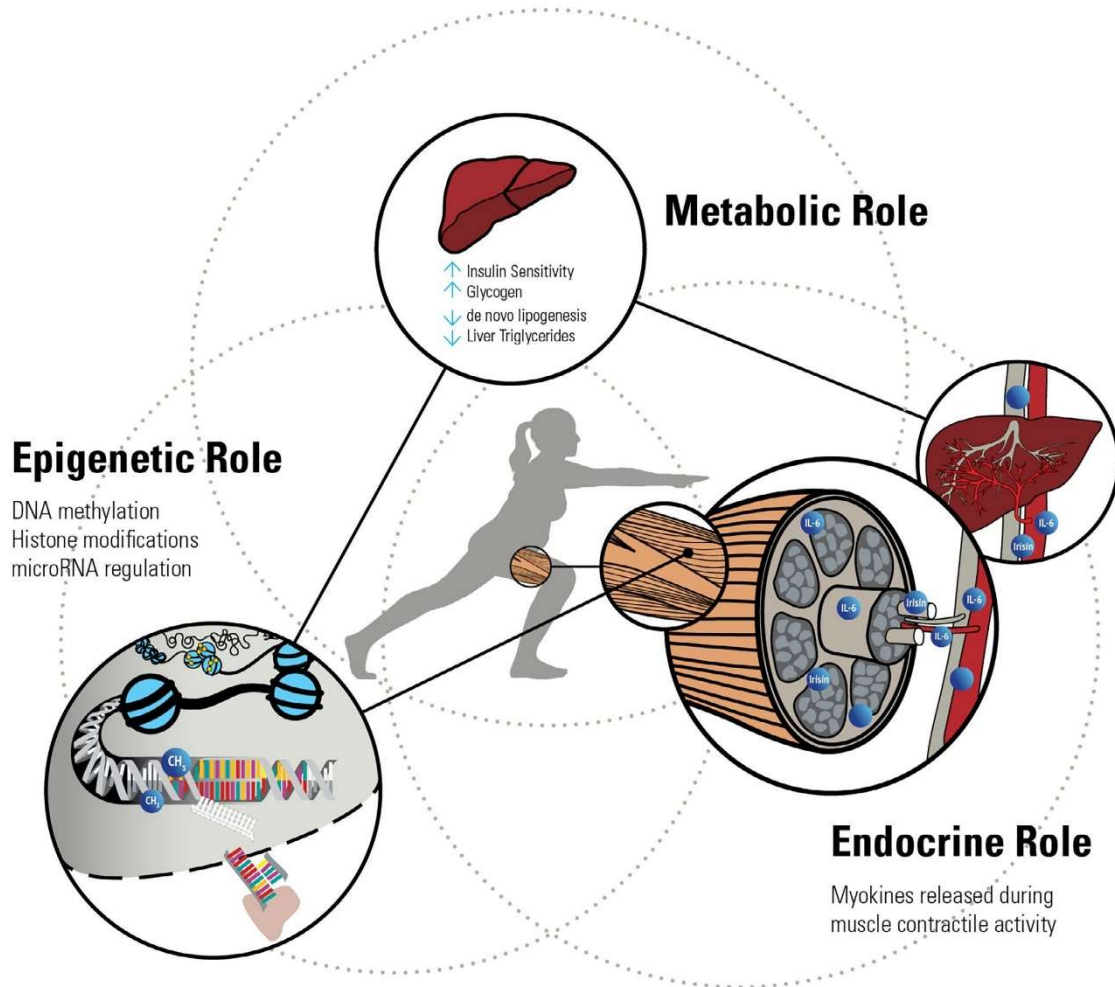


FIGURE 2 Liver-muscle crosstalk. The interaction between skeletal muscle and liver takes place on several intertwined and interconnected levels—metabolic, endocrine and epigenetic. This crosstalk stimulated by physical exercise (PE) has effects on skeletal muscle (SM), but also on non-contractile tissues, such as liver. Generally, exercise promotes energy turnover in SM and improves insulin sensitivity in both SM and liver, and consequently decreases de novo lipogenesis and hepatic lipid accumulation. Moreover, SM contractile activity promotes myokines secretion to the bloodstream. After reaching the liver, myokines can activate signalling pathways and thus regulate metabolic processes. Metabolic processes in the liver can also be modulated by epigenetic-related mechanisms—DNA methylation, post-transcriptional regulation by microRNAs and post-translational modification of histone proteins. These precisely orchestrated metabolic, endocrine and epigenetic mechanisms induced by PE may impact SM and liver adaptive response, thus improving liver ‘fitness’ and promoting beneficial health-related adaptations

Similarly, microRNA-related signalling networks can be regulated by PE, also supporting its therapeutic potential against the development of distinct metabolic diseases at epigenetic level.⁶³ The expression of circulating microRNAs involved in various functions (inflammation, muscle contractility, mitochondrial metabolism, hypoxia/ischaemia adaptations or lipid metabolism), miR-21, miR-146a, miR-210, can be modulated by PE.⁶⁴ Accordingly, high-intensity PE induced increased expression of hepatic miR-122 in diabetic

rats, which correlates with a rescuing-like phenotype of some NAFLD features.⁶⁵ This PE-induced miR-122 upregulation is important in the management of metabolic disorders as miR-122 regulates mitochondrial metabolism and its loss can cause liver dysfunction.⁶⁶ Interestingly, circulatory miR-122 levels are found to be reduced in GDM rats and their foetuses.⁶⁷ Acute endurance exercise decreased miR-23, a negative regulator of PGC-1 α , thus promoting an increased expression of PGC-1 α mRNA and its targets.⁶³ Also,

while low levels of PE resulted in increased SM levels of miR-696 in mice, which further reduced its target PGC-1 α , β -oxidation and mitochondrial DNA content, endurance training downregulated miR-696 levels, thus rescuing inhibited mitochondrial biogenesis.⁶⁸ However, voluntary PE reduced miR-696 levels in mice SM, which seemed not to be correlated with PGC-1 α .⁶⁹ This may suggest that PE type and intensity can differently modulate microRNA roles in mitochondrial biogenesis.

As a master regulator of mitochondrial biogenesis and activity of transcription factors involved in β -oxidation, gluconeogenesis and lipogenesis,⁷⁰ PGC-1 α is an attractive gene candidate for therapeutic interventions. Hence, it drags a lot of attention in PE studies, particularly when it comes to epigenetic regulation. Stimulation of PGC-1 α and subsequently mitochondrial biogenesis through epigenetic modulation could explain the positive modulation of PE in mitochondria-related diseases, such as NAFLD. Still, most studies are focused on PE-related epigenetic adaptations in SM rather than in liver. Further research on liver alterations and muscle-liver axis could provide a thorough explanation on PE modulation of liver diseases via epigenetic mechanisms (Figure 2).

4 | PRENATAL EXERCISE

Although PE is consensually considered as a preventive and therapeutic strategy that mitigates lifestyle-associated metabolic pathologies, questions still arise on whether and how PE can prevent the transmission of maternal metabolic impairments to offspring and break the intergenerational cycle of NAFLD. Maternal PE during pregnancy is a promising, yet challenging approach in managing a possible transmission of maternal metabolic burden to the next generation (Figure 3).

4.1 | Safety and recommendations

During pregnancy, a woman's body undergoes considerable anatomical and physiological alterations, which may result in lifestyle changes and eventual shift towards more sedentary behaviours. However, since the earliest recorded recommendations from the 18th century until nowadays, an active lifestyle during pregnancy has been considered favourable for mothers' and babies' health. Earlier, prenatal PE was only associated with easier labour and reduced foetal size; however,

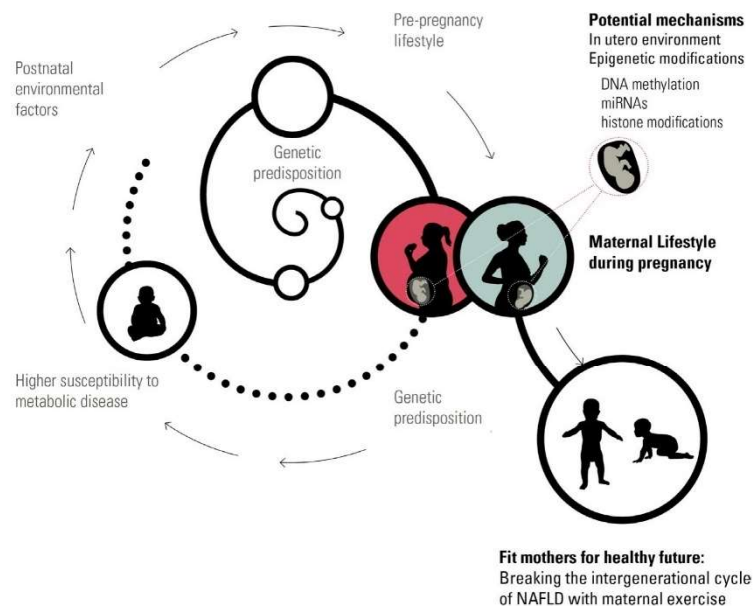


FIGURE 3 Breaking the intergenerational cycle of NAFLD with maternal exercise. Early foetal development is a critical timeframe that may decide the metabolic destiny of the new-growing human being. Genetic predisposition, as well as pre-pregnancy lifestyle, such as physical (in)activity and (im)proper dietary habits, remarkably affects in utero environment in a metabolic and/or epigenetic manner, influencing foetal development. A foetus developing in an adverse in utero environment has an increased susceptibility to NAFLD later in life, especially when further pressured by genetic predisposition and postnatal environmental factors, resulting in more severe NAFLD manifestations in the following generations. However, an active lifestyle during pregnancy provides health-beneficial outcomes in the offspring generation, potentially contributing to breaking the disease-related cycle. It is therefore critical to highlight that maternal exercise during pregnancy represents a promising approach in breaking the intergenerational cycle of NAFLD and preventing transmission of maternal metabolic burden to children

the awareness of the health-related benefits of prenatal PE changed throughout time, being nowadays extended to several dimensions of mothers' and offspring's wellness. Still, the concept of individualized moderation, regarding PE's intensity and volume during pregnancy, remains important in this particular life period.⁷¹

Regular PE in pregnancy is recommended to promote health benefits and associated with minimal risks. Some of the benefits of PE during pregnancy include lower risk for GDM, preterm birth, excessive gestational weight gain, caesarean delivery and macrosomia.⁷² However, considering pregnancy-related physiological and anatomical changes and/or medical complications, some modifications and/or individualized PE programmes are recommended.^{72,73} Recent guidelines consider some activities safe to be performed during pregnancy, such as walking, swimming, stationary cycling, dancing, low-impact aerobic, resistance and stretching exercises,^{72,73} even running/jogging and racquet sports if regularly practiced prior to pregnancy,⁷³ whereas contact sports and activities with high risk of falling should be avoided.⁷² The American College of Obstetricians and Gynecologists and American College of Sports Medicine agree on moderate-intensity PE during pregnancy, recommending if not every day, then at least 20-30 min/d on most days of the week,⁷² or at least 3 d/wk with accumulated 150 min/wk of PE,⁷³ respectively. PE is considered safe and beneficial for health in uncomplicated pregnancies, and it would be expected that prenatal PE might also mitigate maternal, foetal and neonatal metabolic impairments that could trigger the development of NAFLD in both generations.

4.2 | Maternal exercise and offspring metabolism

Recent research regarding prenatal PE and offspring health provides sufficient evidence to support maternal engagement in PE programmes during pregnancy. A recent meta-analysis corroborated the beneficial effects of maternal PE on newborns, as prenatal PE was not associated with any neonatal complications and harmful outcomes, and the risk for macrosomia was reduced.⁷⁴ Moreover, infants of mothers that exercised during pregnancy had improved neuro-motor development, suggesting that they may be more physically active, thus with potentially lower risk to develop obesity later in life.⁷⁵ In fact, moderate prenatal PE can reduce infant adiposity at 6 months of age⁷⁶ and the offspring risk to develop overweight or obesity in childhood and pre-adolescence.⁷⁷

Considering human natural life-span, the study of inter- and transgenerational effects of maternal lifestyle on the next generations is rather complicated. Therefore, rodent models can provide valuable mechanistic information in an appropriate time period. Early animal studies of prenatal PE from

three decades ago demonstrated that offspring of exercised (diabetic) mothers have enhanced glucose tolerance compared with offspring of sedentary mothers.⁷⁸⁻⁸¹ These findings were corroborated by recent studies that include offspring of various ages, from foetuses and neonates to 72 weeks old, and maternal PE at different time points: during pregnancy, before and during pregnancy, or during pregnancy and lactation⁸²⁻⁹³ (for details see Table 1).

Maternal PE can be beneficial against metabolic disturbances when offspring are challenged by HFD. Adult offspring of SCD-fed mothers that exercised before and during pregnancy were protected from offspring HFD-induced obesity.⁹⁴ In a similar study, maternal PE during pregnancy prevented offspring HFD-induced hepatic steatosis, as observed through reduction in hepatic TG accumulation, and increased hepatic PGC-1 α and TFAM expression. However, this effect was noticed in male but not in female offspring.⁸⁹ Actually, maternal prenatal PE seems to modulate offspring metabolism in a gender-specific way. Adult male but not female offspring from exercised mothers had lower fat mass percentage and higher lean mass percentage than offspring of sedentary mothers.⁹⁵ Some of these gender-specific effects may be explained by synergistic effect of maternal PE and HFD feeding. Apparently, prenatal PE showed greater effects in male than in female offspring of HFD-fed obese mothers. Male weaning offspring had lower metabolic risk and improved glucose metabolism. While PE performed during pregnancy by lean mothers improved PGC-1 α expression in offspring of both genders, this effect of PE performed by HFD-fed obese mothers was only observed in male offspring.⁹⁶ Some of these in utero and gender-specific effects could be explained by epigenetic mechanisms; however, the data on offspring liver epigenetic alterations are still lacking.

Prenatal PE is important to improve offspring metabolism even in the presence of maternal HFD, which adverse effects are mitigated by maternal PE.^{93,96,97} Prenatal PE can also counteract the effects of maternal HFD feeding on adult female offspring hepatic glucose metabolism and reduce adiposity and hepatic triglyceride content.⁹³ Surprisingly, maternal prenatal PE can rescue IR in SM of female offspring that were actually prompted by paternal obesity.⁹⁸ Besides the positive modulation of maternal diet-induced insulin sensitivity in both mothers and male offspring, maternal PE prevented an excessive lipid accumulation and hypoxia in placenta.⁹⁹ This PE effect is very important considering the remarkable function of placenta and the fact that possible alterations in intrauterine environment can be detrimental to foetus development. A recent study of our group also reported that PE programmes during pregnancy improved maternal diet-induced GDM and related liver mitochondrial dysfunction and remarkably reduced hepatic triglyceride accumulation in male offspring.⁵³

TABLE 1 Alterations in offspring hepatic metabolism in response to maternal exercise performed before and/or during pregnancy

Reference	Species/Strain and maternal age	Maternal diet	Maternal exercise protocol	Offspring	Effects of maternal exercise on offspring hepatic metabolism
Carlson et al. ⁷⁸	Sprague-Dawley rats	SCD	• Acute PE at GD20.5 - Treadmill: 60 min at 12 m/min • Acute PE at GD21.5 - Treadmill: 60 min at 16 m/min at 10% grade	Foetuses - E20.5 - E21.5	~ hepatic glycogenolysis ~ hepatic glycogen content
Rodgers et al. ⁸⁰			• PE 4 wk before and during pregnancy - Treadmill: 26.8 m/min, 1 h/d, 5 d/wk	28-d-old	~ hepatic glycogen content
Houghton et al. ⁷⁹	Sprague-Dawley rats; 50-60 d of age	SCD	• PE 3 wk before pregnancy; • PE 3 wk before and during pregnancy - Treadmill: 30 m/min, 10° inclination, (60%-70% $\dot{V}O_{2max}$), 1 h/d, 5 d/wk	Foetuses E20	↓ hepatic glycogen content (maternal PE before pregnancy); ~ hepatic glycogen content (maternal PE before and during pregnancy)
Carter et al. ⁸⁴	Sprague-Dawley rats; 12 wk old	SCD	• PE 7-10 d before and during pregnancy, and 12 d after delivery - Free-running wheel	Female offspring; 17 mo old; 18% protein diet; Sedentary	↑ glucose tolerance ↑ glucose disposal ↑ glucose infusion rate ↑ glucose turnover rate ↓ hepatic glucose production
Laker et al. ⁸⁶	C57BL/6 mice; 8 wk old	SCD or HFD	• PE 6 wk before and during pregnancy - Free-running wheel	Offspring from neonatal until 12 mo old; SCD; Sedentary	~ hepatic <i>Pgc-1α</i> methylation: neonatals
Songstad et al. ⁹²	Sprague-Dawley rats; 9 to 11 wk old	SCD	• PE 3 wk before and during pregnancy - Treadmill: HIIT protocol 5 d/wk – 5 min warm up at low speed (at <60% of $\dot{V}O_{2max}$), 10 bouts of 4 min of high-intensity running on a treadmill at 25° inclination (at 85-90% of $\dot{V}O_{2max}$) alternating with two minutes of active recovery (at 50-60% of $\dot{V}O_{2max}$)	Male and female foetuses (E20)	~ hepatic oxidative stress level ~ hepatic total antioxidant capacity ↓ hepatic GPx4.2 gene expression ↓ hepatic eNOS gene expression ↓ hepatic HIF1A gene expression ~ hepatic SOD1, SOD2, iNOS, CAT, HK2, GPx1, GPx2, GPx4.1 gene expression
Quiclet et al. ⁸⁸	Wistar rats; 15 wk old	SCD	• PE 4 wk before and during pregnancy (until GD18) - Treadmill: gradually increased to the speed of 25 m/min (55% of maximal aerobic speed), 1 h/d	Male offspring; - ¾ wk old - 7 mo old SCD; Sedentary	~ glucose tolerance: 3-wk-old offspring ↑ glucose tolerance: 7-mo-old offspring ~ insulin sensitivity ~ hepatic insulin signalling pathway activation (pPKB/pKB ratio): 3-wk-old and 7-mo-old offspring

(Continues)

TABLE 1 (Continued)

Reference	Species/Strain and maternal age	Maternal diet	Maternal exercise protocol	Offspring	Effects of maternal exercise on offspring hepatic metabolism
Sheldon et al. ⁸⁹	Sprague-Dawley rats	SCD	- PE during pregnancy - Free-running wheel	Male and female offspring; - 4 mo old; - 8 mo old; SCD or HFD; Sedentary	~ glucose tolerance ~ hepatic TG: 4-mo-old male offspring; ↓ hepatic TG: 8-mo-old male offspring ~ hepatic TG: female offspring ~ hepatic 1-[14C] palmitate oxidation to CO ₂ ~ hepatic β-HAD activity ~ hepatic CS activity ↑ enhanced mitochondrial biogenesis (PGC-1α, TFAM gene expression): 8-mo-old male offspring ↑ elevated basal autophagic potential (ATG12:5 conjugate, pAMPK/AMPK protein expression): 8-mo-old male offspring
Quicklet et al. ⁸⁷	Wistar rats; 15 wk old	SCD	- PE 4 wk before and during pregnancy (until GD18) - Treadmill: gradually increased to the speed of 25 m/min (55% of maximal aerobic speed), 1 h/d	Male offspring; 3 mo old; SCD or HFHS; Sedentary	~ glucose tolerance ~ insulin sensitivity ~ hepatic insulin signalling pathway activation (pPKB/PKB ratio) ↓ hepatic CS activity ~ hepatic HAD: SCD-fed offspring ↓ hepatic HAD: HFHS-fed offspring ↑ hepatic glycogen content ~ pGSK3/GSK3 ~ hepatic PEPCK ↓ hepatic FAS: SCD-fed offspring ↓↓ hepatic FAS: HFHS-fed offspring

(Continues)

TABLE 1 (Continued)

Reference	Species/Strain and maternal age	Maternal diet	Maternal exercise protocol	Offspring	Effects of maternal exercise on offspring hepatic metabolism
Stanford et al. ⁹³	C57BL/6 mice; 6 wk old	SCD or HFD	• PE 2 wk before and during pregnancy; • PE 2 wk before pregnancy; • PE during pregnancy Free-running wheel	Female offspring: - 8 wk old; - 12 wk old; - 24 wk old; - 36 wk old; - 52 wk old. Sedentary	↑ glucose tolerance: 8 wk old (SCD-fed mothers), 36-wk-old (HFD-fed mothers), 52 wk old (HFD-fed mothers) offspring ~ glucose tolerance: 12 wk old and 24 wk old. ↑ glucose tolerance: 36-wk-old and 52-wk-old offspring of mothers with PE before and during pregnancy ~ glucose tolerance: 36-wk-old and 52-wk-old offspring of mothers with PE before pregnancy or PE during pregnancy ↑ insulin tolerance: 52-wk-old offspring of mothers with PE before and during pregnancy or with PE during pregnancy ~ insulin tolerance: 52-wk-old offspring of mothers with PE before pregnancy ↓ basal rate of hepatic glucose production: 6-, 12-, 24-wk-old offspring ↓ insulin-mediated hepatic glucose production: 12-, 24-wk-old offspring ~ insulin-mediated hepatic glucose production: 6-wk-old offspring ↓ glucagon-mediated hepatic glucose production: 6-, 12-, 24-wk-old offspring ~ glucose infusion rate, rates of glucose appearance, basal rates of glucose disappearance, insulin-stimulated rates of glucose disappearance: 52-wk-old offspring ↓ hepatic TG content: 52-wk-old offspring ↑ hepatic expression of genes involved in pyruvate metabolism, Krebs cycle, and FA transport and oxidation

(Continues)

TABLE 1 (Continued)

Reference	Species/Strain and maternal age	Maternal diet	Maternal exercise protocol	Offspring	Effects of maternal exercise on offspring hepatic metabolism
Cunningham et al ⁸⁵	Wistar rats; 7 to 8 wk old	SCD or WD	- PE 5 wk before and during pregnancy - Free-running wheel	Male and female offspring; 18 wk old; SCD; Sedentary	~ hepatic TG: offspring of exercised SCD-fed mothers ~ hepatic TG: offspring of exercised WD-fed mothers ~ hepatic CS activity ~ protein content of the OXPHOS subunits ↑ OXPHOS subunit CIII: male offspring of exercised SCD-fed mothers ↓ hepatic β-HAD activity: offspring of exercised WD mothers ~ hepatic mitochondrial biogenesis markers (~PPARγ, ↑TFAM, ~PGC-1α) ↑ hepatic markers of autophagy/mitophagy
Siti et al ⁹⁰	Wistar rats; 12 wk old	SCD	- PE 4 wk before and during pregnancy (until GD18) - Treadmill: gradually increased to the speed of 25 m/min with a 10% slope, 1 h/d	Male offspring; 3 wk old	~ hepatic RCR (glutamate/malate) ↓ hepatic RCR (succinate; succinate + glutamate/malate) ~ hepatic OXPHOS subunit CI ↑ hepatic OXPHOS subunits CII, CIII, CIV ↓ hepatic mitochondrial H₂O₂ release (succinate or succinate + glutamate/malate) ~ hepatic mitochondrial H₂O₂ release (rotenone or antimycin A) ↑ hepatic cytochrome a+a3 ~ hepatic cytochrome b, c, c+c1 ↑ hepatic GPx activity ~ hepatic thiols ↑ hepatic % short-chain FA ↓ hepatic % long-chain FA ↓ hepatic unsaturated FA ↑ hepatic saturated FA ↑ hepatic (n-3) FA ↓ hepatic (n-6) FA ↑ hepatic (n-3)/(n-6) FA
Boonpattawong et al ⁸³	C57BL/6 mice;	SCD or WD	- PE 1 wk before and during pregnancy and 3-wk after delivery - Free-running wheel	Male offspring; 17 wk old; SCD or WD; Sedentary	↑ hepatic Mat1a mRNA (one-carbon metabolism) ~ hepatic Mat1a mRNA (one-carbon metabolism) ~ hepatic Mthfr, Mtrr, an Cbs (one-carbon metabolism)

(Continues)

TABLE 1 (Continued)

Reference	Species/Strain and maternal age	Maternal diet	Maternal exercise protocol	Offspring	Effects of maternal exercise on offspring hepatic metabolism
Bae-Gartz et al. ⁸²	C57BL/6 mice; 3-wk old	SCD	- PE during pregnancy (until GD18) - Free-running wheel	Male offspring: - 21-d-old; - 112-d-old. SCD or HFD (only after 70 d of age)	↓ steatosis score: 112-d-old HFD-fed offspring ↓ NAFLD activity score: 112-d-old HFD-fed offspring ↓ hepatic FAS: 21-d-old offspring ↑ hepatic PEPCK protein expression: 21-d-old offspring ↑ hepatic CYP7A1 and SREBP2 protein expression: 21-d-old offspring ↓ hepatic SREBP1 protein expression ↑ tendency for hepatic insulin signalling activation ↑ hepatic PPAR α and PGC-1 α signalling: 21-d-old offspring ~ hepatic TFAM mRNA levels: 21-d-old offspring ~ hepatic PGC-1 α methylation: 21-d-old offspring ↑ hepatic β -oxidation: 21-d-old offspring
Son et al. ⁹¹	C57BL/6J mice; 8 wk old	SCD	- PE during pregnancy (until GD16.5 or GD20.5) - Treadmill: 10–14 m/min, 1 h/d, at 40% VO₂max (E1.5 to E7.5), then 65% VO₂max (E8.5 to E14.5), and 50% VO₂max (E15.5 to E20.5)	Male and female offspring: 3 mo old; SCD or HFD; Sedentary	↑ glucose tolerance: HFD-fed offspring ↓ hepatic lipid accumulation
Stevanović-Silva et al. ⁵³	Sprague-Dawley rats; 7 wk old;	SCD or HFHS	- PE during pregnancy - Treadmill: gradually increased to the speed of 21 m/min, 1 h/d, 6 d/wk - Free-running wheel	Male offspring: 6 wk old; SCD; Sedentary	~ glucose tolerance ↓ TG content ↓ NAFLD score ↓ long-chain acylcarnitines (C16, C18, C18:1) ↑ RCR (glutamate/malate) ~ RCR (succinate) ↓ respiratory rate with oligomycin ~ ADP/O ratio ↑ OXPHOS subunits CI and CIV protein expression ↓ UCP2 protein expression

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ATG, autophagy-related gene; CAT, catalase; Cbs, cystathionine- β -synthase; CYP7A1, cholesterol 7 α -hydroxylase; E, embryonic day; eNOS, endothelial nitric oxide synthase; FA, fatty acids; FAS, fatty acid synthase; GD, gestational day; GPx, glutathione peroxidase; GSK3, glycogen synthase kinase 3; HAD, hydroxyacyl-CoA dehydrogenase; HFD, high-fat diet; HFHS, high-fat high-sucrose diet; HIF1A, hypoxia-inducible factor 1 subunit alpha; HK2, hexokinase 2; iNOS, inducible nitric oxide synthase; Mat1a, methionine adenosyltransferase; Mthfr, methylenetetrahydrofolate reductase; Mtrr, methionine synthase reductase; NAFLD, non-alcoholic fatty liver disease; OXPHOS, oxidative phosphorylation; pAMPK, phosphorylated adenosine monophosphate-activated protein kinase; PE, physical exercise; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; pGSK3, phosphorylated glycogen synthase kinase 3; PKB, protein kinase B; PND, postnatal day; PPAR, peroxisome proliferator-activated receptor; pPKB, phosphorylated protein kinase B; RCR, respiratory control ratio; SCD, standard chow diet; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; SREBP1, sterol regulatory element-binding protein 1; SREBP2, sterol regulatory element-binding protein 2; TFAM, mitochondrial transcription factor A; TG, triglycerides; UCP2, uncoupling protein 2; WD, Western diet.

5 | CONCLUSIONS

Many studies suggest that maternal PE can enhance offspring insulin sensitivity and glucose metabolism, which can be very important in glucose and insulin impairment-related disorders. However, studies analysing the effects of maternal PE as a preventive tool against offspring NAFLD are still lacking. Special focus in future research should also be given to mitochondrial alterations as one of the first key events in NAFLD development. Mechanistic explanation of how PE improves offspring metabolic health is out of reach so far. Gene-environment in utero interactions that lead to epigenetic alterations may be among the mechanisms lowering disease risk. Exercise-induced epigenetic modifications can provide further mechanistic insights into the preventive and therapeutic role of PE against metabolic disorders. In mothers with altered metabolic status (hyperglycaemia, hyperinsulinaemia, dyslipidaemia), PE can rescue these changes, providing a stabilised in utero metabolic milieu that will positively affect foetal developmental programming and eventually reduce the offspring susceptibility to later-life metabolic diseases.¹⁰⁰ Nevertheless, it is hypothesised that maternal PE can have a dose-dependent effect on foetal epigenome, with lower doses considered beneficial, but higher doses detrimental.⁵⁹ Therefore, exercise programmes applied during pregnancy should be carefully designed, with particular emphasis on both mother's and foetal welfare, and following the concept of general moderation, as suggested by recent guidelines. Better understanding of in utero environment and the consequences of its alterations for the offspring could reinforce exercise programmes as interventions to improve health and reduce disease risk in offspring.

ACKNOWLEDGEMENTS

This work was supported by the EU's Horizon 2020 Research and Innovation programme under the Marie Skłodowska-Curie Actions (No. 722619, FOIE GRAS; No. 734719, mt-FOIE GRAS) and by the Portuguese Foundation for Science and Technology (FCT) (UIDB/00617/2020-base; POCI-01-0145-FEDER-016690-PTDC/DTP-DES/7087/2014; POCI-01-0145-FEDER-016657-PTDC/DTP-DES/1082/2014 and SFRH/BD/129645/2017) to JB.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHORS CONTRIBUTIONS

JSS, JB and JM conceptualized the study. JSS, JB and PC performed literature survey. JSS, JB and JM contributed to original draft. JSS, JB, PC, RCC, AA and JM involved in final editing and approval. RCC involved in information visualization. AA and JM involved in funding acquisition.

ORCID

Jelena Stevanović-Silva  <https://orcid.org/0000-0003-4641-1078>

Jorge Belezza  <https://orcid.org/0000-0002-7573-3757>

Pedro Coxito  <https://orcid.org/0000-0003-0573-7418>

Rui Carlos Costa  <https://orcid.org/0000-0002-8881-3174>

António Ascensão  <https://orcid.org/0000-0001-5269-0857>

José Magalhães  <https://orcid.org/0000-0003-4808-8374>

REFERENCES

1. Younossi Z, Tacke F, Arrese M, et al. Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Hepatology*. 2019;69(6):2672-2682.
2. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metab Clin Exp*. 2016;65(8):1038-1048.
3. Stevanović J, Belezza J, Coxito P, Ascensão A, Magalhães J. Physical exercise and liver "fitness": role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease. *Mol Metab*. 2020;32:1-14.
4. Bush H, Golabi P, Younossi ZM. Pediatric non-alcoholic fatty liver disease. *Children*. 2017;4(6):48.
5. Li M, Reynolds CM, Segovia SA, Gray C, Vickers MH. Developmental programming of nonalcoholic fatty liver disease: the effect of early life nutrition on susceptibility and disease severity in later life. *Biomed Res Int*. 2015;2015:437107.
6. Lynch C, Chan CS, Drake AJ. Early life programming and the risk of non-alcoholic fatty liver disease. *J Dev Orig Health Dis*. 2017;8(3):263-272.
7. Bayol SA, Simbi BH, Fowkes RC, Stickland NC. A maternal "junk food" diet in pregnancy and lactation promotes non-alcoholic fatty liver disease in rat offspring. *Endocrinology*. 2010;151(4):1451-1461.
8. Bruce KD, Cagampang FR, Argenton M, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology*. 2009;50(6):1796-1808.
9. Kjaergaard M, Nilsson C, Rosendal A, Nielsen MO, Raun K. Maternal chocolate and sucrose soft drink intake induces hepatic steatosis in rat offspring associated with altered lipid gene expression profile. *Acta Physiol (Oxf)*. 2014;210(1):142-153.
10. Pruis MG, Lendvai A, Bloks VW, et al. Maternal western diet primes non-alcoholic fatty liver disease in adult mouse offspring. *Acta Physiol (Oxf)*. 2014;210(1):215-227.
11. Zou M, Arentson EJ, Teegarden D, Koser SL, Onyskow L, Donkin SS. Fructose consumption during pregnancy and lactation induces fatty liver and glucose intolerance in rats. *Nutr Res*. 2012;32(8):588-598.
12. Gonçalves IO, Oliveira PJ, Ascensão A, Magalhães J. Exercise as a therapeutic tool to prevent mitochondrial degeneration in non-alcoholic steatohepatitis. *Eur J Clin Invest*. 2013;43(11):1184-1194.
13. Keating SE, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J Hepatol*. 2012;57(1):157-166.
14. Pedersen BK, Saltin B. Exercise as medicine – evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sports*. 2015;25(Suppl 3):1-72.

15. Byrne CD, Targher G. NAFLD: a multisystem disease. *J Hepatol*. 2015;62(1 Suppl):S47-S64.
16. VanWagner LB, Rinella ME. Extrahepatic manifestations of non-alcoholic fatty liver disease. *Curr Hepatol Rep*. 2016;15(2):75-85.
17. Clark JM, Alkhuraishi AR, Solga SF, Alli P, Diehl AM, Magnuson TH. Roux-en-Y gastric bypass improves liver histology in patients with non-alcoholic fatty liver disease. *Obes Res*. 2005;13(7):1180-1186.
18. Rosato V, Masarone M, Dallio M, Federico A, Aglitti A, Persico M. NAFLD and extra-hepatic comorbidities: current evidence on a multi-organ metabolic syndrome. *Int J Environ Res Public Health*. 2019;16(18):3415.
19. Wang AY, Dhaliwal J, Mouzaki M. Lean non-alcoholic fatty liver disease. *Clin Nutr*. 2019;38(3):975-981.
20. Martínez de Morentin PB, López M. "Mens sana in corpore sano": exercise and hypothalamic ER stress. *PLoS Biol*. 2010;8(8):e1000464.
21. Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP. Reduced physical activity and risk of chronic disease: the biology behind the consequences. *Eur J Appl Physiol*. 2008;102(4):381-390.
22. Petersen KF, Dufour S, Savage DB, et al. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci USA*. 2007;104(31):12587-12594.
23. Li R, Toan S, Zhou H. Role of mitochondrial quality control in the pathogenesis of nonalcoholic fatty liver disease. *Aging*. 2020;12(7):6467-6485.
24. Lee J, Park JS, Roh YS. Molecular insights into the role of mitochondria in non-alcoholic fatty liver disease. *Arch Pharm Res*. 2019;42(11):935-946.
25. Satapati S, Kucejova B, Duarte JA, et al. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest*. 2015;125(12):4447-4462.
26. Gross B, Pawlak M, Lefebvre P, Staels B. PPARs in obesity-induced T2DM, dyslipidaemia and NAFLD. *Nat Rev Endocrinol*. 2017;13(1):36-49.
27. Aharoni-Simon M, Hann-Obercyger M, Pen S, Madar Z, Tirosh O. Fatty liver is associated with impaired activity of PPAR γ -coactivator 1 α (PGC1 α) and mitochondrial biogenesis in mice. *Lab Invest*. 2011;91(7):1018-1028.
28. Stewart MS, Heerwagen MJ, Friedman JE. Developmental programming of pediatric nonalcoholic fatty liver disease: redefining the "first hit". *Clin Obstet Gynecol*. 2013;56(3):577-590.
29. Mouralidarane A, Soeda J, Visconti-Pugmire C, et al. Maternal obesity programs offspring nonalcoholic fatty liver disease by innate immune dysfunction in mice. *Hepatology*. 2013;58(1):128-138.
30. Xie X, Gao H, Zeng W, et al. Placental DNA methylation of peroxisome-proliferator-activated receptor- γ co-activator-1 α promoter is associated with maternal gestational glucose level. *Clin Sci*. 2015;129(4):385-394.
31. Kruse M, Seki Y, Vuguin PM, et al. High-fat intake during pregnancy and lactation exacerbates high-fat diet-induced complications in male offspring in mice. *Endocrinology*. 2013;154(10):3565-3576.
32. Li J, Huang J, Li JS, Chen H, Huang K, Zheng L. Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets. *J Hepatol*. 2012;56(4):900-907.
33. McCurdy CE, Bishop JM, Williams SM, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest*. 2009;119(2):323-335.
34. Jeznach-Steinhagen A, Ostrowska J, Czerwonogrodzka-Senczynna A, Boniecka I, Shahnazaryan U, Kuryłowicz A. Dietary and pharmacological treatment of nonalcoholic fatty liver disease. *Medicina*. 2019;55(5):166.
35. Wong VW, Chitturi S, Wong GL, Yu J, Chan HL, Farrell GC. Pathogenesis and novel treatment options for non-alcoholic steatohepatitis. *Lancet Gastroenterol Hepatol*. 2016;1(1):56-67.
36. Houghton D, Thoma C, Hallsworth K, et al. Exercise reduces liver lipids and visceral adiposity in patients with nonalcoholic steatohepatitis in a randomized controlled trial. *Clin Gastroenterol Hepatol*. 2017;15(1):96-102.e103.
37. Bacchi E, Negri C, Targher G, et al. Both resistance training and aerobic training reduce hepatic fat content in type 2 diabetic subjects with nonalcoholic fatty liver disease (the RAED2 Randomized Trial). *Hepatology*. 2013;58(4):1287-1295.
38. Johnson NA, Sachinwalla T, Walton DW, et al. Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. *Hepatology*. 2009;50(4):1105-1112.
39. Hallsworth K, Fattakhova G, Hollingsworth KG, et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut*. 2011;60(9):1278-1283.
40. Gonçalves IO, Passos E, Rocha-Rodrigues S, et al. Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion*. 2014;15:40-51.
41. Linden MA, Fletcher JA, Morris EM, et al. Treating NAFLD in OLETF rats with vigorous-intensity interval exercise training. *Med Sci Sports Exerc*. 2015;47(3):556-567.
42. Nobili V, Bedogni G, Alisi A, et al. A protective effect of breastfeeding on the progression of non-alcoholic fatty liver disease. *Arch Dis Child*. 2009;94(10):801-805.
43. Rajia S, Chen H, Morris MJ. Voluntary post weaning exercise restores metabolic homeostasis in offspring of obese rats. *Nutr Metab Cardiovasc Dis*. 2013;23(6):574-581.
44. Bahari H, Caruso V, Morris MJ. Late-onset exercise in female rat offspring ameliorates the detrimental metabolic impact of maternal obesity. *Endocrinology*. 2013;154(10):3610-3621.
45. Caruso V, Bahari H, Morris MJ. The beneficial effects of early short-term exercise in the offspring of obese mothers are accompanied by alterations in the hypothalamic gene expression of appetite regulators and FTO (fat mass and obesity associated) gene. *J Neuroendocrinol*. 2013;25(8):742-752.
46. Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. *Semin Perinatol*. 2015;39(7):512-519.
47. Beleza J, Stevanović-Silva J, Coxito P, et al. Building-up fit muscles for the future: transgenerational programming of skeletal muscle through physical exercise. *Eur J Clin Invest*. 2021:e13515. <https://doi.org/10.1111/eci.13515>
48. Shojaaee-Moradie F, Baynes KC, Pentecost C, et al. Exercise training reduces fatty acid availability and improves the insulin sensitivity of glucose metabolism. *Diabetologia*. 2007;50(2):404-413.
49. Barwell ND, Malkova D, Moran CN, et al. Exercise training has greater effects on insulin sensitivity in daughters of patients with type 2 diabetes than in women with no family history of diabetes. *Diabetologia*. 2008;51(10):1912-1919.
50. Østergård T, Andersen JL, Nyholm B, et al. Impact of exercise training on insulin sensitivity, physical fitness, and muscle oxidative capacity in first-degree relatives of type 2 diabetic patients. *Am J Physiol Endocrinol Metab*. 2006;290(5):E998-E1005.

51. Guo R, Liong EC, So KF, Fung ML, Tipoe GL. Beneficial mechanisms of aerobic exercise on hepatic lipid metabolism in non-alcoholic fatty liver disease. *Hepatobiliary Pancreat Dis Int*. 2015;14(2):139-144.
52. Yan Z, Lira VA, Greene NP. Exercise training-induced regulation of mitochondrial quality. *Exerc Sport Sci Rev*. 2012;40(3):159-164.
53. Stevanović-Silva J, Beleza J, Coxito P, et al. Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring. *Metab Clin Exp*. 2021;116:154704.
54. Beleza J, Rizo-Roca D, Ascensão A, Magalhães J. Targeting mitochondria with sweat: improving mitochondrial function with physical activity. In: Oliveira PJ, ed. *Mitochondrial Biology and Experimental Therapeutics*. Cham: Springer International Publishing; 2018:379-406.
55. Pedersen BK, Steensberg A, Schjerling P. Muscle-derived interleukin-6: possible biological effects. *J Physiol*. 2001;536(Pt 2):329-337.
56. Hong F, Radaeva S, Pan HN, Tian Z, Veech R, Gao B. Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. *Hepatology*. 2004;40(4):933-941.
57. Boström P, Wu J, Jedrychowski MP, et al. A PGC1- α -dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*. 2012;481(7382):463-468.
58. Hoffmann C, Weigert C. Skeletal muscle as an endocrine organ: the role of myokines in exercise adaptations. *Cold Spring Harb Perspect Med*. 2017;7(11):a029793.
59. Chalk TE, Brown WM. Exercise epigenetics and the fetal origins of disease. *Epigenomics*. 2014;6(5):469-472.
60. Barrès R, Yan J, Egan B, et al. Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab*. 2012;15(3):405-411.
61. Zhou D, Hlady RA, Schafer MJ, et al. High fat diet and exercise lead to a disrupted and pathogenic DNA methylome in mouse liver. *Epigenetics*. 2017;12(1):55-69.
62. Sookoian S, Rosselli MS, Gemma C, et al. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor γ coactivator 1 α promoter. *Hepatology*. 2010;52(6):1992-2000.
63. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. *PLoS One*. 2009;4(5):e5610.
64. Baggish AL, Hale A, Weiner RB, et al. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. *J Physiol*. 2011;589(Pt 16):3983-3994.
65. Kalaki-Jouybari F, Shanaki M, Delfan M, Gorgani-Firouzjaei S, Khakdan S. High-intensity interval training (HIIT) alleviated NAFLD feature via miR-122 induction in liver of high-fat high-fructose diet induced diabetic rats. *Arch Physiol Biochem*. 2020;126(3):242-249.
66. Burchard J, Zhang C, Liu AM, et al. microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol Syst Biol*. 2010;6:402.
67. Fornes D, Heinecke F, Roberti SL, White V, Capobianco E, Jawerbaum A. Proinflammation in maternal and fetal livers and circulating miR-122 dysregulation in a GDM rat model induced by intrauterine programming. *Mol Cell Endocrinol*. 2020;510:110824.
68. Aoi W, Naito Y, Mizushima K, et al. The microRNA miR-696 regulates PGC-1 α in mouse skeletal muscle in response to physical activity. *Am J Physiol Endocrinol Metab*. 2010;298(4):E799-E806.
69. Sun Y, Cui D, Zhang Z, Zhang Q, Ji L, Ding S. Voluntary wheel exercise alters the levels of miR-494 and miR-696 in the skeletal muscle of C57BL/6 mice. *Comp Biochem Physiol B Biochem Mol Biol*. 2016;202:16-22.
70. Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1 α , a nodal regulator of mitochondrial biogenesis. *Am J Clin Nutr*. 2011;93(4):884s-890s.
71. Downs DS, Chasan-Taber L, Evenson KR, Leiferman J, Yeo S. Physical activity and pregnancy: past and present evidence and future recommendations. *Res Q Exerc Sport*. 2012;83(4):485-502.
72. Birsner ML, Gyamfi-Bannerman C. Physical activity and exercise during pregnancy and the postpartum period ACOG committee opinion summary, number 804. *Obstet Gynecol*. 2020;135(4):E178-E188.
73. Liguori G. American College of Sports Medicine. *ACSM's Guidelines for Exercise Testing and Prescription*. Philadelphia: Lippincott Williams & Wilkins; 2020.
74. Davenport MH, Meah VL, Ruchat SM, et al. Impact of prenatal exercise on neonatal and childhood outcomes: a systematic review and meta-analysis. *Br J Sports Med*. 2018;52(21):1386-1396.
75. McMillan AG, May LE, Gaines GG, Isler C, Kuehn D. Effects of aerobic exercise during pregnancy on 1-month infant neuromotor skills. *Med Sci Sports Exerc*. 2019;51(8):1671-1676.
76. Patel N, Godfrey KM, Pasupathy D, et al. Infant adiposity following a randomised controlled trial of a behavioural intervention in obese pregnancy. *Int J Obes*. 2017;41(7):1018-1026.
77. Mourtakos SP, Tambalis KD, Panagiotakos DB, et al. Maternal lifestyle characteristics during pregnancy, and the risk of obesity in the offspring: a study of 5,125 children. *BMC Pregnancy Childbirth*. 2015;15:66.
78. Carlson KI, Yang HT, Bradshaw WS, Conlee RK, Winder WW. Effect of maternal exercise on fetal liver glycogen late in gestation in the rat. *J Appl Physiol*. 1986;60(4):1254-1258.
79. Houghton PE, Mottola MF, Mezzapelli J, Vandermolen R, Christopher PD. Glycogen storage in fetuses of trained pregnant rats. *Can J Appl Physiol*. 1997;22(4):384-393.
80. Rodgers CD, Mottola MF, Corbett K, Taylor AW. Skeletal muscle metabolism in the offspring of trained rats. *J Sports Med Phys Fitness*. 1991;31(3):389-395.
81. Vanheest JL, Rodgers CD. Effects of exercise in diabetic rats before and during gestation on maternal and neonatal outcomes. *Am J Physiol*. 1997;273(4):E727-E733.
82. Bae-Gartz I, Kasper P, Großmann N, et al. Maternal exercise conveys protection against NAFLD in the offspring via hepatic metabolic programming. *Sci Rep*. 2020;10(1):15424.
83. Boonpattawong NP, Golbidi S, Tai DC, et al. Exercise during pregnancy mitigates the adverse effects of maternal obesity on adult male offspring vascular function and alters one-carbon metabolism. *Physiol Rep*. 2020;8(18):e14582.
84. Carter LG, Qi NR, De Cabo R, Pearson KJ. Maternal exercise improves insulin sensitivity in mature rat offspring. *Med Sci Sports Exerc*. 2013;45(5):832-840.
85. Cunningham RP, Moore MP, Meers GM, Rueggsegger GN, Booth FW, Rector RS. Maternal physical activity and sex impact markers of hepatic mitochondrial health. *Med Sci Sports Exerc*. 2018;50(10):2040-2048.

86. Laker RC, Lillard TS, Okutsu M, et al. Exercise prevents maternal high-fat diet-induced hypermethylation of the Pgc-1 α gene and age-dependent metabolic dysfunction in the offspring. *Diabetes*. 2014;63(5):1605-1611.
87. Quiclet C, Dubouchaud H, Berthon P, et al. Maternal exercise modifies body composition and energy substrates handling in male offspring fed a high-fat/high-sucrose diet. *J Physiol*. 2017;595(23):7049-7062.
88. Quiclet C, Siti F, Dubouchaud H, et al. Short-term and long-term effects of submaximal maternal exercise on offspring glucose homeostasis and pancreatic function. *Am J Physiol Endocrinol Metab*. 2016;311(2):E508-E518.
89. Sheldon RD, Nicole Blaize A, Fletcher JA, et al. Gestational exercise protects adult male offspring from high-fat diet-induced hepatic steatosis. *J Hepatol*. 2016;64(1):171-178.
90. Siti F, Dubouchaud H, Hininger I, et al. Maternal exercise before and during gestation modifies liver and muscle mitochondria in rat offspring. *J Exp Biol*. 2019;222(Pt 10). <https://doi.org/10.1242/jeb.194969>
91. Son JS, Zhao L, Chen Y, et al. Maternal exercise via exerkine apelin enhances brown adipogenesis and prevents metabolic dysfunction in offspring mice. *Sci Adv*. 2020;6(16):eaaz0359.
92. Songstad NT, Kaspersen KH, Hafstad AD, Basnet P, Ytrehus K, Acharya G. Effects of high intensity interval training on pregnant rats, and the placenta, heart and liver of their fetuses. *PLoS One*. 2015;10(11):e0143095.
93. Stanford KI, Takahashi H, So K, et al. Maternal exercise improves glucose tolerance in female offspring. *Diabetes*. 2017;66(8):2124-2136.
94. Wasinski F, Bacurau RF, Estrela GR, et al. Exercise during pregnancy protects adult mouse offspring from diet-induced obesity. *Nutr Metab*. 2015;12:56.
95. Carter LG, Lewis KN, Wilkerson DC, et al. Perinatal exercise improves glucose homeostasis in adult offspring. *Am J Physiol Endocrinol Metab*. 2012;303(8):E1061-E1068.
96. Raipuria M, Bahari H, Morris MJ. Effects of maternal diet and exercise during pregnancy on glucose metabolism in skeletal muscle and fat of weanling rats. *PLoS One*. 2015;10(4):e0120980.
97. Vega CC, Reyes-Castro LA, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E. Exercise in obese female rats has beneficial effects on maternal and male and female offspring metabolism. *Int J Obes*. 2015;39(4):712-719.
98. Falcão-Tebas F, Marin EC, Kuang J, Bishop DJ, McConell GK. Maternal exercise attenuates the lower skeletal muscle glucose uptake and insulin secretion caused by paternal obesity in female adult rat offspring. *J Physiol*. 2020;598(19):4251-4270.
99. Fernandez-Twinn DS, Gascoin G, Musial B, et al. Exercise rescues obese mothers' insulin sensitivity, placental hypoxia and male offspring insulin sensitivity. *Sci Rep*. 2017;7:44650.
100. Donovan EL, Miller BF. Exercise during pregnancy: developmental origins of disease prevention? *Exerc Sport Sci Rev*. 2011;39(3):111.

How to cite this article: Stevanović-Silva J, Beleza J, Coxito P, Costa RC, Ascensão A, Magalhães J. Fit mothers for a healthy future: Breaking the intergenerational cycle of non-alcoholic fatty liver disease with maternal exercise. *Eur J Clin Invest*. 2021;00:e13596. <https://doi.org/10.1111/eci.13596>

Physical exercise and liver “fitness”: Role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease

Jelena Stevanović¹, Jorge Beleza², Pedro Coxito¹, António Ascensão¹, José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sport, University of Porto, Porto, Portugal

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

Published on: *Molecular Metabolism*,
2020 Feb; 32:1-14.

doi: 10.1016/j.molmet.2019.11.015



Physical exercise and liver “fitness”: Role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease

Jelena Stevanović^{1,*}, Jorge Belezá², Pedro Coxito¹, António Ascensão¹, José Magalhães¹

ABSTRACT

Background: Modern lifestyles, especially high-caloric intake and physical inactivity, contribute to the increased prevalence of non-alcoholic fatty liver disease (NAFLD), which becomes a significant health problem worldwide. Lifestyle changes, however, affect not only parental generation, but also their offspring, reinforcing the need for efficient preventive approaches to deal with this disease. This transgenerational influence of phenotypes dependent on parents (particularly maternal) behaviours may open additional research avenues. Despite persistent attempts to design an effective pharmacological therapy against NAFLD, physical activity, as a non-pharmacological approach, emerges as an exciting strategy.

Scope of Review: Here we briefly review the effect of physical exercise on liver mitochondria adaptations in NAFLD, highlighting the importance of mitochondrial metabolism and transgenerational and epigenetic mechanisms in liver diseases.

Major Conclusions: A deeper look into cellular mechanisms sheds a light on possible effects of physical activity in the prevention and treatment of NAFLD through modulation of function and structure of particular organelles, namely mitochondria. Additionally, despite of increasing evidence regarding the contribution of epigenetic mechanisms in the pathogenesis of different diseases, the role of microRNAs, DNA methylation, and histone modification in NAFLD pathogenesis still needs to be elucidated.

© 2019 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Keywords NAFLD; Physical exercise; Mitochondrial dysfunction; Epigenetics

1. INTRODUCTION

Sedentary lifestyle together with overconsumption of high-caloric diet has been identified as a major cause of metabolic diseases, including the one defined as non-alcoholic fatty liver disease (NAFLD). Usually described as the hepatic manifestation of metabolic syndrome, NAFLD includes a spectrum of conditions, from hepatic steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis, characterized by lipid accumulation in liver cells [1–3]. NAFLD is associated with common metabolic disorders, such as, obesity [4], increased cardiovascular risk [5,6], chronic kidney disease [7], insulin resistance (IR) and type 2 diabetes (T2DM) [8], but it has also been associated with obstructive sleep apnea, osteoporosis, psoriasis, periodontitis, hypothyroidism, male sexual dysfunction, polycystic ovarian syndrome, colorectal cancer, and urolithiasis, proposing NAFLD as a metabolic disease with extra-hepatic manifestations and multi-organ involvement [9,10].

The global prevalence of NAFLD has increased in the last two decades and it is estimated to be about 25% with variations in prevalence in different regions of the world [11,12]. A meta-analytic study of NAFLD global epidemiology showed that the lowest prevalence of NAFLD is observed in Africa, and the highest in Middle East and South America.

Moreover, this same study supports the association of metabolic comorbidities of NAFLD and obesity, T2DM, and metabolic syndrome in the same areas of the globe [11]. The NAFLD prevalence also increases with age, and it is more common in men than in women, in which the prevalence is greatly increased after menopause [13–15]. This gender-specific difference in NAFLD prevalence relies on the possible protective effect of sex hormones, especially estrogen, against the risk of developing NAFLD in women [15].

In this context, it is also important to note that lifestyle may affect not only the parental generation, but also the offspring resistance to develop chronic diseases later in life. Factors to which a mother is exposed during the pregnancy period, such as diet, stress, tobacco and alcohol consumption, or even gestational diabetes mellitus (GDM), may affect fetal development through epigenetic mechanisms that alter fetal phenotype [16,17]. Pregnancy is a period during which a woman's body undergoes multiple metabolic and physiological adaptations necessary to support the demands of pregnancy, as well as to provide physiological environment for fetus development. Changes in maternal lifestyle during pregnancy, especially changes in nutrition and physical activity, may affect those adaptations, and consequently, fetus growth and development [18,19]. Recent data

¹Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sport, University of Porto, Porto, Portugal ²Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

*Corresponding author. Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sport, University of Porto, Rua Dr. Plácido Costa, 91, 4200-450, Porto, Portugal. E-mail: jela.stevanov@gmail.com (J. Stevanović).

Received September 30, 2019 • Revision received November 19, 2019 • Accepted November 22, 2019 • Available online 29 November 2019

<https://doi.org/10.1016/j.molmet.2019.11.015>

Review

Abbreviations			
Drp1	dynamin-related protein 1	Mid51	mitochondrial dynamics protein of 51 kDa
ER	estrogen receptor	miRNA	microRNA
ERE	estrogen response elements	mPTP	mitochondrial permeability transition pores
ET	endurance training	NADH	dihydro-nicotinamide adenine dinucleotide
ETC	electron transport chain	NAFLD	non-alcoholic fatty liver disease
FFA	free fatty acid	NASH	non-alcoholic steatohepatitis
Fgf21	fibroblast growth factor-21	OMM	outer mitochondrial membranes
GCKR	glucokinase regulatory protein	OPA1	optic atrophy 1
Fis1	human fission factor-1	OXPHOS	oxidative phosphorylation
GDM	gestational diabetes mellitus	PGC1 α	peroxisome proliferators-activated receptor-gamma coactivator 1-alpha
HFD	high-fat diet	PNPLA3	patatin-like phospholipase domain-containing 3
IMM	inner mitochondrial membranes	PPAR	peroxisome proliferators-activated receptor
IR	insulin resistance	ROS	reactive oxygen species
LYPLAL1	lysophospholipase-like-1	T2DM	type 2 diabetes mellitus
MBOAT7	membrane bound O-acyltransferase domain-containing-7	TFAM	mitochondrial transcription factor A
MFF	mitochondria fission factor	TG	triglycerides
Mfn1	mitofusin1	TM6SF2	transmembrane 6 superfamily member-2
Mfn2	mitofusin2	UCP2	uncoupling protein-2
Mid49	mitochondrial dynamics protein of 49 kDa	VLDL	very low-density lipoproteins
		VPA	voluntary physical activity

from animal models show that maternal nutrition during pregnancy and lactation may affect the metabolic phenotype of the offspring, making them more prone to alterations in liver lipid metabolism and hepatic steatosis [20–23].

Therefore, to understand the potential clinical and economic burden of NAFLD in the future, recent studies have shed light on possible therapeutic approaches to address this growing challenge. Still, to design the most effective therapy, it is necessary to recognize the underlying mechanisms in the development and progression of NAFLD. As an active metabolic tissue, the liver has important bioenergetics and xenobiotic detoxification roles, and the disruption of its function may lead to different pathological states [24]. Furthermore, given the pivotal role of mitochondrial network in metabolism and cellular energy production, regulation of ion homeostasis and redox signaling as well as in cellular remodeling and adaptation, dysfunction of liver mitochondria seems to have an important contribution to the development of liver diseases. In fact, liver mitochondrial degeneration has been associated with the hepatocyte death and inflammation, contributing to the degenerative processes. Moreover, mitochondria are recognized as important sources and targets of reactive oxygen species (ROS) associated with liver diseases [1]. They have a central role in cellular lipid metabolism and oxidative stress [25], with mitochondrial network dysfunction being proposed as one of the first events that occur during the development of NAFLD. The inability of mitochondria to coordinate subcellular metabolic processes in the liver results in the impaired regulation of hepatic lipid metabolism and consequent lipid accumulation in hepatocytes [25–27]. Recent data further suggest the important role of endoplasmic reticulum, an important organelle for calcium (Ca²⁺) homeostasis and lipid synthesis, in the pathogenesis of NAFLD. The disruption of endoplasmic reticulum homeostasis activates different signalling pathways related to NAFLD progression, including those involved in inflammation and apoptosis [28–30]. Furthermore, not only that the disturbance of mitochondria and endoplasmic reticulum homeostasis leads to signaling and metabolic changes, but also causes the loss of physical or functional interactions between these two extremely relevant organelles related to cellular metabolism. These tight contact points between mitochondria and

endoplasmic reticulum, known as mitochondria-associated endoplasmic reticulum membranes (MAMs), are vital in the regulation of lipid transport, Ca²⁺ homeostasis, inflammatory signaling and cellular survival [31,32] and thus, the disruption of MAMs integrity is proposed to be involved in hepatic metabolic diseases, including NAFLD [33]. Despite the increased knowledge of underlying cellular mechanisms related to NAFLD, the design of a standard effective therapeutic approach for such complex and multifactorial disease seems to be out of reach so far. Pharmacological interventions and bariatric surgery are proposed in the treatment of NAFLD [34,35]; however, lifestyle modifications, particularly physical exercise, have been suggested as a first-line approach. Being considered one of the most relevant environmental risk factors in NAFLD development, it is no wonder that sedentary behavior is an important target in the NAFLD treatment [1,36]. Physical inactivity has been positively associated with the increased prevalence of a very significant number of chronic diseases and premature mortality. According to the World Health Organization (WHO), it is the 4th leading global risk for mortality, followed by overweight and obesity [37]. Around 30% of people worldwide are physically inactive, with women being slightly more inactive than men [38]. The latest Eurobarometer by the European Commission shows that only 7% of people in the European Union regularly exercise, while 46% of Europeans never exercise, mainly due to lack of time [39,40]. Ironically, whereas physical inactivity rises as a global public health problem, evidence on therapeutic benefits of physical activity on health continue to grow. Regular physical exercise is consensually recognized as an important preventive and/or therapeutic non-pharmacological tool against a range of metabolic, cardiovascular, musculoskeletal, pulmonary, neurological, oncological and psychiatric diseases, [36,41]. Among other mechanisms, physical exercise expresses its beneficial effects against pathophysiological conditions by targeting mitochondrial function, morphology, and bioenergetics [42], which clearly makes it a potent and interesting therapeutic stimulus against, at least, those diseases that are characterized by mitochondrial dysfunction. Our group has already reported the advantageous effects of physical exercise on mitochondriopathies related to cardiac dysfunction induced by doxorubicin (DOX) [43–47] or ischemia-

reperfusion [46,47] and brain dysfunction induced by DOX [48,49], as well as those mitochondrial dysfunction associated with high-fat diet (HFD)-induced adiposopathy [50,51] and NAFLD [52–55]. Therefore, taking into account that NAFLD is a multi-organ disease, lifestyle modifications, including physical activity, should be promoted among patients in order to impact both hepatic and extra-hepatic manifestations of this disease [10]. Furthermore, skeletal muscles act as endocrine-type organs, producing myokines during exercise, also expressing multi-organ effect targeting contractile and non-contractile tissues, including liver, adipose tissue, and brain [36], which proves the exercise as a potent protective mechanism against those multi-organ involving diseases.

2. MECHANISMS OF NAFLD DEVELOPMENT

Since 1980, when an advanced stage of fatty liver diseases, usually thought to be exclusively induced by alcohol overconsumption, was identified in people who do not consume excessive amounts of alcohol, NAFLD has been recognized as a serious metabolic disorder. At that moment, although the cause and the related mechanisms of this condition were still unclear, it was mainly identified in moderately obese patients and/or those with associated diseases such as diabetes [56].

Being considered a complex disease, NAFLD results from the interactions of many environmental and genetic factors. The PNPLA3 (patatin-like phospholipase domain-containing 3), APOB (apolipoprotein B), MBOAT7 (membrane bound O-acyltransferase domain containing 7), TM6SF2 (transmembrane 6 superfamily member 2), GSKR (glucokinase regulatory protein), and LYPLAL1 (lysophospholipase-like 1) are only some of genetic *loci* recognized as determinants in the development of NAFLD [57], while lifestyle changes, especially over-nutrition and physical inactivity, are considered main environmental culprits for NAFLD development.

Being a primary organ of glucose and lipid metabolism, the liver is particularly affected by the excessive accumulation of lipids originated from the diet, lipolysis in adipose tissue, or even *de novo* lipogenesis in liver itself, which is obviously aggravated by physical inactivity. Excessive accumulation of lipids, in at least 5% of hepatocytes, represents the first recognizable stage of NAFLD — hepatic steatosis. These accumulated lipids, in the form of micro- or macrovesicles, may cause injury to hepatocytes, leading to the advanced stage of NAFLD, known as non-alcoholic steatohepatitis (NASH), characterized also by inflammation and liver fibrosis. Patients with NASH are at higher risk to further develop cirrhosis and/or hepatocellular carcinoma (HCC) [4,58]. Despite current scientific efforts, the mechanisms of NAFLD pathogenesis remain elusive. One of the first proposed hypothesis explaining NAFLD mechanisms was the ‘two-hit hypothesis’. According to this suggestion, during the first hit, due to the imbalance in lipid metabolism and transport, the accumulation of lipids in hepatocytes occurs and leads to hepatic steatosis. Accordingly, the first hit makes the liver more susceptible to the second hit, associated with a more progressive hepatic damage. During this following stage, inflammatory cytokines are released, levels of oxidative stress increase, and a number of organelles, including mitochondria and endoplasmic reticulum, undergoes dysfunctional modifications characteristic of NASH [59]. Nevertheless, recent studies have challenged this hypothesis suggesting that multiple other ‘hits’, such as IR, adipokines release, nutrition (overconsumption and caloric intake), alteration in intestinal microbiota composition and activity, increased gut permeability, and (epi) genetic constraints may be involved in the pathogenesis of NAFLD and its progression from hepatic steatosis to NASH [60,61].

This ‘multiple hit hypothesis’ has also been referred to explain the development and progression of NAFLD in children and adolescents, the so-called pediatric NAFLD [62], with *in utero* environment suggested as the ‘first hit’ in the development of pediatric NAFLD [63]. The rising global prevalence of pediatric NAFLD [64,65] urges for understanding the onset mechanisms and improvement of current preventive and therapeutic approaches. Data suggest that children with family history of hepatic steatosis are at higher risk to develop this disease [66]; however unhealthy maternal lifestyles and IR during pregnancy, particularly increased in women with GDM, may also cause hepatic lipid excess in fetus. These conditions create a cytotoxic environment in the early fetal life, which “opens the door” for the development and progression of metabolic disorders [63], namely major alterations in the glucose and lipid metabolism. With advancing pregnancy, fasting glucose levels decrease while glucose production in liver increases, subsequently leading to increased fasting insulin levels and IR. Consequently, circulating glucose and free fatty acid levels increase in mothers to provide enough nutrients for fetal development [67]. However, in women who already have family history of diabetes, who are obese and/or have hyperglycemia, these metabolic alterations during pregnancy are even more exacerbated, being the levels of IR even higher and the risk to develop GDM increased. Although the impaired glucose tolerance and IR, characteristic for this type of diabetes, usually disappear after delivery, GDM carries an increased risk for the development of different metabolic disorders in both mothers and children [68,69]. In fact, as fetal metabolism cannot buffer the excess of lipids originated from maternal nutrition and transmitted through placenta [70], exposure to maternal GDM increases the susceptibility to NAFLD in children [71,72]. Moreover, despite the higher prevalence of NAFLD in men than in women in younger to middle age, a few clinical studies have shown that women with prior GDM are more likely to develop NAFLD in middle age [73–75].

3. MITOCHONDRIAL NETWORK FAILURE IN NAFLD

3.1. Lipid metabolism in mitochondria

Being one of the high-metabolic-rate tissues, the liver plays an important role in the metabolism of carbohydrates, proteins and lipids. However, unlike adipose tissue, the liver is not specialized in lipid storage. The pool of free fatty acids (FFAs) is maintained, on the one hand, by the balance between the lipid intake from the diet, lipolysis in adipose tissue, and *de novo* lipogenesis in the liver and, on the other hand, by the removal of liver lipids through secretion into the circulation, β -oxidation [76], or autophagy [77]. In physiological conditions, FFAs enter liver mitochondria where FFAs undergo β -oxidation or remain in the cytoplasm where they are esterified into triglycerides (TGs), which can be secreted into the circulation as very low-density lipoproteins (VLDLs). The increase of FFAs due to excess caloric intake and decreased energy expenditure associated to sedentary behavior leads to the TGs accumulation as lipid droplets in hepatocytes [25], which is characteristic for NAFLD [52,54]. Additionally, the availability of FFAs is also regulated by a selective form of autophagy, also known as “lipophagy”, which appears to have an important function in the regulation of hepatic lipid stores [77]. Excessive lipid storage, however, may inhibit autophagy, leading to a vicious cycle in which decreased autophagy increases lipid accumulation, which further impairs autophagy and promotes over-excessive lipid accumulation [78]. Indeed, decreased autophagy and associated endoplasmic reticulum stress were reported in murine genetic and dietary models of obesity [78,79], as well as in NAFLD-diagnosed patients, dietary NAFLD murine model and *in vitro* human hepatic cells [80]. The

Review

finding that autophagy is involved in breakdown of lipid droplets in liver cells suggests that autophagy impairments may have a critical role in the pathogenesis of NAFLD [78], which supports the role of physical activity as an important tool in the prevention of NAFLD through autophagy promotion [81,82].

Until recently, lipid droplets were considered simple lipid storage vesicle. However, their complex dynamic nature and functions contributed to their distinction as intracellular organelles. Lipid droplets have a neutral lipid core (TG and esters) coated by a phospholipid monolayer and specific scaffolding proteins re-localized from the endoplasmic reticulum or cytosol to their surface. The synthesis of neutral lipid begins within the endoplasmic reticulum membrane bilayer, which further deforms and lipid droplet buds forms an initial lipid droplet. These initial lipid droplets are relatively small (less than 1–100 μm) with their size and number differing among cell types [83,84]. Apart from their role in lipid storage and metabolism, lipid droplets are also involved in numerous cellular events, such as fatty acids trafficking, protein degradation, modulation of nuclear function, virus assembly and pathogen infections, and response to ER stress [83,85]. Due to these various roles of lipid droplets, aberrations in their biology, as well as, lipolysis and autophagy as main catabolic pathways of lipid droplets, are associated to many pathological conditions [86]. Moreover, by isolating lipids, lipid droplets protect cells from excessive lipid accumulation, which could eventually affect membrane integrity and lead to lipotoxicity. This way excessive fatty acids are relatively inert and harmless. However, due to imbalances in processes that maintain FFAs pool in the liver, a non-physiological accumulation of lipid results in changes not only of the number, but also the size of lipid droplets in liver cells. The hallmark of NAFLD is the formation and accumulation of small (microvesicular steatosis) or large lipid droplets (macrovesicular steatosis) in liver cells. In both cases, lipid droplets are considerably larger than those lipid droplets present in liver cells in physiological conditions [84]. The increased size of lipid droplets in pathological conditions arises from several processes: a) TG synthesis in lipid droplets, or b) fusion processes — ripening (diffusion of contents of one lipid droplet into another one) and coalescence (a true fusion of lipid droplets) [84,85]. Besides, some lipid droplets scaffolding proteins, like perilipins, might be involved in physical and metabolic interaction between lipid droplets and mitochondria, and thus in control of local fatty acid flux [87], suggesting that altered interaction between lipid droplets and mitochondria via lipid droplets scaffolding proteins might be present in pathological conditions.

3.2. Mitochondrial bioenergetics

Mitochondria, which occupy almost 20% of hepatocytes area, markedly contribute to the hepatocyte metabolism, especially to β -oxidation and oxidative phosphorylation [88]. Therefore, the lipid accumulation in hepatocytes is only a superficial part of NAFLD pathology. Given their key role in energy production, pH regulation, calcium (Ca^{2+}) and redox homeostasis, liver mitochondria are essential sensors of cell toxicity. Mitochondria control lipid metabolism in liver; however, as in pathological conditions the liver takes over the role of lipid storage, mitochondrial dysfunction is the logical consequence. In fact, lipid accumulation in hepatocytes is known to disrupt liver mitochondrial bioenergetics, which works in a way to maintain cellular homeostasis through multiple pathways [89]. In pathological conditions, increased FFAs accumulation favors an increase of β -oxidation and subsequent generation of NADH, which serves as co-enzyme in the mitochondrial electron transport chain (ETC). However, this increased electron flux through the ETC combined with cytochrome c (Cyt c) release due, at least in part, to tumour necrosis factor- α (TNF- α) membrane

permeabilization promotes an ETC impairment and an over-reduction of the respiratory chain. In these disturbed metabolic conditions, mitochondrial-related 'electron leakage' results in the increased ROS generation, which are able to interact with different macromolecules and affect the normal structure and function of cells and their organelles, including mitochondria themselves [1]. In addition, mitochondrial DNA is more susceptible to DNA damage by ROS than nuclear DNA as it lacks histones, and has only a limited repair capacity [90]. These enhanced oxidative stress conditions trigger the production of inflammatory cytokines, causing inflammation and fibrogenic response, and all of these, together with already extant lipid droplets in hepatocytes, lies in the heart of NASH [91–93].

During the initial phase of NAFLD development, liver mitochondrial bioenergetics alters and undergoes 'remodeling' in order to adapt to nutrients overload and protect against NAFLD progression. In this first stage, mitochondrial respiration increases, but as the disease progresses it becomes inefficient due to mitochondrial electron leakage, leading to the increased ROS production [89,94]. Among other factors [95], the failure of such an important mitochondrial adaptive mechanism is the trigger for progression from simple steatosis to NASH [89,94,96]. These alterations will depend on the progression of the disease and the ability of liver to adapt and possibly store lipids. Still, under chronic conditions, compensatory mechanisms of the liver fail to adapt, and these metabolic pathways are constrained, resulting in lipotoxicity and consequent inflammation and development of NASH [89]. Lipotoxicity-induced liver inflammation has been associated with adipose tissue remodeling and obesity-promoted NAFLD [97]. Impaired metabolic compensation in adipose tissue results in compromised energy homeostasis, and subsequently to obesity and NAFLD progression [98].

In the prolonged state of over-nutrition and excess accumulation of lipids in the liver, mitochondrial bioenergetics collapse and ATP synthesis rates significantly begin to decrease. Indeed, dysfunction of mitochondrial ETC and correspondent decrease in ATP synthesis rates are documented in NAFLD patients and animal models [76,94,99]. Ironically, the flux of TCA cycle in the liver remains elevated, probably in response to the high-energy demands in these conditions, which supplies an inefficient mitochondrial ETC with reducing equivalents and provides conditions for a vicious-cycle of increased ROS production [99].

3.3. Mitochondrial dynamics

Taken together, the above-referred rationale highlights that, although the mechanisms of NAFLD are still not fully clarified, it is consensually accepted that the structural and functional alterations of the mitochondrial network significantly contribute to the pathogenesis of NAFLD [100]. Being morphologically dynamic organelles, mitochondria change shape through fusion and fission-related mechanisms depending on the available energy and supply as well as the levels of stress [101]. Processes of mitochondrial fusion, fission and biogenesis are tightly controlled in response to metabolic stressors. Mitochondrial fission considers the division of one mitochondrion into two mitochondria and it is mediated by the interaction of cytoplasmic mitochondrial fission protein dynamin-like/related protein 1 (DLP1/Drp1) and other mitochondrial fission proteins, such as human fission factor-1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 kDa (MiD49) and 51 kDa (MiD51) [102]. Mitochondria fusion is characterized by the union of two mitochondria into one mitochondrion. Mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) mediate fusion of the outer mitochondrial membranes while optic atrophy 1 (OPA1) fuses the inner mitochondrial membranes and enable

mitochondrial fusion. In normal conditions damaged and/or dysfunctional mitochondria are degraded through mitophagy in order to prevent a release of “toxic” signals from mitochondria into the cytosol. Partly damaged mitochondrion undergoes mitochondrial fission where it is divided into two smaller mitochondria — a healthy one and a damaged one. The damaged mitochondria are further degraded by mitophagy, while healthy mitochondria may eventually fuse contributing to a healthy mitochondrial pool [103].

Mitochondrial dynamics plays an important role in the bioenergetics adaptation to cellular metabolic demands. With alterations in nutrient availability, mitochondria will undergo fusion or fission in order to maintain mitochondrial energy production and bioenergetics [104]. In a rich-nutrient environment, mitochondria fragment to avoid energy waste, by decreasing bioenergetic efficiency and increasing mitochondrial uncoupling, which will eventually lead to the increased nutrient storage [104,105]. During high levels of cellular stress or when damaged, mitochondrial network undergo fission to separate damaged mitochondria from a healthy cellular content and maintain cellular and mitochondrial bioenergetic homeostasis [101]. DLP1/Drp1 mediates mitochondrial fission and some studies show increase in protein expression in animals models of NAFLD [106–108]. In starvation, mitochondria tend to fuse in order to maintain ATP production and, thus, increase bioenergetics efficiency. Mitochondrial elongation during starvation protects cells from autophagy and cell death [104,109]. In the presence of functionally damaged mitochondria, mitochondrial quality control mechanisms are activated and apart from potentially mitophagy-related outcomes, mitochondria may fuse to share RNAs or proteins and compensate for the functional defects. The reduced signaling for mitochondrial fusion detected in NAFLD animal models underlines its potential role in the pathology of NAFLD [106,107]. When mitophagy pathways in hepatocytes are altered, damaged mitochondria accumulate, leading eventually to the release of mitochondrial damage signals into the cytosol resulting in inflammatory cytokines expression and inflammasomes activation, and eventually development of steatohepatitis [103]. Additionally, mitochondrial dynamics is also associated with excessive ROS production and enhanced oxidative damage, therefore prompting mitochondria as a target of oxidative stress levels in metabolic diseases [108,110].

3.4. Mitochondrial permeability transition pore

Once mitochondrial functioning starts failing, their role in Ca^{2+} homeostasis also becomes endangered. Ca^{2+} is recognized as an important factor in the regulation of mitochondrial function. Adequate levels of cytosolic Ca^{2+} are referred as important mediators in the activation of some of the enzymes involved in oxidative phosphorylation. Furthermore, data suggest that extramitochondrial Ca^{2+} is also involved in supplying oxidative phosphorylation with substrates on demand [111,112]. However, in pathological conditions, disrupted Ca^{2+} homeostasis activates mitochondrial buffering ability to uptake it and leads to the overaccumulation of Ca^{2+} in mitochondria, which further affects mitochondrial redox homeostasis and ATP production. Moreover, Ca^{2+} -related involvement in the activation of complex-like pores - mitochondrial permeability transition pores (mPTP), induces the loss of inner mitochondrial membrane permeability. As a consequence, the induction of mPTP stimulates both mitochondrial dysfunction and the release of proapoptotic proteins from mitochondria to the cytosol resulting in the activation of apoptotic-related signaling [113], and eventually of cellular quality control mechanisms, such as autophagy [114,115]. Accordingly, the activation of the mPTP is considered an important factor in the pathogenesis of fatty liver diseases, with susceptibility to mPTP opening significantly increased in

animal models of NAFLD [53,116,117]. Together with other typical metabolic disturbances observed in NAFLD conditions, namely the Ca^{2+} overload and enhanced oxidative stress [116], it seems that high fat and glucose fluctuation may also trigger mPTP, leading to the unregulated flux of molecules into the mitochondrial matrix and, eventually, to mitochondrial swelling, which jeopardizes transmembrane potential and induces the collapse of mitochondrial bioenergetics [118].

3.5. Estrogen signaling and mitochondrial function

Another interesting issue associating NAFLD to impaired mitochondrial function are low levels of estrogen [119]. In fact, estrogen signaling, mediated by estrogen receptor α ($\text{ER}\alpha$), seems to affect mitochondrial function by modulating ATP production, mitochondrial membrane potential, mitochondrial biogenesis, and Ca^{2+} signaling. ERs appear to translocate and to be transitionally present in mitochondria, where they can bind to an estrogen response elements (ERE)-like element of the mitochondrial genome and induce the transcription of mitochondrial DNA genes [120]. The protective effect of estrogen against the risk of developing NAFLD in women has already been proposed [15], while the significance of $\text{ER}\alpha$ in insulin signaling has been noted. Data suggest that $\text{ER}\alpha$ knockout mice express impaired glucose tolerance and IR, downregulation of peroxisome proliferators-activated receptors (PPAR), PPAR α and PPAR Δ , and uncoupling protein-2 (UCP2) expression in skeletal muscle as well as elevated inflammatory signaling in liver [121]. Considering that $\text{ER}\alpha$ can be co-activated by peroxisome proliferators-activated receptor-gamma coactivator 1- α (PGC1 α), the loss of estrogen signaling, present in postmenopausal women, is possibly linked to reduced PGC1 α and associated with liver fat and NAFLD progression, which may explain, at least in part, gender-specific difference in NAFLD development and the increased risk for NAFLD in women after menopause. Furthermore, the loss of estrogen signaling can also exacerbate diet-induced NASH in a rodent model, which highlights the impact of this hormonal-mediated effect in the progression of this pathological condition [122]. Ultimately, an epigenetics-mediated regulation may also explain some of the possible mechanisms that associate estrogen with protective effects against NAFLD risk in women. In a study performed by Zhang et al. [123] and devoted to better understand the role of estrogen signaling in liver of male and female mice diagnosed with NAFLD, *in vivo* and *in vitro* experiments showed that the upregulation of miR-125b might explain, at least in part, the protective mechanism associated to estrogen. The miR-125b, activated by estrogen through $\text{ER}\alpha$, targets fatty acid synthase and possibly alter lipid metabolism in a way that protects against NAFLD [123].

4. PHYSICAL (IN) ACTIVITY-RELATED NAFLD

Being NAFLD one of the most prevalent liver diseases in western countries, the questions of developing effective preventive and/or therapeutic countermeasures against the disease emerge. A design of effective pharmacological treatments was the first starting point, usually including drugs targeting associated metabolic disorders, such as diabetes and IR or pharmacological strategies used in the prevention of mitochondrial degeneration, which is also present in NAFLD. Some of these strategies also included mitochondrial-targeted molecules, antioxidants administration, and anti-obesity drugs; however, all of them showed low levels of effective therapeutic efficiency. Therefore, the US Food and Drug Administration (FDA) has not approved any of them in the treatment for this disease. So far, weight reduction and lifestyle modifications (dietary changes and physical activity) are still

Review

the only ones proposed as effective interventions for the treatment of NAFLD [124,125].

4.1. Physical exercise to prevent NAFLD

Despite the fact that the knowledge regarding the effects of physical exercise on non-contractile tissues is emerging, a research in the area of NAFLD treatment with exercise is still scarce. However, a number of studies with NAFLD patients evidence the importance of lifestyle changes in dealing against this disease [126–128]. Several clinical studies suggest a positive association between sedentary time and the prevalence of NAFLD. Using subjective (questionnaires) or objective (motion sensors) methods to assess physical activity, several studies report that patients with NAFLD spend more of their daily time in sedentary activities than healthy people. In these NAFLD patients, body mass index and weight are positively associated with sedentary lifestyle. Thus, it is not a surprise that the number of steps per day, total daily energy expenditure, and metabolic equivalences (METs) are lower in NAFLD patients than in healthy subjects. Furthermore, these studies showed that higher-intensity exercise is associated with lower prevalence of NAFLD [129–132]. Therefore, increased physical activity seems to be able to prevent the development and progression of NAFLD through different pathways, including the increase of energy expenditure by muscle contractions and through the stimulation of glucose uptake from the circulation into the muscles [131]. Skeletal muscle is the primary organ of disposal of glucose, as the response to insulin. Therefore, the loss of muscle mass, might be involved in the development of IR and IR-related metabolic disorders, including NAFLD. On the other hand, sarcopenia, an aging-related loss of muscle mass, can also occur earlier in life due to lifestyle changes, particularly unhealthy diet and physical inactivity, which are also in the background of NAFLD development [133]. Thus it seems that sarcopenia and NAFLD share similar risk factors and underlying mechanisms. By improving energy balance and insulin sensitivity, physical activity can contribute to the reduction of hepatic lipid accumulation. Better understanding of liver–muscle interaction could improve the management of NAFLD [134].

Taken together, data suggest that increased physical activity is an effective protective non-pharmacological strategy against several metabolic disorders and a therapeutic approach for patients diagnosed with NAFLD. Indeed, regular exercise has been shown to improve liver histological and metabolic features in NAFLD patients. Studies in both NAFLD patients [126–128] and animal models of NAFLD [52,54,55,135] indicate that physical activity is effective in reducing lipid content in hepatocytes and enhancing insulin sensitivity. Although the underlying mechanisms of the protective effects perpetrated by physical activity still remain elusive, some of them may rely on the inter-organ pathways established between liver, skeletal muscles, and adipose tissue. At a glance, skeletal muscles are the main disposal sites for the glucose in post-prandial state. Therefore, by increasing the level of glucose uptake by the muscles and the activity of glycogen synthase in myocytes, the peripheral insulin sensitivity is improved [136]. Also, regular physical activity reduces visceral adiposity in NAFLD patients [126,137], which contributes to lower the influx of fatty acids from visceral adipose tissue to the liver and prevent possible liver lipid accumulation [126].

4.2. Physical exercise during pregnancy

As previously referred, GDM enhances the risk of metabolic impairments in the offspring, but also, an increased likelihood for the development of NAFLD in mothers with GDM sooner than it is expected in non-pregnant women. Considering this transgenerational burden,

strategies that mitigate the development of GDM need to be seriously implemented in order to protect both mother and fetus from this deleterious condition, being physical exercise a non-pharmacological approach that is becoming more and more exciting. Taking into consideration that in uncomplicated pregnancy, there are no impediments to engage in exercise programs and it is considered safe and beneficial during pregnancy, the American College of Obstetricians and Gynecologists (ACOG) recommends that healthy women or even athletes, after the consultation and clinical evaluation by obstetricians/gynecologists, continue to regularly exercise, although some adjustments should be made on the exercise programs and training routines due to the anatomical and physiological changes associated to pregnancy. In fact, non-athlete pregnant women are encouraged to perform at least 30 min of moderate intensity physical exercise every day or, at least, most days of the week [138]. In women diagnosed with GDM, moderate- or high-intensity aerobic or resistance exercise, at least three times per week, lowers fasting blood glucose levels and glycated hemoglobin, and also improves glycemic control [139]. Moreover, it is known that physical exercise in pregnancy can improve physical fitness, cardiovascular function, muscle mass, weight management, sleep quality, and psychologic well-being, as well as, reduce the risk of developing GDM [140]. Therefore, it is expected that exercise may reduce the risk of maternal, fetal, and neonatal complications in GDM that could aggravate hepatic glucose and lipid metabolism and prompt development of NAFLD in both generations.

A deeper look into cellular mechanisms sheds a light on possible effects of physical activity in the prevention and treatment of NAFLD through modulation of function and structure of particular organelles, namely mitochondria.

5. PHYSICAL EXERCISE AND LIVER MITOCHONDRIAL ADAPTATIONS

Depending on the exercise type, intensity and duration, the predominance of substrates utilization may vary during exercise. Generically, the main substrates during low-to moderate-intensity exercise are FFAs and glucose, while with the increase of intensity, glucose becomes a more prominent fuel source, which challenges the energy homeostasis of muscles in several circumstances [42]. Considering the importance of mitochondria in cell metabolism, the effects of exercise in mitochondria are intensively studied. Although it is expected that exercise-related adaptations mainly affect muscle mitochondria, liver mitochondria are also a target during exercise. Given the role of liver in maintaining glucose and lipid metabolism, liver mitochondria are essential for producing energy for these energy-demanding processes. Therefore, as a stimulus for liver mitochondria to produce ATP to maintain homeostasis of those metabolic processes, exercise *per se* also activates systemic pathways that indirectly modulate liver mitochondria bioenergetics, function, and structure [42,141]. In fact, during exercise-related contractions, skeletal-muscle fibers can produce a number of muscle-derived molecules, the so-called myokines that are secreted into the bloodstream and target different non-contractile tissues, such as liver, brain, and adipose tissue. Apart from many other potential pathways, the myokine interaction with tissue-specific receptors on those tissues, modulates molecules that ultimately may activate PGC-1 α , resulting in an improved regulation of mitochondrial biogenesis [126–128]. Still, the mechanisms are rather obscure. Given the known deleterious consequences of distinct pathological conditions on mitochondrial bioenergetics and dynamics, including in the liver, and the health-related relevance of maintaining an adequate mitochondrial network function and structure for cell functioning, the question whether and how exercise

can positively modulate liver mitochondria, and thereby counteract adverse impact of malnutrition and sedentary behavior in the development and progress of NAFLD arises.

So far, there are no consensual suggestions regarding how physical exercise can impact liver mitochondrial signaling pathways in the animal models. Data from several research groups confirm the positive relevance of active lifestyles in the adaptation of liver mitochondria against the harmful consequences of NAFLD [52–55,82,135,142,143]. In fact, two models of exercise (voluntary physical activity (VPA) performed in free-running wheels and endurance training (ET) in the treadmill) in animal models were able to mitigate impairments in mitochondrial bioenergetics and dynamics present in a NASH model induced by a HFD. Both exercise modalities were able to positively modulate liver mitochondria bioenergetics function, reflected by the beneficial changes in the respiratory control ratio [54]. The preservation of liver mitochondrial function can, at least in part, be explained by improvements of mitochondrial membrane structured in the exercise groups. The ratio between phosphatidylcholine and phosphatidylethanolamine, as a marker of membrane integrity, was reduced in sedentary HFD group, suggesting an impaired membrane integrity that was reverted by physical exercise. Nevertheless, cardiolipin, which plays a main role in mitochondrial bioenergetics, decreased in both sedentary and exercise HFD groups [52]. Alterations in the mitochondrial membrane permeability and the induction of mPTP lead to the consequent release of proapoptotic molecules and, ultimately, the progression of both the apoptotic and autophagic signaling [114,115]. These results highlight the relevance of active lifestyle strategies to reduce the susceptibility of liver mitochondria to permeability transition pore opening and to positively modulate apoptotic and autophagic signaling in animal models of NAFLD [53]. Even though, it is important to note that, in some circumstances related with the severity of the deleterious consequences, physical exercise may also stimulates autophagy in various tissues, including liver [82,144], suggesting that one of the mechanisms through which exercise could induce its beneficial effects is the activation of the autophagic signalling [81].

Regarding the positive modulation of physical exercise on liver mitochondrial network remodeling, a study designed by Santos-Alves et al. [145] comparing two chronic exercise modalities (VPA and ET) during 12 weeks in rat model, showed that both models of exercise were able to positively alter the expression of proteins associated with mitochondrial biogenesis and dynamics and increase liver autophagy signaling, thus inducing adaptive remodeling of liver mitochondrial network. On the other hand, another study showed that 5 weeks of endurance treadmill training *per se* was not a sufficiently strong physiological stimulus to significantly alter liver mitochondrial function or protein expression of OXPHOS subunits. Still, endurance training was able to counteract the deleterious impact induced by salicylate and benefit liver mitochondrial function in the context of toxicity [146]. In contrast, an elegant study designed by Fletcher et al. [142] suggests that 4 weeks of endurance training increases OXPHOS activity mediated by complex I-related substrates although without significantly modulating liver mitochondrial biogenesis markers. In accordance, other studies suggest changes in the activity of liver mitochondrial ETC complexes in response to exercise, particularly of complexes I, IV, and V, followed by increase in mitochondrial glutathione content, and without increased ROS production [147]. Likewise, 6 weeks of swimming exercise positively modulated oxidative stress in rats' liver mitochondria by increasing the activity of the antioxidant system and concomitantly decreasing the levels of oxidative stress [148]. Taking together, the increased antioxidant defense and decreased oxidative

stress observed in the referred studies suggest that physical exercise is effective in managing ROS production during exercise and in preserving liver mitochondria redox homeostasis [147,148]. In Otsuka Long-Evans Tokushima Fatty (OLETF) rats, it was shown that, even though both aerobic exercise and treatment with metformin, usually used for the treatment of type 2 diabetes, were effective in modulating NAFLD phenotype regarding fatty acid metabolism and liver mitochondrial function, exercise training *per se* was even more effective, while the combination of drug treatment and a lifestyle change had only a slight synergistic effect [149]. Even though the general perspective highlights a positive impact of physical exercise in liver mitochondrial function and dynamics, some of the discrepancies among these studies may be related to the differences in animal species and strains used and in the features of the distinct exercise models, namely the distinct type, duration, and intensities of exercise programs [24].

6. EPIGENETIC PERSPECTIVE OF NAFLD AND LIFESTYLE MODIFICATIONS

Although the precise underlying mechanisms linking lifestyle changes and development of NAFLD are not completely understood, epigenetic regulation of genes associated with NAFLD development and progression recently came to the fore [150]. Expression of microRNAs (miRNAs) as well as DNA methylation and histone modification by altering DNA accessibility, regulate the activity of genes involved in, among others, lipid metabolism, mitochondrial dysfunction, oxidative stress, and inflammation. In fact, studies on liver biopsy samples from patients diagnosed with NAFLD confirmed the alterations in methylation of genes related to lipid metabolism and mitochondrial biogenesis. Furthermore, the observed differences in DNA methylation status may help to distinguish patients with mild and advanced stages of NAFLD and can be useful to predict progression of NAFLD [151,152]. Despite of increasing evidence regarding the contribution of epigenetic mechanisms in the pathogenesis of different diseases, the role of miRNAs, DNA methylation, and histone modification in NAFLD pathogenesis is still not clear. Global screening of circulating miRNA expressed in liver under NAFLD conditions showed that the up-regulation of miR-122, miR-34a, miR-15b, and miR-16 in circulation were positively associated with NAFLD-related evidence. Increased circulatory levels of miR-122, miR-192, and miR-375 could be proposed as clinical markers of NAFLD progression from hepatic steatosis to NASH. Among these miRNAs, it seems that increased circulatory miR-122, the most abundant miRNA in the liver with an important role in lipid metabolism, has a high impact in NAFLD pathogenesis [153]. Furthermore, NASH was associated with decreased liver expression of miR-122 in NAFLD patients [154]. Similarly, the liver expression of miR-122 was downregulated, together with miR-27 and miR-451, while miR-200a, miR-200b, and miR-429, were upregulated in the liver of a diet-induced rat model of NAFLD [155]. This inverse correlation between circulatory and liver levels of miRNAs may explain their influence in the pathophysiology of NAFLD. Liver lipid-responsive miRNAs miR-34a, which targets one of the members of sirtuin family of NAD-dependent histone deacetylases, is also shown to be upregulated in NASH [154] apparently affecting histone modifications in liver. In addition, histone modifications and DNA methylation regulate gene activity and transcription. Among several transcription factors involved in *de novo* lipogenesis in liver and contributing to liver steatosis, PPAR- γ [156] and its coactivator PGC-1 α drew attention in epigenetics research associated with NAFLD and its progression [156–159]. The involvement of PGC-1 α relies in mitochondrial

Review

biogenesis, β -oxidation, and lipid transport [158,160] and its reduction in liver is associated with NAFLD progression in murine model of NAFLD [161]. It is also an important factor for expression of mitochondrial transcription factor A (TFAM) [160], which plays an important role in replication and gene expression of mitochondrial DNA [143]. Hypermethylation of *PPARGC1A*, but not of *Tfam* promoter, was detected in liver biopsy specimens from NAFLD patients [157].

Given a potency of epigenetics in diseases' pathophysiology, new emphasis is being placed on the influence of epigenetic modifications at the mitochondrial level. miRNAs detected in liver mitochondria may act as sensors of mitochondrial environment leading to the modulation of mitochondrial function and cell signaling [162,163]. Even though, miRNAs regulation is usually related to the cytosol, it seems that mitochondria have a unique pool of miRNAs, independent of total miRNA pool in the cell. The expression of some of these mitochondria-specific miRNAs was increased in liver mitochondria of diabetic mice, such as miR-202–5p, miR-134, miR-155, as well as, miR-494, which affects *Tfam* expression and thus has a role in regulation of mitochondrial DNA [163]. Mitochondrial miRNAs are involved in regulation of transcription, gene expression, gene silencing, cell cycle and cell division, chromatin modification, and *in utero* embryonic development [164]. Additionally, methylation of mitochondrial DNA has been proposed as a biomarker in disease diagnosis, considering that this epigenetic mechanism may be involved in the pathophysiology of some diseases. However, due to the specificity and structural organization of mitochondrial DNA, which lacks in histones, the methylation of mitochondrial DNA may also be a very specific mechanism and further investigation is necessary. So far, some studies have been conducted in areas of drug treatment, oxidative stress, aging, and neurodegenerative diseases [165]. Still, in liver mitochondria of NAFLD patients, methylation of mitochondria encoded NADH dehydrogenase 6 was higher in NASH patients when compared to hepatic steatosis patients [166], suggesting a role of epigenetic alterations not only on nuclear DNA, but also on mitochondrial DNA in the development and progression of NAFLD.

The emerging epigenetic research seems to explain possible underlying mechanisms in NAFLD pathogenesis, but also provides new surprising perspectives on how epigenetic mechanisms play a role in offspring programming. In fact, maternal nutrition has been associated with epigenetic alterations in key genes involved in lipid metabolism and accumulation, such as *Ppara*, *Ppargc*, and *Fgf21*, suggesting that altered DNA methylation patterns in fetal development due to maternal lifestyles may influence offspring health later in life [22,23]. In rodent models, offspring of mothers fed with HFD had altered gene expression, inhibition of hepatic cell cycle and associated DNA methylation [167,168]. *In utero* exposure to maternal HFD affected the genes related to liver dysfunction, immune response, and inflammation, and led to DNA hypermethylation of the genes. Furthermore, these epigenetic alterations, some of them persistent through the life, were associated with prolonged gene expression alterations in the offspring liver, possibly enabling the development of metabolic dysfunctions later in life [169]. Moreover, maternal GDM impacts the epigenome of their offspring, mainly through metabolic pathways affecting fetal growth and development, suggesting that DNA methylation is one of the mechanisms mediating fetal programming. The exposure to GDM in early fetal life is associated with DNA methylation alterations in the genes involved in metabolism, endocrine function, and insulin signaling [170,171].

Ultimately, considering the above-mentioned relevance of epigenetic regulation in the development of NAFLD, epigenetic-related analysis regarding the impact of physical exercise in liver mitochondrial

function and dynamics will be, for sure, a future emerging and growing field of study. Some studies propose DNA methylation status as a marker of progression of NAFLD [151,152], however, this field remains quite open for research regarding NAFLD treatment through changes in lifestyle. Presently, the modulation of the epigenome by changes in lifestyle seems to be able to revert the adverse effect of malnutrition and sedentary lifestyle. Zhou et al. [172] showed that fast food diet increased liver epigenome susceptibility to develop metabolic diseases. On the other hand, physical exercise was able to protect against this fast food diet-induced DNA hypermethylation [172]. Still, most of the studies address the modulating effect of exercise on skeletal muscles epigenome rather than in the liver. For instance, acute exercise was able to decrease promoters methylation of genes involved in mitochondrial biogenesis, PGC-1 α , PPAR- γ , and TFAM in soleus muscles, which suggest that DNA hypomethylation is an early event in contraction-induced gene activation for structural and metabolic adaptations in exercised skeletal muscles [173]. Furthermore, circulating miRNAs can be altered as a physiological response to physical exercise. For instance, in muscle and heart, mir-486 may affect glucose metabolism, while mir-499 is involved in mitochondrial metabolism and apoptosis through Drp1-related signaling. The better understanding of the effect of physical activity on specific circulating miRNAs could justify the use of exercise as a therapeutic strategy [174]. In a HFD-induced animal model of NAFLD, exercise was able to reduce hepatic overexpression of mir-212, which appeared to be involved in lipogenesis and development of NAFLD by targeting FGF-21 [175]. In the study involving HFD-induced obesity in rats, hypoxic training reduced hepatic levels of mir-378, involved in the regulation of lipid metabolism and production of TGs, and, subsequently, increased energy expenditure, β -oxidation in rat liver, and obesity-related morphological and biochemical features. The study also suggests that the probable mechanism is through muscle utilization of fatty acids produced in the liver [176].

In this regard, it is important to note that not only nuclear DNA, but also mitochondrial DNA may undergo epigenetic modifications that are associated with the development of several diseases. In fact, mitochondrial DNA can also be methylated, which attracts attention in the field of disease treatment and prevention. In NASH patients, the methylation level of NADH dehydrogenase 6 encoded by mitochondrial genome was inversely correlated to the level of physical activity [166]. Still, data are scarce and mitochondrial epigenetics and its association to disease risks are still to be elucidated. Therefore, the clarification of the impact of exercise-related epigenetic mechanisms on cellular processes may contribute to this emerging field and promote active lifestyles.

7. CONCLUDING REMARKS

The prevalence of NAFLD and related metabolic disorders (metabolic syndrome, insulin resistance, obesity) is increasing worldwide. Despite numerous pharmacological therapeutic approaches used to counteract multi-organ manifestation of NAFLD, which appear as highly ineffective, physical exercise emerges as a beneficial non-pharmacological preventive and/or therapeutic strategy against this disease. Physical exercise has already been shown as beneficial against some pathological features characteristic for metabolic liver dysfunction, and especially mitochondrial dysfunction. Additionally, considering that the lifestyle changes of parental generation may influence metabolic status of their offspring, widely known benefits of physical exercise, particularly during pregnancy period, should be taken into account. The use of physical activity during pregnancy as a preventive tool against

developing NAFLD in mothers and even against transmitting their impaired metabolic burden to offspring is challenging, being that the mechanisms behind the supposed resistant phenotype are so far not studied. Bearing in mind the potency of epigenetic mechanisms in the development of NAFLD and the transmission of those metabolic impairments to future generation, better understanding of epigenetic-related impact of physical exercise on liver mitochondrial function will be a determining factor in the design of beneficial exercise programs in the future. The further mechanistic look of how physical activity interacts with hepatic mitochondrial “fitness”, particularly via epigenetic mechanisms, may provide necessary clues for proposing active lifestyles during pregnancy as the important preventive strategy to reduce the risk of liver injury through generations. Additionally, a better understanding of muscle–liver axis is expected to delineate mechanisms by which exercise offers benefits in the prevention and treatment of liver diseases.

ACKNOWLEDGEMENT

This work was supported by the EU's Horizon 2020 Research and Innovation program under the Marie Skłodowska-Curie Actions (No.722619, FOIE GRAS; No.734719, mtFOIE GRAS) and by Portuguese Foundation for Science and Technology (FCT) to the Research Center in Physical Activity, Health and Leisure (FCT/UID/DTP/00617/2019; POCI-01-0145-FEDER-016690-PTDC/DTP-DES/7087/2014; POCI-01-0145-FEDER-016657-PTDC/DTP-DES/1082/2014) and to JB (SFRH/BD/129645/2017).

CONFLICT OF INTERESTS

None declared.

REFERENCES

- [1] Goncalves, I.O., Oliveira, P.J., Ascensao, A., Magalhaes, J., 2013. Exercise as a therapeutic tool to prevent mitochondrial degeneration in nonalcoholic steatohepatitis. *European Journal of Clinical Investigation* 43(11):1184–1194.
- [2] Schuppan, D., Schattenberg, J.M., 2013. Non-alcoholic steatohepatitis: pathogenesis and novel therapeutic approaches. *Journal of Gastroenterology and Hepatology* 28(Suppl. 1):68–76.
- [3] Nseir, W., Hellou, E., Assy, N., 2014. Role of diet and lifestyle changes in nonalcoholic fatty liver disease. *World Journal of Gastroenterology* 20(28): 9338–9344.
- [4] Dietrich, P., Hellerbrand, C., 2014. Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best Practice & Research Clinical Gastroenterology* 28(4):637–653.
- [5] Liu, H., Lu, H.Y., 2014. Nonalcoholic fatty liver disease and cardiovascular disease. *World Journal of Gastroenterology* 20(26):8407–8415.
- [6] Athyros, V.G., Tziomalos, K., Katsiki, N., Doumas, M., Karagiannis, A., Mikhailidis, D.P., 2015. Cardiovascular risk across the histological spectrum and the clinical manifestations of non-alcoholic fatty liver disease: an update. *World Journal of Gastroenterology* 21(22):6820–6834.
- [7] Musso, G., Gambino, R., Tabibian, J.H., Ekstedt, M., Kechagias, S., Hamaguchi, M., et al., 2014. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. *PLoS Medicine* 11(7):e1001680.
- [8] Birkenfeld, A.L., Shulman, G.I., 2014. Nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes. *Hepatology* 59(2):713–723.
- [9] Rosato, V., Masarone, M., Dallio, M., Federico, A., Aglitti, A., Persico, M., 2019. NAFLD and extra-hepatic comorbidities: current evidence on a multi-organ metabolic syndrome. *International Journal of Environmental Research and Public Health* 16(18).
- [10] VanWagner, L.B., Rinella, M.E., 2016. Extrahepatic manifestations of nonalcoholic fatty liver disease. *Current Hepatitis Reports* 15(2):75–85.
- [11] Younossi, Z.M., Koenig, A.B., Abdelatif, D., Fazel, Y., Henry, L., Wymer, M., 2016. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64(1): 73–84.
- [12] Rinella, M., Charlton, M., 2016. The globalization of nonalcoholic fatty liver disease: prevalence and impact on world health. *Hepatology* 64(1): 19–22.
- [13] Williams, C.D., Stengel, J., Asike, M.I., Torres, D.M., Shaw, J., Contreras, M., et al., 2011. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 140(1): 124–131.
- [14] Lazo, M., Hernaez, R., Eberhardt, M.S., Bonekamp, S., Kamel, I., Guallar, E., et al., 2013. Prevalence of nonalcoholic fatty liver disease in the United States: the third national health and nutrition examination survey, 1988–1994. *American Journal of Epidemiology* 178(1):38–45.
- [15] Ballestri, S., Nascimbeni, F., Baldelli, E., Marrazzo, A., Romagnoli, D., Lonardo, A., 2017. NAFLD as a sexual dimorphic disease: role of gender and reproductive status in the development and progression of nonalcoholic fatty liver disease and inherent cardiovascular risk. *Advances in Therapy* 34(6): 1291–1326.
- [16] Barua, S., Junaid, M.A., 2015. Lifestyle, pregnancy and epigenetic effects. *Epigenomics* 7(1):85–102.
- [17] Lynch, C., Chan, C.S., Drake, A.J., 2017. Early life programming and the risk of non-alcoholic fatty liver disease. *Journal of Developmental Origins of Health and Disease* 8(3):263–272.
- [18] King, J.C., 2000. Physiology of pregnancy and nutrient metabolism. *American Journal of Clinical Nutrition* 71(5 Suppl.):1218s–1225s.
- [19] Zeng, Z., Liu, F., Li, S., 2017. Metabolic adaptations in pregnancy: a review. *Annals of Nutrition & Metabolism* 70(1):59–65.
- [20] Bayol, S.A., Simbi, B.H., Fowkes, R.C., Stickland, N.C., 2010. A maternal “junk food” diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. *Endocrinology* 151(4):1451–1461.
- [21] Oben, J.A., Mouralidarane, A., Samuelsson, A.M., Matthews, P.J., Morgan, M.L., McKee, C., et al., 2010. Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice. *Journal of Hepatology* 52(6):913–920.
- [22] Puis, M.G., Lendvai, A., Bloks, V.W., Zwier, M.V., Baller, J.F., de Bruin, A., et al., 2014. Maternal western diet primes non-alcoholic fatty liver disease in adult mouse offspring. *Acta Physiologica* 210(1):215–227.
- [23] Wankhade, U.D., Zhong, Y., Kang, P., Alfaro, M., Chintapalli, S.V., Thakali, K.M., et al., 2017. Enhanced offspring predisposition to steatohepatitis with maternal high-fat diet is associated with epigenetic and microbiome alterations. *PLoS One* 12(4):e0175675.
- [24] Ascensao, A., Martins, M.J., Santos-Alves, E., Goncalves, I.O., Portincasa, P., Oliveira, P.J., et al., 2013. Modulation of hepatic redox status and mitochondrial metabolism by exercise: therapeutic strategy for liver diseases. *Mitochondrion* 13(6):862–870.
- [25] Gusdon, A.M., Song, K.X., Qu, S., 2014. Nonalcoholic Fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxidative Medicine and Cellular Longevity* 2014:637027.
- [26] Nassir, F., Ibdah, J.A., 2014. Role of mitochondria in nonalcoholic fatty liver disease. *International Journal of Molecular Sciences* 15(5):8713–8742.
- [27] Fu, A., Shi, X., Zhang, H., Fu, B., 2017. Mitotherapy for fatty liver by intravenous administration of exogenous mitochondria in male mice. *Frontiers in Pharmacology* 8:241.
- [28] Zhang, X.Q., Xu, C.F., Yu, C.H., Chen, W.X., Li, Y.M., 2014. Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease. *World Journal of Gastroenterology* 20(7):1768–1776.

Review

- [29] Ashraf, N.U., Sheikh, T.A., 2015. Endoplasmic reticulum stress and Oxidative stress in the pathogenesis of Non-alcoholic fatty liver disease. *Free Radical Research* 49(12):1405–1418.
- [30] Passos, E., Ascensao, A., Martins, M.J., Magalhaes, J., 2015. Endoplasmic reticulum stress response in non-alcoholic steatohepatitis: the possible role of physical exercise. *Metabolism* 64(7):780–792.
- [31] Vance, J.E., 2014. MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. *Biochimica et Biophysica Acta* 1841(4):595–609.
- [32] Hayashi, T., Rizzuto, R., Hajnoczky, G., Su, T.P., 2009. MAM: more than just a housekeeper. *Trends in Cell Biology* 19(2):81–88.
- [33] Rieusset, J., 2017. Endoplasmic reticulum-mitochondria calcium signaling in hepatic metabolic diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1864(6):865–876.
- [34] Thoma, C., Day, C.P., Trenell, M.I., 2012. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *Journal of Hepatology* 56(1):255–266.
- [35] Dyson, J., Day, C., 2014. Treatment of non-alcoholic fatty liver disease. *Digestive Diseases* 32(5):597–604.
- [36] Fiuzza-Luces, C., Garatachea, N., Berger, N.A., Lucia, A., 2013. Exercise is the real polypill. *Physiology* 28(5):330–358.
- [37] World Health Organization, 2009. Global health risks: mortality and burden of disease attributable to selected major risks. Geneva: World Health Organization.
- [38] Hallal, P.C., Andersen, L.B., Bull, F.C., Guthold, R., Haskell, W., Ekelund, U., 2012. Global physical activity levels: surveillance progress, pitfalls, and prospects. *Lancet* 380(9838):247–257.
- [39] European Commission, 2018. Special Eurobarometer 472. Sport and physical activity.
- [40] Commission, E., 2014. Special Eurobarometer 412. Sport and physical activity. European Commission, Directorate-General for Education and Culture and co.
- [41] Pedersen, B.K., Saltin, B., 2015. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scandinavian Journal of Medicine & Science in Sports* 25(Suppl. 3):1–72.
- [42] Belez, J., Rizo-Roca, D., Ascensão, A., Magalhães, J., 2018. Targeting mitochondria with sweat: improving mitochondrial function with physical activity. *Mitochondrial biology and experimental therapeutics*. Springer. p. 379–406.
- [43] Ascensao, A., Magalhaes, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., et al., 2005. Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. *International Journal of Cardiology* 100(3):451–460.
- [44] Ascensao, A., Magalhaes, J., Soares, J.M., Ferreira, R., Neuparth, M.J., Marques, F., et al., 2005. Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *American Journal of Physiology - Heart and Circulatory Physiology* 289(2):H722–H731.
- [45] Ascensao, A., Lumini-Oliveira, J., Machado, N.G., Ferreira, R.M., Goncalves, I.O., Moreira, A.C., et al., 2011. Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. *Clinical Science* 120(1):37–49.
- [46] Ascensao, A., Ferreira, R., Oliveira, P.J., Magalhaes, J., 2006. Effects of endurance training and acute Doxorubicin treatment on rat heart mitochondrial alterations induced by in vitro anoxia-reoxygenation. *Cardiovascular Toxicology* 6(3–4):159–172.
- [47] Ascensao, A., Magalhaes, J., Soares, J.M., Ferreira, R., Neuparth, M.J., Marques, F., et al., 2006. Endurance training limits the functional alterations of rat heart mitochondria submitted to in vitro anoxia-reoxygenation. *International Journal of Cardiology* 109(2):169–178.
- [48] Marques-Aleixo, I., Santos-Alves, E., Balca, M.M., Rizo-Roca, D., Moreira, P.I., Oliveira, P.J., et al., 2015. Physical exercise improves brain cortex and cerebellum mitochondrial bioenergetics and alters apoptotic, dynamic and auto(mito)phagy markers. *Neuroscience* 301:480–495.
- [49] Marques-Aleixo, I., Santos-Alves, E., Balca, M.M., Moreira, P.I., Oliveira, P.J., Magalhaes, J., et al., 2016. Physical exercise mitigates doxorubicin-induced brain cortex and cerebellum mitochondrial alterations and cellular quality control signaling. *Mitochondrion* 26:43–57.
- [50] Rocha-Rodrigues, S., Rodriguez, A., Becerril, S., Ramirez, B., Goncalves, I.O., Belez, J., et al., 2017. Physical exercise remodels visceral adipose tissue and mitochondrial lipid metabolism in rats fed a high-fat diet. *Clinical and Experimental Pharmacology and Physiology* 44(3):386–394.
- [51] Rocha-Rodrigues, S., Rodriguez, A., Gouveia, A.M., Goncalves, I.O., Becerril, S., Ramirez, B., et al., 2016. Effects of physical exercise on myokines expression and brown adipose-like phenotype modulation in rats fed a high-fat diet. *Life Sciences* 165:100–108.
- [52] Goncalves, I.O., Maciel, E., Passos, E., Torrella, J.R., Rizo, D., Viscor, G., et al., 2014. Exercise alters liver mitochondria phospholipidomic profile and mitochondrial activity in non-alcoholic steatohepatitis. *The International Journal of Biochemistry & Cell Biology* 54:163–173.
- [53] Goncalves, I.O., Passos, E., Diogo, C.V., Rocha-Rodrigues, S., Santos-Alves, E., Oliveira, P.J., et al., 2016. Exercise mitigates mitochondrial permeability transition pore and quality control mechanisms alterations in nonalcoholic steatohepatitis. *Applied Physiology Nutrition and Metabolism* 41(3):298–306.
- [54] Goncalves, I.O., Passos, E., Rocha-Rodrigues, S., Diogo, C.V., Torrella, J.R., Rizo, D., et al., 2014. Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion* 15:40–51.
- [55] Goncalves, I.O., Passos, E., Rocha-Rodrigues, S., Torrella, J.R., Rizo, D., Santos-Alves, E., et al., 2015. Physical exercise antagonizes clinical and anatomical features characterizing Lieber-DeCarli diet-induced obesity and related metabolic disorders. *Clinical Nutrition* 34(2):241–247.
- [56] Ludwig, J., Viggiano, T.R., McGill, D.B., Oh, B.J., 1980. Nonalcoholic steatohepatitis: mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clinic Proceedings* 55(7):434–438.
- [57] Del Campo, J.A., Gallego-Duran, R., Gallego, P., Grande, L., 2018. Genetic and epigenetic regulation in nonalcoholic fatty liver disease (NAFLD). *International Journal of Molecular Sciences* 19(3).
- [58] Drew, L., 2017. Fighting the fatty liver. *Nature* 550(7675):S102–S103.
- [59] Rowell, R.J., Anstee, Q.M., 2015. An overview of the genetics, mechanisms and management of NAFLD and ALD. *Clinical Medicine* 15(Suppl. 6):S77–S82.
- [60] Takaki, A., Kawai, D., Yamamoto, K., 2013. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). *International Journal of Molecular Sciences* 14(10):20704–20728.
- [61] Buzzetti, E., Pinzani, M., Tsochatzis, E.A., 2016. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 65(8):1038–1048.
- [62] Clemente, M.G., Mandato, C., Poeta, M., Vajro, P., 2016. Pediatric non-alcoholic fatty liver disease: Recent solutions, unresolved issues, and future research directions. *World Journal of Gastroenterology* 22(36):8078–8093.
- [63] Stewart, M.S., Heerwagen, M.J., Friedman, J.E., 2013. Developmental programming of pediatric nonalcoholic fatty liver disease: redefining the “first hit”. *Clinical Obstetrics and Gynecology* 56(3):577–590.
- [64] Anderson, E.L., Howe, L.D., Jones, H.E., Higgins, J.P., Lawlor, D.A., Fraser, A., 2015. The prevalence of non-alcoholic fatty liver disease in children and adolescents: a systematic review and meta-analysis. *PLoS One* 10(10):e0140908.
- [65] Vos, M.B., Abrams, S.H., Barlow, S.E., Caprio, S., Daniels, S.R., Kohli, R., et al., 2017. NASPGHAN clinical practice guideline for the diagnosis and treatment of nonalcoholic fatty liver disease in children: recommendations from the Expert Committee on NAFLD (ECON) and the North American Society of Pediatric Gastroenterology. *Hepatology and Nutrition (NASPGHAN)* 64(2):319.

- [66] Long, M.T., Gurary, E.B., Massaro, J.M., Ma, J., Hoffmann, U., Chung, R.T., et al., 2019. Parental non-alcoholic fatty liver disease increases risk of non-alcoholic fatty liver disease in offspring. *Liver International* 39(4):740–747.
- [67] Lain, K.Y., Catalano, P.M., 2007. Metabolic changes in pregnancy. *Clinical Obstetrics and Gynecology* 50(4):938–948.
- [68] American Diabetes Association, 2004. Gestational diabetes mellitus. *Diabetes Care* 27(Suppl. 1):S88–S90.
- [69] Coustan, D.R., 2013. Gestational diabetes mellitus. *Clinical Chemistry* 59(9):1310–1321.
- [70] Brumbaugh, D.E., Friedman, J.E., 2014. Developmental origins of nonalcoholic fatty liver disease. *Pediatric Research* 75(1–2):140–147.
- [71] Patel, S., Lawlor, D.A., Callaway, M., Macdonald-Wallis, C., Sattar, N., Fraser, A., 2016. Association of maternal diabetes/glycosuria and pre-pregnancy body mass index with offspring indicators of non-alcoholic fatty liver disease. *BMC Pediatrics* 16:47.
- [72] Brumbaugh, D.E., Tearse, P., Cree-Green, M., Fenton, L.Z., Brown, M., Scherzinger, A., et al., 2013. Intrahepatic fat is increased in the neonatal offspring of obese women with gestational diabetes. *The Journal of Pediatrics* 162(5):930–936 e931.
- [73] Forbes, S., Taylor-Robinson, S.D., Patel, N., Allan, P., Walker, B.R., Johnston, D.G., 2011. Increased prevalence of non-alcoholic fatty liver disease in European women with a history of gestational diabetes. *Diabetologia* 54(3):641–647.
- [74] Ajmera, V.H., Gunderson, E.P., VanWagner, L.B., Lewis, C.E., Carr, J.J., Terrault, N.A., 2016. Gestational diabetes mellitus is strongly associated with non-alcoholic fatty liver disease. *American Journal of Gastroenterology* 111(5):658–664.
- [75] Foghsgaard, S., Andreassen, C., Vedtofte, L., Andersen, E.S., Bahne, E., Strandberg, C., et al., 2017. Nonalcoholic fatty liver disease is prevalent in women with prior gestational diabetes mellitus and independently associated with insulin resistance and waist circumference. *Diabetes Care* 40(1):109–116.
- [76] Nassir, F., Rector, R.S., Hammoud, G.M., Ibdah, J.A., 2015. Pathogenesis and prevention of hepatic steatosis. *Gastroenterology and Hepatology* 11(3):167–175.
- [77] Martinez-Lopez, N., Singh, R., 2015. Autophagy and lipid droplets in the liver. *Annual Review of Nutrition* 35:215–237.
- [78] Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., et al., 2009. Autophagy regulates lipid metabolism. *Nature* 458(7242):1131–1135.
- [79] Yang, L., Li, P., Fu, S., Calay, E.S., Hotamisligil, G.S., 2010. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metabolism* 11(6):467–478.
- [80] Gonzalez-Rodriguez, A., Mayoral, R., Agra, N., Valdecantos, M.P., Pardo, V., Miñolena-Colina, M.E., et al., 2014. Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD. *Cell Death & Disease* 5:e1179.
- [81] Dasarthy, S., 2017. Are exercise benefits in nonalcoholic fatty liver disease due to increased autophagy? *Exercise and Sport Sciences Reviews* 45(3):125.
- [82] Rosa-Caldwell, M.E., Lee, D.E., Brown, J.L., Brown, L.A., Perry Jr., R.A., Greene, E.S., et al., 2017. Moderate physical activity promotes basal hepatic autophagy in diet-induced obese mice. *Applied Physiology Nutrition and Metabolism* 42(2):148–156.
- [83] Welte, M.A., 2015. Expanding roles for lipid droplets. *Current Biology* 25(11):R470–R481.
- [84] Gluchowski, N.L., Becuwe, M., Walther, T.C., Farese Jr., R.V., 2017. Lipid droplets and liver disease: from basic biology to clinical implications. *Nature Reviews Gastroenterology & Hepatology* 14(6):343–355.
- [85] Wilfling, F., Haas, J.T., Walther, T.C., Farese Jr., R.V., 2014. Lipid droplet biogenesis. *Current Opinion in Cell Biology* 29:39–45.
- [86] Onal, G., Kutlu, O., Gozuacik, D., Dokmeci Emre, S., 2017. Lipid droplets in health and disease. *Lipids in Health and Disease* 16(1):128.
- [87] Wang, H., Sreenivasan, U., Hu, H., Saladino, A., Polster, B.M., Lund, L.M., et al., 2011. Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *The Journal of Lipid Research* 52(12):2159–2168.
- [88] Wei, Y., Rector, R.S., Thyfault, J.P., Ibdah, J.A., 2008. Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World Journal of Gastroenterology* 14(2):193–199.
- [89] Patterson, R.E., Kalavaiapalli, S., Williams, C.M., Nautiyal, M., Mathew, J.T., Martinez, J., et al., 2016. Lipotoxicity in steatohepatitis occurs despite an increase in tricarboxylic acid cycle activity. *American Journal of Physiology. Endocrinology and Metabolism* 310(7):E484–E494.
- [90] Kauppila, J.H., Stewart, J.B., 2015. Mitochondrial DNA: radically free of free-radical driven mutations. *Biochimica et Biophysica Acta* 1847(11):1354–1361.
- [91] Leung, T.M., Nieto, N., 2013. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *Journal of Hepatology* 58(2):395–398.
- [92] Liang, W., Menke, A.L., Driessen, A., Koek, G.H., Lindeman, J.H., Stoop, R., et al., 2014. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PLoS One* 9(12):e115922.
- [93] Satapati, S., Kucejova, B., Duarte, J.A., Fletcher, J.A., Reynolds, L., Sunny, N.E., et al., 2015. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *Journal of Clinical Investigation* 125(12):4447–4462.
- [94] Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., et al., 2015. Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metabolism* 21(5):739–746.
- [95] Pierantonelli, I., Svegliati-Baroni, G., 2019. Nonalcoholic fatty liver disease: basic pathogenetic mechanisms in the progression from NAFLD to NASH. *Transplantation* 103(1):e1–e13.
- [96] Grattagliano, I., Montezinho, L.P., Oliveira, P.J., Fruhbeck, G., Gomez-Ambrosi, J., Montecucco, F., et al., 2019. Targeting mitochondria to oppose the progression of nonalcoholic fatty liver disease. *Biochemical Pharmacology* 160:34–45.
- [97] Ishtiaq, S.M., Rashid, H., Hussain, Z., Arshad, M.I., Khan, J.A., 2019. Adiponectin and PPAR: a setup for intricate crosstalk between obesity and non-alcoholic fatty liver disease. *Reviews in Endocrine & Metabolic Disorders* 20(3):253–261.
- [98] Taketani, H., Nishikawa, T., Nakajima, H., Kodo, K., Sugimoto, S., Aoi, W., et al., 2019. Aging-associated impairment in metabolic compensation by subcutaneous adipose tissue promotes diet-induced fatty liver disease in mice. *Diabetes, Metabolic Syndrome and Obesity* 12:1473–1492.
- [99] Satapati, S., Sunny, N.E., Kucejova, B., Fu, X., He, T.T., Mendez-Lucas, A., et al., 2012. Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *The Journal of Lipid Research* 53(6):1080–1092.
- [100] Paradies, G., Paradies, V., Ruggiero, F.M., Petrosillo, G., 2014. Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. *World Journal of Gastroenterology* 20(39):14205–14218.
- [101] Nasrallah, C.M., Horvath, T.L., 2014. Mitochondrial dynamics in the central regulation of metabolism. *Nature Reviews Endocrinology* 10(11):650–658.
- [102] Hall, A.R., Burke, N., Dongworth, R.K., Hausenloy, D.J., 2014. Mitochondrial fusion and fission proteins: novel therapeutic targets for combating cardiovascular disease. *British Journal of Pharmacology* 171(8):1890–1906.
- [103] Mansouri, A., Gattolliat, C.H., Asselah, T., 2018. Mitochondrial dysfunction and signaling in chronic liver diseases. *Gastroenterology* 155(3):629–647.
- [104] Schrepfer, E., Scorrano, L., 2016. Mitofusins, from mitochondria to metabolism. *Molecular Cell* 61(5):683–694.
- [105] Jheng, H.F., Tsai, P.J., Guo, S.M., Kuo, L.H., Chang, C.S., Su, I.J., et al., 2012. Mitochondrial fission contributes to mitochondrial dysfunction and

Review

- insulin resistance in skeletal muscle. *Molecular and Cellular Biology* 32(2): 309–319.
- [106] Du, J., Zhang, X., Han, J., Man, K., Zhang, Y., Chu, E.S., et al., 2017. Pro-inflammatory CXCR3 impairs mitochondrial function in experimental non-alcoholic steatohepatitis. *Theranostics* 7(17):4192–4203.
- [107] Xu, J., Cao, K., Li, Y., Zou, X., Chen, C., Szeto, I.M., et al., 2014. Bitter melon inhibits the development of obesity-associated fatty liver in C57BL/6 mice fed a high-fat diet. *Journal of Nutrition* 144(4):475–483.
- [108] Galloway, C.A., Lee, H., Brookes, P.S., Yoon, Y., 2014. Decreasing mitochondrial fission alleviates hepatic steatosis in a murine model of nonalcoholic fatty liver disease. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 307(6):G632–G641.
- [109] Gomes, L.C., Di Benedetto, G., Scorrano, L., 2011. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nature Cell Biology* 13(5):589–598.
- [110] Yu, T., Sheu, S.S., Robotham, J.L., Yoon, Y., 2008. Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. *Cardiovascular Research* 79(2):341–351.
- [111] Gellerich, F.N., Gizatullina, Z., Trumbeckaite, S., Nguyen, H.P., Pallas, T., Arandarcikaite, O., et al., 2010. The regulation of OXPHOS by extramitochondrial calcium. *Biochimica et Biophysica Acta* 1797(6–7):1018–1027.
- [112] Williams, G.S., Boyman, L., Chikando, A.C., Khairallah, R.J., Lederer, W.J., 2013. Mitochondrial calcium uptake. *Proceedings of the National Academy of Sciences of the U S A* 110(26):10479–10486.
- [113] Figueira, T.R., Barros, M.H., Camargo, A.A., Castilho, R.F., Ferreira, J.C., Kowaltowski, A.J., et al., 2013. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. *Antioxidants and Redox Signaling* 18(16):2029–2074.
- [114] Lemasters, J.J., 2007. Modulation of mitochondrial membrane permeability in pathogenesis, autophagy and control of metabolism. *Journal of Gastroenterology and Hepatology* 22(Suppl. 1):S31–S37.
- [115] Rasola, A., Bernardi, P., 2011. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. *Cell Calcium* 50(3):222–233.
- [116] Teodoro, J.S., Rolo, A.P., Duarte, F.V., Simoes, A.M., Palmeira, C.M., 2008. Differential alterations in mitochondrial function induced by a choline-deficient diet: understanding fatty liver disease progression. *Mitochondrion* 8(5–6):367–376.
- [117] Navarro, C.D.C., Figueira, T.R., Francisco, A., Dal'Bo, G.A., Ronchi, J.A., Rovani, J.C., et al., 2017. Redox imbalance due to the loss of mitochondrial NAD(P)-transhydrogenase markedly aggravates high fat diet-induced fatty liver disease in mice. *Free Radical Biology and Medicine* 113:190–202.
- [118] Yin, X., Zheng, F., Pan, Q., Zhang, S., Yu, D., Xu, Z., et al., 2015. Glucose fluctuation increased hepatocyte apoptosis under lipotoxicity and the involvement of mitochondrial permeability transition opening. *Journal of Molecular Endocrinology* 55(3):169–181.
- [119] Mooga, V.P., White, C.R., Giordano-Mooga, S., 2018. Estrogen and mitochondrial function in disease. *Mitochondrial diseases*. IntechOpen.
- [120] Gupte, A.A., Pownall, H.J., Hamilton, D.J., 2015. Estrogen: an emerging regulator of insulin action and mitochondrial function. *Journal of Diabetes Research* 2015:916585.
- [121] Ribas, V., Nguyen, M.T., Henstridge, D.C., Nguyen, A.K., Beaven, S.W., Watt, M.J., et al., 2010. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice. *American Journal of Physiology. Endocrinology and Metabolism* 298(2): E304–E319.
- [122] Besse-Patin, A., Leveille, M., Oropeza, D., Nguyen, B.N., Prat, A., Estall, J.L., 2017. Estrogen signals through peroxisome proliferator-activated receptor-gamma coactivator 1alpha to reduce oxidative damage associated with diet-induced fatty liver disease. *Gastroenterology* 152(1):243–256.
- [123] Zhang, Z.C., Liu, Y., Xiao, L.L., Li, S.F., Jiang, J.H., Zhao, Y., et al., 2015. Upregulation of miR-125b by estrogen protects against non-alcoholic fatty liver in female mice. *Journal of Hepatology* 63(6):1466–1475.
- [124] LaBrecque, D.R., Abbas, Z., Anania, F., Ferenci, P., Khan, A.G., Goh, K.L., et al., 2014. World Gastroenterology Organisation global guidelines: non-alcoholic fatty liver disease and nonalcoholic steatohepatitis. *Journal of Clinical Gastroenterology* 48(6):467–473.
- [125] Tsochatzis, E.A., Papatheodoridis, G.V., 2011. Is there any progress in the treatment of non-alcoholic fatty liver disease? *World Journal of Gastrointestinal Pharmacology and Therapeutics* 2(1):1–5.
- [126] Houghton, D., Thoma, C., Hallsworth, K., Cassidy, S., Hardy, T., Burt, A.D., et al., 2017. Exercise reduces liver lipids and visceral adiposity in patients with nonalcoholic steatohepatitis in a randomized controlled trial. *Clinical Gastroenterology and Hepatology* 15(1):96–102 e103.
- [127] Shojaei-Moradie, F., Cuthbertson, D.J., Barrett, M., Jackson, N.C., Herring, R., Thomas, E.L., et al., 2016. Exercise training reduces liver fat and increases rates of VLDL clearance but not VLDL production in NAFLD. *Journal of Clinical Endocrinology & Metabolism* 101(11):4219–4228.
- [128] Sullivan, S., Kirk, E.P., Mittendorfer, B., Patterson, B.W., Klein, S., 2012. Randomized trial of exercise effect on intrahepatic triglyceride content and lipid kinetics in nonalcoholic fatty liver disease. *Hepatology* 55(6):1738–1745.
- [129] Zelber-Sagi, S., Nitzan-Kaluski, D., Goldsmith, R., Webb, M., Zivbel, I., Goldiner, I., et al., 2008. Role of leisure-time physical activity in nonalcoholic fatty liver disease: a population-based study. *Hepatology* 48(6):1791–1798.
- [130] Kwak, M.S., Kim, D., Chung, G.E., Kim, W., Kim, Y.J., Yoon, J.H., 2015. Role of physical activity in nonalcoholic fatty liver disease in terms of visceral obesity and insulin resistance. *Liver International* 35(3):944–952.
- [131] Hallsworth, K., Thoma, C., Moore, S., Ploetz, T., Anstee, Q.M., Taylor, R., et al., 2015. Non-alcoholic fatty liver disease is associated with higher levels of objectively measured sedentary behaviour and lower levels of physical activity than matched healthy controls. *Frontline Gastroenterology* 6(1):44–51.
- [132] Gerber, L., Otgonsuren, M., Mishra, A., Escheik, C., Binerdinc, A., Stepanova, M., et al., 2012. Non-alcoholic fatty liver disease (NAFLD) is associated with low level of physical activity: a population-based study. *Alimentary Pharmacology and Therapeutics* 36(8):772–781.
- [133] Kim, J.A., Choi, K.M., 2019. Sarcopenia and fatty liver disease. *Hepatology International* 13(6):674–687.
- [134] Kim, H.Y., Choi, J.Y., Park, Y.M., 2019. Relative skeletal muscle mass and non-alcoholic fatty liver disease: from association to causation. *Hepatobiliary Surgery and Nutrition* 8(5):509–511.
- [135] Rector, R.S., Uptergrove, G.M., Morris, E.M., Borengasser, S.J., Laughlin, M.H., Booth, F.W., et al., 2011. Daily exercise vs. caloric restriction for prevention of nonalcoholic fatty liver disease in the OLETF rat model. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 300(5):G874–G883.
- [136] Roberts, C.K., Little, J.P., Thyfault, J.P., 2013. Modification of insulin sensitivity and glycemic control by activity and exercise. *Medicine & Science in Sports & Exercise* 45(10):1868–1877.
- [137] Zhang, H.J., He, J., Pan, L.L., Ma, Z.M., Han, C.K., Chen, C.S., et al., 2016. Effects of moderate and vigorous exercise on nonalcoholic fatty liver disease: a randomized clinical trial. *JAMA Internal Medicine* 176(8):1074–1082.
- [138] ACOG Committee Opinion No. 650, 2015. Physical activity and exercise during pregnancy and the postpartum period. *Obstetrics & Gynecology* 126(6):e135–e142.
- [139] Harrison, A.L., Shields, N., Taylor, N.F., Frawley, H.C., 2016. Exercise improves glycaemic control in women diagnosed with gestational diabetes mellitus: a systematic review. *Journal of Physiotherapy* 62(4):188–196.
- [140] Prather, H., Spitznagle, T., Hunt, D., 2012. Benefits of exercise during pregnancy. *PM sR* 4(11):845–850 quiz 850.

- [141] Fletcher, J.A., Linden, M.A., Sheldon, R.D., Meers, G.M., Morris, E.M., Butterfield, A., et al., 2016. Fibroblast growth factor 21 and exercise-induced hepatic mitochondrial adaptations. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 310(10):G832–G843.
- [142] Fletcher, J.A., Meers, G.M., Linden, M.A., Kearney, M.L., Morris, E.M., Thyfault, J.P., et al., 2014. Impact of various exercise modalities on hepatic mitochondrial function. *Medicine & Science in Sports & Exercise* 46(6): 1089–1097.
- [143] Lezi, E., Lu, J., Burns, J.M., Swerdlow, R.H., 2013. Effect of exercise on mouse liver and brain bioenergetic infrastructures. *Experimental Physiology* 98(1):207–219.
- [144] He, C., Bassik, M.C., Moresi, V., Sun, K., Wei, Y., Zou, Z., et al., 2012. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* 481(7382):511–515.
- [145] Santos-Alves, E., Marques-Aleixo, I., Rizo-Roca, D., Torrella, J.R., Oliveira, P.J., Magalhaes, J., et al., 2015. Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling. *Life Sciences* 135:124–130.
- [146] Ascensao, A., Goncalves, I.O., Lumini-Oliveira, J., Marques-Aleixo, I., Dos Passos, E., Rocha-Rodrigues, S., et al., 2012. Endurance training and chronic intermittent hypoxia modulate in vitro salicylate-induced hepatic mitochondrial dysfunction. *Mitochondrion* 12(6):607–616.
- [147] Sun, L., Shen, W., Liu, Z., Guan, S., Liu, J., Ding, S., 2010. Endurance exercise causes mitochondrial and oxidative stress in rat liver: effects of a combination of mitochondrial targeting nutrients. *Life Sciences* 86(1–2):39–44.
- [148] Lima, F.D., Stamm, D.N., Della-Pace, I.D., Dobrachinski, F., de Carvalho, N.R., Royes, L.F., et al., 2013. Swimming training induces liver mitochondrial adaptations to oxidative stress in rats submitted to repeated exhaustive swimming bouts. *PLoS One* 8(2):e55668.
- [149] Linden, M.A., Fletcher, J.A., Morris, E.M., Meers, G.M., Kearney, M.L., Crissey, J.M., et al., 2014. Combining metformin and aerobic exercise training in the treatment of type 2 diabetes and NAFLD in OLETF rats. *American Journal of Physiology. Endocrinology and Metabolism* 306(3): E300–E310.
- [150] Lee, J.H., Friso, S., Choi, S.W., 2014. Epigenetic mechanisms underlying the link between non-alcoholic fatty liver diseases and nutrition. *Nutrients* 6(8): 3303–3325.
- [151] Murphy, S.K., Yang, H., Moylan, C.A., Pang, H., Dellinger, A., Abdelmalek, M.F., et al., 2013. Relationship between methylome and transcriptome in patients with nonalcoholic fatty liver disease. *Gastroenterology* 145(5):1076–1087.
- [152] Zeybel, M., Hardy, T., Robinson, S.M., Fox, C., Anstee, Q.M., Ness, T., et al., 2015. Differential DNA methylation of genes involved in fibrosis progression in non-alcoholic fatty liver disease and alcoholic liver disease. *Clinical Epigenetics* 7:25.
- [153] Pirola, C.J., Fernandez Gianotti, T., Castano, G.O., Mallardi, P., San Martino, J., Mora Gonzalez Lopez Ledesma, M., et al., 2015. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. *Gut* 64(5):800–812.
- [154] Cheung, O., Puri, P., Eicken, C., Contos, M.J., Mirshahi, F., Maher, J.W., et al., 2008. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 48(6):1810–1820.
- [155] Alisi, A., Da Sacco, L., Bruscalupi, G., Piemonte, F., Panera, N., De Vito, R., et al., 2011. Mimome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. *Laboratory Investigation* 91(2):283–293.
- [156] Cheung, O., Sanyal, A.J., 2008. Abnormalities of lipid metabolism in nonalcoholic fatty liver disease. *Seminars in Liver Disease* 28(4):351–359.
- [157] Sookoian, S., Rosselli, M.S., Gemma, C., Burgueno, A.L., Fernandez Gianotti, T., Castano, G.O., et al., 2010. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter. *Hepatology* 52(6):1992–2000.
- [158] Schmidt, S.F., Mandrup, S., 2011. Gene program-specific regulation of PGC-1[alpha] activity. *Genes & Development* 25(14):1453–1458.
- [159] Ahrens, M., Ammerpohl, O., von Schonfels, W., Kolarova, J., Bens, S., Itzel, T., et al., 2013. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. *Cell Metabolism* 18(2):296–302.
- [160] Aharoni-Simon, M., Hann-Obercyger, M., Pen, S., Madar, Z., Tirosh, O., 2011. Fatty liver is associated with impaired activity of PPARgamma-coactivator 1alpha (PGC1alpha) and mitochondrial biogenesis in mice. *Laboratory Investigation* 91(7):1018–1028.
- [161] Estall, J.L., Kahn, M., Cooper, M.P., Fisher, F.M., Wu, M.K., Laznik, D., et al., 2009. Sensitivity of lipid metabolism and insulin signaling to genetic alterations in hepatic peroxisome proliferator-activated receptor-gamma coactivator-1alpha expression. *Diabetes* 58(7):1499–1508.
- [162] Kren, B.T., Wong, P.Y., Sarver, A., Zhang, X., Zeng, Y., Steer, C.J., 2009. MicroRNAs identified in highly purified liver-derived mitochondria may play a role in apoptosis. *RNA Biology* 6(1):65–72.
- [163] Bian, Z., Li, L.M., Tang, R., Hou, D.X., Chen, X., Zhang, C.Y., et al., 2010. Identification of mouse liver mitochondria-associated miRNAs and their potential biological functions. *Cell Research* 20(9):1076–1078.
- [164] Sripatha, L., Tomar, D., Prajapati, P., Singh, R., Singh, A.K., Singh, R., 2012. Systematic analysis of small RNAs associated with human mitochondria by deep sequencing: detailed analysis of mitochondrial associated miRNA. *PLoS One* 7(9):e44873.
- [165] Iacobazzi, V., Castegna, A., Infantino, V., Andria, G., 2013. Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. *Molecular Genetics and Metabolism* 110(1–2):25–34.
- [166] Pirola, C.J., Gianotti, T.F., Burgueno, A.L., Rey-Funes, M., Loidl, C.F., Mallardi, P., et al., 2013. Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* 62(9):1356–1363.
- [167] Dudley, K.J., Sloboda, D.M., Connor, K.L., Beltrand, J., Vickers, M.H., 2011. Offspring of mothers fed a high fat diet display hepatic cell cycle inhibition and associated changes in gene expression and DNA methylation. *PLoS One* 6(7):e21662.
- [168] Keleher, M.R., Zaidi, R., Shah, S., Oakley, M.E., Pavlatos, C., El Idrissi, S., et al., 2018. Maternal high-fat diet associated with altered gene expression, DNA methylation, and obesity risk in mouse offspring. *PLoS One* 13(2): e0192606.
- [169] Seki, Y., Suzuki, M., Guo, X., Glenn, A.S., Vuguin, P.M., Fiallo, A., et al., 2017. In utero exposure to a high-fat diet programs hepatic hypermethylation and gene dysregulation and development of metabolic syndrome in male mice. *Endocrinology* 158(9):2860–2872.
- [170] Petropoulos, S., Guillemin, C., Ergaz, Z., Dimov, S., Suderman, M., Weinstein-Fudim, L., et al., 2015. Gestational diabetes alters offspring DNA methylation profiles in human and rat: identification of key pathways involved in endocrine system disorders, insulin signaling, diabetes signaling, and ILK signaling. *Endocrinology* 156(6):2222–2238.
- [171] Ruchat, S.M., Houde, A.A., Voisin, G., St-Pierre, J., Perron, P., Baillargeon, J.P., et al., 2013. Gestational diabetes mellitus epigenetically affects genes predominantly involved in metabolic diseases. *Epigenetics* 8(9):935–943.
- [172] Zhou, D., Hlady, R.A., Schafer, M.J., White, T.A., Liu, C., Choi, J.H., et al., 2017. High fat diet and exercise lead to a disrupted and pathogenic DNA methylome in mouse liver. *Epigenetics* 12(1):55–69.
- [173] Barres, R., Yan, J., Egan, B., Treebak, J.T., Rasmussen, M., Fritz, T., et al., 2012. Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism* 15(3):405–411.

Review

- [174] Xu, T., Liu, Q., Yao, J., Dai, Y., Wang, H., Xiao, J., 2015. Circulating microRNAs in response to exercise. *Scandinavian Journal of Medicine & Science in Sports* 25(2):e149–e154.
- [175] Xiao, J., Bei, Y., Liu, J., Dimitrova-Shumkovska, J., Kuang, D., Zhou, Q., et al., 2016. miR-212 downregulation contributes to the protective effect of exercise against non-alcoholic fatty liver via targeting FGF-21. *Journal of Cellular and Molecular Medicine* 20(2):204–216.
- [176] Lu, Y.L., Jing, W., Feng, L.S., Zhang, L., Xu, J.F., You, T.J., et al., 2014. Effects of hypoxic exercise training on microRNA expression and lipid metabolism in obese rat livers. *Journal of Zhejiang University - Science B* 15(9):820–829.

CHAPTER III

Experimental work

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

Jelena Stevanović-Silva¹, Jorge Beleza², Pedro Coxito¹, Susana Pereira^{1,3}, Hugo Rocha⁴, Tiago Bordeira Gaspar^{5,6,7,8}, Fátima Gärtner^{5,9,10}, Rossana Correia^{11,12}, Maria João Martins^{5,13}, Tiago Guimarães^{13,14}, Sandra Martins^{14,15}, Paulo Oliveira³, António Ascensão¹, José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, 4200-450, Porto, Portugal;

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain;

³ CNC — Center for Neuroscience and Cell Biology, UC-Biotech, University of Coimbra, 3060-197, Cantanhede, Portugal;

⁴ Newborn Screening, Metabolism and Genetics Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-053, Porto, Portugal;

⁵ Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135 Porto, Portugal;

- ⁶ Cancer Signalling and Metabolism Group, Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), 4200-135 Porto, Portugal;
- ⁷ Medical Faculty of University of Porto (FMUP), 4200-139 Porto, Portugal;
- ⁸ Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto, 4050-313, Porto, Portugal;
- ⁹ Department of Molecular Pathology and Immunology, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313, Porto, Portugal
- ¹⁰ Glycobiology in Cancer Group, Institute of Molecular Pathology and Immunology of University of Porto (Ipatimup), University of Porto, 4200-135, Porto, Portugal
- ¹¹ HEMS — Histology and Electron Microscopy Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135, Porto, Portugal;
- ¹² Ipatimup — Institute of Molecular Pathology and Immunology of the University of Porto, 4200-135, Porto, Portugal;
- ¹³ Department of Biomedicine, Biochemistry Unit, Faculty of Medicine, University of Porto, 4200-319, Porto, Portugal;
- ¹⁴ Department of Clinical Pathology, São João Hospital Centre, EPE, 4200-319, Porto, Portugal;
- ¹⁵ EPIUnit, Institute of Public Health, University of Porto, 4050-091 Porto, Portugal.

Published on: *Metabolism – Clinical and Experimental*,
2021;116:154704.

doi: 10.1016/j.metabol.2021.154704



Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

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



Jelena Stevanović-Silva^{a,*}, Jorge Beleza^b, Pedro Coxito^a, Susana Pereira^{a,c}, Hugo Rocha^d,
Tiago Bordeira Gaspar^{e,f,g,h}, Fátima Gärtner^{e,i,j}, Rossana Correia^{k,l}, Maria João Martins^{e,m}, Tiago Guimarães^{m,n},
Sandra Martins^{n,o}, Paulo J. Oliveira^c, António Ascensão^a, José Magalhães^a

^a Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sport, University of Porto, 4200-450, Porto, Portugal

^b Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

^c CNC – Center for Neuroscience and Cell Biology, UC-Biotech, University of Coimbra, 3060-197 Cantanhede, Portugal

^d Newborn Screening, Metabolism and Genetics Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-053 Porto, Portugal

^e Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135 Porto, Portugal

^f Cancer Signalling and Metabolism Group, Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), 4200-135 Porto, Portugal

^g Medical Faculty of University of Porto (FMUP), 4200-139 Porto, Portugal

^h Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto, 4050-313 Porto, Portugal

ⁱ Department of Molecular Pathology and Immunology, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313, Porto, Portugal

^j Glycobiology in Cancer Group, Institute of Molecular Pathology and Immunology of University of Porto (Ipatimup), University of Porto, 4200-135 Porto, Portugal

^k HEMS – Histology and Electron Microscopy Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135, Porto, Portugal

^l Ipatimup – Institute of Molecular Pathology and Immunology of the University of Porto, 4200-135 Porto, Portugal

^m Department of Biomedicine, Biochemistry Unit, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal

ⁿ Department of Clinical Pathology, São João Hospital Centre, EPE, 4200-319 Porto, Portugal

^o EPIUnit, Institute of Public Health, University of Porto, 4050-091 Porto, Portugal

ARTICLE INFO

Article history:

Received 6 November 2020

Accepted 27 December 2020

Keywords:

Pregnancy

Physical activity

Fetal programming

Liver mitochondria

Gestational diabetes

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.

© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 phosphorylation; RCR, respiratory control ratio; S/R, succinate/rotenone; T2DM, type 2 diabetes mellitus; TG, triglycerides; UCP2, uncoupling protein-2.

* Corresponding author at: R. Dr. Plácido da Costa 91, 4200-450 Porto, Portugal.

E-mail address: jela.stevanov@gmail.com (J. Stevanović-Silva).

<https://doi.org/10.1016/j.metabol.2021.154704>

0026-0495/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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'Arbresle, 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'Arbresle, 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 Veterinária* – 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-

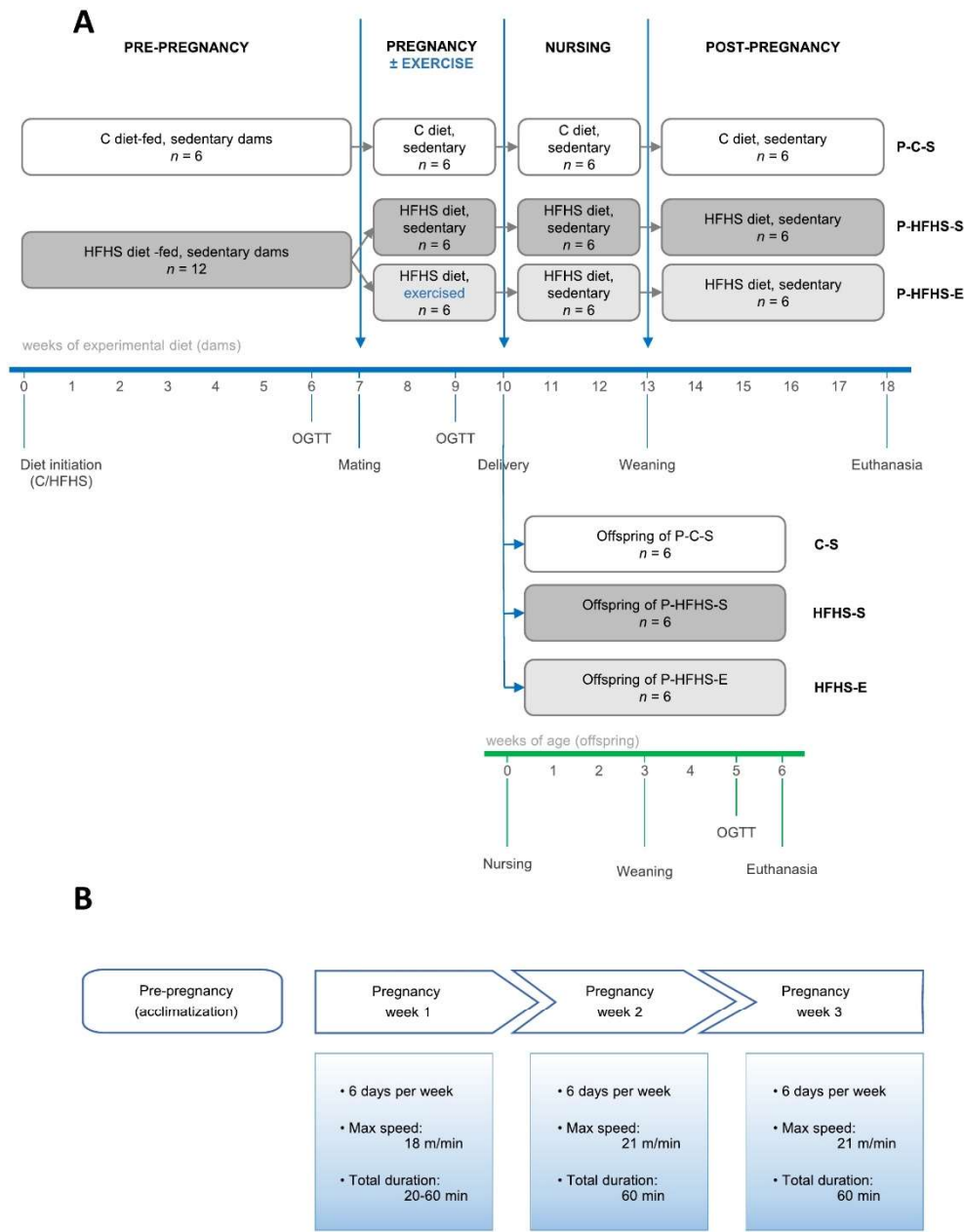


Fig. 1. Experimental (A) and exercise (B) protocol. C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; P – pregnant animals in maternal generation; S – sedentary animals in maternal generation; E – exercising animals in maternal generation. OGTT – oral glucose tolerance test.

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 O₂; maintenance: 2.5% isoflurane, 0.4 L/m O₂) 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 butyl esters by incubation with 60 µL of 3 N butanolic 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 KH_2PO_4 , 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 $\pm$ 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

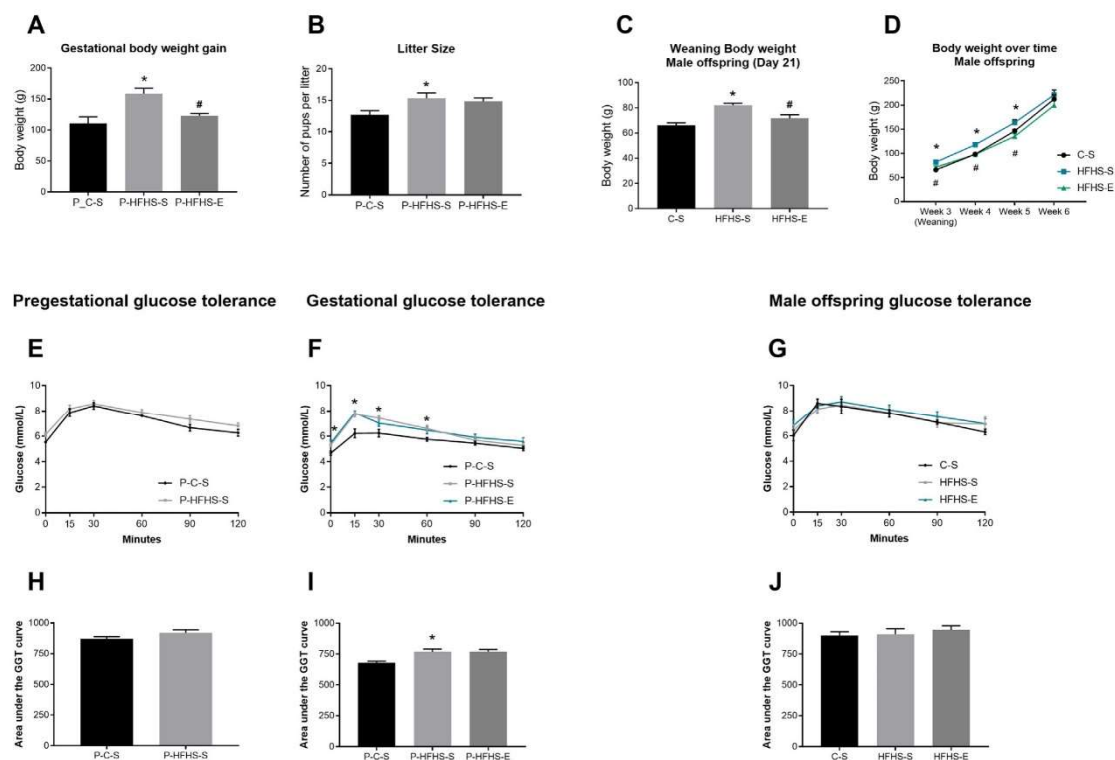


Fig. 2. Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal body weight gain during gestation period (A), litter size (B) and offspring body weight changes from weaning (C) to the euthanasia point at 6 weeks of age (D), maternal glucose tolerance before pregnancy (E, H) 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 $\pm$ 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.

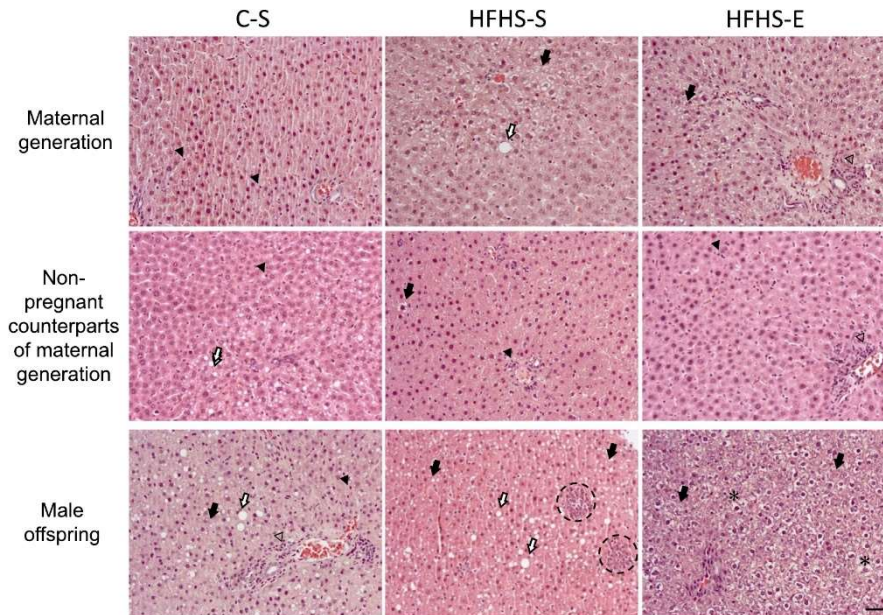


Fig. 3. Representative light micrographs ($\times 200$) of liver tissue from all groups and generations: C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. The presence of microvesicular steatosis (black arrow), macrovesicular 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.

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

Values are means $\pm$ 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 not 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

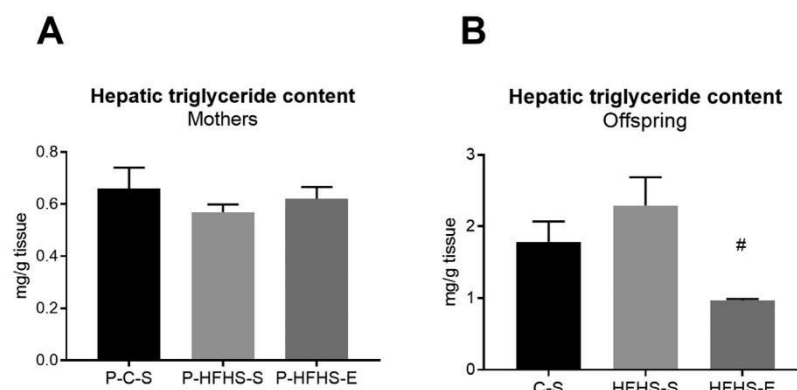


Fig. 4. Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal (A) and offspring (B) liver triglyceride content; P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised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

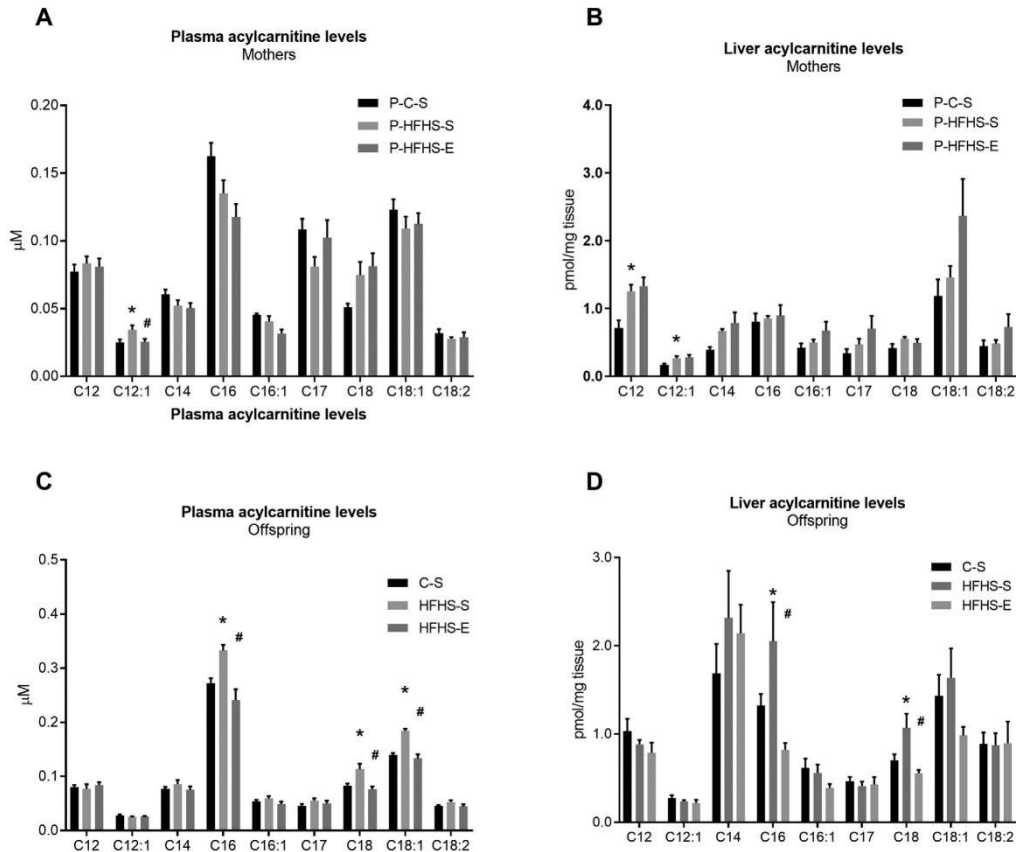


Fig. 5. Effect of maternal high-fat high-sucrose diet and gestational exercise on acylcarnitine levels in (A) plasma and (B) liver in the maternal generation on acylcarnitine levels in plasma (C) and liver (D) in the offspring generation. C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means $\pm$ 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.

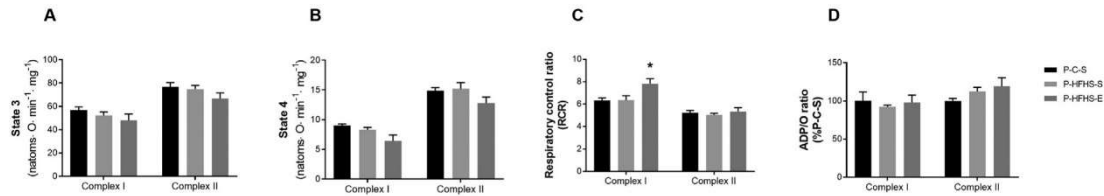
Glucose intolerance during pregnancy is another important marker of GDM. To confirm that GDM-related glucose intolerance was developed only at the onset of the pregnancy and not before, we performed OGTT before and during pregnancy. The HFHS-diet consumption before

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].

Mothers



Offspring

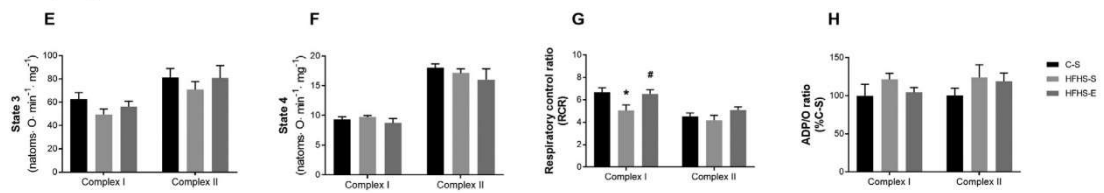


Fig. 6. Effect of maternal high-fat high-sucrose diet and gestational exercise on liver mitochondria (A) state 3 respiration, (B) state 4 respiration, (C), respiratory control ratio (RCR), and (D) ADP/O ratio in the maternal generation; and (E) state 3 respiration, (F) state 4 respiration, (G), respiratory control ratio (RCR), and (H) ADP/O ratio in the offspring 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 $\pm$ 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, fibroblast 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-

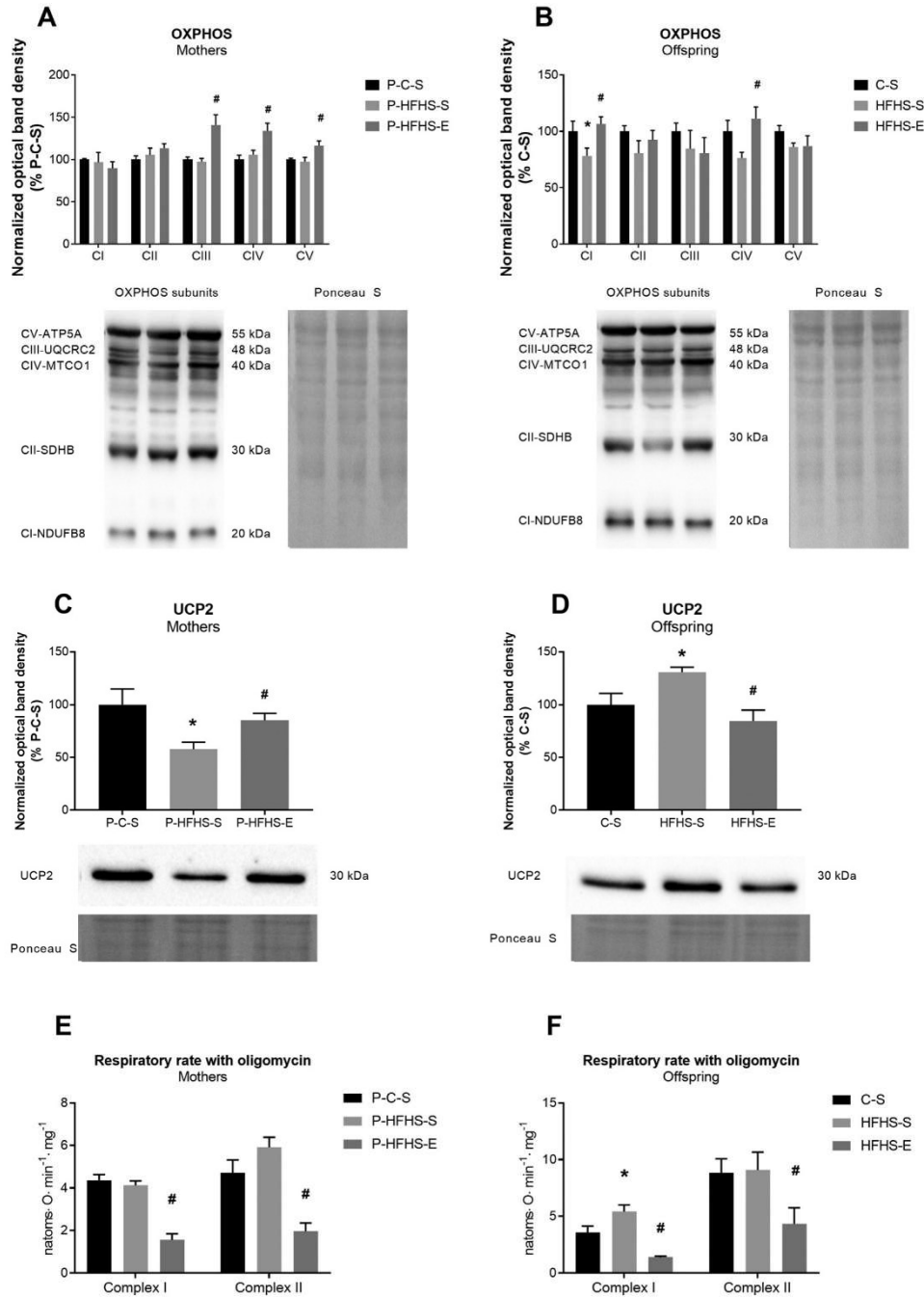


Fig. 7. Effect of maternal high-fat high-sucrose diet and gestational exercise on OXPHOS subunits in (A) maternal generation and (B) offspring generation and UCP2 content in (C) maternal generation and (D) offspring generation; and respiratory rate with oligomycin in (E) maternal generation and (F) offspring 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 $\pm$ 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.

PPAR α -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 mitochondrial 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>.

Funding

This work was supported by the EU's Horizon 2020 Research and Innovation program under the Marie Skłodowska-Curie Actions (No.722619, FOIE GRAS; No.734719, mtFOIE GRAS) and by the Portuguese Foundation for Science and Technology (FCT) (UIDB/00617/2020-base; POCI-01-0145-FEDER-016690-PTDC/DTP-DES/7087/2014; POCI-01-0145-FEDER-016657-PTDC/DTP-DES/1082/2014), to JB (SFRH/BD/129645/2017), to SP (SFRH/BPD/116061/2016).

CRediT authorship contribution statement

Jelena Stevanović Silva: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing,

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.

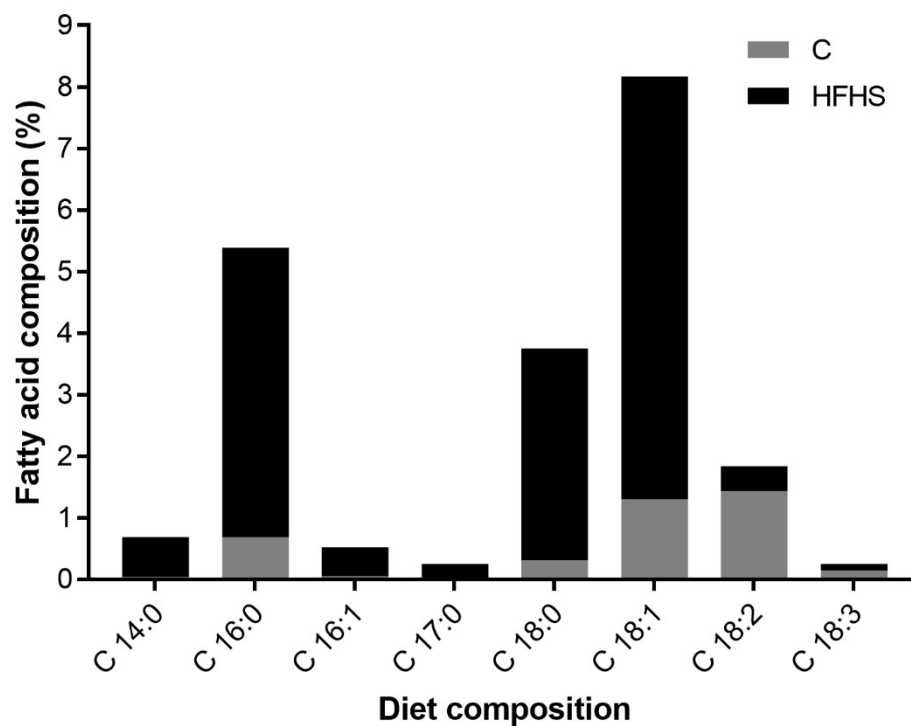
Declaration of competing interest

No conflict of interest.

References

- [1] Saklayen MG. The global epidemic of the metabolic syndrome. *Curr Hypertens Rep*. 2018;20:12. <https://doi.org/10.1007/s11906-018-0812-z>.
- [2] Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol*. 2018;15:11–20. <https://doi.org/10.1038/nrgastro.2017.109>.
- [3] World Health Organization. Global Report on Diabetes. 2016.
- [4] Ajmera VH, Gunderson EP, VanWagner LB, Lewis CE, Carr JJ, Terrault NA. Gestational diabetes mellitus is strongly associated with non-alcoholic fatty liver disease. *Am J Gastroenterol*. 2016;111:658–64. <https://doi.org/10.1038/ajg.2016.57>.
- [5] Donnelly SR, Hinkle SN, Rawal S, Grunnet LG, Chavarro JE, Vaag A, et al. Prospective study of gestational diabetes and fatty liver scores 9 to 16 years after pregnancy. *J Diabetes*. 2019;11:895–905. <https://doi.org/10.1111/1753-0407.12934>.
- [6] Mehmood S, Margolis M, Ye C, Maple-Brown L, Hanley AJ, Connelly PW, et al. Hepatic fat and glucose tolerance in women with recent gestational diabetes. *BMJ Open Diabetes Res Care*. 2018;6:e000549. <https://doi.org/10.1136/bmjdc-2018-000549>.
- [7] Tan EK, Tan EL. Alterations in physiology and anatomy during pregnancy. *Best Pract Res Clin Obstet Gynaecol*. 2013;27:791–802. <https://doi.org/10.1016/j.bpobgyn.2013.08.001>.
- [8] Pereira TJ, Moyce BL, Kereliuk SM, Dolinsky VW. Influence of maternal overnutrition and gestational diabetes on the programming of metabolic health outcomes in the offspring: experimental evidence. *Biochemistry and cell biology = Biochimie et biologie cellulaire*. 2015;93:438–51. <https://doi.org/10.1139/bcb-2014-0141>.
- [9] Patel KR, White FV, Deutsch GH. Hepatic steatosis is prevalent in stillborns delivered to women with diabetes mellitus. *J Pediatr Gastroenterol Nutr*. 2015;60:152–8. <https://doi.org/10.1097/mpg.0000000000000520>.
- [10] Pereira TJ, Fonseca MA, Campbell KE, Moyce BL, Cole LK, Hatch GM, et al. Maternal obesity characterized by gestational diabetes increases the susceptibility of rat offspring to hepatic steatosis via a disrupted liver metabolome. *J Physiol*. 2015;593:3181–97. <https://doi.org/10.1113/jp270429>.
- [11] Brawerman GM, Dolinsky VW. Therapies for gestational diabetes and their implications for maternal and offspring health: evidence from human and animal studies. *Pharmacol Res*. 2018;130:52–73. <https://doi.org/10.1016/j.phrs.2018.02.002>.
- [12] Gonzalez CD, Alvarinas J, Bolanos R, Di Girolamo G. Hepatic elimination of drugs in gestational diabetes. *Curr Clin Pharmacol*. 2018;13:21–7. <https://doi.org/10.2174/1574884713666180326104613>.
- [13] Committee Opinion No ACOG. 650: physical activity and exercise during pregnancy and the postpartum period. *Obstet Gynecol*. 2015;126:e135–42. <https://doi.org/10.1097/aog.0000000000001214>.
- [14] Gonçalves IO, Maciel E, Passos E, Torrella JR, Rizo D, Viscor G, et al. Exercise alters liver mitochondria phospholipidomic profile and mitochondrial activity in non-alcoholic steatohepatitis. *Int J Biochem Cell Biol*. 2014;54:163–73. <https://doi.org/10.1016/j.biocel.2014.07.011>.
- [15] Gonçalves IO, Passos E, Rocha-Rodrigues S, Diogo CV, Torrella JR, Rizo D, et al. Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion*. 2014;15:40–51. <https://doi.org/10.1016/j.mito.2014.03.012>.
- [16] Rector RS, Uptergrove GM, Morris EM, Borengasser SJ, Laughlin MH, Booth FW, et al. Daily exercise vs. caloric restriction for prevention of nonalcoholic fatty liver disease in the OLETF rat model. *Am J Physiol Gastrointest Liver Physiol*. 2011;300:G874–83. <https://doi.org/10.1152/ajpgi.00510.2010>.
- [17] Sheldon RD, Nicole Blaize A, Fletcher JA, Pearson KJ, Donkin SS, Newcomer SC, et al. Gestational exercise protects adult male offspring from high-fat diet-induced hepatic steatosis. *J Hepatol*. 2016;64:171–8. <https://doi.org/10.1016/j.jhep.2015.08.022>.
- [18] Stanford KI, Takahashi H, So K, Alves-Wagner AB, Prince NB, Lehnig AC, et al. Maternal exercise improves glucose tolerance in female offspring. *Diabetes*. 2017;66:2124–36. <https://doi.org/10.2337/db17-0098>.
- [19] Wasinski F, Bacurau RF, Estrela GR, Klempin F, Arakaki AM, Batista RO, et al. Exercise during pregnancy protects adult mouse offspring from diet-induced obesity. *Nutrition & metabolism*. 2015;12:56. <https://doi.org/10.1186/s12986-015-0052-z>.
- [20] Gusdon AM, Song KX, Qu S. Nonalcoholic fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxid Med Cell Longev*. 2014;2014:637027. <https://doi.org/10.1155/2014/637027>.
- [21] Stevanović J, Beleza J, Coxito P, Ascensão A, Magalhães J. Physical exercise and liver “fitness”: role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease. *Molecular metabolism*. 2020;32:1–14. <https://doi.org/10.1016/j.molmet.2019.11.015>.
- [22] Zhang L. Voluntary oral administration of drugs in mice. *Protocol Exchange*. 2011;10.
- [23] Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, et al. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PLoS one*. 2014;9:e115922. <https://doi.org/10.1371/journal.pone.0115922>.
- [24] Rashed MS, Ozand PT, Bucknall MP, Little D. Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry. *Pediatr Res*. 1995;38:324–31. <https://doi.org/10.1203/00006450-199509000-00009>.
- [25] Petucci C, Rojas-Betancourt S, Gardell SJ. Comparison of tissue harvest protocols for the quantitation of acylcarnitines in mouse heart and liver by mass spectrometry. *Metabolomics*. 2012;8:784–92. <https://doi.org/10.1007/s11306-011-0370-8>.
- [26] Romero-Calvo I, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustín O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem*. 2010;401:318–20. <https://doi.org/10.1016/j.ab.2010.02.036>.
- [27] Estrany ME, Piroz AM, Lladó I, Gianotti M. Isocaloric intake of a high-fat diet modifies adiposity and lipid handling in a sex dependent manner in rats. *Lipids Health Dis*. 2011;10:52. <https://doi.org/10.1186/1476-511x-10-52>.
- [28] Effect of diet and physical activity based interventions in pregnancy on gestational weight gain and pregnancy outcomes: meta-analysis of individual participant data from randomised trials. *BMJ (Clinical research ed)*. 2017;358:j3119. <https://doi.org/10.1136/bmj.j3119>.
- [29] Wang J, Wen D, Liu X, Liu Y. Impact of exercise on maternal gestational weight gain: an updated meta-analysis of randomized controlled trials. *Medicine*. 2019;98:e16199. <https://doi.org/10.1097/md.00000000000016199>.
- [30] Carter LG, Ngo Tenlep SY, Woollett LA, Pearson KJ. Exercise improves glucose disposal and insulin signaling in pregnant mice fed a high fat diet. *Journal of diabetes & metabolism*. 2015;6. <https://doi.org/10.4172/2155-156.1000634>.
- [31] Smooty J, Larcombe AN, Chivers EK, Matthews VB, Gorman S. Maternal high fat diet compromises survival and modulates lung development of offspring, and impairs lung function of dams (female mice). *Respir Res*. 2019;20:21. <https://doi.org/10.1186/s12931-019-0976-3>.
- [32] Suarez-Trujillo A, Chen Y, Aduwari C, Cummings S, Kuang S, Buhman KK, et al. Maternal high-fat diet exposure during gestation, lactation, or gestation and lactation differentially affects intestinal morphology and proteome of neonatal mice. *Nutrition research (New York, NY)*. 2019;66:48–60. <https://doi.org/10.1016/j.nutres.2019.03.014>.
- [33] Morris DW. State-dependent optimization of litter size. *Oikos*. 1998;83:518–28. <https://doi.org/10.2307/3546679>.
- [34] Herrera E, Desoye G. Maternal and fetal lipid metabolism under normal and gestational diabetic conditions. *Horm Mol Biol Clin Invest*. 2016;26:109–27. <https://doi.org/10.1515/hmbci-2015-0025>.
- [35] Kc K, Shakya S, Zhang H. Gestational diabetes mellitus and macrosomia: a literature review. *Ann Nutr Metab*. 2015;66(Suppl. 2):14–20. <https://doi.org/10.1159/000371628>.
- [36] Straczkowski M, Kowalska I, Dzienis-Straczowska S, Kinalski M, Górski J, Kinalska I. The effect of exercise training on glucose tolerance and skeletal muscle triacylglycerol content in rats fed with a high-fat diet. *Diabetes Metab*. 2001;27:19–23.
- [37] Visiedo F, Bugatto F, Sánchez V, Cózar-Castellano I, Bartha JL, Perdomo G. High glucose levels reduce fatty acid oxidation and increase triglyceride accumulation in human placenta. *Am J Physiol Endocrinol Metab*. 2013;305:E205–12. <https://doi.org/10.1152/ajpendo.00032.2013>.
- [38] Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology (Baltimore, Md)*. 2009;50:1796–808. <https://doi.org/10.1002/hep.23205>.
- [39] Kruse M, Seki Y, Vuguin PM, Du XQ, Fiallo A, Glenn AS, et al. High-fat intake during pregnancy and lactation exacerbates high-fat diet-induced complications in male offspring in mice. *Endocrinology*. 2013;154:3565–76. <https://doi.org/10.1210/en.2012-1877>.
- [40] Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*. 2008;7:45–56. <https://doi.org/10.1016/j.cmet.2007.10.013>.
- [41] Zabielski P, Hady HR, Chacinska M, Roszczyc K, Gorski J, Blachnio-Zabielska AU. The effect of high fat diet and metformin treatment on liver lipids accumulation and their impact on insulin action. *Sci Rep*. 2018;8:7249. <https://doi.org/10.1038/s41598-018-25397-6>.
- [42] Grijalva J, Vakili K. Neonatal liver physiology. *Semin Pediatr Surg*. 2013;22:185–9. <https://doi.org/10.1053/j.sempedsurg.2013.10.006>.

- [43] Xu G, Hansen JS, Zhao XJ, Chen S, Hoene M, Wang XL, et al. Liver and muscle contribute differently to the plasma acylcarnitine pool during fasting and exercise in humans. *J Clin Endocrinol Metab*. 2016;101:5044–52. <https://doi.org/10.1210/jc.2016-1859>.
- [44] Schooneman MG, Ten Have GA, van Vlies N, Houten SM, Deutz NE, Soeters MR. Transorgan fluxes in a porcine model reveal a central role for liver in acylcarnitine metabolism. *Am J Physiol Endocrinol Metab*. 2015;309:E256–64. <https://doi.org/10.1152/ajpendo.00503.2014>.
- [45] Pedersen BK, Steensberg A, Fischer C, Keller C, Keller P, Plomgaard P, et al. Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil*. 2003;24:113–9. <https://doi.org/10.1023/a:1026070911202>.
- [46] Rocha-Rodrigues S, Rodríguez A, Gouveia AM, Gonçalves IO, Becerril S, Ramírez B, et al. Effects of physical exercise on myokines expression and brown adipose-like phenotype modulation in rats fed a high-fat diet. *Life Sci*. 2016;165:100–8. <https://doi.org/10.1016/j.lfs.2016.09.023>.
- [47] Arias-Loste MT, Ranchal I, Romero-Gómez M, Crespo J, Irisin, a link among fatty liver disease, physical inactivity and insulin resistance. *Int J Mol Sci*. 2014;15:23163–78. <https://doi.org/10.3390/ijms151223163>.
- [48] van Vlies N, Ferdinandusse S, Turkenburg M, Wanders RJ, Vaz FM. PPAR alpha-activation results in enhanced carnitine biosynthesis and OCTN2-mediated hepatic carnitine accumulation. *Biochim Biophys Acta*. 1767;2007:1134–42. <https://doi.org/10.1016/j.bbabi.2007.07.001>.
- [49] Borengasser SJ, Lau F, Kang P, Blackburn ML, Ronis MJ, Badger TM, et al. Maternal obesity during gestation impairs fatty acid oxidation and mitochondrial SIRT3 expression in rat offspring at weaning. *PLoS one*. 2011;6:e24068. <https://doi.org/10.1371/journal.pone.0024068>.
- [50] Chung E, Joiner HE, Skelton T, Looten KD, Manczak M, Reddy PH. Maternal exercise upregulates mitochondrial gene expression and increases enzyme activity of fetal mouse hearts. *Physiol Rep*. 2017;5. <https://doi.org/10.14814/phy2.13184>.
- [51] Liu J, Lee I, Feng HZ, Galen SS, Hüttemann PP, Perkins GA, et al. Aerobic exercise pre-conception and during pregnancy enhances oxidative capacity in the hindlimb muscles of mice offspring. *J Strength Cond Res*. 2018;32:1391–403. <https://doi.org/10.1519/jsc.0000000000002416>.
- [52] Siti F, Dubouchaud H, Hininger I, Quiclet C, Vial G, Galinier A, et al. Maternal exercise before and during gestation modifies liver and muscle mitochondria in rat offspring. *J Exp Biol*. 2019;222. <https://doi.org/10.1242/jeb.194969>.
- [53] Villani G, Greco M, Papa S, Attardi G. Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. *J Biol Chem*. 1998;273:31829–36. <https://doi.org/10.1074/jbc.273.48.31829>.
- [54] Beleza J, Albuquerque J, Santos-Alves E, Fonseca P, Santocildes G, Stevanovic J, et al. Self-paced free-running wheel mimics high-intensity interval training impact on rats' functional, physiological, biochemical, and morphological features. *Front Physiol*. 2019;10:593. <https://doi.org/10.3389/fphys.2019.00593>.
- [55] Bayol SA, Simbi BH, Fowkes RC, Stickland NC. A maternal “junk food” diet in pregnancy and lactation promotes nonalcoholic fatty liver disease in rat offspring. *Endocrinology*. 2010;151:1451–61. <https://doi.org/10.1210/en.2009-1192>.
- [56] Serviddio G, Bellanti F, Tamborra R, Rollo T, Capitano N, Romano AD, et al. Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. *Gut*. 2008;57:957–65. <https://doi.org/10.1136/gut.2007.147496>.
- [57] Armstrong MB, Towle HC. Polyunsaturated fatty acids stimulate hepatic UCP-2 expression via a PPARalpha-mediated pathway. *Am J Physiol Endocrinol Metab*. 2001;281:E1197–204. <https://doi.org/10.1152/ajpendo.2001.281.6.E1197>.
- [58] Cardoso AR, Kakimoto PA, Kowaltowski AJ. Diet-sensitive sources of reactive oxygen species in liver mitochondria: role of very long chain acyl-CoA dehydrogenases. *PLoS one*. 2013;8:e77088. <https://doi.org/10.1371/journal.pone.0077088>.
- [59] Català-Niell A, Estrany ME, Proenza AM, Gianotti M, Lladó I. Skeletal muscle and liver oxidative metabolism in response to a voluntary isocaloric intake of a high fat diet in male and female rats. *Cell Physiol Biochem*. 2008;22:327–36. <https://doi.org/10.1159/000149811>.
- [60] Greggio C, Jha P, Kulkarni SS, Lagarrigue S, Broskey NT, Boutant M, et al. Enhanced respiratory chain supercomplex formation in response to exercise in human skeletal muscle. *Cell Metab*. 2017;25:301–11. <https://doi.org/10.1016/j.cmet.2016.11.004>.
- [61] Lapuente-Brun E, Moreno-Loshuertos R, Acín-Pérez R, Latorre-Pellicer A, Colás C, Balsa E, et al. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. *Science (New York, NY)*. 2013;340:1567–70. <https://doi.org/10.1126/science.1230381>.
- [62] Acín-Pérez R, Bayona-Bafaluy MP, Fernández-Silva P, Moreno-Loshuertos R, Pérez-Martos A, Bruno C, et al. Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol Cell*. 2004;13:805–15. [https://doi.org/10.1016/s1097-2765\(04\)00124-8](https://doi.org/10.1016/s1097-2765(04)00124-8).
- [63] Diaz F, Fukui H, Garcia S, Moraes CT. Cytochrome c oxidase is required for the assembly/stability of respiratory complex I in mouse fibroblasts. *Mol Cell Biol*. 2006;26:4872–81. <https://doi.org/10.1128/mcb.01767-05>.
- [64] Cheng G, Polito CC, Haines JK, Shafizadeh SF, Fiorini RN, Zhou X, et al. Decrease of intracellular ATP content downregulated UCP2 expression in mouse hepatocytes. *Biochem Biophys Res Commun*. 2003;308:573–80. [https://doi.org/10.1016/s0006-291x\(03\)01049-8](https://doi.org/10.1016/s0006-291x(03)01049-8).
- [65] Ma SG, Yu WN, Jin Y, Hong B, Hu W. Evaluation of serum ischemia-modified albumin levels in pregnant women with and without gestational diabetes mellitus. *Gynecological endocrinology* : the official journal of the International Society of Gynecological Endocrinology. 2012;28:837–40. <https://doi.org/10.3109/09513590.2012.683069>.
- [66] Westbrook RH, Dusheiko G, Williamson C. Pregnancy and liver disease. *J Hepatol*. 2016;64:933–45. <https://doi.org/10.1016/j.jhep.2015.11.030>.

Experimental study I: Supplementary data

Supplementary data 1 (S1). The difference in fatty acid composition between Control diet (C) and High-fat high-sucrose diet (HFHS).

Gestational exercise antagonizes the impact of maternal high-fat high-sucrose diet on liver mitochondrial alterations and quality control signalling in male offspring

Jelena Stevanović-Silva¹, Jorge Beleza², Pedro Coxito¹, Rui Fernandes³, Paulo J. Oliveira⁴, António Ascensão¹, José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal;

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain;

³ Histology and Electron Microscopy (HEMS), Institute for Research and Innovation in Health Sciences (i3S), University of Porto, Porto, Portugal;

⁴ CNC-Center for Neuroscience and Cell Biology, CIBB - Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, (3004-504) Coimbra, Portugal.

Submitted in: *Cellular and Molecular Life Sciences*

Abstract

Maternal high-caloric nutrition and related gestational diabetes mellitus (GDM) are relevant modulators of the intrauterine environment, increasing the risk for liver metabolic alterations, particularly in mitochondrial bioenergetics and related signalling processes in mothers and offspring. In contrast, as a non-pharmacological approach against metabolic disorders, exercise is highly recommended in GDM treatment. We analysed whether gestational exercise (GE) protects mothers from diet-induced GDM metabolic consequences and mitigates liver mitochondrial deleterious alterations in their 6-week-old male offspring.

Female Sprague-Dawley rats were fed with control or high-fat high-sucrose (HFHS) diet and kept sedentary or submitted to GE. Male offspring were sedentary and fed with control diet.

Sedentary HFHS mothers and their offspring showed impaired hepatic mitochondrial biogenesis and morphological evidence of mitochondrial remodelling. In contrast, GE-related beneficial effects were demonstrated by upregulation of mitochondrial biogenesis signalling markers (peroxisome proliferator-activated receptor-gamma co-activator-1 α , mitochondrial transcription factor A) and mitochondrial fusion proteins (mitofusin 1, mitofusin 2) and downregulation of mitochondrial fission dynamin-related protein 1. Alterations in miR-34a, miR-130b, and miR-494, associated with epigenetic regulation of mitochondrial biogenesis, suggested that GE is a more critical modulator of intergenerational changes in miRs expression than the maternal diet. Auto/mitophagic and apoptotic signalling markers were not substantially affected by maternal diet/GE in the offspring, whereas they were modulated in mothers at the mitochondrial but not tissue level.

In conclusion, GE seems to positively modulate the altered hepatic mitochondrial biogenesis and dynamics markers and quality control signalling associated with maternal HFHS diet-related GDM in mothers and offspring.

Keywords: Gestational diabetes, mitochondrial biogenesis, mitochondrial dynamics, epigenetics, gestational exercise, fetal programming

Abbreviations: C - control diet, ChREBP - carbohydrate response element binding protein, DRP1 - dynamin related protein 1, E – exercised animals; ER α - estrogen receptor alpha, GDM - gestational diabetes mellitus, GE – gestational exercise, HFHS - high-fat high-sucrose diet, IR - insulin resistance, LC3 - microtubule-associated protein-1 light chain 3, MFN1 - mitofusin 1, MFN2 - mitofusin 2, mtDNA - mitochondrial DNA, NAFLD - non-alcoholic fatty liver disease, nDNA - nuclear DNA, OPA1 - optic atrophy 1, OXPHOS - oxidative phosphorylation, P – pregnant animals; PCA - principal component analysis, PEPCK - phosphoenolpyruvate carboxykinase, PINK1 - PTEN Induced Kinase 1, PGC-1 α - peroxisome proliferator-activated receptor gamma co-activator-1alpha, S – sedentary animals; SREBP-1 - sterol regulator element binding protein 1, TFAM - mitochondrial transcription factor A, TOM20 - translocase of outer membrane 20.

INTRODUCTION

The intrauterine environment can disturb susceptible cell proliferation and differentiation processes, leading to some (ir)reversible alterations in embryonal and fetal development. Maternal nutrition alterations result in a specific in utero milieu that strongly impacts the offspring's early life development. Moreover, the nutritional environment during pre-conception and pregnancy can be a significant risk factor for metabolic disorders in adulthood [1]. Exposure to maternal hyperglycemia and insulin resistance (IR) in pregnancy, characteristic of gestational diabetes mellitus (GDM), represents a risk factor for the development of various metabolic disturbances in the offspring, including obesity, adiposity, altered glucose metabolism, hypertension, and non-alcoholic fatty liver disease (NAFLD) [2]. Also, the increased metabolic risk may be transmitted to the following three generations [3]. Maternal metabolic status can program skeletal muscle mitochondrial dysfunction across three generations, probably through germline alterations and aberrant oocytes [4]. Given the essential role of mitochondria in metabolism and cellular quality control signalling, mitochondrial dysfunction may be one of the first events characterizing the onset of metabolic diseases. Indeed, mitochondrial impairment has been observed in liver tissue under deleterious conditions of IR, diabetes, NAFLD, or drug-induced liver injury [5-7]. Importantly, mitochondrial inheritance tends to be almost exclusively uniparental. Although paternal mitochondrial DNA (mtDNA) can be transferred to the offspring in some exceptional cases, maternal inheritance remains more dominant [8, 9]. This sheds light on the importance of maternal mitochondrial health at determining offspring mitochondrial fate, thereby influencing the phenotype of future generations.

However, data on GDM-related alterations in liver mitochondrial function of mothers and offspring are limited. Mitochondrial biogenesis, mitophagy, and dynamic processes modulate mitochondrial function to maintain mitochondrial homeostasis and plasticity to adapt to metabolic demands. These processes can be compromised in IR, diabetes, and liver diseases [10, 11]. Despite similar pathophysiological mechanisms between GDM and non-gestational diabetes, characterized by IR and hyperglycaemia, the context of pregnancy probably

brings a new aspect on underlying mechanisms, which urges a need for research on GDM-related pathophysiology. Since GDM consequences are potentially transmitted across generations, their management becomes an important matter to be considered. Although some pharmacological approaches against GDM are recommended, lifestyle changes involving nutritional and physical exercise habits are considered essential first-line non-pharmacological preventive and therapeutic approaches against GDM [12, 13]. In fact, physical exercise has been recommended as a beneficial strategy against diabetes and liver diseases [11, 14, 15]. Exercise has also been proposed to regulate the mitochondrial life cycle by enhancing mitochondrial biogenesis and dynamics, and thus improving metabolic function [16]. Using this same diet-induced GDM model, we previously reported that gestational exercise (GE) antagonized some of the impairments observed in mitochondrial function from GDM mothers, and that these benefits were maintained for a prolonged period after exercise training cessation. Moreover, GE diminished the adverse effects of maternal diet on mitochondrial respiration and prevented lipid accumulation in male offspring liver [17]. However, some signalling processes related to mitochondrial physiology, namely mitochondrial biogenesis and dynamics, (auto)mitophagy and apoptotic signaling that are usually pivotal at determining the hepatic protective effects of exercise against in NAFLD models, are still to be elucidated in mothers with GDM and, particularly, their offspring. Therefore, in the present study, we aimed to better understand the relevance of an GE against GDM-induced deleterious consequences in both generations. We investigated effects of maternal diet and GE on liver mitochondrial dynamics and biogenesis as well as quality control signalling in male offspring and their eventual regulation through microRNA mechanisms. Additionally, we followed up the effects of diet-induced GDM and GE on liver mitochondrial alterations in mothers 8 weeks after the pregnancy and exercise cessation.

MATERIAL AND METHODS

Animal model

Female 7-week-old Sprague-Dawley rats were randomly divided into two diet groups: control (C) and high-fat, high-sucrose (HFHS) diet (E157452-047 and D12451 (II) mod., Ssniff, Soest, Germany) group. After 7 weeks, dams were mated, and pregnant (P) HFHS-fed dams divided into sedentary animals (S) and animals submitted to exercise protocol during pregnancy (E). This resulted in three experimental groups (n = 6): P-C-S: sedentary C-fed dams; P-HFHS-S: sedentary HFHS-fed dams; and P-HFHS-E: exercised HFHS-fed dams. The P-HFHS-E dams were exercised on a motor-driven treadmill (LE8700, Panlab Harvard Apparatus, Holliston, USA) 6 days/week, 40-60 min/day for 3 weeks, as previously described [17], and additionally, they had access to the free-running wheels during pregnancy. The sedentary groups were placed on a static treadmill to be exposed to the same environmental conditions. Dams were delivered naturally, and the offspring was weaned at 3 weeks of age. Male pups (one from each litter) were assigned to three different experimental groups depending on the maternal treatment (n = 6): C-S: offspring of C-fed sedentary dams; HFHS-S: offspring of HFHS-fed sedentary dams; and HFHS-E: offspring of HFHS-fed exercised mothers. All the offspring were C-fed and sedentary. Dams were sacrificed at 8 weeks after delivery and the offspring at 6 weeks of age.

All animals were housed in a normal environment (21-22°C; 50-60% humidity, 12h light/dark cycles), receiving food and water ad libitum. 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 Veterinária; No.0421/000/000/2018) approved the experimental protocol, 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.

Animal sacrifice and tissue sampling

Animals were anaesthetized (induction at 5% isoflurane, 1 L/m O₂; maintaining at 2.5% isoflurane, 0.4 L/m O₂) after overnight fasting. The liver was rapidly

excised, rinsed with ice-cold PBS, weighed, separated for microscopy, and mitochondria isolation. Mitochondria were isolated as previously described [17]. Liver tissue and isolated mitochondria suspension were stored at -80°C for later semi-quantification by Western blotting. The blood collected from inferior vena cava was centrifuged ($3000 \times g$, 10 min). Collected plasma was stored at -80°C for later biochemical analysis.

Transmission electron microscopy

For evaluation of ultrastructural alterations induced by diet and exercise, liver tissues were fixed in 2,5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate Buffer 0.1M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–60 nm thickness) were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan at the HEMS / IBMC - Institute for Molecular and Cell Biology of the University of Porto

Hepatic glycogen content determination

Liver tissue (30 mg) was homogenized in 600 μl of ddH₂O, boiled for 10 min at 95°C , and centrifuged at $15,500 \times g$ for 10 min at 4°C . The supernatant was diluted in PBS (1:6) and each sample (30 μl) was either digested in one volume of 1.2U/ml amyloglucosidase (Sigma-Aldrich, A1602) or diluted in one volume of PBS. After setting up, the microplate (with blanks, glucose standards (0.01, 0.02, 0.04, 0.08, 0.16 mg/ml), and digested/diluted samples) was incubated for 60 min at 37°C , and then for 5 min at room temperature. Afterwards, 100 μl of Glucose assay reagent (Sigma-Aldrich, G3293) was added and the plate was incubated for 15 min at room temperature. The absorbance was measured at 340 nm. The glycogen content was calculated based on the glucose standard curve and the

difference in the absorbance between digested and untreated samples. The protocol was adapted from Tennessen et al. [18].

Western blotting

Previously isolated liver mitochondria were used for analysis, as well as liver tissue homogenized in RIPA lysis buffer (20188, Millipore) supplemented with protease and phosphate inhibitors cocktails (P2714, Sigma-Aldrich; 524636, Millipore) and centrifuged. Equivalent amounts (20 µg) of liver mitochondria or tissue denatured in sample loading buffer were separated by SDS-PAGE electrophoresis (12% gels) and transferred to PVDF membranes. Membranes were blocked with non-fat dry milk or BSA (in Tris-buffered saline with 0.1% Tween20), and afterwards incubated with primary antibodies: anti-Bax (Cell Signaling, #2772), anti-Bcl2 (Cell Signaling, #2870), anti-Beclin1 (Cell Signaling, #3495), anti-carbohydrate response element binding protein (ChREBP) (Santa Cruz, sc-515922), anti-Cytochrome C (BD Biosciences, 556433), anti-dynamin related protein 1 (DRP1) (Cell Signaling, #8570), anti-estrogen receptor alpha (ERα) (Santa Cruz, sc-787), anti-microtubule-associated protein-1 light chain 3 (LC3) (MBL, PD014), anti-mitofusin 1 (MFN1) (Santa Cruz, sc-50440), anti-mitofusin 2 (MFN2) (Santa Cruz, sc-50331), anti-optic atrophy 1 (OPA1) (Abcam, ab119685), anti-Parkin (Cell Signaling, #4211), anti-phosphoenolpyruvate carboxykinase, cytosolic (PEPCK-C) (Santa Cruz, sc-377027), anti-peroxisome proliferator-activated receptor gamma co-activator-1alpha (PGC-1α) (Abcam, ab54481), anti-PTEN Induced Kinase 1 (PINK1) (Abcam, ab23707), anti-sterol regulator element binding protein 1 (SREBP-1) (Abcam, ab3259), anti-mitochondrial transcription factor A (TFAM) (Santa Cruz, sc-23588), anti-translocase of outer membrane 20 (TOM20) (Santa Cruz, sc-11415). Later, membranes were incubated with secondary antibodies: horseradish peroxidase-conjugated anti-goat, anti-mouse, or anti-rabbit (Santa Cruz, sc-2354, sc-2005, sc-2357, respectively). Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, #1705061) and acquired by ChemiDoc-XRS Image System (Bio-Rad). Bands' density was determined by using the Image Lab software 6.0.1 (Bio-Rad). Ponceau S staining was used to normalize differences in protein

loading and transfer [19]. The final data were expressed as the percentage variation of the control values (% P-C-S and % C-S for the mothers and offspring, respectively).

Real-time PCR analysis

For quantitative assessment of mRNA expression, total RNA was extracted from rats' liver (30 mg of tissue) using PureLink™ RNA Mini Kit (12183018A, ThermoFisher Scientific) and following manufacturer's instruction. Total RNA was then converted to cDNA using Xpert cDNA Synthesis Mastermix (#GK81, GRISP). Real-time PCR was performed for quantitative assessment of mRNA expression using the fluorescent dye, PowerUp Sybr Green Master Mix (A25742, ThermoFisher Scientific) on a Step One Plus thermocycler (Applied Biosystems). The relative expression of target genes was normalized to the housekeeping gene using fluorescence threshold cycle values (Ct) calculated for each sample. For mRNA transcripts, the following primers were used: *Pgc-1α* (forward 5'-AAAAGCTTGACTGGCGTCAT-3'; reverse 5'-TCAGGAAGATCTGGGCAAAG-3'), *Tfam* (forward 5'-GCTAAACACCCAGATGCAAAA-3'; reverse 5'-CGAGGTCTTTTTGGTTTTCC-3'); *Mfn1* (forward 5'-TGGTCACACAACCAACTGCT-3'; reverse 5'-GGGCCAAAATACGTGCACAA-3'); *Mfn2* (forward 5'-GTGACGTGTTGGGTGTGAT-3'; reverse 5'-GGACATCTCGTTTCTAGCTGGT-3'); *Drp1* (forward 5'-CCAGGAATGACCAAGGTCCC-3'; reverse 5'-CCTCGTCCATCAGGTCCAAC-3'); 18S rRNA (forward 5'-CATTCGAACGTCTGCCCTAT-3'; reverse 5'-GTTTCTCAGGCTCCCTCTCC-3'). For miRNA analysis, small RNA was extracted from the rats' liver (20 mg of tissue) using mirVana miRNA Isolation Kit (AM1560, Invitrogen) according to the manufacturer's protocols. The cDNA was obtained using the TaqMa Advanced miRNA Assay (A25576, Applied Biosystems). The relative expressions of miR-122-5p, miR-34a-5p, miR-130b-3p, and miR-494-3p were determined using the TaqMan Fast Advanced Master Mix (Applied Biosystem). The primers used were assay ID rno480899_mir, rno481304_mir; rno481304_mir; rno481191_mir. Relative mRNA and miRNA

expressions were reported as fold values of the control group (% P-C-S and % C-S for the mothers and offspring, respectively).

Quantification of mitochondrial DNA copy number

Total DNA was isolated from rats' livers (30 mg) using GRS Genomic DNA Kit BroadRange (#GK06.0100, GRISP). For the detection of liver nuclear DNA (nDNA) and mtDNA, specific primer sequences were used: GAPDH (forward 5'-GGAAAGACAGGTGTTTTGCA-3'; reverse 5'-AGGTCAGAGTGAGCAGGACA-3') and Rnr2 (forward: 5'-AGCTATTAATGGTTCGTTTGT-3'; reverse 5'-AGGAGGCTCCATTTCTCTTGT-3'), respectively [20]. Quantitative real-time PCR was conducted using PowerUp SYBR® Green Master Mix (A25742, ThermoFisher Scientific) on a Step One Plus thermocycler (Applied Biosystems) using paired reverse and forward primers with mitochondrial and nuclear products performed separately, under the following conditions 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were presented as mtDNA/nDNA ($\Delta Ct = Ct(nDNA) - Ct(mtDNA)$; relative mtDNA content = $2 \times 2^{\Delta Ct}$).

Statistical analysis

The results are expressed as the mean $\pm$ SEM (Standard Error of the Mean). Statistical analysis was performed in GraphPad Prism 9.0 software (GraphPad software, San Diego, CA, USA). Data were analysed using one-way ANOVA test followed by Sidak post-hoc test, with the significance level set at 5%. The follow-up statistics were performed with the program RStudio Team (Integrated Development for R. RStudio, MA, USA).

RESULTS

Histology

Although some phenotypic patterns were observed, the ultrastructural examination of the different male liver tissue samples did not show any statistically significant alterations between groups (Table 1). The male offspring of sedentary HFHS-fed mothers showed a trend towards a lower number of

mitochondrial structures per field and, at the same time, an increased mitochondrial diameter ($p < 0.1$). On the contrary, GE partially prevented these alterations and reduced the number of lipid droplets in the offspring's livers. Interestingly, the presence of glycogen in the liver of HFHS-S offspring was not detected, whereas C-S and HFHS-E offspring did not differ in histologically observed glycogen accumulation. Due to technical problems with the samples, the animals from the maternal generation were not histologically examined.

Table 1. Semiquantitative analysis of ultrastructural examination of offspring liver tissue.

	C-S	HFHS-S	HFHS-E
No. lipid droplets	251.4 ± 38.8	253.2 ± 40.4	195.0 ± 32.0
No. mitochondrial structures	2434.0 ± 73.7	2075.0 ± 315.4	2183.6 ± 288.2
Mitochondria diameter	0.72 ± 0.02	0.86 ± 0.02	0.78 ± 0.08
Megamitochondria	-	-	-
No. abnormal mitochondria	7.4 ± 2.0	3.6 ± 1.2	26.3 ± 9.0 [#]
No. intramitochondrial granules	+	+	+
Intramitochondrial crystalline inclusion	0.8 + 0.8	3.2 + 1.0	1.0 + 0.3
Glycogen accumulation	+	-	++

C – maternal control diet; HFHS – maternal 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 mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Regulators of glucose/lipid metabolism

Considering the role of transcriptional factors SREBP-1 and ChREBP in glucose and lipid metabolism, and PEPCK-C in gluconeogenesis, their protein amount was examined (Fig. 1). In mothers, no increase in mature/native SREBP-1 ratio was observed due to HFHS-feeding, while GE induced its decrease (Fig. 1a). In HFHS-E offspring, the same effect of GE was observed; however, exposure to maternal HFHS diet increased SREBP-1 ratio in the offspring liver (Fig. 1d). Although the HFHS diet in mothers lowered PEPCK content (Fig. 1c), a reduced PEPCK content in the offspring was only observed when mothers were exercised (Fig. 1f). GE had the same stimulatory effect on plasma insulin in mothers (Fig.

1h) and hepatic glycogen accumulation in both generations (Fig. 1g, 1i), the latter parameter agreeing to the offspring hepatic glycogen accumulation histologically observed.

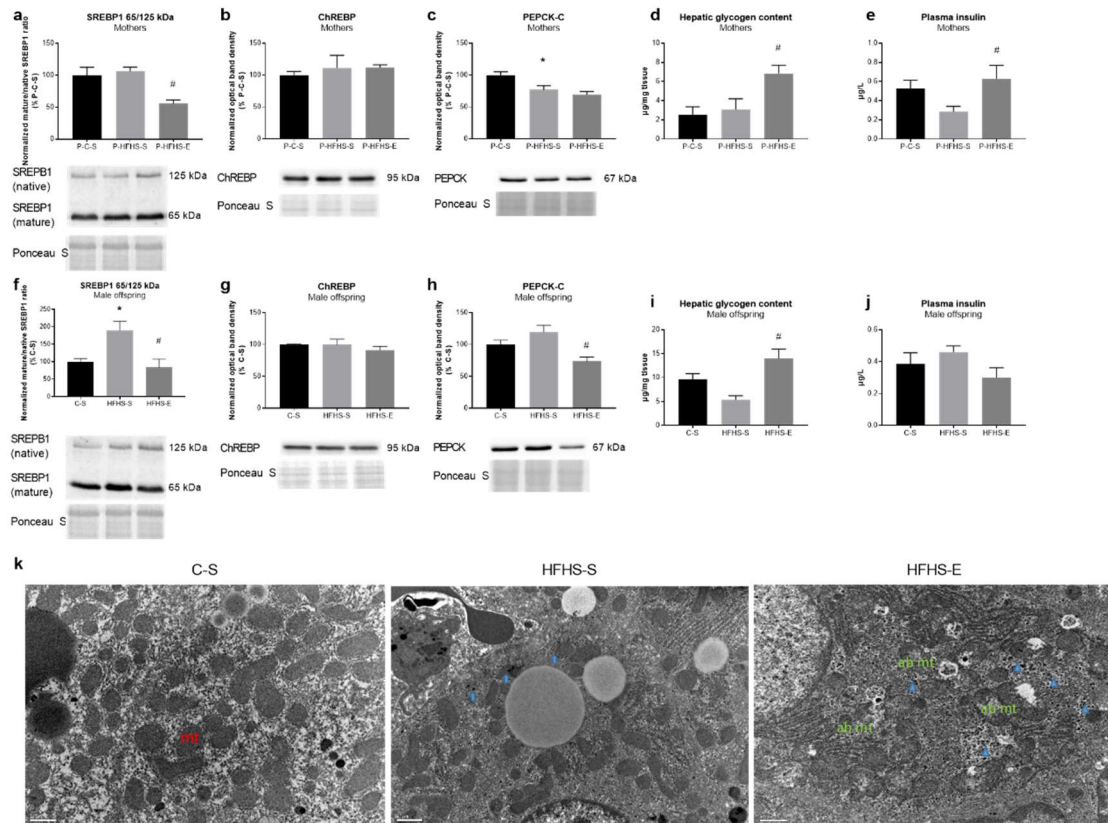


Figure 1. Effects of maternal HFHS and GE on regulators of liver glucose and lipid metabolism in mothers: (a) ratio of mature (65 kDa) and native (125 kDa) SREBP-1 form, (b) relative content of ChREBP, (c) relative content of PEPCK, (d) hepatic glycogen content, (e) plasma insulin levels; and in offspring: (f) ratio of mature and native SREBP-1 form, (g) relative content of ChREBP, (h) relative content of PEPCK, (i) hepatic glycogen content, (j) plasma insulin levels; (k) representative transmission electron microscopy images of liver parenchyma of male offspring at intermediate magnification (12,000×): the presence of an increase of a number of mitochondria (mt), enlarged intramitochondrial granules (arrows), higher abnormal mitochondria (ab mt), glycogen accumulation (arrowheads); scale bar=1 μm. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Mitochondrial biogenesis end-points

The protein expression of key regulators mitochondrial biogenesis (PGC-1 α and TFAM) were assessed. Non-statistically significant decreases in PGC-1 α and TFAM protein content, due to maternal HFHS diet and sedentary lifestyle, were observed in both generations, while GE stimulated their protein expression. Statistical differences were found between P-HFHS-S and P-HFHS-E regarding TFAM protein content in both mothers and offspring and PGC-1 α in offspring (Fig. 2a, 2d). These effects of GE were also observed in transcripts (Fig. 2b, 2e). Furthermore, *Tfam* mRNA levels were remarkably reduced in HFHS-S offspring ($p \leq 0.01$), which was reverted by GE ($p \leq 0.05$) (Fig. 2f). In contrast to maternal, offspring mitochondrial DNA copy number was similarly altered by maternal diet and/or exercise (Fig. 2f). On the other hand, maternal diet and exercise did not affect TOM20 content (Fig. 2a, 2d). To further identify diet and exercise effects, principal component analysis (PCA), performed by using markers of mitochondrial biogenesis in the offspring of HFHS-fed mothers, showed a clear difference based on the maternal physical activity levels (Fig. 2h), which is not as noticeable in mothers (Fig. 2g). This effect could be a consequence of physical activity cessation after pup delivery.

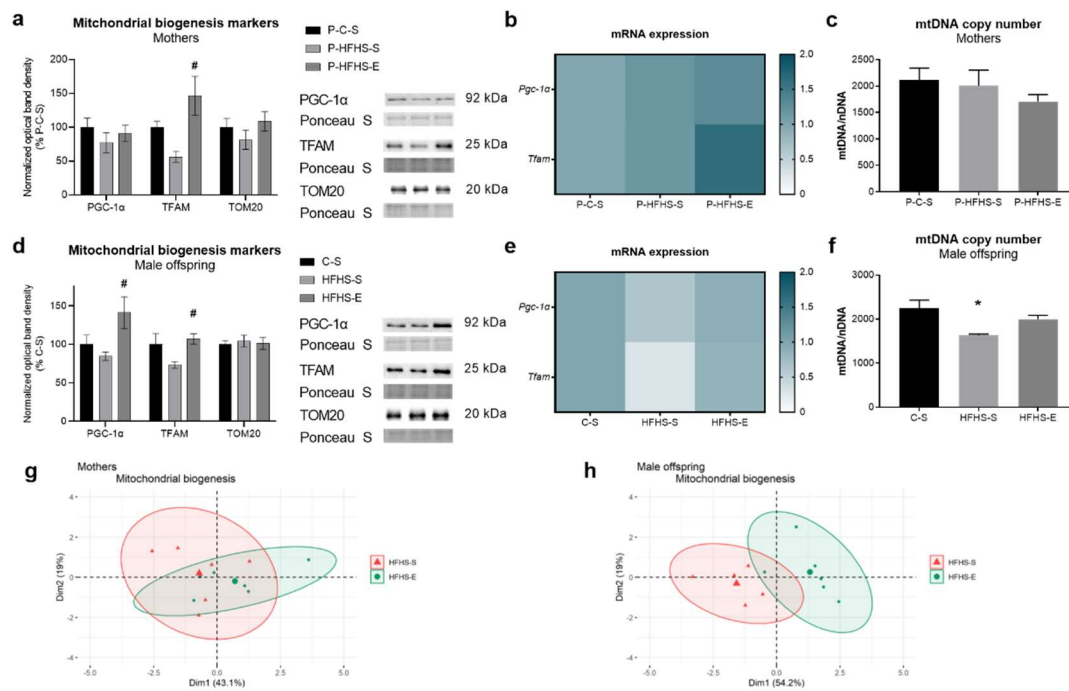


Figure 2. Effects of maternal HFHS and GE on different markers of liver mitochondrial biogenesis in mothers: (a) relative content of PGC-1 α , TFAM, and TOM20, (b) heat map showing the relative expression levels for *Pgc-1 α* and *Tfam* transcripts (c) mitochondrial DNA copy number, (g) principal component analysis of hepatic mitochondrial biogenesis markers; and in offspring: (d) relative content of PGC-1 α , TFAM, and TOM20. (e) heat map showing the relative mRNA expression levels of *Pgc-1 α* and *Tfam*, (f) mitochondrial DNA copy number, (h) principal component analysis of hepatic mitochondrial biogenesis markers. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Mitochondrial dynamics

The liver mitochondrial content of proteins involved in mitochondrial fusion (MFN1, MFN2, and OPA1) and mitochondrial fission (DRP1) was analysed. In HFHS dams, a sedentary lifestyle induced a reduction in the content of mitochondrial fusion proteins, whereas GE reverted this effect in MFN1 and MFN2 contents, but not in OPA1. DRP1 protein expression was 26% increased by HFHS diet in sedentary dams, whereas GE significantly reduced its content (Fig. 3a). Furthermore, mRNA levels of *Mfn1*, *Mfn2*, and *Drp1* tended to decrease

due to HFHS diet and to increase in the exercising animals, although without a significant statistical difference (Fig. 3b). Regarding the male offspring, most of mitochondrial fusion proteins' content was not significantly affected by maternal diet and exercise; yet, GE slightly mitigated the decrease in OPA1 content imposed by the maternal HFHS diet. Similarly to their mothers, the offspring of sedentary HFHS mothers had increased mitochondrial fission-related DRP1 protein expression, inhibited by maternal GE despite maternal HFHS diet (Fig. 3c). Intrauterine exposure to maternal HFHS diet resulted in decreased transcripts for *Mfn1* ($p \leq 0.05$), *Mfn2* ($p = 0.06$), and *Drp1* ($P \leq 0.01$) in male offspring, which was prevented by GE (Fig. 3d). Like in mitochondrial biogenesis PCA (Fig. 2g, 2h), the same effects of GE were observed regarding the mitochondrial dynamics' markers (Fig. 3e, 3f), undoubtedly showing the strong impact of GE despite altered intrauterine environment due to maternal HFHS diet.

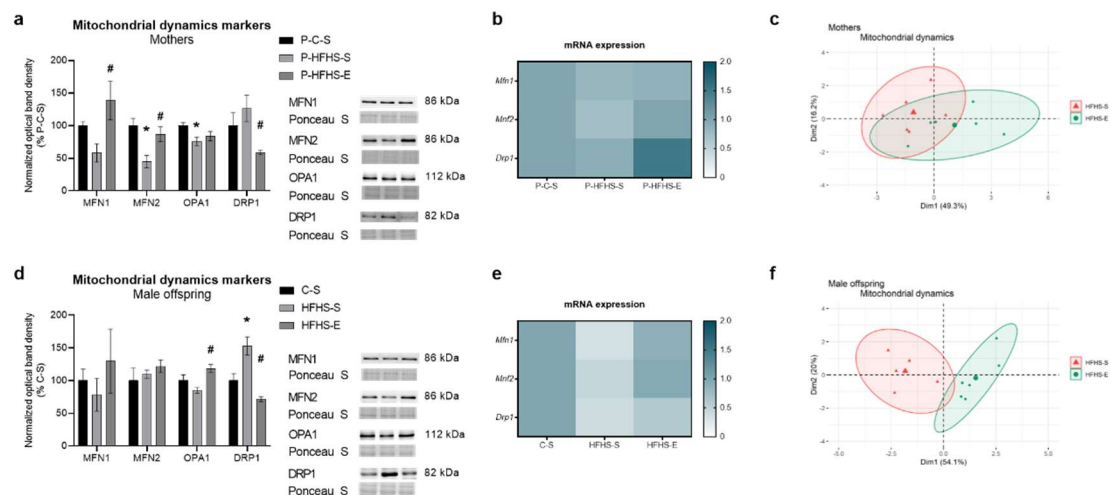


Figure 3. Effects of maternal HFHS and GE on different markers of liver mitochondrial dynamics in mothers: (a) relative mitochondrial content of MFN1, MFN2, OPA1, and DRP1, (b) heat map showing the relative transcripts for *Mfn1*, *Mfn2*, and *Drp1*, (c) principal component analysis of hepatic mitochondrial dynamics markers; and in the offspring: (d) relative mitochondrial content of MFN1, MFN2, OPA1, and DRP1, (e) heat map showing the relative hepatic transcripts for *Mfn1*, *Mfn2*, and *Drp1*, (f) principal component analysis of hepatic mitochondrial dynamics markers. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Estrogen receptor- α protein expression

Since ER signalling has been associated with regulating metabolic function and is necessary for exercise-related protection against liver diseases [21], we determined the protein levels of ER α in whole liver and liver mitochondria, as it can also locate in mitochondria regulating mitochondrial biogenesis [22]. HFHS feeding in mothers reduced the mitochondrial content of ER α , which was prevented by exercise during pregnancy; yet, this was not observed at the whole liver tissue level (Fig. 4a). In male offspring, no alterations were observed in ER α protein expression (Fig. 4b).

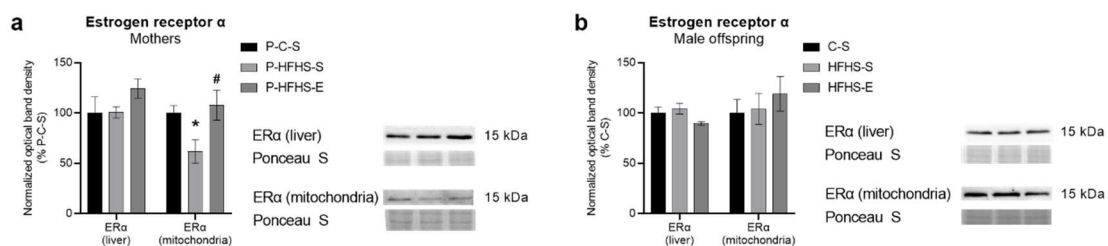


Figure 4. Effects of maternal HFHS and GE on the protein expression of ER α in the liver tissue and mitochondria of (a) maternal generation and (b) offspring generation. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Auto(mito)phagy signalling

Being autophagy and mitophagy both important mechanisms for cell quality control processes, coupled with mitochondrial biogenesis and dynamics, we assessed the content of proteins PINK1 and Parkin, and LC3-II, pro-autophagic Beclin1, and anti-autophagic and anti-apoptotic Bcl2. The hepatic levels of these proteins were not significantly affected by maternal diet/GE in mothers or offspring. HFHS feeding reduced Parkin content in dams, while GE increased it by 38% (Fig. 5b). The same effect was observed in the pro-autophagic Beclin1 content (Fig. 5g). The offspring of exercised HFHS mothers showed increased PINK1 expression (Fig. 5d). However, the offspring of sedentary HFHS mothers had a higher LC3-I protein content, and the same trend in LC3-II, which was

rescued by GE (Fig. 5f). The Beclin/Bcl2 ratio was not altered in any of the groups studied.

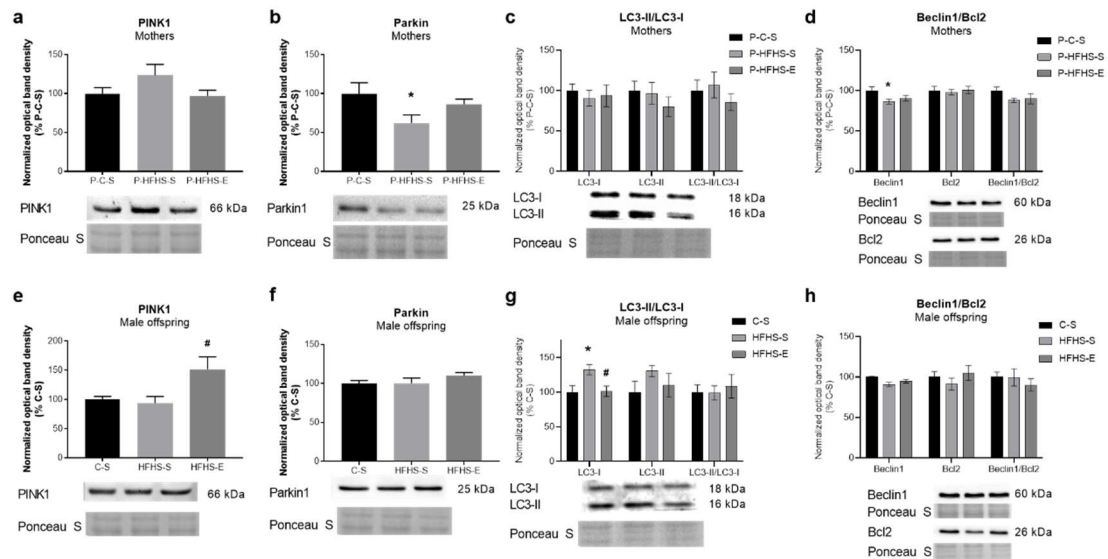


Figure 5. Effects of maternal HFHS and GE on the protein expression of different markers of auto(mito)phagy signalling in liver and liver mitochondria in mothers: (a) relative content of PINK1, (b) relative content of Parkin, (c) relative content of LC3-I and LC3-II and their ratio, (d) relative content of Beclin1 and Bcl2 and their ratio; and in offspring: (e) relative content of PINK1, (f) relative content of Parkin, (g) relative content of LC3-I and LC3-II and their ratio, (h) relative content of Beclin1 and Bcl2 and their ratio. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Apoptotic signalling

Analysis of the content of some pro-apoptotic proteins at the tissue level showed no alterations in any generation (Fig. 6). Nevertheless, the same proteins were remarkably modulated at the mitochondrial level, particularly in the maternal generation, suggesting a delicate adjustment of these mechanisms to diet and physical activity levels. Mitochondrial pro-apoptotic Bax and its ratio over Bcl2 increased by 38% in HFHS-fed animals, which was prevented by exercise (Fig. 6c). Opposite effects were observed in mitochondrial cytochrome c content (Fig. 6e), suggesting that cytochrome c release occurred in the P-HFHS-S group and was prevented even with a short exercise period. In the offspring, alterations of

the intrauterine environment did not affect apoptotic signalling pathway. Maternal HFHS increased Bax and Bcl2 content, which was reverted by GE (Fig. 6d). Nevertheless, it seems that maternal diet and exercise were not sufficiently strong factors to modulate some of the proteins involved in apoptotic pathways in the offspring.

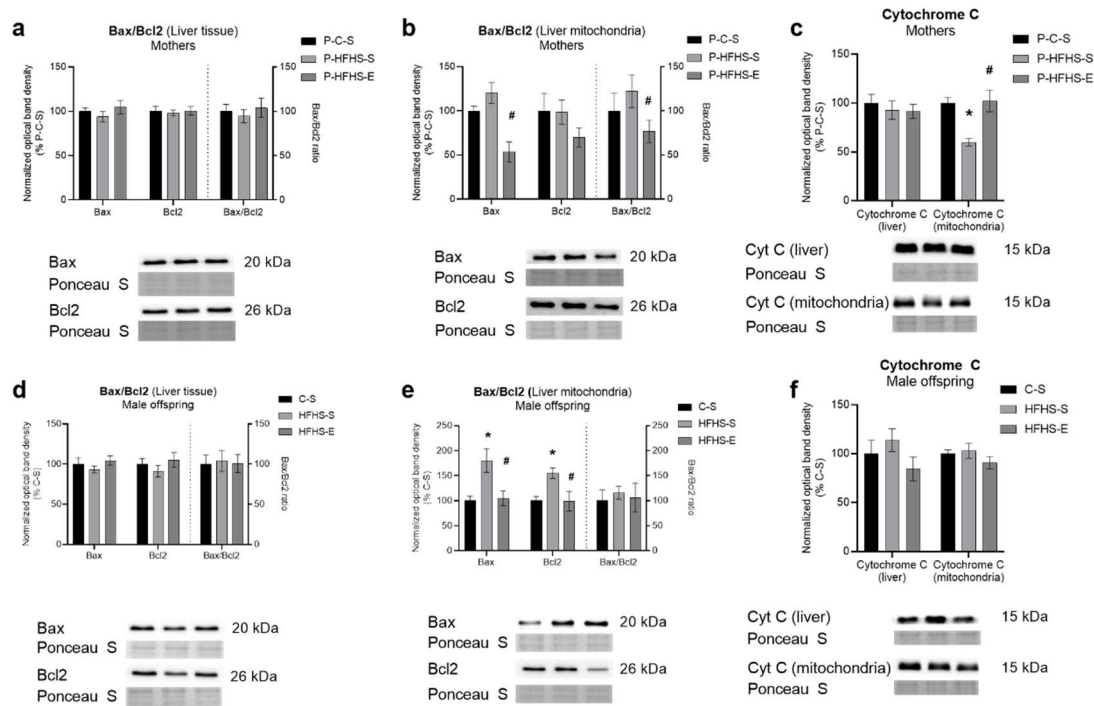


Figure 6. Effects of maternal HFHS and GE on the protein expression of different markers of apoptotic signalling in mothers: (a) relative content of liver Bax and Bcl2 and their ratio, (b) relative content of liver mitochondrial Bax and Bcl2 and their ratio, (c) relative content of liver and liver mitochondrial cytochrome c; and in offspring: (d) relative content of liver Bax and Bcl2 and their ratio, (e) relative content of liver mitochondrial Bax and Bcl2 and their ratio, (f) relative content of liver and liver mitochondrial cytochrome c. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

Expression of miR-122, miR-34a, miR-130b, and miR-494 in the liver

Recent data published by our group [17] and the data here presented suggest that mitochondrial bioenergetics and network regulation were modulated by maternal diet/GE, particularly in the offspring. Epigenetic regulation could help

explain the intergenerational effect of maternal diet/GE. We examined microRNAs that regulate hepatic lipid metabolism and/or mitochondrial biogenesis. Even though miR-122 is one of the most abundant hepatic microRNAs involved in lipid metabolism [14], no alterations were observed in mothers or offspring (Fig. 7a, 7e). However, miR-34a, known to inhibit PGC-1 α activation [23], was reduced with GE in both mothers and their male offspring (Fig. 7b, 7f), which corroborates with PGC-1 α regulation (Fig. 2). With similar roles, miR-130b expression was stimulated by HFHS diet, but prevented by GE (Fig. 7c, 7g) following regulation of PGC-1 α . Interestingly, miR-494, targeting TFAM [24], was increased by HFHS diet in mothers and only slightly in offspring, whereas GE decreased its levels only in the offspring generation (Fig. 7d, 7h).

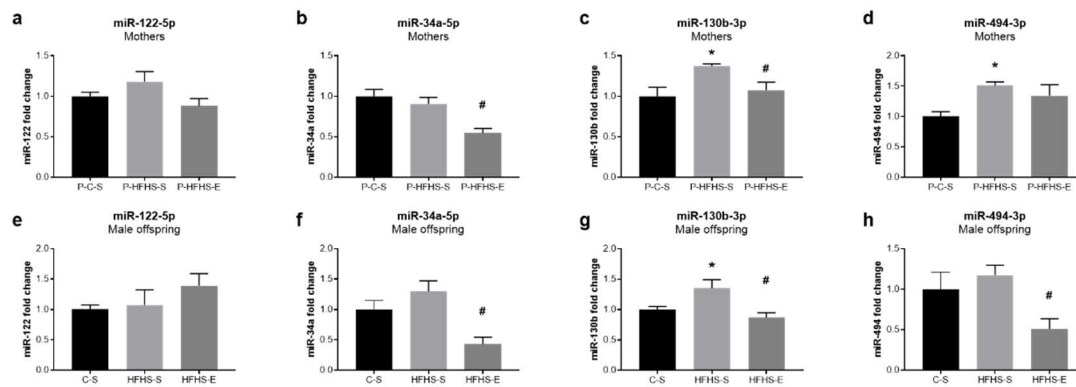


Figure 7. Effects of maternal HFHS and GE on the micro-RNA expression in maternal livers: (a) relative expression of miR-122, (b) relative expression of miR-34a, (c) relative expression of miR-130b, (d) relative expression of miR-494; and in offspring: (e) relative expression of miR-122, (f) relative expression of miR-34a, (g) relative expression of miR-130b; (h) Relative expression of miR-494. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. * vs. P-C-S ($p < 0.05$) or vs. C-S ($p < 0.05$) in mothers or offspring, respectively. # vs. P-HFHS-S ($p < 0.05$) or vs. HFHS-S ($p < 0.05$) in mothers or offspring, respectively.

DISCUSSION

Maternal nutrition alterations during pregnancy and/or GDM may induce metabolic alterations during early embryonic and fetal life and trigger chronic metabolic disorders in offspring [4, 25, 26]. However, maternal GE can prevent these adverse effects on metabolic health [27, 28] and even reduce the risk for

NAFLD and obesity in high-fat diet (HFD)-fed offspring [29, 30]. Recently, our group suggested that GE mitigated liver lipid accumulation and mitochondrial dysfunction in male offspring of GDM mothers and prevented some HFHS deleterious consequences in mothers, most probably those restricted to GDM [17]. Therefore, we aimed to clarify putative mechanisms behind these GE-modulated metabolic and mitochondrial manifestations, focusing on mitochondrial biogenesis and dynamics, and quality control signalling in mothers and male offspring.

Considering previously observed effects of maternal diet/GE on hepatic triglyceride accumulation [17], we analysed SREBP-1 and ChREBP content, as vital transcription factors involved in *de novo* lipogenesis. Decline in mature/native SREBP-1 ratio in exercising mothers and their male offspring supports the decreased hepatic triglyceride accumulation observed in our previous study [17], as SREBP-1 knocking down lowered hepatic triglycerides content [31]. However, ChREBP was not affected in our study. Yet, SREBP-1 is activated by insulin and ChREBP by glucose independently of insulin [32, 33], and as increased SREBP-1 is related to IR [34], it could be speculated that GDM-characteristic IR was a more important factor in affecting the male offspring lipid metabolism. In contrast to our expectations, miR-122, as a liver-specific microRNA targeting SREBP-1 [14], was not affected by maternal diet/GE. In diet-induced diabetic and NAFLD rats, exercise improved NAFLD phenotype by inducing hepatic miR-122 and downregulating its target genes [14]. Reduced hepatic miR-122 has been reported in offspring of HFD-fed mice [35] and male fetuses of GDM mothers [36]. Whether our study's offspring miR-122 content was not susceptible to specific in utero conditions or whether there is a reversal effect of the offspring C diet post-weaning consumption deserves further studies. In a healthy liver, miR-122 and miR-34a cooperatively regulate and balance cell survival and proliferation [37], with miR-34a being upregulated in the liver of HFD mice [38] and steatohepatitis patients [39]. Despite non-statistically significant increase of miR-34a in the offspring of GDM mothers, GE remarkably inhibited its expression in both generations. Similarly, swimming suppressed brain miR-34a expression and thus rescued impaired autophagy and mitochondrial

dynamics in brain aging disorders [40], whereas voluntary exercise in HFD rats reduced testicular miR-34a and related apoptosis [41]. Still, the effect of exercise on miR-34a levels is not fully understood, particularly at the intergenerational level.

Being involved in hepatic glycogenesis and gluconeogenesis, SREBP-1 knockdown resulted in the upregulation of genes associated with these processes [31]. The increased hepatic glycogen accumulation in exercised HFHS mothers and their offspring with reduced SREBP-1 gene expression corroborates this finding. Hepatic gluconeogenesis is found to increase in NAFLD and diabetes [42-44], although this effect was prevented with exercise [42]. Chronic exercise modulated PEPCK expression by improving insulin sensitivity [42], whereas acute exercise increased hepatic glycogen content and reduced PEPCK levels in obese and diabetic animals [45], which agrees with our data. Gluconeogenesis tends to increase during pregnancy regardless of GDM [45], which is opposite from our observations. Yet, as reliance on gluconeogenesis is lower when glucose is directly available from external resources [44], low PEPCK levels after 18-week of sucrose-rich diet are not surprising, with 3 weeks of exercise probably not being enough to counteract this effect. Nevertheless, miR-34a changes can explain, at least partially, offspring hepatic PEPCK regulation and glycogen accumulation. Hepatic miR-34a overexpression was associated with increased PEPCK mRNA level [46]. Also, miR-34a downregulation restored fibroblast growth factor-19 [46] and -21 signalling [47], which further restored glycogen loss in diabetic animals [48], and eventually suppressed SREBP-1 [49] and PEPCK [50], respectively. Also, enhanced PGC-1 α has been detected in miR-34a knock-out mice [23], which corroborates our data. In fact, lipogenic SREBP-1c can interfere and inhibit PGC-1 α recruitment, thus suppressing hepatic PEPCK [51], which our study also supports. Therefore, SREBP-1 alterations, at least partly and indirectly regulated by miR-34a in the offspring of exercising mothers, could explain previously observed changes in hepatic lipid accumulation of male offspring [17].

In fact, excessive lipid accumulation is associated with impaired activity of PGC-1 α and, thus, decreased mitochondrial biogenesis, which consequently leads to

decreased β -oxidation and respiratory function [52, 53], which we also observed in the liver of male offspring of HFHS mothers [17]. Mitochondrial biogenesis is important to enhance mitochondrial capacity, alleviating hepatic lipid accumulation [54]. Impaired mitochondrial biogenesis, observed as reduced PGC-1 α and TFAM and mtDNA content, was found in NAFLD mice [52] and diabetic patients [55, 56], which might be related to their reduced exercise capacity [57]. Mitochondrial biogenesis is also impaired in diabetic women's placenta, which appears to be sex-specific, with PGC-1 α , TFAM, and mtDNA content reduced in the placenta of male offspring [58], bringing a possible risk of metabolic disturbances in adulthood. Indeed, our data agree with these findings. Although in GDM mothers, GE increased PGC-1 α content to some extent, prolonged HFHS consumption and exercise cessation were the probable reason for not reverting it to the control level. Still, its downstream target TFAM suggested potentially impaired mitochondrial biogenesis in GDM mothers, which was reverted by exercise. Similarly, in the offspring, GE promoted PGC-1 α and TFAM expression and the generation of new mitochondria in the offspring, as supported by increased mtDNA copy number. Additionally, liver PGC-1 α upregulation can reduce hepatic triglyceride accumulation and prevent impaired mitochondrial respiration [56, 57], the effects we reported previously [17] and which could be, at least partly, attributed to PGC-1 α overexpression. Mitochondrial biogenesis can be further regulated by the translocation of ER α into mitochondria. A prolonged over-stimulation or deficiency of ER-mediated mitochondrial biogenesis can lead to over-accumulation or deficiency of ATP and ROS, and generally, mitochondrial dysfunction [22]. Interestingly, maternal diet/GE changes rather affected ER α content in liver mitochondria than on the tissue level, suggesting the importance of ER α -related mitochondrial biogenesis during pregnancy. Also, exercise could increase ER α expression in visceral adipose tissue [59], while ER α signalling was involved in exercise-related beneficial effects against hepatic steatosis, with some of them being limited with ER α loss [21]. Still, this association could not be made in the offspring, probably due to their young age before their estrogen synthesis and regulation began [60]. Expression of miR-130b and miR-494 that target PGC-1 α and TFAM,

respectively, was also modulated by maternal diet/GE. Maternal GDM stimulated miR-130b expression and SREBP-1 ratio in both generations while downregulated PGC-1 α and TFAM, with the latter suggested to be directly regulated by this microRNA [61].

MiR-130b overexpression has been associated with impaired mitochondrial biogenesis in human placenta in diabetic conditions [62] and with a parallel decline in PGC-1 α and TFAM content [62, 63]. Although some exercise modalities can decrease circulating miR-130b [64, 65], the effects of exercise on liver miR-130b expression are not known. Up to our knowledge, this is the first study showing that reduction in hepatic miR-130b levels due to GE is associated with alterations in PGC-1 α in mothers and male offspring. Exercise can downregulate skeletal muscle miR-494 expression as well [24, 66], and thus upregulate TFAM expression [24], which was also evident in the offspring of exercised HFHS-mothers. Although peripheral blood miR-494 is reportedly downregulated in GDM women [67], GDM mothers had upregulated hepatic miR-494 regardless of exercise, suggesting that miR-494 effects might be tissue-specific and that exercise cessation influenced its expression.

The regulation of mitochondrial biogenesis is closely coupled with mitochondrial dynamics and mitophagy to remove damaged mitochondria and maintain viable ones [68]. Maternal nutrient oversupply can lead to alterations in the balance between mitochondrial fission and fusion and eventual metabolic compromise [69]. Hyperglycaemia and hyperlipidemia can shift mitochondria towards fission over fusion, favoring mitochondrial fragmentation and decreasing mitochondrial generation ATP capacity [70]. In fact, a similar effect of GDM-related hyperglycemia was noticed in liver mitochondria of GDM dams and their offspring, which agrees with observed content of PGC-1 α . PGC-1 α pathway regulates MFN2 [71], which deficiency has been associated with IR and diabetes [56, 70], followed by PGC-1 α deficiency as well [56]. Reduced MFN2 levels and related mitochondrial damage and apoptosis were observed in the placenta of early miscarriages [72], suggesting that precise regulation of mitochondrial dynamics is necessary for some developmental processes [73]. Our data suggest that mitochondria may favor fission to remove dysfunctional mitochondria and

preserve bioenergetics, in GDM dams and their offspring. Regardless of the different regulation of mitochondrial fusion in two generations, GE improved mitochondrial biogenesis and dynamics, thus stimulating adaptive response for mitochondrial renewal. Exercise can stimulate mitochondrial fusion [11, 74]. In high-energy demanding conditions, such as exercise, mitochondria tend to fuse and extend their network providing benefits to cellular homeostasis [75]. Interestingly, among fusion proteins, the offspring of exercised mothers had only significantly higher OPA1 levels, partly explained by the improved mitochondrial oxidative phosphorylation (OXPHOS) capacity observed in this offspring group [17]. Under high levels of OXPHOS, OPA1 activity at the mitochondrial membranes, and thus, mitochondrial inner membrane fusion activity is stimulated, whereas mitochondrial outer membrane fusion seems to be insensitive to OXPHOS regulation [76]. On the other side, OPA1 might be important for regulating OXPHOS, as OPA1-depleted cells expressed impairments in OXPHOS [77, 78]. GE also prevented increasing mitochondrial fission protein content imposed by maternal GDM in both generations. As the activation of DRP1-mediated fission can respond to the presence of damaged mitochondria, exercise might be an important factor shaping offspring mitochondria in the very early stage of fetal development.

PINK1 regulates mitophagy through Parkin and DRP1 activation in response to mitochondrial damage [79]. In sedentary dams, HFHS diet upregulated DRP1 content, but downregulated Parkin expression, without affecting PINK1. Yet, PINK1 activation is not mandatory for mitophagy since DRP1 could eventually successfully contribute to remove damaged mitochondria [79]. Decreased Parkin levels, and thus decreased stimulation of mitophagy, may suggest an impaired removal of damaged mitochondria enforced by HFHS and sedentary lifestyle in dams. In HFD-induced steatohepatitis Parkin has also been shown to be reduced in sedentary animals, whereas exercise restored mitophagy signalling [11]. We did not observe such effect of GE in dams, which could be ascribed to the exercise cessation after pregnancy and strengthens the need for regular physical activity. Still, intrauterine exposure to GE stimulated offspring PINK1 protein expression. PINK1 can ensure the rapid segregation of damaged mitochondria

and faster mitophagy, without necessary over-activation of DRP1 [79]. Besides, PINK1 accumulation can reduce mtDNA replication to limit the damaged mtDNA transmission [80], which agrees with our results. Therefore, data suggest that GE is an important factor during intrauterine development that maintains a healthy mitochondrial pool in male offspring. The increase of MFN1 and MFN2 content with the regulation of PINK1/Parkin by GE may represent a rescue mechanism to compensate for the mitochondrial damage by maternal GDM. However, we did not observe any alteration in hepatic apoptotic signalling. Likewise, Bax expression was not affected in the placenta of diabetic women [72, 81]. Interestingly, Bax content was differently regulated at the mitochondrial and tissue level. In fact, regulation of mitochondrial fusion and fission is associated upstream with apoptosis [72, 81]; therefore, it is not surprising that alterations in apoptotic signalling were only observed at the liver mitochondrial, but not at the tissue level. Bax translocation and accumulation in mitochondria under apoptotic stimuli can be an early event contributing to the cytochrome c release from mitochondria and initiation of the intrinsic apoptotic signalling [82]. Accordingly, the cytochrome c content in mothers was greatly reduced in liver mitochondria, but not at the tissue level, which GE successfully prevented. Although the mitochondrial isolation protocol may lead to loss of some cytochrome c, which may be more extensive in some groups than in others, this result suggests that physiologically, an increased cytochrome c release occurs in HFHS-fed mothers, which concomitantly increases apoptotic signaling. Still, Bax accumulation might not be the only cause of the lower mitochondrial content of cytochrome c, as fission-related DRP1 is also involved in apoptotic signalling through cytochrome c [81]. Mitochondrial DRP1 levels were indeed increased in sedentary HFHS dams, which added to OPA1 depletion [83] might have facilitated a potential cytochrome c release and contributed to a slight pro-apoptotic image in GDM dams. Yet, such changes were not observed in the male offspring, and despite eventual regulation of Bax and Bcl2 protein expression, maternal diet/GE did not modulate apoptotic signalling, at least in this age, sex, and tissue.

Generally, GE alleviated hepatic metabolic disturbances in GDM mothers despite prolonged HFHS feeding and exercise cessation. The impact of GE was more

obvious and influential in the following generation. GE prevented the over-excessive placental transfer of maternal food-derived nutrients to the fetal liver, thus reducing the nutritional pressure on offspring liver mitochondria and enabling normal development of liver structure and function and mitochondrial biogenesis dynamics, and quality control signalling. In general, our results revealed that changes caused by diet and GE are more evident in liver mitochondria rather than in the whole liver tissue, either directly or via in utero environment. In the absence of interventions, these mitochondrial alterations and the prolonged harmful lifestyle may lead to a more exacerbated metabolic phenotype.

REFERENCES

- [1] Langley-Evans SC, McMullen S (2010) Developmental origins of adult disease. *Med Princ Pract* 19:87-98. <https://doi.org/10.1159/000273066>.
- [2] Bianco ME, Josefson JL (2019) Hyperglycemia During Pregnancy and Long-Term Offspring Outcomes. *Curr Diab Rep* 19:143. <https://doi.org/10.1007/s11892-019-1267-6>.
- [3] Ponzio BF, Carvalho MH, Fortes ZB, do Carmo Franco M (2012) Implications of maternal nutrient restriction in transgenerational programming of hypertension and endothelial dysfunction across F1-F3 offspring. *Life Sci* 90:571-7. <https://doi.org/10.1016/j.lfs.2012.01.017>.
- [4] Saben JL, Boudoures AL, Asghar Z, Thompson A, Drury A, Zhang W, et al. (2016) Maternal Metabolic Syndrome Programs Mitochondrial Dysfunction via Germline Changes across Three Generations. *Cell Rep* 16:1-8. <https://doi.org/10.1016/j.celrep.2016.05.065>.
- [5] Vacca M, Allison M, Griffin JL, Vidal-Puig A (2015) Fatty Acid and Glucose Sensors in Hepatic Lipid Metabolism: Implications in NAFLD. *Semin Liver Dis* 35:250-61. <https://doi.org/10.1055/s-0035-1562945>.
- [6] Pessayre D, Fromenty B, Berson A, Robin MA, Lett  ron P, Moreau R, et al. (2012) Central role of mitochondria in drug-induced liver injury. *Drug Metab Rev* 44:34-87. <https://doi.org/10.3109/03602532.2011.604086>.
- [7] Szendroedi J, Phielix E, Roden M (2011) The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat Rev Endocrinol* 8:92-103. <https://doi.org/10.1038/nrendo.2011.138>.
- [8] Parakatselaki ME, Ladoukakis ED (2021) mtDNA Heteroplasmy: Origin, Detection, Significance, and Evolutionary Consequences. *Life (Basel)* 11 <https://doi.org/10.3390/life11070633>.
- [9] Rius R, Cowley MJ, Riley L, Puttick C, Thorburn DR, Christodoulou J (2019) Biparental inheritance of mitochondrial DNA in humans is not a common phenomenon. *Genet Med* 21:2823-6. <https://doi.org/10.1038/s41436-019-0568-0>.

- [10] Rovira-Llopis S, Bañuls C, Diaz-Morales N, Hernandez-Mijares A, Rocha M, Victor VM (2017) Mitochondrial dynamics in type 2 diabetes: Pathophysiological implications. *Redox Biol* 11:637-45. <https://doi.org/10.1016/j.redox.2017.01.013>.
- [11] Gonçalves IO, Passos E, Diogo CV, Rocha-Rodrigues S, Santos-Alves E, Oliveira PJ, et al. (2016) Exercise mitigates mitochondrial permeability transition pore and quality control mechanisms alterations in nonalcoholic steatohepatitis. *Appl Physiol Nutr Metab* 41:298-306. <https://doi.org/10.1139/apnm-2015-0470>.
- [12] 14. Management of Diabetes in Pregnancy: Standards of Medical Care in Diabetes-2021. *Diabetes Care* 44:S200-s10. <https://doi.org/10.2337/dc21-S014>.
- [13] ACOG Practice Bulletin No. 190 Summary: Gestational Diabetes Mellitus. *Obstet Gynecol* 131:406-8. <https://doi.org/10.1097/aog.0000000000002498>.
- [14] Kalaki-Jouybari F, Shanaki M, Delfan M, Gorgani-Firouzjaee S, Khakdan S (2020) High-intensity interval training (HIIT) alleviated NAFLD feature via miR-122 induction in liver of high-fat high-fructose diet induced diabetic rats. *Arch Physiol Biochem* 126:242-9. <https://doi.org/10.1080/13813455.2018.1510968>.
- [15] Sabag A, Way KL, Keating SE, Sultana RN, O'Connor HT, Baker MK, et al. (2017) Exercise and ectopic fat in type 2 diabetes: A systematic review and meta-analysis. *Diabetes Metab* 43:195-210. <https://doi.org/10.1016/j.diabet.2016.12.006>.
- [16] Yan Z, Lira VA, Greene NP (2012) Exercise training-induced regulation of mitochondrial quality. *Exerc Sport Sci Rev* 40:159-64. <https://doi.org/10.1097/JES.0b013e3182575599>.
- [17] Stevanović-Silva J, Beleza J, Coxito P, Pereira S, Rocha H, Gaspar TB, et al. (2021) Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring. *Metabolism* 116:154704. <https://doi.org/10.1016/j.metabol.2021.154704>.
- [18] Tennessen JM, Barry WE, Cox J, Thummel CS (2014) Methods for studying metabolism in *Drosophila*. *Methods* 68:105-15. <https://doi.org/10.1016/j.ymeth.2014.02.034>.
- [19] Romero-Calvo I, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustín O, et al. (2010) Reversible Ponceau staining as a loading

control alternative to actin in Western blots. *Anal Biochem* 401:318-20. <https://doi.org/10.1016/j.ab.2010.02.036>.

[20] Carabelli J, Burgueño AL, Rosselli MS, Gianotti TF, Lago NR, Pirola CJ, et al. (2011) High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. *J Cell Mol Med* 15:1329-38. <https://doi.org/10.1111/j.1582-4934.2010.01128.x>.

[21] Winn NC, Jurrissen TJ, Grunewald ZI, Cunningham RP, Woodford ML, Kanaley JA, et al. (2019) Estrogen receptor- α signaling maintains immunometabolic function in males and is obligatory for exercise-induced amelioration of nonalcoholic fatty liver. *Am J Physiol Endocrinol Metab* 316:E156-e67. <https://doi.org/10.1152/ajpendo.00259.2018>.

[22] Chen JQ, Cammarata PR, Baines CP, Yager JD (2009) Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *Biochim Biophys Acta* 1793:1540-70. <https://doi.org/10.1016/j.bbamcr.2009.06.001>.

[23] Lee J, Padhye A, Sharma A, Song G, Miao J, Mo YY, et al. (2010) A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. *J Biol Chem* 285:12604-11. <https://doi.org/10.1074/jbc.M109.094524>.

[24] Yamamoto H, Morino K, Nishio Y, Ugi S, Yoshizaki T, Kashiwagi A, et al. (2012) MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. *Am J Physiol Endocrinol Metab* 303:E1419-27. <https://doi.org/10.1152/ajpendo.00097.2012>.

[25] Agarwal P, Brar N, Morriseau TS, Kereliuk SM, Fonseca MA, Cole LK, et al. (2019) Gestational Diabetes Adversely Affects Pancreatic Islet Architecture and Function in the Male Rat Offspring. *Endocrinology* 160:1907-25. <https://doi.org/10.1210/en.2019-00232>.

[26] Puijs MG, Lendvai A, Bloks VW, Zwier MV, Baller JF, de Bruin A, et al. (2014) Maternal western diet primes non-alcoholic fatty liver disease in adult mouse offspring. *Acta Physiol (Oxf)* 210:215-27. <https://doi.org/10.1111/apha.12197>.

[27] Stanford KI, Lee MY, Getchell KM, So K, Hirshman MF, Goodyear LJ (2015) Exercise before and during pregnancy prevents the deleterious effects of

maternal high-fat feeding on metabolic health of male offspring. *Diabetes* 64:427-33. <https://doi.org/10.2337/db13-1848>.

[28] Laker RC, Lillard TS, Okutsu M, Zhang M, Hoehn KL, Connelly JJ, et al. (2014) Exercise prevents maternal high-fat diet-induced hypermethylation of the *Pgc-1 α* gene and age-dependent metabolic dysfunction in the offspring. *Diabetes* 63:1605-11. <https://doi.org/10.2337/db13-1614>.

[29] Sheldon RD, Nicole Blaize A, Fletcher JA, Pearson KJ, Donkin SS, Newcomer SC, et al. (2016) Gestational exercise protects adult male offspring from high-fat diet-induced hepatic steatosis. *J Hepatol* 64:171-8. <https://doi.org/10.1016/j.jhep.2015.08.022>.

[30] Wasinski F, Bacurau RF, Estrela GR, Klempin F, Arakaki AM, Batista RO, et al. (2015) Exercise during pregnancy protects adult mouse offspring from diet-induced obesity. *Nutr Metab (Lond)* 12:56. <https://doi.org/10.1186/s12986-015-0052-z>.

[31] Ruiz R, Jideonwo V, Ahn M, Surendran S, Tagliabracci VS, Hou Y, et al. (2014) Sterol regulatory element-binding protein-1 (SREBP-1) is required to regulate glycogen synthesis and gluconeogenic gene expression in mouse liver. *J Biol Chem* 289:5510-7. <https://doi.org/10.1074/jbc.M113.541110>.

[32] Linden AG, Li S, Choi HY, Fang F, Fukasawa M, Uyeda K, et al. (2018) Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice. *J Lipid Res* 59:475-87. <https://doi.org/10.1194/jlr.M081836>.

[33] Xu X, So JS, Park JG, Lee AH (2013) Transcriptional control of hepatic lipid metabolism by SREBP and ChREBP. *Semin Liver Dis* 33:301-11. <https://doi.org/10.1055/s-0033-1358523>.

[34] Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL (2000) Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and *ob/ob* mice. *Mol Cell* 6:77-86.

[35] Benatti RO, Melo AM, Borges FO, Ignacio-Souza LM, Simino LA, Milanski M, et al. (2014) Maternal high-fat diet consumption modulates hepatic lipid metabolism and microRNA-122 (miR-122) and microRNA-370 (miR-370)

expression in offspring. *Br J Nutr* 111:2112-22. <https://doi.org/10.1017/s0007114514000579>.

[36] Fornes D, Heinecke F, Roberti SL, White V, Capobianco E, Jawerbaum A (2020) Proinflammation in maternal and fetal livers and circulating miR-122 dysregulation in a GDM rat model induced by intrauterine programming. *Mol Cell Endocrinol* 510:110824. <https://doi.org/10.1016/j.mce.2020.110824>.

[37] McDaniel K, Herrera L, Zhou T, Francis H, Han Y, Levine P, et al. (2014) The functional role of microRNAs in alcoholic liver injury. *J Cell Mol Med* 18:197-207. <https://doi.org/10.1111/jcmm.12223>.

[38] Pogribny IP, Starlard-Davenport A, Tryndyak VP, Han T, Ross SA, Rusyn I, et al. (2010) Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab Invest* 90:1437-46. <https://doi.org/10.1038/labinvest.2010.113>.

[39] Cheung O, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW, et al. (2008) Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 48:1810-20. <https://doi.org/10.1002/hep.22569>.

[40] Kou X, Li J, Liu X, Chang J, Zhao Q, Jia S, et al. (2017) Swimming attenuates d-galactose-induced brain aging via suppressing miR-34a-mediated autophagy impairment and abnormal mitochondrial dynamics. *J Appl Physiol* (1985) 122:1462-9. <https://doi.org/10.1152/japplphysiol.00018.2017>.

[41] Heydari H, Ghiasi R, Hamidian G, Ghaderpour S, Keyhanmanesh R (2021) Voluntary exercise improves sperm parameters in high fat diet receiving rats through alteration in testicular oxidative stress, mir-34a/SIRT1/p53 and apoptosis. *Horm Mol Biol Clin Investig* <https://doi.org/10.1515/hmbci-2020-0085>.

[42] Chang SP, Chen YH, Chang WC, Liu IM, Cheng JT (2006) Merit of physical exercise to reverse the higher gene expression of hepatic phosphoenolpyruvate carboxykinase in obese Zucker rats. *Life Sci* 79:240-6. <https://doi.org/10.1016/j.lfs.2005.12.044>.

[43] Sunny NE, Parks EJ, Browning JD, Burgess SC (2011) Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab* 14:804-10. <https://doi.org/10.1016/j.cmet.2011.11.004>.

- [44] Barthel A, Schmol D (2003) Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 285:E685-92. <https://doi.org/10.1152/ajpendo.00253.2003>.
- [45] Butte NF (2000) Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am J Clin Nutr* 71:1256s-61s. <https://doi.org/10.1093/ajcn/71.5.1256s>.
- [46] Fu T, Choi SE, Kim DH, Seok S, Suino-Powell KM, Xu HE, et al. (2012) Aberrantly elevated microRNA-34a in obesity attenuates hepatic responses to FGF19 by targeting a membrane coreceptor β -Klotho. *Proc Natl Acad Sci U S A* 109:16137-42. <https://doi.org/10.1073/pnas.1205951109>.
- [47] Fu T, Seok S, Choi S, Huang Z, Suino-Powell K, Xu HE, et al. (2014) MicroRNA 34a inhibits beige and brown fat formation in obesity in part by suppressing adipocyte fibroblast growth factor 21 signaling and SIRT1 function. *Mol Cell Biol* 34:4130-42. <https://doi.org/10.1128/mcb.00596-14>.
- [48] Kir S, Beddow SA, Samuel VT, Miller P, Previs SF, Suino-Powell K, et al. (2011) FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science* 331:1621-4. <https://doi.org/10.1126/science.1198363>.
- [49] Zhang Y, Lei T, Huang JF, Wang SB, Zhou LL, Yang ZQ, et al. (2011) The link between fibroblast growth factor 21 and sterol regulatory element binding protein 1c during lipogenesis in hepatocytes. *Mol Cell Endocrinol* 342:41-7. <https://doi.org/10.1016/j.mce.2011.05.003>.
- [50] Kong LJ, Feng W, Wright M, Chen Y, Dallas-yang Q, Zhou YP, et al. (2013) FGF21 suppresses hepatic glucose production through the activation of atypical protein kinase C α . *Eur J Pharmacol* 702:302-8. <https://doi.org/10.1016/j.ejphar.2012.11.065>.
- [51] Yamamoto T, Shimano H, Nakagawa Y, Ide T, Yahagi N, Matsuzaka T, et al. (2004) SREBP-1 interacts with hepatocyte nuclear factor-4 α and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *J Biol Chem* 279:12027-35. <https://doi.org/10.1074/jbc.M310333200>.
- [52] Aharoni-Simon M, Hann-Obercyger M, Pen S, Madar Z, Tirosh O (2011) Fatty liver is associated with impaired activity of PPAR γ -coactivator 1 α (PGC1 α)

and mitochondrial biogenesis in mice. *Lab Invest* 91:1018-28. <https://doi.org/10.1038/labinvest.2011.55>.

[53] Jana BA, Chintamaneni PK, Krishnamurthy PT, Wadhwani A, Mohankumar SK (2019) Cytosolic lipid excess-induced mitochondrial dysfunction is the cause or effect of high fat diet-induced skeletal muscle insulin resistance: a molecular insight. *Mol Biol Rep* 46:957-63. <https://doi.org/10.1007/s11033-018-4551-7>.

[54] Wang SW, Sheng H, Bai YF, Weng YY, Fan XY, Lou LJ, et al. (2020) Neohesperidin enhances PGC-1 α -mediated mitochondrial biogenesis and alleviates hepatic steatosis in high fat diet fed mice. *Nutr Diabetes* 10:27. <https://doi.org/10.1038/s41387-020-00130-3>.

[55] Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. (2003) PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267-73. <https://doi.org/10.1038/ng1180>.

[56] Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* 100:8466-71. <https://doi.org/10.1073/pnas.1032913100>.

[57] Hernández-Alvarez MI, Thabit H, Burns N, Shah S, Brema I, Hatunic M, et al. (2010) Subjects with early-onset type 2 diabetes show defective activation of the skeletal muscle PGC-1 α /Mitofusin-2 regulatory pathway in response to physical activity. *Diabetes Care* 33:645-51. <https://doi.org/10.2337/dc09-1305>.

[58] Jiang S, Teague AM, Tryggestad JB, Aston CE, Lyons T, Chernausek SD (2017) Effects of maternal diabetes and fetal sex on human placenta mitochondrial biogenesis. *Placenta* 57:26-32. <https://doi.org/10.1016/j.placenta.2017.06.001>.

[59] Metz L, Gerbaix M, Masgrau A, Guillet C, Walrand S, Boisseau N, et al. (2016) Nutritional and exercise interventions variably affect estrogen receptor expression in the adipose tissue of male rats. *Nutr Res* 36:280-9. <https://doi.org/10.1016/j.nutres.2015.12.003>.

- [60] Bell MR (2018) Comparing Postnatal Development of Gonadal Hormones and Associated Social Behaviors in Rats, Mice, and Humans. *Endocrinology* 159:2596-613. <https://doi.org/10.1210/en.2018-00220>.
- [61] Chen Z, Luo J, Ma L, Wang H, Cao W, Xu H, et al. (2015) MiR130b-Regulation of PPAR γ Coactivator-1 α Suppresses Fat Metabolism in Goat Mammary Epithelial Cells. *PLoS One* 10:e0142809. <https://doi.org/10.1371/journal.pone.0142809>.
- [62] Jiang S, Teague AM, Tryggestad JB, Chernausek SD (2017) Role of microRNA-130b in placental PGC-1 α /TFAM mitochondrial biogenesis pathway. *Biochem Biophys Res Commun* 487:607-12. <https://doi.org/10.1016/j.bbrc.2017.04.099>.
- [63] Zhang J, Jazii FR, Haghighi MM, Alvares D, Liu L, Khosraviani N, et al. (2020) miR-130b is a potent stimulator of hepatic very-low-density lipoprotein assembly and secretion via marked induction of microsomal triglyceride transfer protein. *Am J Physiol Endocrinol Metab* 318:E262-e75. <https://doi.org/10.1152/ajpendo.00276.2019>.
- [64] Makarova JA, Maltseva DV, Galatenko VV, Abbasi A, Maximenko DG, Grigoriev AI, et al. (2014) Exercise immunology meets MiRNAs. *Exerc Immunol Rev* 20:135-64.
- [65] Radom-Aizik S, Zaldivar F, Jr., Oliver S, Galassetti P, Cooper DM (2010) Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. *J Appl Physiol* (1985) 109:252-61. <https://doi.org/10.1152/jappphysiol.01291.2009>.
- [66] Sun Y, Cui D, Zhang Z, Zhang Q, Ji L, Ding S (2016) Voluntary wheel exercise alters the levels of miR-494 and miR-696 in the skeletal muscle of C57BL/6 mice. *Comp Biochem Physiol B Biochem Mol Biol* 202:16-22. <https://doi.org/10.1016/j.cbpb.2016.07.002>.
- [67] He Y, Bai J, Liu P, Dong J, Tang Y, Zhou J, et al. (2017) miR-494 protects pancreatic β -cell function by targeting PTEN in gestational diabetes mellitus. *Excli j* 16:1297-307. <https://doi.org/10.17179/excli2017-491>.

- [68] Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C (2010) New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *J Cell Sci* 123:2533-42. <https://doi.org/10.1242/jcs.070490>.
- [69] Lahera V, de Las Heras N, López-Farré A, Manucha W, Ferder L (2017) Role of Mitochondrial Dysfunction in Hypertension and Obesity. *Curr Hypertens Rep* 19:11. <https://doi.org/10.1007/s11906-017-0710-9>.
- [70] Bonnard C, Durand A, Peyrol S, Chanseaux E, Chauvin MA, Morio B, et al. (2008) Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* 118:789-800. <https://doi.org/10.1172/jci32601>.
- [71] Liesa M, Palacín M, Zorzano A (2009) Mitochondrial dynamics in mammalian health and disease. *Physiol Rev* 89:799-845. <https://doi.org/10.1152/physrev.00030.2008>.
- [72] Pang W, Zhang Y, Zhao N, Darwiche SS, Fu X, Xiang W (2013) Low expression of Mfn2 is associated with mitochondrial damage and apoptosis in the placental villi of early unexplained miscarriage. *Placenta* 34:613-8. <https://doi.org/10.1016/j.placenta.2013.03.013>.
- [73] Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* 160:189-200. <https://doi.org/10.1083/jcb.200211046>.
- [74] Cartoni R, Léger B, Hock MB, Praz M, Crettenand A, Pich S, et al. (2005) Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. *J Physiol* 567:349-58. <https://doi.org/10.1113/jphysiol.2005.092031>.
- [75] Srinivasan S, Guha M, Kashina A, Avadhani NG (2017) Mitochondrial dysfunction and mitochondrial dynamics-The cancer connection. *Biochim Biophys Acta Bioenerg* 1858:602-14. <https://doi.org/10.1016/j.bbabbio.2017.01.004>.
- [76] Mishra P, Carelli V, Manfredi G, Chan DC (2014) Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to

- oxidative phosphorylation. *Cell Metab* 19:630-41. <https://doi.org/10.1016/j.cmet.2014.03.011>.
- [77] Chen H, Chomyn A, Chan DC (2005) Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 280:26185-92. <https://doi.org/10.1074/jbc.M503062200>.
- [78] Kushnareva YE, Gerencser AA, Bossy B, Ju WK, White AD, Waggoner J, et al. (2013) Loss of OPA1 disturbs cellular calcium homeostasis and sensitizes for excitotoxicity. *Cell Death Differ* 20:353-65. <https://doi.org/10.1038/cdd.2012.128>.
- [79] Pryde KR, Smith HL, Chau KY, Schapira AH (2016) PINK1 disables the anti-fission machinery to segregate damaged mitochondria for mitophagy. *J Cell Biol* 213:163-71. <https://doi.org/10.1083/jcb.201509003>.
- [80] Zhang Y, Wang ZH, Liu Y, Chen Y, Sun N, Gucek M, et al. (2019) PINK1 Inhibits Local Protein Synthesis to Limit Transmission of Deleterious Mitochondrial DNA Mutations. *Mol Cell* 73:1127-37.e5. <https://doi.org/10.1016/j.molcel.2019.01.013>.
- [81] Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, et al. (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 1:515-25. [https://doi.org/10.1016/s1534-5807\(01\)00055-7](https://doi.org/10.1016/s1534-5807(01)00055-7).
- [82] Breckenridge DG, Stojanovic M, Marcellus RC, Shore GC (2003) Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. *J Cell Biol* 160:1115-27. <https://doi.org/10.1083/jcb.200212059>.
- [83] Arnoult D, Grodet A, Lee YJ, Estaquier J, Blackstone C (2005) Release of OPA1 during apoptosis participates in the rapid and complete release of cytochrome c and subsequent mitochondrial fragmentation. *J Biol Chem* 280:35742-50. <https://doi.org/10.1074/jbc.M505970200>.

Exercise performed during pregnancy positively modulates liver metabolism and promotes mitochondrial biogenesis of female offspring in an animal model of diet-induced gestational diabetes

Jelena Stevanović-Silva¹, Jorge Beleza², Pedro Coxito¹, Hugo Rocha³, Tiago Bordeira Gaspar^{4,5,6,7}, Fátima Gärtner^{4,8,9}, Rossana Correia^{10,11}, Rui Fernandes^{10,12}, Paulo Oliveira¹³, António Ascensão¹, José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal;

² Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain;

³ Newborn Screening, Metabolism and Genetics Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-053, Porto, Portugal;

⁴ Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135 Porto, Portugal;

⁵ Cancer Signalling and Metabolism Group, Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), 4200-135 Porto, Portugal;

- ⁶ Medical Faculty of University of Porto (FMUP), 4200-139 Porto, Portugal;
- ⁷ Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto, 4050-313, Porto, Portugal;
- ⁸ Department of Molecular Pathology and Immunology, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313, Porto, Portugal
- ⁹ Glycobiology in Cancer Group, Institute of Molecular Pathology and Immunology of University of Porto (Ipatimup), University of Porto, 4200-135, Porto, Portugal
- ¹⁰ HEMS — Histology and Electron Microscopy Institute for Research and Innovation in Health Sciences (i3S), University of Porto, 4200-135, Porto, Portugal;
- ¹¹ Ipatimup — Institute of Molecular Pathology and Immunology of the University of Porto, 4200-135, Porto, Portugal;
- ¹² IBMC — Institute for molecular and Cell biology of Porto, 4200-135, Porto, Portugal;
- ¹³ CNC-Center for Neuroscience and Cell Biology, CIBB - Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, 3004-504, Coimbra, Portugal.

Submitted in: *Biochimica et Biophysica Acta - Molecular Basis of Disease*

Abstract

Gestational diabetes mellitus (GDM) is associated with a high-risk for metabolic complications in offspring. However, exercise is recognized as a non-pharmacological strategy against metabolic disorders and is recommended in GDM treatment. This study aimed to investigate whether gestational exercise (GE) could modulate maternal high-fat high-sucrose (HFHS) diet-related hepatic metabolic and mitochondrial outcomes in female offspring of mothers with HFHS-induced GDM.

Female Sprague-Dawley rats were fed with control or HFHS diet and kept sedentary or submitted to GE. Their female offspring were fed with control diet and kept sedentary. Hepatic lipid accumulation, lipid metabolism regulators, mitochondrial biogenesis and dynamics markers, and microRNAs associated to the regulation of these markers were evaluated.

Female offspring of GDM mothers showed increased body weight at early age, whereas GE prevented this effect of maternal HFHS-feeding and reduced hepatic lipid accumulation. GE stimulated hepatic mRNA transcription and protein expression of mitochondrial biogenesis markers (peroxisome proliferator-activated receptor-gamma co-activator-1alpha and mitochondrial transcription factor A) and mRNA transcription of mitochondrial dynamics markers (mitofusin-1, mitofusin-2, and dynamin-related protein-1) that were altered by maternal GDM, while mitochondrial dynamics markers protein expression was not affected by maternal diet/GE except for optic atrophy-1. MicroRNAs associated with these processes (miR-122, miR-34a, miR-130b, miR-494), and the expression of auto/mitophagy- and apoptosis-related proteins were not substantially influenced by altered intrauterine environment.

Our findings suggest that GE is an important regulator of the intrauterine environment positively affecting liver metabolism and promoting liver mitochondrial biogenesis in female offspring despite eventual effects of maternal HFHS-feeding and related GDM.

Keywords: gestational exercise; fetal programming; pregnancy; physical exercise.

Abbreviations: ALT – Alanine aminotransferase; AST – Aspartate aminotransferase; BW - Body weight; C – Control diet; ChREBP - Carbohydrate response element binding protein; E – Exercised animals; ER α - Estrogen receptor alpha; ETC - Electron transport chain; FA - Fatty acid; GDM - Gestational diabetes mellitus; GE - Gestational exercise; GTT - Glucose tolerance test; HDL – High-density lipoproteins; HFHS - High-fat, high-sucrose diet; IR - Insulin resistance; LC3 - Microtubule-associated protein-1 light chain 3; LDL – Low-density lipoproteins; MFN1 - Mitofusin 1; MFN2 - Mitofusin 2; NAFLD - Non-alcoholic fatty liver disease; NAS - NAFLD activity score; nDNA - Nuclear DNA; OPA1 - Optic atrophy 1; OXPHOS - Oxidative phosphorylation; PCA – Principal Component Analysis; PEPCK - Phosphoenolpyruvate carboxykinase; PGC-1 α - Peroxisome proliferator-activated receptor gamma co-activator-1alpha; S – Sedentary animals; SREBP-1 - Sterol regulator element binding protein 1; TFAM - Mitochondrial transcription factor A; TOM20 - Translocase of outer membrane 20; UCP2 - Uncoupling protein 2.

INTRODUCTION

Maternal lifestyle can affect metabolic status of their newborns, which can even be prolonged throughout their lifetime. Offspring liver health can be considerably threatened by maternal nutrition and (related) metabolic disorders, including insulin resistance (IR), diabetes, and liver diseases [1]. The most common metabolic condition occurring during pregnancy - gestational diabetes mellitus (GDM), can have short- or long-time consequences on mothers and offspring. GDM-associated adverse intrauterine environment has metabolic complications and possible impact the fetal genome, eventually potentiating the development of the metabolic disturbance in the offspring [2,3]. Mitochondria may undergo alterations that influence fetal metabolic health because of their important role in hepatic metabolism. In fact, the fetal liver has fewer mitochondria and limited or absent gluconeogenesis potential compared with the adult liver. If maternal lifestyle results in excess liver accumulation of fuels, the fetus might face harmful alterations in hepatic β -oxidation, Krebs cycle activity, and de novo lipogenesis that affect mitochondrial health. Eventually, this pathological scenario may enhance fetal hepatic lipid accumulation, priming the fetal liver for (mitochondrial) failure and risk for liver diseases in adulthood [4].

Therefore, it is of great clinical and social relevance to propose efficient therapeutic strategies against this maternal condition, thus preventing the fetus' transmission of maternal metabolic disturbances. Usually, pharmaceuticals used against IR or diabetes are also recommended for the GDM treatment [5]. However, the effect of maternal pharmaceutical intervention during pregnancy on fetal drug tolerance, metabolism, and elimination are not completely understood. While during early pregnancy, drug metabolites in amniotic fluid originate from the maternal blood, during later gestation, they can also be excreted through fetal urine and re-ingested by swallowing. As drug-induced alterations in mothers and fetus may affect the delivery and distribution of oxygen and nutrients [6], physicians need to balance between maternal therapy and potential effects on the fetus [7]. This emphasizes the need to replace pharmacological therapies during pregnancy with safer alternatives. Gestational exercise (GE) is recommended as the first-line strategy against GDM [8,9]. Exercise has been

proven as an effective preventive and/or therapeutic non-pharmacological tool against metabolic diseases not related to pregnancy [10-12]. Chronic exercise can induce hepatic mitochondrial biogenesis, dynamics, and autophagy signalling, eventually contributing to hepatocyte renewal and beneficial mitochondrial adaptations, thus mitigating the phenotype associated with high-caloric consumption-related conditions [13]. Additionally, GE counteracts non-healthy maternal lifestyle (high-caloric diet consumption and sedentarism) or abates and delays more progressive disease development if the offspring follows the maternal lifestyle [14,15]. These GE effects can be explained by the effective turnover of energy originating from the maternal diet, thus reducing the excessive placental fuel transfer and consequent pressure on fetal hepatic mitochondria. Therefore, considering that the focus of intergenerational inheritance of metabolic diseases is mainly focused on male offspring, and that the data about the impact of GDM on liver (mitochondrial) status of young female offspring are still rare, our study assessed whether GE prevents the deleterious effects of maternal diet, contributing to a more resistant mitochondrial phenotype in their female offspring.

MATERIAL AND METHODS

Animals

The experimental protocol has been previously described with details [16]. Briefly, female Sprague-Dawley rats (aged 7 weeks), purchased from Charles River (L'Arbresle, France), were fed with control (C) diet (E157452-047, Ssniff, Soest, Germany) or high-fat, high-sucrose (HFHS) diet (D12451 (II) mod., Ssniff, Soest, Germany) groups. After 7 weeks, the dams were mated with male rats fed with C diet. During and after pregnancy, the animals were fed with respective diets. The mothers fed with C diet were sedentary during the whole experimental protocol. Half of the pregnant HFHS-fed dams were submitted to GE protocol during 3 weeks of pregnancy (E) while the rest remained sedentary (S). The exercising HFHS mothers performed the physical exercise program only during 3 weeks of pregnancy. The animals were exercised on a motor-driven treadmill (LE8700, Panlab Harvard Apparatus, Holliston, MA, USA) 6 days per week, 40-60 minutes per day during the dark cycle for 3 weeks, with a gradual increase of

training duration in the 1st week and a gradual increase of training intensity during the following 2 weeks. However, in the last few days of the protocol (E19-E21 pregnancy day), the intensity and duration of the exercise were adjusted according to each animal's need. Additionally, the exercising mothers had access to running wheels inside their cages for the whole period of pregnancy until delivery to mimic voluntary physical activity. One week before the exercise protocol, animals from the exercising group were adapted to the treadmill. The sedentary groups were also placed on a static treadmill to be exposed to the same environmental conditions. Mothers were allowed to deliver naturally. At birth, litter size was reduced to three male and three female pups to avoid food competition. The offspring was separated from the mothers after 3 weeks of nursing. One female pup from each litter was assigned to three different experimental groups (n = 6 per group): C-S - offspring of sedentary C-fed mothers; HFHS-S - offspring of sedentary HFHS-fed mothers; and HFHS-E - offspring of exercised HFHS-fed mothers. The offspring from all experimental groups were fed with C diet and sedentary and were sacrificed at 6 weeks of age. All animals were housed in a normal environment (21-22°C; 50-60% humidity, 12 h light/dark cycles), receiving food and water ad libitum. The Ethical Committee of the "Instituto de Investigação e Inovação em Saúde – i3S", University of Porto, and National Government Authority (Direção Geral de Alimentação e Veterinária – No.0421/000/000/2018) approved the experimental protocol, and 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.

Glucose tolerance test

To confirm gestational glucose metabolism of the female offspring, oral glucose tolerance test (GTT) was performed at 5 weeks of age. Glucose was administered to the rats at the dose of 2 g/kg of body weight (BW) in the form of non-flavoured jelly, according to the procedure described in Zhang [17]. Rats were previously trained to eat non-flavoured jelly without glucose. Moreover, considering that a

prolonged fasting-induced hypoglycaemia during pregnancy could influence the fetus development, the fasting period prior to gestational GTT in mothers was reduced to 6 hours, and the same procedure was, thus, performed in offspring GTT.

Animal sacrifice and tissue sampling

Animals were anaesthetized (induction at 5% isoflurane, 1 L/m O₂; maintaining at 2.5% isoflurane, 0.4 L/m O₂) between 8:00 and 10:00 am after overnight fasting. Blood was collected from inferior vena cava and centrifuged (3000 x g, 10 min, 4°C). Collected plasma was stored at -80°C. Plasma was later used for biochemical and metabolomics analysis. The abdominal cavity was opened to remove the liver, which was subsequently rinsed with ice-cold PBS, weighed, and separated for light and electron microscopy, Western blotting and PCR analysis.

Light and transmission electron 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. 4µm tissue sections in adhesive slides were stained with hematoxylin/eosin. Stained slides were blindly evaluated using the Carl-Zeiss Axio Observer light microscope equipped with Zen2 software by two veterinary pathologists (TBG and FG). Histologic evaluation considered the main histologic features of non-alcoholic fatty liver disease (NAFLD) in rodent models [18]. 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. All parameters were evaluated in a four-level scoring system, in which 0 corresponds to the 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).

For evaluation of ultrastructural alterations, liver tissues were fixed in 2,5% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate Buffer 0.1M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin

(40–60 nm) sections were prepared on RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA) and mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each. Afterwards, they were examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan at the Institute for Molecular and Cell Biology of the University of Porto.

For the determination of hepatic triglyceride content, Triglyceride Quantification Kit (BioVision, Milpitas, USA) was used following the manufacturer's instructions.

Analysis of acylcarnitine levels in plasma and liver homogenates

Plasma acylcarnitines were detected using Whatman 903 paper (Guthrie cards) and quantified by tandem mass spectrometry (MS/MS), through standard protocols [19]. Hepatic acylcarnitine quantification was performed using the protocol adapted from Petucci et al. [20]. Liver tissue was homogenised in ice-cold 80% methanol (1:10 ratio), vortexed, centrifuged at 14000 rpm (10°C, 5 minutes) and supernatant was collected. In a 96-well plate, 140 µL of supernatant and 100 µL of methanol containing deuterated acylcarnitine internal standard solutions (Cambridge Isotope labs, USA) were added to each well. The mixture was dried using nitrogen flow and derivatised to the corresponding buthyl esters by incubation with 60 µL of 3N buthanolic HCl (70°C, 15 min). Samples were dried 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).

Hepatic glycogen content determination

Following the adapted protocol by Tennessen et al. [21], liver tissue (30 mg) was homogenized in 600 µL of ddH₂O, boiled (95°C, 10 min), and centrifuged (15,500 x g, 10 min, 4°C). After diluting the supernatant in PBS (1:6), to each sample (30 µL) was added one volume of either 1.2U/ml amyloglucosidase (Sigma-Aldrich, A1602) or PBS. The glucose standards were prepared (0.01, 0.02, 0.04, 0.08, 0.16 mg/ml). Samples, previously digested with amyloglucosidase or diluted in PBS, were incubated at 37°C for 60 min, and afterwards for 5 min at RT. To each

microplate well, Glucose assay reagent (Sigma-Aldrich, G3293) was added (100 μ l) and after the incubation, for 15 min at RT the absorbance was measured at 340 nm. The difference in the absorbance between samples digested with amyloglucosidase and untreated samples was used to calculate the glycogen content.

Western blotting

Liver tissue was homogenized in RIPA lysis buffer (20188, Millipore) with protease and phosphate inhibitor cocktails (P2714, Sigma-Aldrich; 524636, Millipore) and centrifuged. Equivalent amounts of liver tissue were denatured in sample loading buffer and separated by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE, 12% gels), and subsequently transferred to PVDF membranes. Membranes were blocked with non-fat dry milk or BSA in Tris-buffered saline with 0.1% Tween20, and then incubated with the following primary antibodies: anti-Bax (Cell Signaling, #2772), anti-Bcl2 (Cell Signaling, #2870), anti-Beclin1 (Cell Signaling, #3495), anti-carbohydrate response element binding protein (ChREBP) (Santa Cruz, sc-515922), anti-Cytochrome C (BD Biosciences, 556433), anti-dynamin related protein 1 (DRP1) (Cell Signaling, #8570), anti-estrogen receptor alpha (ER α) (Santa Cruz, sc-787), anti-microtubule-associated protein-1 light chain 3 (LC3) (MBL, PD014), anti-mitofusin 1 (MFN1) (Santa Cruz, sc-50440), anti-mitofusin 2 (MFN2) (Santa Cruz, sc-50331), anti-optic atrophy 1 (OPA1) (Abcam, ab119685), anti-oxidative phosphorylation (OXPHOS) cocktail (Abcam, ab110413), anti-Parkin (Cell Signaling, #4211), anti-phosphoenolpyruvate carboxykinase (PEPCK) (Santa Cruz, sc-377027), anti-peroxisome proliferator-activated receptor gamma co-activator-1alpha (PGC-1 α) (Abcam, ab54481), anti-PINK1 (Abcam, ab23707), anti-sterol regulator element binding protein 1 (SREBP-1) (Abcam, ab3259), anti-mitochondrial transcription factor A (TFAM) (Santa Cruz, sc-23588), anti-translocase of outer membrane 20 (TOM20) (Santa Cruz, sc-11415), anti-uncoupling protein 2 (UCP2) (Sigma Aldrich, SAB2501087). Later, membranes were incubated with the following secondary antibodies: horseradish peroxidase-conjugated anti-goat (Santa Cruz, sc-2354), anti-mouse (Santa Cruz, sc-2005), or anti-rabbit (Santa Cruz, sc-2357).

Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, #1705061) and acquired by ChemiDoc-XRS Image System (Bio-Rad). Band density was determined with the Image Lab software 6.0.1 (Bio-Rad). Ponceau S staining was used for normalization of differences in protein loading or transfer [22], and the final data were expressed as the percentage variation of the control values (% C-S).

Real-time PCR analysis

Total RNA was extracted from rats' liver (30 mg) using PureLink™ RNA Mini Kit (12183018A, ThermoFisher Scientific). Total RNA was then converted to cDNA using Xpert cDNA Synthesis Mastermix (#GK81, GRISP, Portugal). Real-time PCR was performed for quantitative assessment of mRNA expression using the fluorescent dye, PowerUp Sybr Green Master Mix (A25742, ThermoFisher Scientific) on a Step One Plus thermocycler (Applied Biosystems). The relative expression of target genes was normalized to the housekeeping gene using fluorescence threshold cycle values (Ct) calculated for each sample. For mRNA transcripts, the following primers were used: *Pgc-1α* (forward 5'-AAAAGCTTGACTGGCGTCAT-3'; reverse 5'-TCAGGAAGATCTGGGCAAAG-3'), *Tfam* (forward 5'-GCTAAACACCCAGATGCAAAA-3'; reverse 5'-CGAGGTCTTTTGGTTTTCC-3'); *Mfn1* (forward 5'-TGGTCACACAACCAACTGCT-3'; reverse 5'-GGGCCAAAATACGTGCACAA-3'); *Mfn2* (forward 5'-GTGACGTGTTGGGTGTGAT-3'; reverse 5'-GGACATCTCGTTTCTAGCTGGT-3'); *Drp1* (forward 5'-CCAGGAATGACCAAGGTCCC-3'; reverse 5'-CCTCGTCCATCAGGTCCAAC-3'); 18S rRNA (forward 5'-CATTCTGAACGTCTGCCCTAT-3'; reverse 5'-GTTTCTCAGGCTCCCTCTCC-3').

For miRNA analysis, small RNAs were extracted from the liver (20 mg) using mirVana miRNA Isolation Kit (AM1560, Invitrogen) according to the manufacturer's protocols. The cDNA was obtained using the TaqMa Advanced miRNA Assay (A25576, Applied Biosystems) following the manufacturer's protocols. The relative expressions of miR-122-5p, miR-34a-5p, miR-130b-3p, and miR-494-3p were determined on a Step One Plus thermocycler (Applied

Biosystems) using the TaqMan Fast Advanced Master Mix (Applied Biosystem). The following primers were used: assay ID rno480899_mir, rno481304_mir; rno481304_mir; rno481191_mir. Relative mRNA and miRNA expressions were reported as fold value of the control group (% C-S).

Quantification of mitochondrial DNA copy number

Total DNA was isolated from liver (30 mg) using GRS Genomic DNA Kit BroadRange (#GK06.0100, GRISP, Portugal). Specific primers for nuclear DNA (nDNA) were: forward 5'-GGAAAGACAGGTGTTTTGCA-3'; reverse 5'-AGGTCAGAGTGAGCAGGACA-3'; and for mitochondrial DNA (mtDNA): forward: 5'-AGCTATTAATGGTTCGTTTGT-3'; reverse 5'-AGGAGGCTCCATTCTCTTGT-3' [23]. Quantitative Real-time PCR was conducted using PowerUp SYBR® Green Master Mix (A25742, ThermoFisher Scientific) on a Step One Plus thermocycler (Applied Biosystems). Mitochondrial and nuclear products were performed separately, but under the same conditions (95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 1 min). The results were presented as mtDNA/nDNA ($\Delta C_t = C_t(nDNA) - C_t(mtDNA)$; relative mtDNA content = $2 \times 2^{\Delta C_t}$).

Statistical analysis

The results are expressed as the mean $\pm$ SEM (Standard Error of the Mean). Statistical analysis was performed in GraphPad Prism 8.0 software (GraphPad software, San Diego, CA, USA). Data were analysed using one-way ANOVA test followed by Sidak post-hoc test, with the significance level set at 5%. The follow-up statistics were performed with the program RStudio Team (Integrated Development for R. RStudio, MA).

RESULTS

Animal characteristics

At sacrifice (6 weeks of age), the female offspring from three experimental groups did not show any alterations in BW, liver weight, or the analysed plasma parameters (Table 1). However, we observed a non-statistically significant

increase in BW and blood glucose, triglycerides, cholesterol, and aspartate aminotransferase (AST) levels in the offspring of sedentary HFHS diets, which were reverted by GE.

Table 1. Animal data and plasma biochemical analyses

	C-S	HFHS-S	HFHS-E
Body weight (g)	168.80 ± 5.76	174.50 ± 2.90	162.00 ± 5.41
Liver weight (g)	6.14 ± 0.38	6.42 ± 0.31	5.63 ± 0.19
Liver weight/body weight (mg·g⁻¹)	36.42 ± 1.74	37.11 ± 1.14	32.97 ± 1.35
Glucose (mg/dL)	167.60 ± 8.79	182.80 ± 13.27	183.00 ± 7.75
Triglycerides (mg/dL)	47.17 ± 3.64	59.00 ± 7.16	57.00 ± 4.73
Cholesterol (mg/dL)	91.67 ± 3.44	100.75 ± 6.55	86.83 ± 3.71
HDL (mg/dL)	65.33 ± 3.18	59.00 ± 2.74	58.60 ± 2.11
LDL (mg/dL)	30.71 ± 1.41	30.50 ± 2.42	26.33 ± 1.67
AST (U/L)	81.40 ± 2.42	94.80 ± 16.41	77.20 ± 2.90
ALT (U/L)	38.71 ± 1.84	39.40 ± 1.96	33.80 ± 2.57

Values are means ± SEM (n = 6).

C – maternal control diet; HFHS – maternal 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.

Interestingly, compared to the offspring of either sedentary C-fed mothers or exercised HFHS-fed mothers, the female offspring of sedentary HFHS-fed mothers had a higher BW at weaning (Fig.1A). The same effect of maternal diet/exercise was maintained until sacrifice, when similar trends were observed (Fig.1B). Even though HFHS diet feeding impaired the maternal glucose tolerance [16], such diet effect was not observed in their 5-week-old female offspring (Fig. 1C,D).

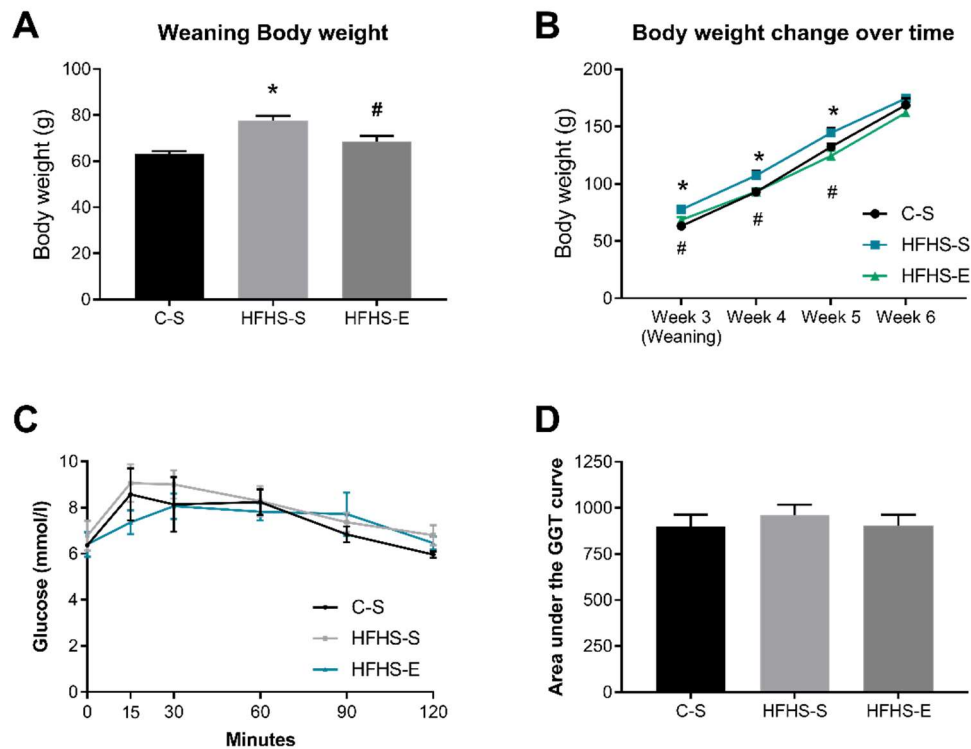


Figure 1. Effect of maternal HFHS diet and GE on female offspring body weight changes from (A) weaning period to (B) the sacrifice point at 6 weeks of age; and (C, D) offspring glucose tolerance at 5 weeks of age. GE – gestational exercise; C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers; GTT – glucose tolerance test. Values are means $\pm$ SEM ($n = 6$). * vs. C-S ($p < 0.05$). # vs. HFHS-S ($p < 0.05$).

Histological analysis and liver triglyceride content

Offspring of exercised HFHS-fed mothers showed lower NAFLD activity score (NAS) and liver triglyceride content compared to the offspring from sedentary C-fed or HFHS-fed mothers even though the effect of diet was not observed (Table 2, Fig. 2), suggesting that GE regulated placental lipid transfer to the fetuses. However, for rodent steatosis models, it is suggested that hepatic triglyceride score would be a more precise measure of steatosis than histological analysis [18]. Indeed, we observed a clearer image of hepatic lipid accumulation, consistent with some other liver parameters. As observed on electron microscopy, the number of lipid droplets showed a non-statistically significant decrease in the offspring of sedentary HFHS mothers (Table 2, Fig. 2), even though lipid droplets with larger diameter were observed in this group (data not

shown), and an opposite effect, more similar to the control group, was observed in the offspring of exercised mothers.

An increasing trend in mitochondrial number and diameter and a clear rise in intramitochondrial crystalline inclusion, resulting from maternal HFHS diet, was prevented with GE.

Table 2. NAFLD activity score recorded from offspring livers of all experimental groups, observed blindly under light microscope, hepatic triglyceride content, and semiquantitative analysis of ultrastructural examination of offspring liver tissue.

	C-S	HFHS-S	HFHS-E
NAS	4.9 ± 0.8	5.4 ± 0.7	3.5 ± 0.2 [#]
Hepatic triglyceride content (mg/g tissue)	1.6 ± 0.1	1.8 ± 0.4	0.9 ± 0.05 [#]
No. lipid droplets	154.7 ± 26.3	98.0 ± 23.7	189.6 ± 38.2
No. mitochondria	2579.7 ± 169.3	3038.0 ± 292.9	2419.8 ± 508.8
Mitochondria diameter	0.59 ± 0.02	0.82 ± 0.02 [*]	0.62 ± 0.04 [#]
Megamitochondria	-	-	-
No. abnormal mitochondria	38.5 ± 9.1	28.0 ± 11.0	25.25 ± 2.0
No. intramitochondrial granules	+	+	+
Intramitochondrial crystalline inclusion	0.0 ± 0.0	3.3 ± 0.3 [*]	1.0 ± 0.4 [#]
Glycogen accumulation	+	++	+

NAS – NAFLD activity score; C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means ± SEM (n = 6). * vs. C-S (p < 0.05). # vs. HFHS-S (p < 0.05).

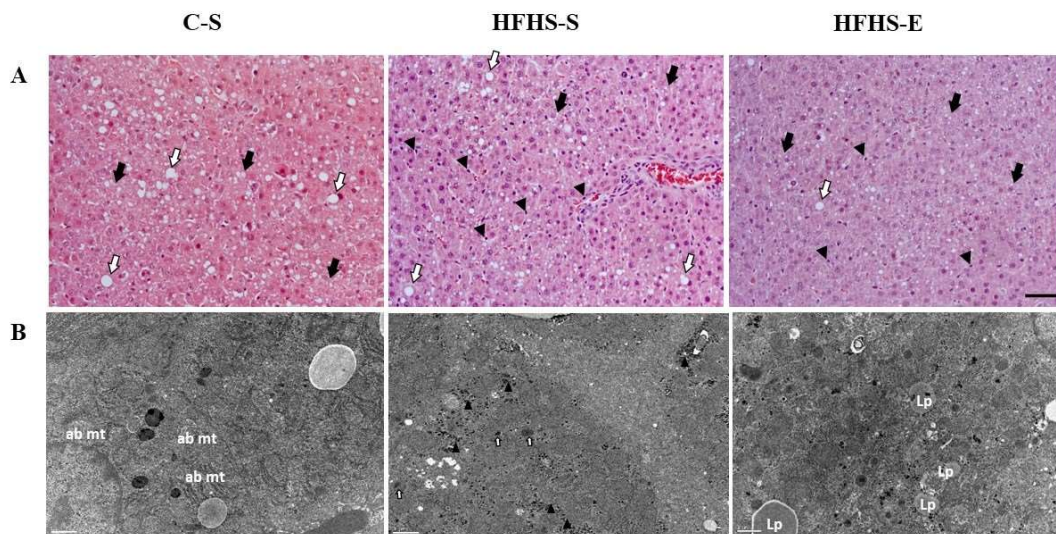


Figure 2. Effect of maternal HFHS diet and GE on (A) representative light micrographs ($\times 200$) of liver tissue (microvesicular steatosis (black arrow), macrovesicular steatosis (white arrow), lobular inflammation (black arrowhead); Scale bar = $50\mu\text{m}$.) and (B) representative transmission electron micrographs ($\times 12\,000$) of liver parenchyma (increase of mitochondrial number (ab mt), enlarged intramitochondrial crystalline inclusion (arrow), glycogen accumulation (arrowhead), lipid droplets (Lp); Scale bar = $1\mu\text{m}$.). GE – gestational exercise; C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers.

Plasma and liver acylcarnitine levels

As acylcarnitine profiling can describe changes in β -oxidation, plasma and liver samples were used to measure acylcarnitine levels. As can be depicted from Fig. 3B, the liver was more sensitive than plasma to maternal HFHS diet and/or GE. Maternal HFHS feeding and sedentarism led to increased levels of long chain acylcarnitines in plasma (Fig. 3A), but especially in the liver (Fig. 3B). However, GE was able to prevent the abnormal acylcarnitine accumulation, and consequent mitochondrial abnormalities. Furthermore, the increased levels of acylcarnitines in the offspring livers correlate to the increased fatty acid (FA) content of the HFHS diet consumed by mothers [16], suggesting that the fetal liver suffers adaptations to the conditions of altered maternal diet.

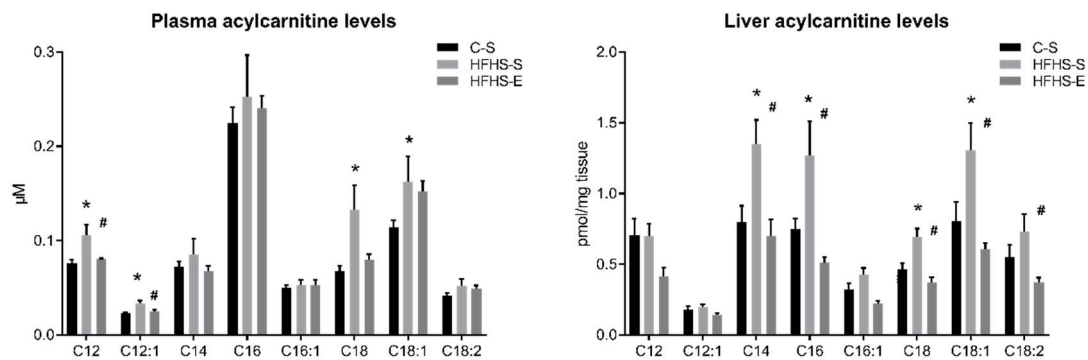


Figure 3. Effect of maternal HFHS diet and GE on acylcarnitine levels in (A) plasma and (B) liver in the female offspring. GE – gestational exercise; C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means $\pm$ SEM ($n = 6$). * vs. C-S ($p < 0.05$); # vs. HFHS-S ($p < 0.05$).

OXPHOS complex subunit and UCP2 protein expression

Even though no statistically significant changes were observed in the protein expression of electron transport chain (ETC) complexes, some general trends were identified in OXPHOS complex subunits (Fig. 4A), suggesting that they can be differently regulated in female offspring by maternal diet and exercise. On the other hand, hepatic UCP2 protein was significantly reduced in the offspring of exercised HFHS-fed mothers, although HFHS feeding alone in mothers did not affect its content.

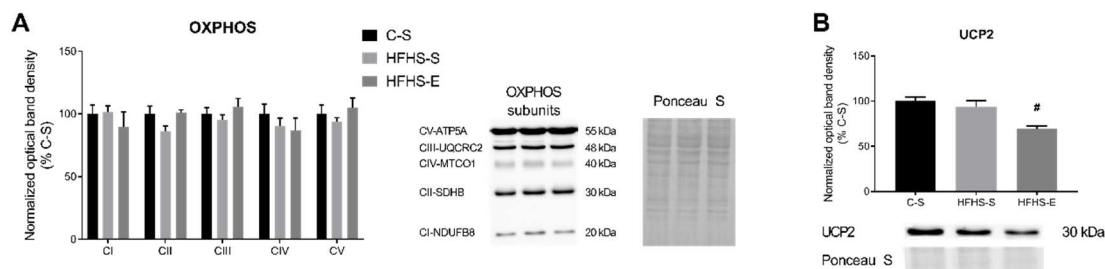


Figure 4. Effect of maternal HFHS diet and GE on liver (A) Relative contents of OXPHOS subunits and (B) Relative content UCP2. GE – gestational exercise; C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers; CI – complex I CI-NDUFB8 subunit; 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. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the C-S. Values are means $\pm$ SEM ($n = 6$). # vs. HFHS-S ($p < 0.05$).

Regulators of glucose/lipid metabolism

Transcriptional factors SREBP-1 and ChREBP are involved in the regulation of glucose and lipid metabolism, and PEPCK in gluconeogenesis, thus, the protein expression of these genes was examined in the female offspring (Fig. 5). Intrauterine exposure to maternal HFHS feeding only upregulated hepatic ChREBP protein expression (Fig. 5B), whereas GE reduced ChREBP and PEPCK content (Fig. 5B, C). Maternal diet and/or exercise neither affect plasma insulin levels (Fig. 5E) nor hepatic glycogen accumulation, although non-statistical differences were observed (Fig. 5D).

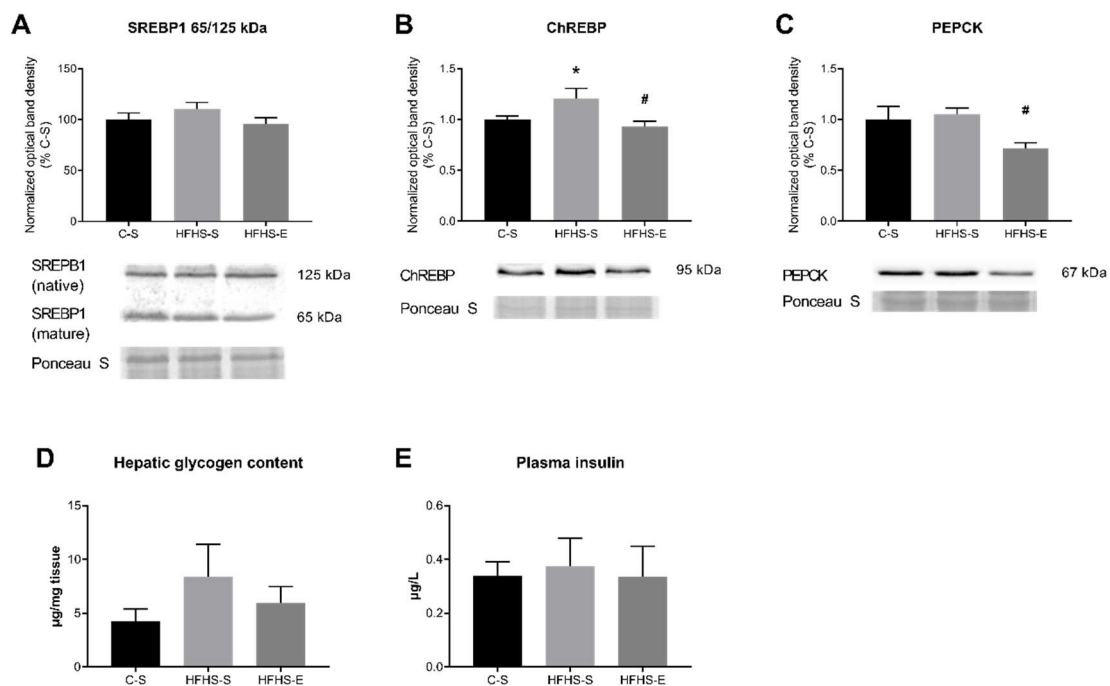


Figure 5. Effects of maternal HFHS diet and GE on the protein expression of different markers of liver glucose and lipid metabolism (A) Ratio of mature (64 kDa) and native (125 kDa) SREBP-1 form; (B) Relative content of ChREBP; (C) Relative content of PEPCK; (D) Hepatic glycogen content; (E). Western blot bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of C-S. GE – gestational exercise; C –mothers fed with control diet; HFHS –mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers; ChREBP - carbohydrate response element binding protein; PEPCK - phosphoenolpyruvate carboxykinase; SREBP-1 - sterol regulator element binding protein 1. Values are means $\pm$ SEM (n = 6). * vs. C-S (p < 0.05); # vs. HFHS-S (p < 0.05).

Mitochondrial biogenesis end-points

To analyse alterations in liver mitochondrial biogenesis, the gene expression and protein content of key promoters of mitochondrial biogenesis PGC1 α and TFAM, as well as the protein content of mitochondrial protein importer TOM20 and mtDNA copy number were assessed. A non-statistical decrease in PGC1 α and TFAM protein in female offspring of HFHS-fed mothers were identified (Fig. 6A,B). Despite this, GE remarkably upregulated mRNA and protein levels of PGC1 α and TFAM (Fig. 6A,B). On the other hand, the mitochondrial protein import, as observed as TOM20 protein content (Fig. 6C) and mtDNA copy number (Fig. 6C), were not affected by maternal diet/GE. Interestingly, principal component analysis using markers of mitochondrial biogenesis in the offspring of HFHS-fed mothers showed a difference based on the maternal physical activity levels (Fig. 6D). We also assessed ER α protein content, as it can also regulate mitochondrial biogenesis, particularly upon its translocation to mitochondria [24]; however, no alterations in the liver of female offspring were observed (Fig. 6E).

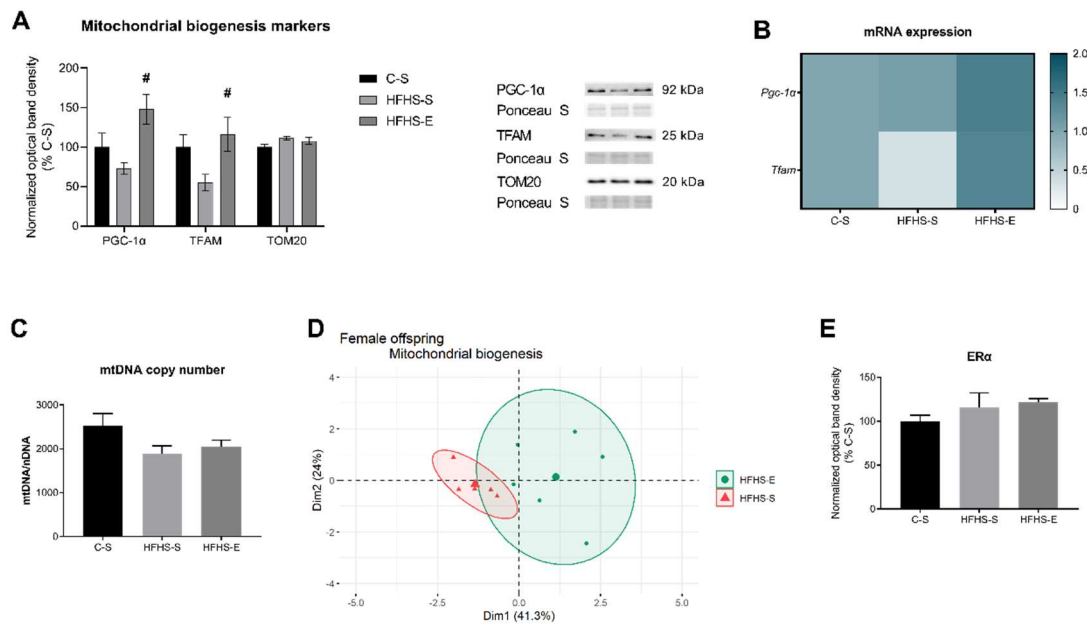


Figure 6. Effects of maternal HFHS diet and GE on the protein expression of different markers of liver mitochondrial biogenesis: (A) Relative content of PGC-1 α , TFAM, and TOM20; (B) Heat map showing the relative transcript levels for *Pgc-1 α* and *Tfam*; (C) Mitochondrial DNA copy number; (D) Principal component analysis of hepatic mitochondrial biogenesis markers; (E) Relative content of ER α . Western blot bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of C-S. GE – gestational exercise; C –mothers fed with control diet; HFHS –mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers; ER α – estrogen receptor-alpha; PGC-1 α - peroxisome proliferator-activated receptor gamma co-activator-1alpha; TFAM - mitochondrial transcription factor A; TOM20 - translocase of outer membrane 20. Values are means $\pm$ SEM (n = 6). * vs. C-S (p < 0.05); # vs. HFHS-S (p < 0.05).

Mitochondrial dynamics

Being closely associated with mitochondrial biogenesis, mitochondrial dynamics is a key feature for maintaining mitochondrial integrity. We analysed the mRNA and protein content of mitochondrial fusion proteins MFN1, MFN2, and OPA1 and mitochondrial fission protein DRP1. As observed on Fig. 7A, the content of mitochondrial fusion and fission proteins was unaffected by maternal diet and/or exercise, except for the OPA1 protein levels. The maternal HFHS-diet-induced increase of inner mitochondrial fusion protein content was restored to control values by GE. This suggests that the inner mitochondrial membrane, containing

the ETC, might be more sensitive to alterations imposed by dietary manipulations in the mother. Nevertheless, mRNA levels of *Mfn1*, *Mfn2*, and *Drp1* decreased in the offspring of sedentary HFHS mothers (*Mfn2*: $p < 0.05$), whereas GE significantly rescued this change in all 3 genes ($p < 0.05$) (Fig. 7B). Concordantly, principal component analysis using markers of mitochondrial dynamics showed a clear distinction between the offspring of HFHS-fed mothers based on the maternal physical activity levels (Fig. 7C), pointing that GE has a substantial impact on the intrauterine environment.

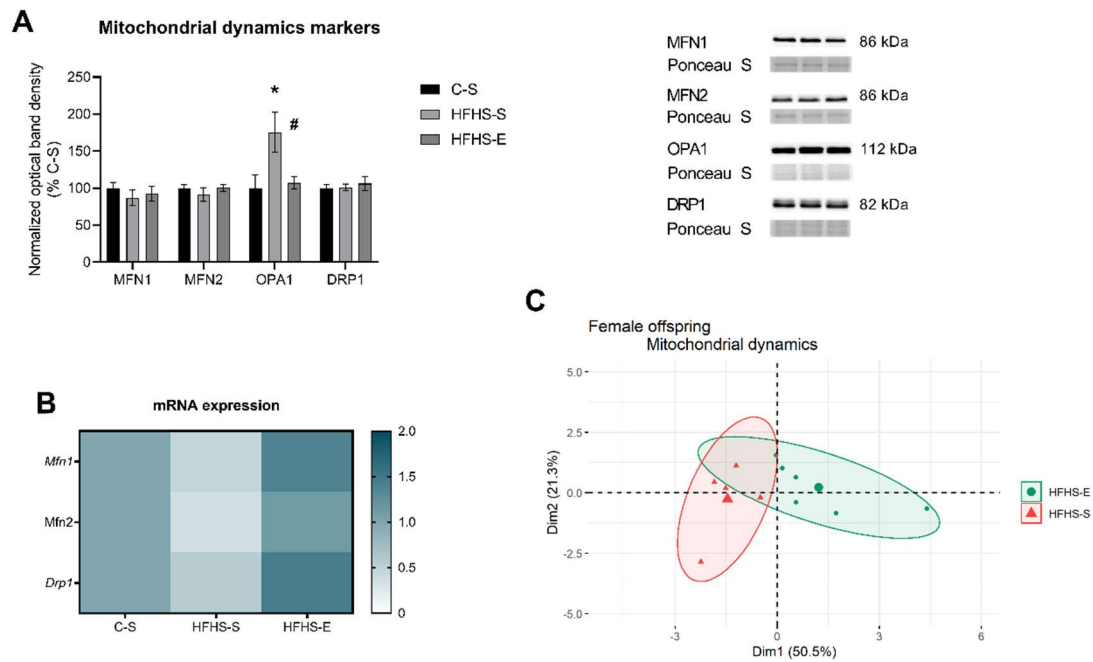


Figure 7. Effects of maternal HFHS diet and GE on the protein expression of different markers of liver mitochondrial dynamics: (A) Relative protein content of MFN1, MFN2, OPA1, and DRP1; (B) Heat map showing the transcripts for *Mfn1*, *Mfn2*, and *Drp1*; (C) Principal component analysis of hepatic mitochondrial dynamics markers; Western blot bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of C-S. GE – gestational exercise; C –mothers fed with control diet; HFHS –mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers; DRP1 - dynamin related protein 1; MFN1 – mitofusin1; MFN2 – mitofusin2; OPA1 - optic atrophy 1. Values are means $\pm$ SEM ($n = 6$). * vs. C-S ($p < 0.05$); # vs. HFHS-S ($p < 0.05$).

Auto(mito)phagy and apoptotic signalling

Processes of mitochondrial biogenesis and dynamics are closely coupled with auto(mito)phagy mechanisms. Therefore, we also analysed the content of mitochondrial quality control proteins PINK1, Parkin, and LC3, as well as pro-autophagic Beclin1 and pro-apoptotic Bax and anti-autophagic and anti-apoptotic Bcl2. We also measured the amount of cytochrome c present in the whole liver homogenates. The only significant alteration was measured in Parkin protein expression (Fig. 8A), which was increased by the maternal diet, whereas GE reverted it to the control levels. However, liver autophagy signalling (Fig. 8C) and apoptotic signalling (Fig. 8D) or cytochrome c (Fig. 8) were not altered by maternal HFHS diet and/or exercise, suggesting that this is likely not one main mechanism which an altered intrauterine environment modulates fetal liver metabolism in female offspring.

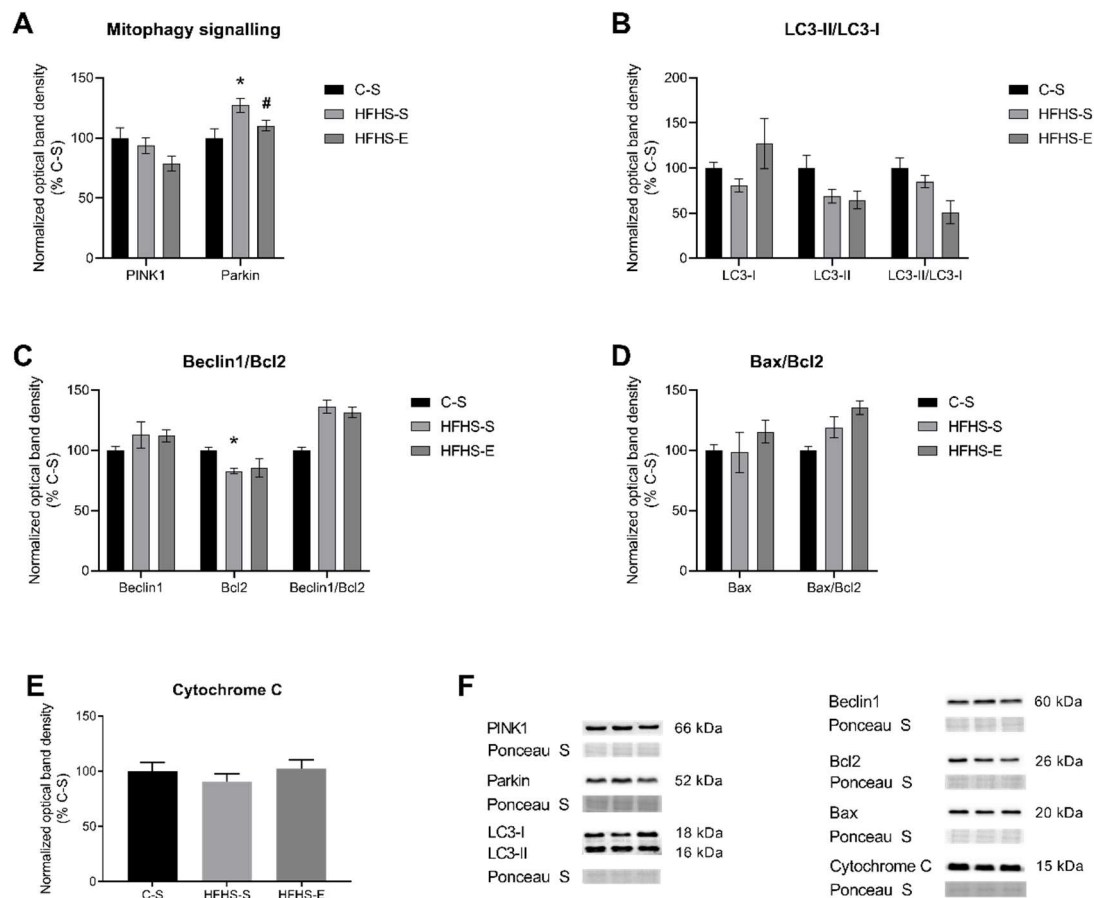


Figure 8. Effect of maternal HFHS diet and GE on the hepatic protein expression of different markers of mitophagy signalling: (A) Relative content of PINK1 and Parkin, (B) Relative content of LC3-I and LC3-II, and their ratio; autophagy signalling: (C) Relative content of Beclin1 and Bcl2 and their ratio; and apoptotic signalling: (D) Relative content of Bax and its ratio with Bcl2, (E) Relative content of Cytochrome C; (F) Typical immunoblots of respective proteins are presented. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of C-S. GE – gestational exercise; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. Values are means $\pm$ SEM (n = 6). * vs. C-S (p < 0.05). # vs. HFHS-S (p < 0.05).

Expression of miR-122, miR-34a, miR-130b, and miR-494 in the liver

To assess whether epigenetic mechanisms were involved in regulating the intrauterine effect of maternal diet and exercise, we examined microRNAs known to regulate hepatic lipid metabolism and/or mitochondrial biogenesis, such as miR-122, miR-34a, miR-130b, and miR-494. However, no significant alterations

of these miRNAs were identified in the liver of the female offspring, except for miR-34a (Fig.9). In this case, GE considerably prevented an increasing trend of miR-34a in the female offspring previously exposed to a maternal HFHS diet.

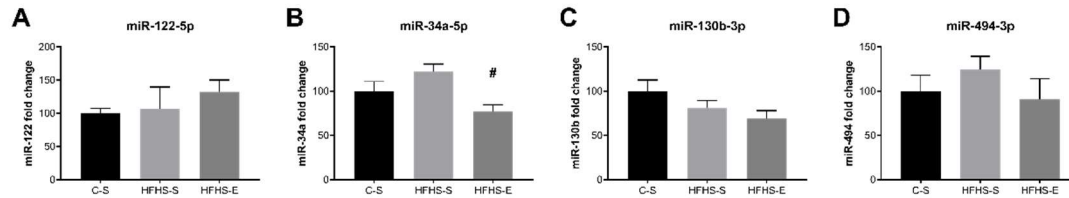


Figure 9. Effects of maternal HFHS diet and GE on the micro-RNA expression in the liver: (A) Relative expression of miR-122. (B) Relative expression of miR-34a. GE – gestational exercise; C –mothers fed with control diet; HFHS –mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. (C) Relative expression of miR-130b. (D) Relative expression of miR-494. Values are means $\pm$ SEM (n = 6). * vs. C-S (p < 0.05). # vs. HFHS-S (p < 0.05).

DISCUSSION

Considering various pregnancy-related metabolic adaptations in response to fetal demands, and GDM-related possible intergenerational transmission of metabolic burden, design of an effective treatment to protect offspring metabolic health is a complicated task. Given the consensual beneficial effects of exercise against liver diseases, it is expected that GE would improve metabolic state of the offspring exposed to maternal GDM-related metabolic manifestations. Data on liver mitochondrial changes due to maternal HFHS diet and related GDM and GE as a non-pharmacological tool against detrimental intrauterine environment is lacking, especially in female offspring. Therefore, we analysed whether GE could modulate hepatic lipid accumulation and mitochondrial biogenesis and dynamics in the female offspring of mothers with diet-induced GDM.

Macrosomia is one of the most frequent complications in offspring of GDM mothers, since an increased blood glucose is transferred through the placenta to the fetus, and stored as body fat [25]. Compared to the offspring of non-GDM mothers, 3-week-old female offspring of GDM mothers showed increased BW, which maintained for the following 2 weeks. GE prevented this excessive BW increase, yet, not reverting it completely to the control levels, implying that high-caloric intake during lactation can still program offspring development. Indeed,

high-fat diet consumption only during lactation can also increase the offspring BW [26].

Maternal high-caloric diet before and during pregnancy can predispose offspring to NAFLD development [27-29]. However, in our study, HFHS-S offspring did not differ from C-S offspring regarding NAS or liver triglyceride content, although a tendency for less lipid droplets but of a larger size was found. Still, in most of those studies, offspring were fed with high-fat diet, which probably potentiated an existing milder phenotype induced by maternal nutrition. Accordingly, no changes were observed in lipogenic transcription factor SREBP1 content, nor in miR-122, which targets SREBP1 and FA synthesis [30]. However, ChREBP was upregulated in the HFHS-S offspring. Both SREBP1 and ChREBP are needed for coordinated glycolytic and lipogenic regulation, but differently stimulated – by insulin and glucose, respectively [31], thus we could speculate that maternal GDM-related hyperglycemia was more important factor affecting the female offspring lipid metabolism. High-carbohydrate diet could induce transcription of both ChREBP and PEPCK [32], which we only observed in ChREBP content, whereas GE downregulated expression of both proteins. Decreased ChREBP content could partly explain lower lipid accumulation in HFHS-E offspring, since hepatic ChREBP inhibition is shown to alleviate hepatic steatosis [33]. Lower PEPCK content could be explained by downregulated miR-34a by GE. In fact, exercise prevented miR-34a overexpression, associated with the suppression of fibroblast growth factor-21 signalling, which suppresses PEPCK further [34]. The regulation of female offspring miR-34a levels also corresponded to the trends observed in hepatic glycogen accumulation, as miR-34a can suppress fibroblast growth factor-19 signalling, important in glycogen regulation [35,36].

It was also proposed that ER α can protect female mice from NAFLD [37] whereas in male mice, ER α signalling was necessary for exercise-related beneficial effects against NAFLD [38]. In our study, GE did not alter hepatic ER α content in female offspring, despite reduced hepatic lipid accumulation in these animals compared to HFHS-S offspring, suggesting that GE prevented hepatic lipid accumulation in 6-week-old offspring before the activation of ER α -related signalling. One limitation was that we used total liver extracts and we cannot conclude whether

a differential localization of the receptor existed. In rats, after the initial postnatal raise in estradiol concentrations from maternal or adrenal origin, its levels decline until the 6th week of age, after which hormone synthesis regulation begins [39]. Since exercise can reduce the FAs availability [40], one of the possible explanations could be that GE was efficient in energy turnover, so that less dietary FAs from the maternal diet were transported and accumulated in the fetal liver, which hepatic acylcarnitine levels in HFHS-E offspring also support.

In fact, the maternal HFHS diet was enriched with long-chain FAs (C14:0, C16:0, C16:1, C18:0, C18:1, C18:2) [16], which correlates to the acylcarnitines increased in the livers of HFHS-S offspring – C14:0, C16:0, C18:0, C18:1, and C18:2, with non-statistical increases in C16:1. This was not followed by plasma levels of some acylcarnitines, suggesting that the fetal liver is primarily primed by maternal excessive calories intake. Actually, FAs from maternal diet are transferred to the fetal liver, which still has an incomplete capacity to cope with the excessive nutrient supplies. With an increase of available nutrients, β -oxidation can be initially increased, but without a concomitant increase of Krebs cycle fluxes, β -oxidation flux rapidly becomes a surplus, resulting in acylcarnitine accumulation. In accordance with others [41], our results support that maternal HFHS diet and sedentary behaviour can induce liver acylcarnitine accumulation and consequent mitochondrial failure in the offspring liver. Also, exercise can increase plasma acylcarnitines levels [42,43], which is opposite to what we observed in HFHS-E offspring. Still, it should be considered that, in our study, the effect of the exercise on the offspring is indirect – via intrauterine environment. These acylcarnitine levels in HFHS-S offspring could indicate eventual alterations in β -oxidation, which together with OXPHOS could imply mitochondrial pathogenesis [41]. However, OXPHOS subunits expression was not affected by maternal diet/GE. Expression of UCP2, that has been related to mitochondrial proton leak and increased susceptibility to NAFLD [44], was reduced by GE, suggesting lower NAFLD risk, which correlated with NAS and hepatic triglyceride content in HFHS-E offspring. In contrast, others reported that maternal high-caloric diet upregulated UCP2 in offspring [45], which we also observed in male offspring of GDM mothers [16]. In fact, with an increase of energy supplies

originating from maternal diet, observed as an increased offspring liver acylcarnitines accumulation, it would be expected that liver mitochondria activate UCP2 signalling and related uncoupling [44]. Thus, although sex-specific responses to maternal diet on the intrauterine environment are evident, it seems that GE promotes protective mechanisms against these changes in a sex-independent manner.

We previously suggested that 6-week-old male offspring of GDM mothers are at early stage of liver disease before any histological observations [16], confirming previous observations that show that liver mitochondrial dysfunction may be an early sensitive marker of liver disease [46]. To better understand female offspring mitochondrial alterations resulting from maternal GDM, despite no observed changes in lipid accumulation, we analysed PGC-1 α , a master regulator of mitochondrial biogenesis, that promotes mtDNA transcription via stimulation of TFAM expression [47]. Reduced hepatic PGC1 α levels have been reported in sedentary high-fat diet-fed mice [48] and IR obese subjects [49]. We found that maternal HFHS diet slightly decreased the offspring's hepatic Pgc1 α and Tfam mRNA and protein content. In contrast, HFHS-E offspring had increased PGC1 α expression, suggesting that GE is a powerful tool to mitigate the consequences of maternal HFHS diet on mitochondrial biogenesis. Exercise has been proposed to increase the hepatic PGC1 α expression [50] and concomitantly stimulate β -oxidation and reduce triglyceride accumulation [51], which corroborates with PGC1 α expression, and hepatic triglycerides and acylcarnitines in HFHS-E offspring. Still, no correlation was found between mitochondrial biogenesis and miRNA regulation, as miR-130b and miR-494, which target PGC1- α and TFAM, respectively [52,53], were not altered in our study.

Besides mitochondrial biogenesis, mitochondrial dynamics processes - fusion and fission - are also involved in regulating a healthy mitochondrial network [54]; and their dysregulation has been associated with maternal nutrient oversupply [55]. Accordingly, our data showed that maternal GDM downregulated both fusion- and fission-related transcripts in female offspring, which GE remarkably rescued. Such effect was not observed in protein content suggesting the complex interaction of post-transcriptional processes. As a higher-energy consuming

process than transcription, translation is strictly controlled, and proteins are produced only at levels required for their specific functions [56]. In our study, only OPA1 protein content was upregulated by maternal GDM, which was prevented by GE. Since OPA1 requires MFNs for fusion [57], whose protein levels were not altered in this study, and considering its complex post-transcriptional regulation, high OPA1 levels could be ascribed to alterations in alternative splicing or proteolysis. These post-transcriptional mechanisms may generate distinct OPA1 isoforms and explain the effect of OPA1 overexpression on mitochondrial network fragmentation [54], which is supported by a non-significant increase in mtDNA copy number found in our study. OPA1 is also proposed as a mitophagic factor [58], since its overexpression can upregulate Parkin [59], which corroborates with hepatic Parkin levels observed in HFHS-S female offspring. Even though both mitophagy markers, PINK1 and Parkin, accumulate on damaged mitochondria and target them for autophagic degradation by Parkin-dependent ubiquitination [60], PINK1 can enhance mitophagy, but is not necessary for the process [61]. Therefore, our data regarding PINK1 and Parkin are not as surprising and suggest that a mitochondrial abnormality exists, as also seen in raised intramitochondrial crystalline inclusions, a common abnormality in NAFLD [62]. Nevertheless, GE rescued all these changes, thus protecting the hepatic mitochondrial health of female offspring despite maternal GDM. The absence in response of some hepatic autophagic and apoptotic markers in female offspring can suggest that maternal diet/GE does not have a strong impact on these processes, at least at the tissue level, whereas the effects in mitochondria might be more evident.

CONCLUSIONS

The findings of this study, specifically focused on the maternal diet/GE-related intrauterine effects on female offspring, suggest that GE performed during pregnancy is a potent factor mitigating maternal diet-induced GDM-related detrimental consequences on the hepatic tissue homeostasis of female offspring. Yet, in contrast with previous data published by our group in male offspring [16], in this female offspring group, maternal diet did not induce hepatic lipid

accumulation associated with NAFLD development, which suggests a sex-specific response to maternal high-caloric diet. On the other hand, the maternal diet-induced evident harmful alterations in mitochondrial biogenesis and dynamics were prevented by GE. Therefore, exercise during pregnancy appears to be an effective first-line non-pharmacological strategy to prevent offspring metabolic disorders originating from maternal GDM and should be considered an essential component of clinical counseling.

REFERENCES

- [1] Bianco ME, Josefson JL (2019) Hyperglycemia During Pregnancy and Long-Term Offspring Outcomes. *Curr Diab Rep* 19:143. <https://doi.org/10.1007/s11892-019-1267-6>.
- [2] Franzago M, Fraticelli F, Stuppia L, Vitacolonna E (2019) Nutrigenetics, epigenetics and gestational diabetes: consequences in mother and child. *Epigenetics* 14:215-35. <https://doi.org/10.1080/15592294.2019.1582277>.
- [3] Monteiro LJ, Norman JE, Rice GE, Illanes SE (2016) Fetal programming and gestational diabetes mellitus. *Placenta* 48 Suppl 1:S54-s60. <https://doi.org/10.1016/j.placenta.2015.11.015>.
- [4] Baker PR, 2nd, Friedman JE (2018) Mitochondrial role in the neonatal predisposition to developing nonalcoholic fatty liver disease. *J Clin Invest* 128:3692-703. <https://doi.org/10.1172/jci120846>.
- [5] Brawerman GM, Dolinsky VW (2018) Therapies for gestational diabetes and their implications for maternal and offspring health: Evidence from human and animal studies. *Pharmacol Res* 130:52-73. <https://doi.org/10.1016/j.phrs.2018.02.002>.
- [6] Ring JA, Ghabrial H, Ching MS, Smallwood RA, Morgan DJ (1999) Fetal hepatic drug elimination. *Pharmacol Ther* 84:429-45. [https://doi.org/10.1016/s0163-7258\(99\)00046-7](https://doi.org/10.1016/s0163-7258(99)00046-7).
- [7] Blumenfeld YJ, Reynolds-May MF, Altman RB, El-Sayed YY (2010) Maternal-fetal and neonatal pharmacogenomics: a review of current literature. *J Perinatol* 30:571-9. <https://doi.org/10.1038/jp.2009.183>.
- [8] (2018) ACOG Practice Bulletin No. 190 Summary: Gestational Diabetes Mellitus. *Obstetrics & Gynecology* 131:406-8. <https://doi.org/10.1097/aog.0000000000002498>.
- [9] Association AD (2020) 14. Management of Diabetes in Pregnancy: Standards of Medical Care in Diabetes—2021. *Diabetes Care* 44:S200-S10. <https://doi.org/10.2337/dc21-S014>.
- [10] Fletcher JA, Meers GM, Linden MA, Kearney ML, Morris EM, Thyfault JP, et al. (2014) Impact of various exercise modalities on hepatic mitochondrial function.

- Med Sci Sports Exerc 46:1089-97.
<https://doi.org/10.1249/mss.0000000000000223>.
- [11] Gonçalves IO, Passos E, Rocha-Rodrigues S, Diogo CV, Torrella JR, Rizo D, et al. (2014) Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion* 15:40-51.
<https://doi.org/10.1016/j.mito.2014.03.012>.
- [12] Sampath Kumar A, Maiya AG, Shastry BA, Vaishali K, Ravishankar N, Hazari A, et al. (2019) Exercise and insulin resistance in type 2 diabetes mellitus: A systematic review and meta-analysis. *Ann Phys Rehabil Med* 62:98-103.
<https://doi.org/10.1016/j.rehab.2018.11.001>.
- [13] Santos-Alves E, Marques-Aleixo I, Rizo-Roca D, Torrella JR, Oliveira PJ, Magalhães J, et al. (2015) Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling. *Life Sci* 135:124-30.
<https://doi.org/10.1016/j.lfs.2015.06.007>.
- [14] Sheldon RD, Nicole Blaize A, Fletcher JA, Pearson KJ, Donkin SS, Newcomer SC, et al. (2016) Gestational exercise protects adult male offspring from high-fat diet-induced hepatic steatosis. *J Hepatol* 64:171-8.
<https://doi.org/10.1016/j.jhep.2015.08.022>.
- [15] Wasinski F, Bacurau RF, Estrela GR, Klempin F, Arakaki AM, Batista RO, et al. (2015) Exercise during pregnancy protects adult mouse offspring from diet-induced obesity. *Nutr Metab (Lond)* 12:56. <https://doi.org/10.1186/s12986-015-0052-z>.
- [16] Stevanović-Silva J, Beleza J, Coxito P, Pereira S, Rocha H, Gaspar TB, et al. (2021) Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring. *Metabolism* 116:154704.
<https://doi.org/10.1016/j.metabol.2021.154704>.
- [17] Zhang L (2011) Voluntary oral administration of drugs in mice.
- [18] Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, et al. (2014) Establishment of a general NAFLD scoring system for rodent models and

comparison to human liver pathology. *PLoS One* 9:e115922. <https://doi.org/10.1371/journal.pone.0115922>.

[19] Rashed MS, Ozand PT, Bucknall MP, Little D (1995) Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry. *Pediatr Res* 38:324-31. <https://doi.org/10.1203/00006450-199509000-00009>.

[20] Petucci C, Rojas-Betancourt S, Gardell SJ (2012) Comparison of tissue harvest protocols for the quantitation of acylcarnitines in mouse heart and liver by mass spectrometry. *Metabolomics* 8:784-92. <https://doi.org/10.1007/s11306-011-0370-8>.

[21] Tennessen JM, Barry WE, Cox J, Thummel CS (2014) Methods for studying metabolism in *Drosophila*. *Methods* 68:105-15. <https://doi.org/10.1016/j.ymeth.2014.02.034>.

[22] Romero-Calvo I, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustin O, et al. (2010) Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 401:318-20. <https://doi.org/10.1016/j.ab.2010.02.036>.

[23] Carabelli J, Burgueño AL, Rosselli MS, Gianotti TF, Lago NR, Pirola CJ, et al. (2011) High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. *J Cell Mol Med* 15:1329-38. <https://doi.org/10.1111/j.1582-4934.2010.01128.x>.

[24] Chen JQ, Cammarata PR, Baines CP, Yager JD (2009) Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *Biochim Biophys Acta* 1793:1540-70. <https://doi.org/10.1016/j.bbamcr.2009.06.001>.

[25] Kc K, Shakya S, Zhang H (2015) Gestational diabetes mellitus and macrosomia: a literature review. *Ann Nutr Metab* 66 Suppl 2:14-20. <https://doi.org/10.1159/000371628>.

[26] Lippert RN, Hess S, Klemm P, Burgeno LM, Jahans-Price T, Walton ME, et al. (2020) Maternal high-fat diet during lactation reprograms the dopaminergic circuitry in mice. *J Clin Invest* 130:3761-76. <https://doi.org/10.1172/jci134412>.

- [27] Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. (2009) Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology* 50:1796-808. <https://doi.org/10.1002/hep.23205>.
- [28] Thompson MD, Cismowski MJ, Trask AJ, Lallier SW, Graf AE, Rogers LK, et al. (2016) Enhanced Steatosis and Fibrosis in Liver of Adult Offspring Exposed to Maternal High-Fat Diet. *Gene Expr* 17:47-59. <https://doi.org/10.3727/105221616x692135>.
- [29] Wankhade UD, Zhong Y, Kang P, Alfaro M, Chintapalli SV, Piccolo BD, et al. (2018) Maternal High-Fat Diet Programs Offspring Liver Steatosis in a Sexually Dimorphic Manner in Association with Changes in Gut Microbial Ecology in Mice. *Sci Rep* 8:16502. <https://doi.org/10.1038/s41598-018-34453-0>.
- [30] Kalaki-Jouybari F, Shanaki M, Delfan M, Gorgani-Firouzjaee S, Khakdan S (2020) High-intensity interval training (HIIT) alleviated NAFLD feature via miR-122 induction in liver of high-fat high-fructose diet induced diabetic rats. *Arch Physiol Biochem* 126:242-9. <https://doi.org/10.1080/13813455.2018.1510968>.
- [31] Linden AG, Li S, Choi HY, Fang F, Fukasawa M, Uyeda K, et al. (2018) Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice. *J Lipid Res* 59:475-87. <https://doi.org/10.1194/jlr.M081836>.
- [32] Jois T, Howard V, Youngs K, Cowley MA, Sleeman MW (2016) Dietary Macronutrient Composition Directs ChREBP Isoform Expression and Glucose Metabolism in Mice. *PLoS One* 11:e0168797. <https://doi.org/10.1371/journal.pone.0168797>.
- [33] Dentin R, Benhamed F, Hainault I, Fauveau V, Foulle F, Dyck JR, et al. (2006) Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* 55:2159-70. <https://doi.org/10.2337/db06-0200>.
- [34] Kong LJ, Feng W, Wright M, Chen Y, Dallas-yang Q, Zhou YP, et al. (2013) FGF21 suppresses hepatic glucose production through the activation of atypical protein kinase C α . *Eur J Pharmacol* 702:302-8. <https://doi.org/10.1016/j.ejphar.2012.11.065>.

- [35] Fu T, Choi SE, Kim DH, Seok S, Suino-Powell KM, Xu HE, et al. (2012) Aberrantly elevated microRNA-34a in obesity attenuates hepatic responses to FGF19 by targeting a membrane coreceptor β -Klotho. *Proc Natl Acad Sci U S A* 109:16137-42. <https://doi.org/10.1073/pnas.1205951109>.
- [36] Kir S, Beddow SA, Samuel VT, Miller P, Previs SF, Suino-Powell K, et al. (2011) FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science* 331:1621-4. <https://doi.org/10.1126/science.1198363>.
- [37] Zhang ZC, Liu Y, Xiao LL, Li SF, Jiang JH, Zhao Y, et al. (2015) Upregulation of miR-125b by estrogen protects against non-alcoholic fatty liver in female mice. *J Hepatol* 63:1466-75. <https://doi.org/10.1016/j.jhep.2015.07.037>.
- [38] Winn NC, Jurrissen TJ, Grunewald ZI, Cunningham RP, Woodford ML, Kanaley JA, et al. (2019) Estrogen receptor- α signaling maintains immunometabolic function in males and is obligatory for exercise-induced amelioration of nonalcoholic fatty liver. *Am J Physiol Endocrinol Metab* 316:E156-e67. <https://doi.org/10.1152/ajpendo.00259.2018>.
- [39] Bell MR (2018) Comparing Postnatal Development of Gonadal Hormones and Associated Social Behaviors in Rats, Mice, and Humans. *Endocrinology* 159:2596-613. <https://doi.org/10.1210/en.2018-00220>.
- [40] Shojaee-Moradie F, Baynes KC, Pentecost C, Bell JD, Thomas EL, Jackson NC, et al. (2007) Exercise training reduces fatty acid availability and improves the insulin sensitivity of glucose metabolism. *Diabetologia* 50:404-13. <https://doi.org/10.1007/s00125-006-0498-7>.
- [41] Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7:45-56. <https://doi.org/10.1016/j.cmet.2007.10.013>.
- [42] Hansen JS, Zhao X, Irmeler M, Liu X, Hoene M, Scheler M, et al. (2015) Type 2 diabetes alters metabolic and transcriptional signatures of glucose and amino acid metabolism during exercise and recovery. *Diabetologia* 58:1845-54. <https://doi.org/10.1007/s00125-015-3584-x>.

- [43] Xu G, Hansen JS, Zhao XJ, Chen S, Hoene M, Wang XL, et al. (2016) Liver and Muscle Contribute Differently to the Plasma Acylcarnitine Pool During Fasting and Exercise in Humans. *J Clin Endocrinol Metab* 101:5044-52. <https://doi.org/10.1210/jc.2016-1859>.
- [44] Serviddio G, Bellanti F, Tamborra R, Rollo T, Capitanio N, Romano AD, et al. (2008) Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. *Gut* 57:957-65. <https://doi.org/10.1136/gut.2007.147496>.
- [45] Bayol SA, Simbi BH, Fowkes RC, Stickland NC (2010) A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. *Endocrinology* 151:1451-61. <https://doi.org/10.1210/en.2009-1192>.
- [46] Gonçalves IO, Oliveira PJ, Ascensão A, Magalhães J (2013) Exercise as a therapeutic tool to prevent mitochondrial degeneration in nonalcoholic steatohepatitis. *Eur J Clin Invest* 43:1184-94. <https://doi.org/10.1111/eci.12146>.
- [47] Alam TI, Kanki T, Muta T, Ukaji K, Abe Y, Nakayama H, et al. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res* 31:1640-5. <https://doi.org/10.1093/nar/gkg251>.
- [48] Hirschey MD, Shimazu T, Jing E, Grueter CA, Collins AM, Aouizerat B, et al. (2011) SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. *Mol Cell* 44:177-90. <https://doi.org/10.1016/j.molcel.2011.07.019>.
- [49] Croce MA, Eagon JC, LaRiviere LL, Korenblat KM, Klein S, Finck BN (2007) Hepatic lipin 1beta expression is diminished in insulin-resistant obese subjects and is reactivated by marked weight loss. *Diabetes* 56:2395-9. <https://doi.org/10.2337/db07-0480>.
- [50] Laye MJ, Rector RS, Borengasser SJ, Naples SP, Uptergrove GM, Ibdah JA, et al. (2009) Cessation of daily wheel running differentially alters fat oxidation capacity in liver, muscle, and adipose tissue. *J Appl Physiol* (1985) 106:161-8. <https://doi.org/10.1152/japplphysiol.91186.2008>.
- [51] Morris EM, Meers GM, Booth FW, Fritsche KL, Hardin CD, Thyfault JP, et al. (2012) PGC-1 α overexpression results in increased hepatic fatty acid oxidation

- with reduced triacylglycerol accumulation and secretion. *Am J Physiol Gastrointest Liver Physiol* 303:G979-92. <https://doi.org/10.1152/ajpgi.00169.2012>.
- [52] Jiang S, Teague AM, Tryggestad JB, Chernaused SD (2017) Role of microRNA-130b in placental PGC-1 α /TFAM mitochondrial biogenesis pathway. *Biochem Biophys Res Commun* 487:607-12. <https://doi.org/10.1016/j.bbrc.2017.04.099>.
- [53] Yamamoto H, Morino K, Nishio Y, Ugi S, Yoshizaki T, Kashiwagi A, et al. (2012) MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. *Am J Physiol Endocrinol Metab* 303:E1419-27. <https://doi.org/10.1152/ajpendo.00097.2012>.
- [54] Liesa M, Palacín M, Zorzano A (2009) Mitochondrial dynamics in mammalian health and disease. *Physiol Rev* 89:799-845. <https://doi.org/10.1152/physrev.00030.2008>.
- [55] Lahera V, de Las Heras N, López-Farré A, Manucha W, Ferder L (2017) Role of Mitochondrial Dysfunction in Hypertension and Obesity. *Curr Hypertens Rep* 19:11. <https://doi.org/10.1007/s11906-017-0710-9>.
- [56] Wegler C, Ölander M, Wiśniewski JR, Lundquist P, Zettl K, Åsberg A, et al. (2020) Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genom Bioinform* 2:lqz010. <https://doi.org/10.1093/nargab/lqz010>.
- [57] Cipolat S, Martins de Brito O, Dal Zilio B, Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. *Proc Natl Acad Sci U S A* 101:15927-32. <https://doi.org/10.1073/pnas.0407043101>.
- [58] Yoo SM, Jung YK (2018) A Molecular Approach to Mitophagy and Mitochondrial Dynamics. *Mol Cells* 41:18-26. <https://doi.org/10.14348/molcells.2018.2277>.
- [59] Hu X, Dai Y, Zhang R, Shang K, Sun X (2018) Overexpression of Optic Atrophy Type 1 Protects Retinal Ganglion Cells and Upregulates Parkin Expression in Experimental Glaucoma. *Front Mol Neurosci* 11:350. <https://doi.org/10.3389/fnmol.2018.00350>.

- [60] Ashrafi G, Schwarz TL (2013) The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ* 20:31-42. <https://doi.org/10.1038/cdd.2012.81>.
- [61] Steer EK, Dail MK, Chu CT (2015) Beyond mitophagy: cytosolic PINK1 as a messenger of mitochondrial health. *Antioxid Redox Signal* 22:1047-59. <https://doi.org/10.1089/ars.2014.6206>.
- [62] Caldwell SH, de Freitas LA, Park SH, Moreno ML, Redick JA, Davis CA, et al. (2009) Intramitochondrial crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* 49:1888-95. <https://doi.org/10.1002/hep.22851>.

CHAPTER IV

General Discussion

The findings of the overall animal study included in this dissertation revealed that GE is an important modulator of hepatic mitochondrial metabolism, offering crucial adaptations against deleterious consequences of maternal high fat-high sucrose diet and consequent GDM-triggered hostile *in utero* environment in the male and female offspring as well as in the mothers even after prolonged period of high-caloric diet feeding and reduced physical exercise. Interestingly, some of the mitochondrial metabolic adaptations are sex-specific with male offspring expressing a phenotype more similar to their mothers. The three experimental studies were organized to elucidate the adverse effects of GDM and the counteracting beneficial effects of GE on mitochondrial respiratory function later in life and in their young male offspring (experimental study I), as well as to provide information on the metabolic and epigenetic mechanisms of action of the maternal diet and exercise on both male and female offspring (experimental studies II and III). It is our belief that data obtained in the present study contribute to the better understanding of developmental origin of liver metabolic diseases and of maternal GE on offspring liver metabolism, reinforcing the importance of physical exercise performed during pregnancy as an indispensable strategy in improving offspring's health.

Model of gestational diabetes mellitus and gestational exercise

Considering that GDM is a metabolic disorder with negative consequences on metabolism later in life and that programs the offspring susceptibility for metabolic disease, it was important to establish an animal model of GDM and implement gestational physical exercise to mitigate GDM-related metabolic effects. Undoubtedly, our data have shown that maternal HFHS diet together with physical inactivity led to the typical features of GDM in mothers – 1) increased glucose intolerance during the period of pregnancy, but not before pregnancy independently of previous lifestyle (study I); 2) increased gestational body weight gain (study I); and 3) fetal macrosomia (studies I and III). In accordance with data from several clinical trials and meta-analyses (Ming et al., 2018; Wiebe et al., 2015), GE prevented the excessive increase of body weight in mothers during pregnancy and in their offspring (studies I and III). Nevertheless, despite similar

expectations to prevent impaired glucose tolerance during pregnancy, the GE program implemented in our study did not achieve this goal (study I). In line, others suggested that the beneficial effects of exercise on metabolism can be affected by HFD and that 4 weeks of exercise improved glucose tolerance in the control diet group but not in the HFD group (Strackowski et al., 2001). Considering the specificity of metabolism during the pregnancy period and the eventual earlier switch to IR in HFHS group (Stewart et al., 2013), the two weeks of exercise preceding the glucose tolerance test were probably not efficient enough to regulate HFHS diet-induced changes in glucose tolerance. Nevertheless, considering that GE rescued liver TG accumulation and mitochondrial metabolism in the mothers and offspring (studies I, II, and III), it could be speculated that the physical exercise is more potent to prevent metabolic disturbance in the last trimester of the pregnancy, when fetal demands increase followed by increased lipolysis and IR. Unlike our study, where glucose tolerance was assessed in the end of the second trimester, others showed that GDM-related glucose intolerance was alleviated in women exercising throughout the pregnancy when the assessment of glucose tolerance was performed in the third trimester (Cordero et al., 2015; Young & Treadway, 1992). In fact, physical exercise performed only during the second trimester was not enough to promote enhancement in glucose metabolism in exercised women (Ramírez-Vélez, 2012).

Alterations in hepatic lipid and glucose metabolism

To the best of our knowledge, this experimental protocol is the first animal study to investigate the effects of GDM and, particularly, GE on maternal liver mitochondrial metabolism later in life, i.e., after GDM and physical exercise cessation. Bearing in mind that GDM increases the risk of developing NAFLD later in life (Ajmera et al., 2016; Foghsgaard et al., 2017; Lavrentaki et al., 2019), TG accumulation was assessed in liver 8 weeks after pregnancy and physical exercise protocol cessation (study I). Surprisingly, HFHS-fed mothers had lower trends of NAFLD score than the control-fed animals, even though their non-pregnant counterparts demonstrated that prolonged HFHS feeding increased NAFLD score, which can be delayed by 3 weeks of exercise at some moment

during the lifetime. Therefore, it seems that pregnancy *per se*, with its particular metabolic alteration, represents a specific factor modulating liver metabolism in a way that protects mother and fetus from additional environmental insults, such as HFHS. Some of the mechanisms involved may include accelerated breakdown of lipids in the third trimester in order to answer to the fetal demands (Lain & Catalano, 2007) or increased gestational level of estrogen, which protective role against NAFLD development in women has been recognized (Carulli et al., 2006; Polyzos et al., 2013). Nevertheless, *in utero* environment seems to be affected by all these changes. Increasing trends in hepatic TG content and NAFLD score due to maternal HFHS diet was observed in the male offspring (studies I), but not in the female offspring (study III), whereas GE reduced both parameters in all offspring regardless of maternal diet effect.

One of the explanations for these trends and for the sex-specific differences between offspring could be the very high levels of estradiol found in neonate rats, from maternal or adrenal origin, as females present slightly higher concentration than males (Bell, 2018). Additionally, the regulation of miR-125b by estrogen via estrogen receptor alpha (ER α) protects female mice from hepatic steatosis, but not male mice (Zhang et al., 2015). Therefore, we assessed the ER α content, also suggested to be important for the beneficial effects of exercise against lipid accumulation (Winn et al., 2019). However, we could not find its association with hepatic TG content and NAFLD score in male and female offspring (studies I and III). The absence of alterations in ER α expression in the 6-week-old offspring (studies II and III) could be explained, at least in part, by the fact that at that age, after reducing the levels of estradiol of maternal or adrenal origin, rats' estradiol concentration is lower (Bell, 2018). Mitochondrial function, biogenesis, and dynamics can be under estrogenic control (Klinge, 2020), which support an interesting finding in study II that mothers have alterations in ER α levels in liver mitochondria, but not on the whole tissue level. The observed mitochondrial ER α changes match with the trends detected in markers of mitochondrial biogenesis and fusion on the liver mitochondrial level. Even though the male and female offspring follow the maternal pattern in liver mitochondrial biogenesis and fusion, which is also expected considering the actual and dominant concept of maternal

mitochondrial inheritance (Luo et al., 2018; Parakatselaki & Ladoukakis, 2021; Rius et al., 2019), at least at this age these changes in mitochondrial homeostasis cannot be related to the ER α alterations in the offspring. Yet, another study showed that maternal HFD upregulated hepatic ER α in the male offspring at adulthood, but not in the female offspring (Miranda et al., 2018), suggesting that male offspring might be predisposed for estrogen receptor signalling later in life, which deserves further research.

To clarify the putative mechanisms underlying intrauterine effects on lipid metabolism in mothers and offspring, we assessed protein expression of transcriptional factors involved in hepatic lipid (and glucose) metabolism, such as SREBP-1 and ChREBP (Linden et al., 2018), as well as PEPCK, which is a key rate-limiting gluconeogenic enzyme (Yabaluri & Bashyam, 2010). Whereas in mothers we could only observe SREBP-1 downregulation by GE, in the offspring there is an evident sex specificity influence in the regulation of these processes. Interestingly, SREBP-1 mediates insulin-related induction of lipogenic genes, whereas ChREBP mediates glucose-related induction of these genes (Linden et al., 2018), even though nutritional regulation of lipogenic genes may be completely independent on insulin (Matsuzaka et al., 2004). Fetal liver SREBP-1 was found to be elevated in late gestation, upon *in utero* insulin injection inducing premature rise in insulin, during the lactation, and until weaning (Bobard et al., 2005). In our study, the increase of mature/native SREBP-1 ratio in the offspring of sedentary HFHS mothers, also prevented by GE during pregnancy, is concomitant with hepatic lipid accumulation, although the effects were only observed in male offspring. Similar sex-dependent changes were also observed in the intrauterine growth restriction rat model, being suggested that developmental programming and differential alterations of liver structure may be, at least partly, underlying mechanisms of lipid dysregulation (Choi et al., 2007). On the contrary, ChREBP was upregulated only in the HFHS-S female offspring, but not in males. Thus, it could be speculated that maternal hyperglycemia, characteristic for GDM, has sex-specific effects, and that hepatic lipid metabolism in the male offspring is rather mediated via insulin and SREBP-1, whereas in the female offspring it is mediated via glucose and ChREBP activation. Although

maternal *in utero* hyperglycemia can cause fetal hyperinsulinemia (Kc et al., 2015), only male offspring, but not female had obvious trends (although not significant) in insulin plasma due to maternal lifestyle. This might be a consequence, at least in part, of the post-weaning C diet. Likewise, it has been suggested that GDM is a high-risk factor for male children, but not for female, adiposity accumulation (Alur, 2019), and that maternal nutrition impacts glucose homeostasis programming in female offspring and body weight and adiposity in male offspring (Dearden et al., 2018). Among other yet unknown putative mechanisms, these sex differences and the female offspring glucose metabolic susceptibility to maternal high-fat high-sucrose diet could be related with paraventricular hypothalamus gene expression (Dearden & Balthasar, 2014), which impairment might result in dysfunctional glucose homeostasis (Myers & Olson, 2012). Also, SREBP-1 is able to repress genes of gluconeogenesis, such as PEPCK (Chakravarty et al., 2001; Yamamoto et al., 2004), which is concomitant with the PEPCK content in the male, but not in the female offspring, suggesting that there must be additional mechanisms, including those related with the epigenetic landscape, promoting PEPCK downregulation in the offspring of exercising mothers. Regarding mothers, PEPCK downregulation in HFHS-fed animals was reduced regardless of physical activity levels, suggesting that activation of gluconeogenesis was not necessary in these animals, as expected when glucose is available from external resources (Barthel & Schmoll, 2003), such as HFHS diet. High hepatic gluconeogenesis rates have been reported in animals or patients diagnosed with diabetes and NAFLD (Barthel & Schmoll, 2003; Chang et al., 2006; Sunny et al., 2011). On the other hand, physical exercise improves insulin sensitivity, which could prevent these alterations in gluconeogenesis in obese and diabetic rats and downregulate hepatic PEPCK (Chang et al., 2006). In fact, although in mothers 3-weeks of physical exercise were not enough to counteract 18-week of HFHS diet, this GE emerged as a very potent factor in reducing gluconeogenesis rates in the offspring. Yet, gluconeogenesis is not apparent in the fetus as PEPCK appears only after the birth (Kalhan & Parimi, 2000). Therefore, the effects of maternal diet and/or

exercise on this metabolic pathway is indirect, through other metabolic pathways or even some epigenetic mechanisms, rather than through a direct *in utero* effect.

Hepatic mitochondrial function, biogenesis, and dynamics

Hepatic mitochondrial respiratory function involves pathways considered vital in maintaining hepatic gluconeogenesis and lipogenesis, such as β -oxidation, Krebs cycle, and the respiratory electron transport chain. In response to nutrient overstimulation, although mitochondrial network adapts and remodels to counteract the changes, less favorable hepatic metabolic condition, such as IR, will continue to prevail (Surugihalli et al., 2019). In fact, impaired mitochondrial energetics has been associated with T2DM and liver diseases (García-Berumen et al., 2019; Lambert et al., 2014; Satapati et al., 2012), and mitochondrial dysfunction has even proposed as one of the primary events in the NAFLD development (Gonçalves et al., 2013).

In the present study, data from acylcarnitine profiling, used to ascribe alterations in β -oxidation (Koves et al., 2008), showed an interesting, but not surprising, match with the fatty acid composition of the maternal HFHS diet in the offspring generation (study I and III). As offspring were exposed to HFHS diet only indirectly, through placenta or maternal nursing, and fed with C diet post-weaning, the observed alterations in acylcarnitines must be related to maternal diet/GE. The increase in plasma acylcarnitines has been observed in different metabolic disorders – prediabetic and diabetic conditions, metabolic syndrome, insulin resistance, and obesity (Bene et al., 2013; Floegel et al., 2013; Mai et al., 2013). In contrast, data from mothers revealed that acylcarnitines were not altered by nutrition and/or GE (study I). In line with our results, others showed that even a prolonged HFD did not influence hepatic acylcarnitines (Koves et al., 2008; Zabielski et al., 2018). Additionally, it should be considered that during pregnancy, due to increased fetal demands, maternal hepatic metabolism changes and its concomitant metabolic state shifts from anabolic to catabolic (Lain & Catalano, 2007; Zeng et al., 2017). Yet, it has been reported that GDM-related hyperglycemia can decrease β -oxidation of fatty acids in placenta and result in higher lipid accumulation (Visiedo et al., 2013). Therefore, it can be

hypothesized that decreased β -oxidation would spare placenta fatty acids and allowed greater availability of those substrates for the fetus. Indeed, although only in the male offspring of GDM mothers, the rise in acylcarnitine levels in plasma and liver is in line with hepatic lipid accumulation (study I). Yet, in the female offspring of GDM mothers no alterations were observed in hepatic TG content despite increase in acylcarnitine levels (study III). A study conducted by Devanathan et al. (2016) reported a similar effect in diabetic Goto-Kakizaki rats, i.e., heart acylcarnitines were increased in both sexes, but TG accumulation was sex-specific – increased in male and reduced in female rats. This suggests that fatty acids in females were shuttled from TG synthesis, or that synthesized TG were converted into other lipid products, yet the study did not provide a clear mechanism for this sex-specificity (Devanathan et al., 2016). Nevertheless, exercise prevented an excessive increase of acylcarnitines due to maternal metabolic disturbances. In fact, GE was effective regarding energy turnover from maternal diet; thus, less fatty acids could have been transferred to the fetus, resulting in less acylcarnitine accumulation in the offspring plasma and liver (study I and III).

Accumulation of acylcarnitines may induce disruption in β -oxidation (Visiedo et al., 2013). Although an initial increase of β -oxidation may have occurred in the animals submitted to HFD and low physical exercise levels, β -oxidation rate may outpace the activity of the Krebs cycle, which might have led to disruption of β -oxidation flux and mitochondrial accumulation of acylcarnitines, consequently contributing to mitochondrial failure in sedentary animals (Koves et al., 2008; Muoio & Koves, 2007). Furthermore, β -oxidation can directly affect hepatic mitochondrial respiratory function (Bjørndal et al., 2018). Concordantly with these data, mitochondrial respiratory function was adversely affected by maternal GDM conditions in the liver of male offspring (study I). In the male offspring, besides compromised mitochondrial RCR (in the presence of complex-I-linked substrates), maternal GDM induced a decreased expression of OXPHOS complex I-related proteins, and slightly lessened the expression of other respiratory complexes, suggesting that adverse *in utero* environment, due to the maternal diet/GE, reduces hepatic oxidative capacity. In the sedentary GDM

mothers, such effect was not observed (study I). Interestingly, whereas the studies using male rats have demonstrated that acute or chronic HFD feeding can impair hepatic mitochondrial function (Ciapaite et al., 2007; Garnol et al., 2014; Gonçalves et al., 2014; Morris et al., 2013), studies performed on female rats fail to reveal these alterations in mitochondrial respiration under such feeding conditions (Cardoso et al., 2013; Català-Niell et al., 2008). Even though studies on hepatic mitochondrial respiration in the offspring generation are scarce, a decreased OXPHOS protein expression or activity was found in the offspring of obese or HFD-fed mothers (Borengasser et al., 2011; Bruce et al., 2009). In the present study, GE not only rescued the compromised mitochondrial respiratory efficiency, together with an increase in OXPHOS complex I and IV-related protein expression in the male offspring, but even provided adaptative mechanisms to the exercised GDM mothers. In fact, even after 8 weeks of exercise cessation, respiratory function and OXPHOS complex III and IV, and ATP synthase protein expression remained elevated in the exercised GDM dams (study I). The impairment of mitochondrial respiratory chain in the offspring of sedentary HFHS-fed mothers was also followed by increased proton leakage rates (as observed in upregulation of UCP2 and respiration in the presence of oligomycin) (study I), which is a common consequence in fatty liver disease (Serviddio et al., 2008) and is also reported in the offspring of mothers fed with high-caloric diet (Bayol et al., 2010). In such conditions of energy excess, liver mitochondrial substrate oxidation shifts from processes coupled to ATP synthesis to uncoupled pathways involving UCP2 (Serviddio et al., 2008), which can additionally be stimulated by fatty acids (Armstrong & Towle, 2001), as the increased acylcarnitine accumulation due to the maternal adverse lifestyle may be a stimulus for the UCP2 accumulation and the consequent proton leakage. On the other hand, mothers had an opposite response to the diet and exercise regarding UCP2 levels, which suggest different regulatory mechanisms in two generations. Indeed, in complicated pregnancy, such as GDM, there is a higher risk for acute ischemia (Ma et al., 2012; Westbrook et al., 2016), and eventual ATP depletion, which is associated to reduced UCP2 levels (Cheng et al., 2003). Regardless of intergenerational differences in response to maternal sedentary lifestyle and

high-energy diet, it was clear that GE rescued any of the altered constraints in the mitochondrial function in both generations.

Unfortunately, as one of the limitations of this study, data on hepatic mitochondrial oxygen consumption from female offspring were not completely collected due to unexpected technical constraints; thus, no conclusion on eventual sex-specificity regarding mitochondrial respiration was possible to obtain in this study. Nevertheless, the analysis of the mitochondrial respiratory assays in a limited number of the female offspring ($n = 2$ per group) showed the same pattern as observed in the males. Despite obvious effects of the maternal diet/GE, considering such a small sample size, the results from this experimental group were not included in the present study.

To clarify the mechanisms underlying mitochondrial modulation, mitochondrial biogenesis and dynamics were also assessed. The master regulator of mitochondrial biogenesis, PGC-1 α , its downstream target TFAM, and the content of mtDNA, which transcription is regulated by TFAM (Puigserver & Spiegelman, 2003), were decreased in diabetic patients (Mootha et al., 2003; Patti et al., 2003), probably due to the reduced exercise capacity of these individuals (Hernández-Alvarez et al., 2010). In our study, in contrast with data obtained in mothers, the transcription levels of mitochondrial biogenesis and dynamics markers were modified by maternal diet/GE in both male and female offspring (studies II and III). This suggests that the modulatory stimuli imposed by diet and physical exercise activate different mechanisms in the maternal generation and, through *in utero* environment, in the offspring generation. Nevertheless, the mitochondrial protein content followed the same trends in both generations. Hence, maternal HFHS diet impaired mitochondrial biogenesis, observed through mRNA and protein levels of PGC-1 α , TFAM and mtDNA copy number, but maternal GE remarkably mitigated these diet-induced deleterious consequences (studies II and III). As the stimulation of PGC-1 α gene in HFD rats was reported to enhance liver mitochondrial respiration and prevent excessive hepatic lipid accumulation (Morris et al., 2013), this can explain, at least in part, the observed boosted mitochondrial respiration and lower TG content in the male offspring and their mothers (study I).

The preservation of the pool of healthy mitochondria depends not only on the processes of mitochondrial biogenesis, but also on mechanisms of mitochondrial fusion and fission, and mitophagy (Seo et al., 2010). In the present study, GE prevented the maternal diet-induced decline of mitochondrial biogenesis markers' transcriptional levels in the offspring generation (studies II and III), but such effects of diet and exercise were not detected in the mothers (study II). Regarding the protein content of some mitochondrial dynamics markers (MFN1, MFN2, OPA1, and DRP1), it seems that maternal HFHS diet shifted the mitochondrial network towards fission, whereas GE prevented this trend and rather stimulated mitochondrial fusion. However, such effects were observed only in mothers and their male, but not in female offspring. The last, only had alterations of OPA1 content and in a completely opposite manner compared to their mothers or male offspring. Yet, it should be considered that, as previously mentioned, one of the weaknesses of this study is that liver mitochondria were not isolated from female offspring; therefore, markers of mitochondrial dynamics were analysed at liver tissue level and not on isolated mitochondrial, as it was in the mothers and male offspring. Regarding these processes of mitochondrial dynamics, the inconsistency between DRP1 mRNA expression and protein content could be explained by the complex interaction of post-transcriptional processes that strictly control protein levels. In fact, being the protein synthesis, a much higher energy demanding process compared to transcription, its more meticulously and rigorously controlled and proteins synthesized only when their specific function is required (Wegler et al., 2020).

Besides mitochondrial biogenesis and dynamics, cellular bioenergetics is also mediated by mitochondrial quality control signalling, including the recycling of damaged mitochondria (Michel et al., 2012). Regarding mitophagy markers PINK1 and Parkin, we observed an interesting sex and intergenerational specificity. While in male offspring only PINK1 was modulated by GE (study II), Parkin was more sensitive to maternal diet/GE both in mothers and female offspring, although with an opposite effect between generations (study II and III). The impact of GE in the male offspring induced PINK1 upregulation, together with mitochondrial fusion stimulation, which could be considered a rescue mechanism

counteracting the mitochondrial impairment by maternal high-caloric diet. On the other hand, the downregulation of Parkin in mothers fed with the HFHS diet and with sedentary lifestyle, reinforces its role as compromising factors of the clearance of damaged mitochondria, which is similar to that reported in HFD-fed sedentary male rats (Gonçalves et al., 2016). In the young female offspring, Parkin-associated mitophagy was stimulated as a response to the mitochondrial damage (observed also through intramitochondrial crystalline inclusions accumulation) induced by maternal HFHS diet, but was prevented by GE. Nevertheless, there is a lack in sex-specific comparative studies in offspring of mothers with altered nutrition and/or metabolic dysfunction during pregnancy, being studies mainly focused on male offspring. Up to our knowledge, this is the first study that compares male and female offspring of GDM mothers, and that follows the post-GDM alterations in dams. Therefore, it is of critical relevance to compare sex differences in future studies. In general, our data suggest that *in utero* environment promotes sex-specific regulation of mitophagy that its different from the observed in the mothers and has direct impact of their lifestyle. Similarly, autophagic and apoptotic signalling was only affected in mothers, particularly at mitochondrial level as no alterations were observed in the whole liver tissue, suggesting disrupted apoptosis due to HFHS diet and its rescue by GE. As no significant impact in these markers was evident in the offspring generation, regardless of sex, it seems that the *in utero* environment was not a strong modulator of these processes in this generation and/or that regular post-weaning diet was sufficient to restore any eventual impairment.

Potential epigenetic mechanisms involved in the regulation of intergenerational hepatic metabolism

In order to better understand mechanisms underlying intergenerational transmission of metabolic burden and mediating sex-specific programming, several microRNAs, known to be involved in the regulation of hepatic metabolism and mitochondrial biogenesis through maternal diet/GE, were analyzed.

Although considered the most abundant microRNA in the liver tissue (Kalaki-Jouybari et al., 2020), data from the present study cannot ascertain the role of

miR-122 in the eventual alterations on lipid metabolism imposed by maternal diet/GE (studies II and III). Interestingly, maternal lifestyle, specifically GE, modulated those microRNAs that are associated with regulation of mitochondrial biogenesis. In fact, particularly evident in the male offspring and partly in mothers, GE prevented any eventual modifications in miR-34a, miR-130b, and miR-494 induced by maternal diet, therefore supporting directly or indirectly the regular processes of mitochondrial biogenesis. In these experimental groups, GE emerged as a very powerful stimuli, even stronger than the maternal diet itself, in regulating microRNAs-related mechanisms (study II). However, this was not as clear in the female offspring, except for miR-34a and trends in miR-494 expression (study III). Being these microRNAs coded on autosomal chromosomes, eventual sexual dimorphism in this study could be more related to other mechanisms. Placental microRNA regulation could also affect developmental processes, yet sex-specific studies are lacking. For instance, placental miR-130b expression regulates mitochondrial biogenesis through PGC-1 α and TFAM, but differences between sexes were not reported (Jiang et al., 2017). Moreover, sex differences in hepatic miR-494 are expected only in old rats, while aging may induce changes in hepatic miR-34a between adult and old rats, regardless of sex (Kwekel et al., 2017). Also, inverse association between maternal pre-pregnancy body mass index and placental miR-34a expression was observed only in girls, but not in boys (Tsamou et al., 2017). Still, differences in the development of placenta and its microRNA expression as well as variation are expected between animal and human studies (Hayder et al., 2018), which reinforces the need of more sex-related studies in various *in vitro* and *in vivo* models in order to better understand this complex epigenetic regulation.

Sexual dimorphism of biological processes involved in GDM-related liver mitochondrial health in the offspring

Mitochondrial inheritance is almost exclusively maternal (Luo et al., 2018; Parakatselaki & Ladoukakis, 2021; Rius et al., 2019), thus the interest in determining the impact of maternal mitochondrial “health” on offspring mitochondrial future is increasing. Due to this uniparental inheritance,

mitochondria have a strong sex-specific effect. Generally, several mechanisms and properties in liver mitochondria express higher activity rate, efficacy, and/or content in females than in males, such as ROS production, ADP-stimulated respiration, HFD-induced OXPHOS capacity, protein content, cardiolipin content, and mtDNA copy number, while there are no differences in mitochondrial biogenesis factors (Ventura-Clapier et al., 2017). It is even proposed that males have lower respiratory function than females, irrespective of physical activity levels, and that they are more reliant on exercise to preserve the mitochondrial health, whereas females have high respiratory capacities and low mitophagy flux, even if sedentary (Von Schulze et al., 2018).

The sexual dimorphism observed in physiological processes can be related with differences in sex chromosomes, hormonal regulation, epigenetic mechanisms, or even placenta (Dearden et al., 2018; Ventura-Clapier et al., 2017). Genes of the mitochondrial genome and X chromosome are more time under selection in females, thus better optimized than in males (Tower, 2006; Ventura-Clapier et al., 2017). On the other hand, it was hypothesised that nuclear genes that are inherited from the father could regulate activity of mitochondria derived from mothers (Mittwoch, 2004). Also, considering the vital role of placenta in the transfer of nutrients to the fetus, it is expected that maternal nutritional state may differently affect male and female placenta; however, more research is still needed. Changes in morphology and function of male placenta was shown to be more susceptible to *in utero* programming in obese rats (Kim et al., 2014), whereas in humans these effects were rather seen in female placenta (Mandò et al., 2016).

However, in our study we could not directly associate any of the sex-specific differences with the proposed mechanisms, namely the hormonal regulation through ER α and miRNAs.

Considering the young age of the offspring and the regulation of their sex hormones, particularly estrogens, we could not support observations that females are generally more protected than males from metabolic disorders and hepatic lipid accumulation due to the protective actions of estrogen (Ballestri et al., 2017). As previously discussed, data from rats suggest that steroidogenesis at birth is

low and high estradiol concentrations of maternal or adrenal origin reach the peak at 15 days of age, after which remain low until the regulation of estrous cycle in females at about 6 weeks of age (Bell, 2018), which was actually the terminal time point in our study.

Being epigenetic regulation of the genome possibly altered by maternal nutritional status and affecting embryonic development, it is considered as a possible mechanism through which *in utero* environment alterations can be translated into permanent gene expression changes of the offspring (Dearden et al., 2018). Yet, as previously discussed, no clear sex-specific differences in epigenetic regulation were observed in our study, except that female offspring were less susceptible to maternal lifestyle-related *in utero* alterations.

The clear sex specificity was observed in the regulation of transcriptional factors related with lipid and glucose metabolism. In fact, it was shown that male children of obese or diabetic mothers are more susceptible to adiposity (Andres et al., 2015; Oostvogels et al., 2017; Regnault et al., 2013), which was confirmed by animal studies suggesting that female offspring are more vulnerable to glucose homeostasis alterations due to maternal nutritional status (Dearden & Balthasar, 2014; Fuente-Martín et al., 2012; Pankey et al., 2017). In our study, we observed that male offspring of sedentary GDM mothers increased hepatic lipid accumulation and mature/native SREBP1 ratio, which suggests tendencies towards adiposity. On the other hand, only ChREBP upregulation was evident in female offspring, supporting the beforementioned hypothesis, being GE an effective strategy to prevents these alterations.

Nevertheless, future research on the effects of maternal lifestyle (diet and GE) on both sexes is necessary to elucidate the way alterations in the early life environment can differently impact metabolic health of male and female offspring.

CHAPTER V

General Conclusions

Considering the overall results of the different experimental studies included in the present thesis, the following main conclusions can be made:

- I) GE was efficient in preventing the metabolic and mitochondrial adverse consequences in maternal generation that were related to the HFHS-diet induced GDM and not necessarily to the prolonged HFHS diet consumption.
- II) GE prevented the deterioration of *in utero* conditions due to the maternal HFHS diet and related GDM and protected both male and female offspring from such harmful *in utero* environment. Moreover, GE emerged as a more potent factor for the embryonic and fetal development than maternal diet regarding the modulation of hepatic metabolic pathways.
- III) Hepatic lipid accumulation, characteristic from chronic liver diseases, was reduced by GE in both male and female offspring, but sex specificity was observed in the regulation of lipid and glucose metabolism as a response to maternal high-caloric feeding before and during pregnancy and lactation. This prompted alterations in lipid metabolism in the male and in glucose metabolism in the female offspring.
- IV) GE rescued liver mitochondrial respiratory function impairments derived from maternal HFHS feeding in the male offspring. Moreover, promoted positive mitochondrial adaptations in the maternal generation even after exercise cessation and prolonged HFHS feeding.
- V) Impairments in mitochondrial biogenesis and dynamics imposed by maternal GDM were prevented by GE, suggesting the relevance of GE performed during pregnancy in preserving mitochondrial homeostasis and healthy mitochondrial network, particularly in the next generation.
- VI) Auto/mitophagy and apoptotic signalling pathways in the offspring liver were not affected by maternal diet/GE, although these processes were observed mitochondrial level in the mothers, suggesting that liver mitochondria rather than the whole tissue are more sensitive to lifestyle alterations.

VII) Epigenetic regulation via microRNA-related mechanisms was more sensitive to GE than to the maternal diet, particularly in the male offspring and mothers.

In general, this work highlights the role of GE as a first-line non-pharmacological strategy preventing GDM-related metabolic complications in mothers and, particularly, in their offspring. Physical exercise performed during the sensitive period of pregnancy, particularly if additionally burdened by a high-caloric diet, can alleviate the parameters that are closely related to the GDM development and not only to the high-caloric diet consumption. Even though some of the liver mitochondrial pathways studied in the mothers were rescued by the GE program and even maintained despite HFHS feeding persistence and physical exercise cessation, the GE could not prevent all the metabolic changes. Thus, it can be speculated that a synergistic effect of metabolic adaptations during pregnancy and HFHS diet-induced GDM compromised mitochondrial homeostasis in a manner that 3 weeks of GE program could not completely prevent, and it was even aggravated by prolonged HFHS diet. However, GE prevented the excessive transfer of HFHS-derived nutrients through placenta to the fetal liver, reducing the nutritional pressure on offspring liver development and metabolism. Therefore, GE prevented the metabolic consequences induced by maternal HFHS-related GDM, and thus can be considered as a very powerful modulator of *in utero* environment. Taking into consideration the beneficial influence that GE might have on mothers' and, especially, children health, data from the present study reinforces and encourages the implementation of physical exercise programs during pregnancy in the clinical counselling.

As a support to the beforementioned general conclusion, the principal component analysis, that included absolutely all variables used in the preparation of this dissertation, revealed a clear distinction between the three experimental groups in each generation and sex (Fig. 1). Interestingly, the experimental groups in the maternal generation appeared to be separated into three completely distinct clusters (Fig. 1A). It could be speculated that the pregnancy *per se* imposes metabolic alterations that in synergistic interaction with HFHS diet or the

combination of HFHS diet and GE brings distinctive characteristics to each experimental group. On the other hand, the principal component analysis of both male and female offspring showed a clear distinction between the HFHS groups, with the offspring of exercising mothers being more similar to the offspring from the control group (Fig. 1B. C). Still, the effect of GE is better observed in the male than female offspring. However, it should be taken into consideration that the experiments in the female offspring were performed exclusively at the whole tissue levels, unlike the male offspring, in which some of the parameters were also analysed at the mitochondrial level. This might suggest that mitochondrial alterations due to the maternal diet/GE could eventually contribute to the clearer experimental groups clustering in the male offspring. Yet, the presence of sex-specificity should not be underestimated and deserves future research.

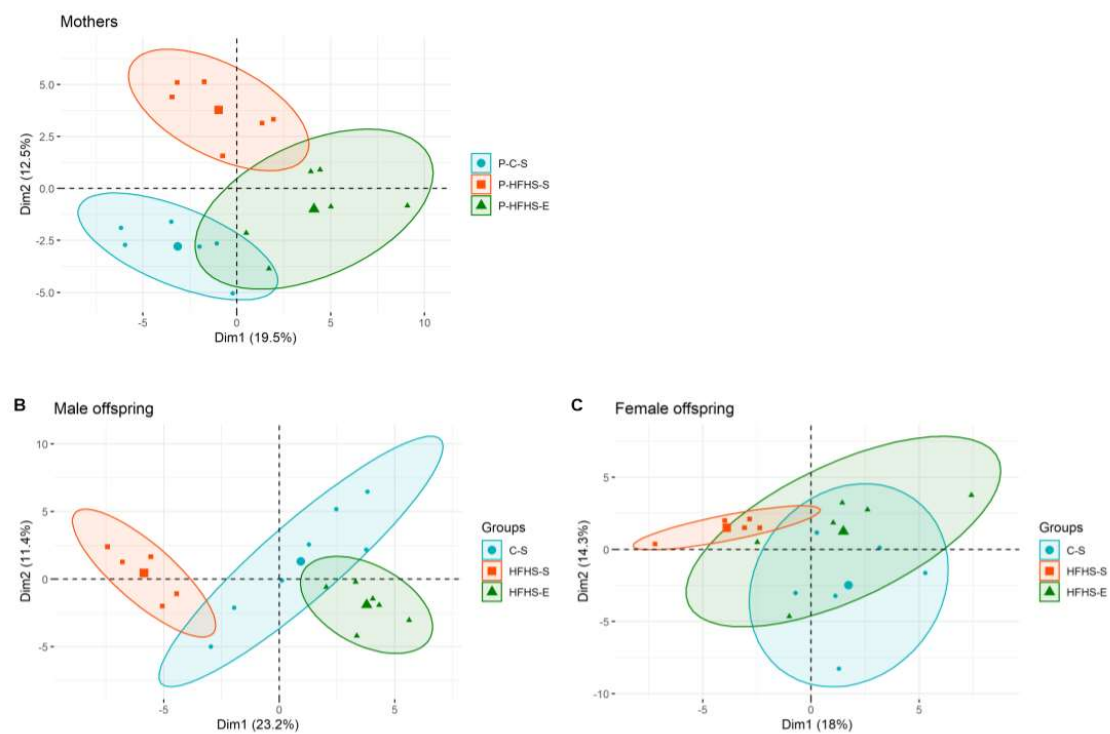


Figure 1. Principal component analysis of all parameters studied in this dissertation in (A) mothers, (B) male offspring, and (C) female offspring.

P – pregnant animals, C –mothers fed with control diet; HFHS –mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers.

REFERENCES

- Adeva-Andany, M. M., Pérez-Felpete, N., Fernández-Fernández, C., Donapetry-García, C., & Pazos-García, C. (2016). Liver glucose metabolism in humans. *Biosci Rep*, 36(6). <https://doi.org/10.1042/bsr20160385>
- Agarwal, M. M., Dhatt, G. S., & Othman, Y. (2015). Gestational diabetes: differences between the current international diagnostic criteria and implications of switching to IADPSG. *J Diabetes Complications*, 29(4), 544-549. <https://doi.org/10.1016/j.jdiacomp.2015.03.006>
- Agarwal, P., Morriseau, T. S., Kereliuk, S. M., Doucette, C. A., Wicklow, B. A., & Dolinsky, V. W. (2018). Maternal obesity, diabetes during pregnancy and epigenetic mechanisms that influence the developmental origins of cardiometabolic disease in the offspring. *Crit Rev Clin Lab Sci*, 55(2), 71-101. <https://doi.org/10.1080/10408363.2017.1422109>
- Aharoni-Simon, M., Hann-Obercyger, M., Pen, S., Madar, Z., & Tirosh, O. (2011). Fatty liver is associated with impaired activity of PPAR γ -coactivator 1 α (PGC1 α) and mitochondrial biogenesis in mice. *Lab Invest*, 91(7), 1018-1028. <https://doi.org/10.1038/labinvest.2011.55>
- Ajmera, V. H., Gunderson, E. P., VanWagner, L. B., Lewis, C. E., Carr, J. J., & Terrault, N. A. (2016). Gestational Diabetes Mellitus Is Strongly Associated With Non-Alcoholic Fatty Liver Disease. *Am J Gastroenterol*, 111(5), 658-664. <https://doi.org/10.1038/ajg.2016.57>
- Almeida, M. C., Dorés, J., Vicente, L., Paiva, S., Ruas, L. (2017). Consenso “diabetes gestacional”: Atualização 2017. *Revista Portuguesa de Diabetes*, 12(1), 24-38.
- Alur, P. (2019). Sex Differences in Nutrition, Growth, and Metabolism in Preterm Infants. *Front Pediatr*, 7, 22. <https://doi.org/10.3389/fped.2019.00022>
- American College of Obstetricians and Gynecologists (2020). Physical activity and exercise during pregnancy and the postpartum period ACOG committee opinion summary, number 804. *Obstetrics and Gynecology*, 135(4), E178-E188.
- American College of Sports Medicine. (2020). ACSM's guidelines for exercise testing and prescription. Lippincott Williams & Wilkins.

- American Diabetes Association. Gestational Diabetes Mellitus. (2003). *Diabetes Care*, 26 (suppl 1), s103-s105. <https://doi.org/10.2337/diacare.26.2007.S103>
- American Diabetes Association. Standards of Medical Care in Diabetes—2016: Summary of Revisions. (2016). *Diabetes Care*, 39(Supplement 1), S4-S5. <https://doi.org/10.2337/dc16-S003>
- Andres, A., Hull, H. R., Shankar, K., Casey, P. H., Cleves, M. A., & Badger, T. M. (2015). Longitudinal body composition of children born to mothers with normal weight, overweight, and obesity. *Obesity (Silver Spring)*, 23(6), 1252-1258. <https://doi.org/10.1002/oby.21078>
- Ansari, J., Carvalho, B., Shafer, S. L., & Flood, P. (2016). Pharmacokinetics and Pharmacodynamics of Drugs Commonly Used in Pregnancy and Parturition. *Anesth Analg*, 122(3), 786-804. <https://doi.org/10.1213/ane.0000000000001143>
- Armstrong, M. B., & Towle, H. C. (2001). Polyunsaturated fatty acids stimulate hepatic UCP-2 expression via a PPAR α -mediated pathway. *Am J Physiol Endocrinol Metab*, 281(6), E1197-1204. <https://doi.org/10.1152/ajpendo.2001.281.6.E1197>
- Auvinen, A. M., Luiro, K., Jokelainen, J., Järvelä, I., Knip, M., Auvinen, J., & Tapanainen, J. S. (2020). Type 1 and type 2 diabetes after gestational diabetes: a 23 year cohort study. *Diabetologia*, 63(10), 2123-2128. <https://doi.org/10.1007/s00125-020-05215-3>
- Bach, D., Pich, S., Soriano, F. X., Vega, N., Baumgartner, B., Oriola, J., Dagaard, J. R., Lloberas, J., Camps, M., Zierath, J. R., Rabasa-Lhoret, R., Wallberg-Henriksson, H., Laville, M., Palacín, M., Vidal, H., Rivera, F., Brand, M., & Zorzano, A. (2003). Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. *J Biol Chem*, 278(19), 17190-17197. <https://doi.org/10.1074/jbc.M212754200>
- Bahari, H., Caruso, V., & Morris, M. J. (2013). Late-onset exercise in female rat offspring ameliorates the detrimental metabolic impact of maternal obesity. *Endocrinology*, 154(10), 3610-3621. <https://doi.org/10.1210/en.2013-1059>

- Ballestri, S., Nascimbeni, F., Baldelli, E., Marrazzo, A., Romagnoli, D., & Lonardo, A. (2017). NAFLD as a Sexual Dimorphic Disease: Role of Gender and Reproductive Status in the Development and Progression of Nonalcoholic Fatty Liver Disease and Inherent Cardiovascular Risk. *Adv Ther*, 34(6), 1291-1326. <https://doi.org/10.1007/s12325-017-0556-1>
- Barker, D. J., & Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*, 1(8489), 1077-1081. [https://doi.org/10.1016/s0140-6736\(86\)91340-1](https://doi.org/10.1016/s0140-6736(86)91340-1)
- Barthel, A., & Schmolli, D. (2003). Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab*, 285(4), E685-692. <https://doi.org/10.1152/ajpendo.00253.2003>
- Bayol, S. A., Simbi, B. H., Fowkes, R. C., & Stickland, N. C. (2010). A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. *Endocrinology*, 151(4), 1451-1461. <https://doi.org/10.1210/en.2009-1192>
- Beleza, J., Stevanović-Silva, J., Coxito, P., Costa, R. C., Ascensão, A., Torrella, J. R., & Magalhães, J. (2021). Building-up fit muscles for the future: Transgenerational programming of skeletal muscle through physical exercise. *Eur J Clin Invest*, e13515. <https://doi.org/10.1111/eci.13515>
- Bell, M. R. (2018). Comparing Postnatal Development of Gonadal Hormones and Associated Social Behaviors in Rats, Mice, and Humans. *Endocrinology*, 159(7), 2596-2613. <https://doi.org/10.1210/en.2018-00220>
- Benard, G., Faustin, B., Passerieux, E., Galinier, A., Rocher, C., Bellance, N., Delage, J. P., Casteilla, L., Letellier, T., & Rossignol, R. (2006). Physiological diversity of mitochondrial oxidative phosphorylation. *Am J Physiol Cell Physiol*, 291(6), C1172-1182. <https://doi.org/10.1152/ajpcell.00195.2006>
- Bene, J., Márton, M., Mohás, M., Bagosi, Z., Bujtor, Z., Oroszlán, T., Gasztonyi, B., Wittmann, I., & Melegh, B. (2013). Similarities in serum acylcarnitine patterns in type 1 and type 2 diabetes mellitus and in metabolic syndrome. *Ann Nutr Metab*, 62(1), 80-85. <https://doi.org/10.1159/000345759>

- Bhat, G., Baba, C. S., Pandey, A., Kumari, N., & Choudhuri, G. (2012). Life style modification improves insulin resistance and liver histology in patients with non-alcoholic fatty liver disease. *World J Hepatol*, 4(7), 209-217. <https://doi.org/10.4254/wjh.v4.i7.209>
- Bjørndal, B., Alterås, E. K., Lindquist, C., Svardal, A., Skorve, J., & Berge, R. K. (2018). Associations between fatty acid oxidation, hepatic mitochondrial function, and plasma acylcarnitine levels in mice. *Nutr Metab (Lond)*, 15, 10. <https://doi.org/10.1186/s12986-018-0241-7>
- Bobard, A., Hainault, I., Ferré, P., Foufelle, F., & Bossard, P. (2005). Differential regulation of sterol regulatory element-binding protein 1c transcriptional activity by insulin and liver X receptor during liver development. *J Biol Chem*, 280(1), 199-206. <https://doi.org/10.1074/jbc.M406522200>
- Bonnard, C., Durand, A., Peyrol, S., Chanseau, E., Chauvin, M. A., Morio, B., Vidal, H., & Rieusset, J. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest*, 118(2), 789-800. <https://doi.org/10.1172/jci32601>
- Borengasser, S. J., Lau, F., Kang, P., Blackburn, M. L., Ronis, M. J., Badger, T. M., & Shankar, K. (2011). Maternal obesity during gestation impairs fatty acid oxidation and mitochondrial SIRT3 expression in rat offspring at weaning. *PLoS One*, 6(8), e24068. <https://doi.org/10.1371/journal.pone.0024068>
- Bruce, K. D., Cagampang, F. R., Argenton, M., Zhang, J., Ethirajan, P. L., Burdge, G. C., Bateman, A. C., Clough, G. F., Poston, L., Hanson, M. A., McConnell, J. M., & Byrne, C. D. (2009). Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology*, 50(6), 1796-1808. <https://doi.org/10.1002/hep.23205>
- Brumbaugh, D. E., & Friedman, J. E. (2014). Developmental origins of nonalcoholic fatty liver disease. *Pediatr Res*, 75(1-2), 140-147. <https://doi.org/10.1038/pr.2013.193>
- Brumbaugh, D. E., Tearse, P., Cree-Green, M., Fenton, L. Z., Brown, M., Scherzinger, A., Reynolds, R., Alston, M., Hoffman, C., Pan, Z., Friedman,

- J. E., & Barbour, L. A. (2013). Intrahepatic fat is increased in the neonatal offspring of obese women with gestational diabetes. *J Pediatr*, 162(5), 930-936.e931. <https://doi.org/10.1016/j.jpeds.2012.11.017>
- Buckley, B. S., Harreiter, J., Damm, P., Corcoy, R., Chico, A., Simmons, D., Vellinga, A., & Dunne, F. (2012). Gestational diabetes mellitus in Europe: prevalence, current screening practice and barriers to screening. A review. *Diabet Med*, 29(7), 844-854. <https://doi.org/10.1111/j.1464-5491.2011.03541.x>
- Burchard, J., Zhang, C., Liu, A. M., Poon, R. T., Lee, N. P., Wong, K. F., Sham, P. C., Lam, B. Y., Ferguson, M. D., Tokiwa, G., Smith, R., Leeson, B., Beard, R., Lamb, J. R., Lim, L., Mao, M., Dai, H., & Luk, J. M. (2010). microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol Syst Biol*, 6, 402. <https://doi.org/10.1038/msb.2010.58>
- Bush, N. C., Chandler-Laney, P. C., Rouse, D. J., Granger, W. M., Oster, R. A., & Gower, B. A. (2011). Higher maternal gestational glucose concentration is associated with lower offspring insulin sensitivity and altered beta-cell function. *J Clin Endocrinol Metab*, 96(5), E803-809. <https://doi.org/10.1210/jc.2010-2902>
- Cardoso, A. R., Kakimoto, P. A., & Kowaltowski, A. J. (2013). Diet-sensitive sources of reactive oxygen species in liver mitochondria: role of very long chain acyl-CoA dehydrogenases. *PLoS One*, 8(10), e77088. <https://doi.org/10.1371/journal.pone.0077088>
- Carulli, L., Lonardo, A., Lombardini, S., Marchesini, G., & Loria, P. (2006). Gender, fatty liver and GGT. *Hepatology*, 44(1), 278-279. <https://doi.org/10.1002/hep.21218>
- Caruso, V., Bahari, H., & Morris, M. J. (2013). The beneficial effects of early short-term exercise in the offspring of obese mothers are accompanied by alterations in the hypothalamic gene expression of appetite regulators and FTO (fat mass and obesity associated) gene. *J Neuroendocrinol*, 25(8), 742-752. <https://doi.org/10.1111/jne.12053>

- Català-Niell, A., Estrany, M. E., Proenza, A. M., Gianotti, M., & Lladó, I. (2008). Skeletal muscle and liver oxidative metabolism in response to a voluntary isocaloric intake of a high fat diet in male and female rats. *Cell Physiol Biochem*, 22(1-4), 327-336. <https://doi.org/10.1159/000149811>
- Ceccarelli, S., Panera, N., Gnani, D., & Nobili, V. (2013). Dual role of microRNAs in NAFLD. *Int J Mol Sci*, 14(4), 8437-8455. <https://doi.org/10.3390/ijms14048437>
- Chakravarty, K., Leahy, P., Becard, D., Hakimi, P., Foretz, M., Ferre, P., Foufelle, F., & Hanson, R. W. (2001). Sterol regulatory element-binding protein-1c mimics the negative effect of insulin on phosphoenolpyruvate carboxykinase (GTP) gene transcription. *J Biol Chem*, 276(37), 34816-34823. <https://doi.org/10.1074/jbc.M103310200>
- Chang, E., Hafner, H., Varghese, M., Griffin, C., Clemente, J., Islam, M., Carlson, Z., Zhu, A., Hak, L., Abrishami, S., Gregg, B., & Singer, K. (2019). Programming effects of maternal and gestational obesity on offspring metabolism and metabolic inflammation. *Sci Rep*, 9(1), 16027. <https://doi.org/10.1038/s41598-019-52583-x>
- Chang, S. P., Chen, Y. H., Chang, W. C., Liu, I. M., & Cheng, J. T. (2006). Merit of physical exercise to reverse the higher gene expression of hepatic phosphoenolpyruvate carboxykinase in obese Zucker rats. *Life Sci*, 79(3), 240-246. <https://doi.org/10.1016/j.lfs.2005.12.044>
- Cheng, G., Polito, C. C., Haines, J. K., Shafizadeh, S. F., Fiorini, R. N., Zhou, X., Schmidt, M. G., & Chavin, K. D. (2003). Decrease of intracellular ATP content downregulated UCP2 expression in mouse hepatocytes. *Biochem Biophys Res Commun*, 308(3), 573-580. [https://doi.org/10.1016/s0006-291x\(03\)01409-8](https://doi.org/10.1016/s0006-291x(03)01409-8)
- Cheung, O., Puri, P., Eicken, C., Contos, M. J., Mirshahi, F., Maher, J. W., Kellum, J. M., Min, H., Luketic, V. A., & Sanyal, A. J. (2008). Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology*, 48(6), 1810-1820. <https://doi.org/10.1002/hep.22569>
- Choi, G. Y., Tosh, D. N., Garg, A., Mansano, R., Ross, M. G., & Desai, M. (2007). Gender-specific programmed hepatic lipid dysregulation in intrauterine

- growth-restricted offspring. *Am J Obstet Gynecol*, 196(5), 477.e471-477.
<https://doi.org/10.1016/j.ajog.2007.02.024>
- Ciapaite, J., Bakker, S. J., Van Eikenhorst, G., Wagner, M. J., Teerlink, T., Schalkwijk, C. G., Fodor, M., Ouwens, D. M., Diamant, M., Heine, R. J., Westerhoff, H. V., & Krab, K. (2007). Functioning of oxidative phosphorylation in liver mitochondria of high-fat diet fed rats. *Biochim Biophys Acta*, 1772(3), 307-316.
<https://doi.org/10.1016/j.bbadis.2006.10.018>
- Cordero, Y., Mottola, M. F., Vargas, J., Blanco, M., & Barakat, R. (2015). Exercise Is Associated with a Reduction in Gestational Diabetes Mellitus. *Med Sci Sports Exerc*, 47(7), 1328-1333.
<https://doi.org/10.1249/mss.0000000000000547>
- Coustan, D. R. (2013). Gestational diabetes mellitus. *Clin Chem*, 59(9), 1310-1321. <https://doi.org/10.1373/clinchem.2013.203331>
- Dabelea, D., Mayer-Davis, E. J., Lamichhane, A. P., D'Agostino, R. B., Jr., Liese, A. D., Vehik, K. S., Narayan, K. M., Zeitler, P., & Hamman, R. F. (2008). Association of intrauterine exposure to maternal diabetes and obesity with type 2 diabetes in youth: the SEARCH Case-Control Study. *Diabetes Care*, 31(7), 1422-1426. <https://doi.org/10.2337/dc07-2417>
- Dearden, L., & Balthasar, N. (2014). Sexual dimorphism in offspring glucose-sensitive hypothalamic gene expression and physiological responses to maternal high-fat diet feeding. *Endocrinology*, 155(6), 2144-2154.
<https://doi.org/10.1210/en.2014-1131>
- Dearden, L., Bouret, S. G., & Ozanne, S. E. (2018). Sex and gender differences in developmental programming of metabolism. *Mol Metab*, 15, 8-19.
<https://doi.org/10.1016/j.molmet.2018.04.007>
- Delhaes, F., Giza, S. A., Koreman, T., Eastabrook, G., McKenzie, C. A., Bedell, S., Regnault, T. R. H., & de Vrijer, B. (2018). Altered maternal and placental lipid metabolism and fetal fat development in obesity: Current knowledge and advances in non-invasive assessment. *Placenta*, 69, 118-124.
<https://doi.org/10.1016/j.placenta.2018.05.011>

- DeSisto, C. L., Kim, S. Y., & Sharma, A. J. (2014). Prevalence estimates of gestational diabetes mellitus in the United States, Pregnancy Risk Assessment Monitoring System (PRAMS), 2007-2010. *Prev Chronic Dis*, 11, E104. <https://doi.org/10.5888/pcd11.130415>
- Devanathan, S., Whitehead, T. D., Fettig, N., Gropler, R. J., Nemanich, S., & Shoghi, K. I. (2016). Sexual dimorphism in myocardial acylcarnitine and triglyceride metabolism. *Biol Sex Differ*, 7, 25. <https://doi.org/10.1186/s13293-016-0077-7>
- Draijer, L., Benninga, M., & Koot, B. (2019). Pediatric NAFLD: an overview and recent developments in diagnostics and treatment. *Expert Rev Gastroenterol Hepatol*, 13(5), 447-461. <https://doi.org/10.1080/17474124.2019.1595589>
- Feghali, M., Venkataramanan, R., & Caritis, S. (2015). Pharmacokinetics of drugs in pregnancy. *Semin Perinatol*, 39(7), 512-519. <https://doi.org/10.1053/j.semperi.2015.08.003>
- Feig, D. S., Zinman, B., Wang, X., & Hux, J. E. (2008). Risk of development of diabetes mellitus after diagnosis of gestational diabetes. *Cmaj*, 179(3), 229-234. <https://doi.org/10.1503/cmaj.080012>
- Fernandes, R. A., & Zanesco, A. (2010). Early physical activity promotes lower prevalence of chronic diseases in adulthood. *Hypertens Res*, 33(9), 926-931. <https://doi.org/10.1038/hr.2010.106>
- Ferreira, A. F., Silva, C. M., Antunes, D., Sousa, F., Lobo, A. C., & Moura, P. (2018). Gestational Diabetes Mellitus: Is There an Advantage in Using the Current Diagnostic Criteria? *Acta medica portuguesa*, 31(7-8), 416-424.
- Fiuza-Luces, C., Santos-Lozano, A., Joyner, M., Carrera-Bastos, P., Picazo, O., Zugaza, J. L., Izquierdo, M., Ruilope, L. M., & Lucia, A. (2018). Exercise benefits in cardiovascular disease: beyond attenuation of traditional risk factors. *Nat Rev Cardiol*, 15(12), 731-743. <https://doi.org/10.1038/s41569-018-0065-1>
- Fletcher, J. A., Meers, G. M., Linden, M. A., Kearney, M. L., Morris, E. M., Thyfault, J. P., & Rector, R. S. (2014). Impact of various exercise modalities

- on hepatic mitochondrial function. *Med Sci Sports Exerc*, 46(6), 1089-1097.
<https://doi.org/10.1249/mss.0000000000000223>
- Floegel, A., Stefan, N., Yu, Z., Mühlenbruch, K., Drohan, D., Joost, H. G., Fritsche, A., Häring, H. U., Hrabě de Angelis, M., Peters, A., Roden, M., Prehn, C., Wang-Sattler, R., Illig, T., Schulze, M. B., Adamski, J., Boeing, H., & Pischon, T. (2013). Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. *Diabetes*, 62(2), 639-648. <https://doi.org/10.2337/db12-0495>
- Foghsgaard, S., Andreasen, C., Vedtofte, L., Andersen, E. S., Bahne, E., Strandberg, C., Buhl, T., Holst, J. J., Svare, J. A., Clausen, T. D., Mathiesen, E. R., Damm, P., Gluud, L. L., Knop, F. K., & Vilsbøll, T. (2017). Nonalcoholic Fatty Liver Disease Is Prevalent in Women With Prior Gestational Diabetes Mellitus and Independently Associated With Insulin Resistance and Waist Circumference. *Diabetes Care*, 40(1), 109-116. <https://doi.org/10.2337/dc16-1017>
- Forbes, S., Taylor-Robinson, S. D., Patel, N., Allan, P., Walker, B. R., & Johnston, D. G. (2011). Increased prevalence of non-alcoholic fatty liver disease in European women with a history of gestational diabetes. *Diabetologia*, 54(3), 641-647. <https://doi.org/10.1007/s00125-010-2009-0>
- Fornes, D., Heinecke, F., Roberti, S. L., White, V., Capobianco, E., & Jawerbaum, A. (2020). Proinflammation in maternal and fetal livers and circulating miR-122 dysregulation in a GDM rat model induced by intrauterine programming. *Mol Cell Endocrinol*, 510, 110824. <https://doi.org/10.1016/j.mce.2020.110824>
- Fornes, D., White, V., Higa, R., Heinecke, F., Capobianco, E., & Jawerbaum, A. (2018). Sex-dependent changes in lipid metabolism, PPAR pathways and microRNAs that target PPARs in the fetal liver of rats with gestational diabetes. *Mol Cell Endocrinol*, 461, 12-21. <https://doi.org/10.1016/j.mce.2017.08.004>
- Fu, G., Brkić, J., Hayder, H., & Peng, C. (2013). MicroRNAs in Human Placental Development and Pregnancy Complications. *Int J Mol Sci*, 14(3), 5519-5544. <https://doi.org/10.3390/ijms14035519>

- Fu, T., Choi, S. E., Kim, D. H., Seok, S., Suino-Powell, K. M., Xu, H. E., & Kemper, J. K. (2012). Aberrantly elevated microRNA-34a in obesity attenuates hepatic responses to FGF19 by targeting a membrane coreceptor β -Klotho. *Proc Natl Acad Sci U S A*, 109(40), 16137-16142. <https://doi.org/10.1073/pnas.1205951109>
- Fu, T., Seok, S., Choi, S., Huang, Z., Suino-Powell, K., Xu, H. E., Kemper, B., & Kemper, J. K. (2014). MicroRNA 34a inhibits beige and brown fat formation in obesity in part by suppressing adipocyte fibroblast growth factor 21 signaling and SIRT1 function. *Mol Cell Biol*, 34(22), 4130-4142. <https://doi.org/10.1128/mcb.00596-14>
- Fuente-Martín, E., Granado, M., García-Cáceres, C., Sanchez-Garrido, M. A., Frago, L. M., Tena-Sempere, M., Argente, J., & Chowen, J. A. (2012). Early nutritional changes induce sexually dimorphic long-term effects on body weight gain and the response to sucrose intake in adult rats. *Metabolism*, 61(6), 812-822. <https://doi.org/10.1016/j.metabol.2011.11.003>
- García-Berumen, C. I., Ortiz-Avila, O., Vargas-Vargas, M. A., Del Rosario-Tamayo, B. A., Guajardo-López, C., Saavedra-Molina, A., Rodríguez-Orozco, A. R., & Cortés-Rojó, C. (2019). The severity of rat liver injury by fructose and high fat depends on the degree of respiratory dysfunction and oxidative stress induced in mitochondria. *Lipids Health Dis*, 18(1), 78. <https://doi.org/10.1186/s12944-019-1024-5>
- Garnol, T., Endlicher, R., Kučera, O., Drahota, Z., & Cervinková, Z. (2014). Impairment of mitochondrial function of rat hepatocytes by high fat diet and oxidative stress. *Physiol Res*, 63(2), 271-274. <https://doi.org/10.33549/physiolres.932645>
- Garrison, A. (2015). Screening, diagnosis, and management of gestational diabetes mellitus. *Am Fam Physician*, 91(7), 460-467.
- Giancotti, A., Monti, M., Nevi, L., Safarikia, S., D'Ambrosio, V., Brunelli, R., Pajno, C., Corno, S., Di Donato, V., Musella, A., Chiappetta, M. F., Bosco, D., Panici, P. B., Alvaro, D., & Cardinale, V. (2019). Functions and the Emerging Role of the Foetal Liver into Regenerative Medicine. *Cells*, 8(8). <https://doi.org/10.3390/cells8080914>

- Gluckman, P. D., Hanson, M. A., Cooper, C., & Thornburg, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*, 359(1), 61-73. <https://doi.org/10.1056/NEJMra0708473>
- Godfrey, K. M., Haugen, G., Kiserud, T., Inskip, H. M., Cooper, C., Harvey, N. C., Crozier, S. R., Robinson, S. M., Davies, L., & Hanson, M. A. (2012). Fetal liver blood flow distribution: role in human developmental strategy to prioritize fat deposition versus brain development. *PLoS One*, 7(8), e41759. <https://doi.org/10.1371/journal.pone.0041759>
- Gonçalves, I. O., Maciel, E., Passos, E., Torrella, J. R., Rizo, D., Viscor, G., Rocha-Rodrigues, S., Santos-Alves, E., Domingues, M. R., Oliveira, P. J., Ascensão, A., & Magalhães, J. (2014). Exercise alters liver mitochondria phospholipidomic profile and mitochondrial activity in non-alcoholic steatohepatitis. *Int J Biochem Cell Biol*, 54, 163-173. <https://doi.org/10.1016/j.biocel.2014.07.011>
- Gonçalves, I. O., Oliveira, P. J., Ascensão, A., & Magalhães, J. (2013). Exercise as a therapeutic tool to prevent mitochondrial degeneration in nonalcoholic steatohepatitis. *Eur J Clin Invest*, 43(11), 1184-1194. <https://doi.org/10.1111/eci.12146>
- Gonçalves, I. O., Passos, E., Diogo, C. V., Rocha-Rodrigues, S., Santos-Alves, E., Oliveira, P. J., Ascensão, A., & Magalhães, J. (2016). Exercise mitigates mitochondrial permeability transition pore and quality control mechanisms alterations in nonalcoholic steatohepatitis. *Appl Physiol Nutr Metab*, 41(3), 298-306. <https://doi.org/10.1139/apnm-2015-0470>
- Gonçalves, I. O., Passos, E., Rocha-Rodrigues, S., Diogo, C. V., Torrella, J. R., Rizo, D., Viscor, G., Santos-Alves, E., Marques-Aleixo, I., Oliveira, P. J., Ascensão, A., & Magalhães, J. (2014). Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion*, 15, 40-51. <https://doi.org/10.1016/j.mito.2014.03.012>
- Goyal, D., Limesand, S. W., & Goyal, R. (2019). Epigenetic responses and the developmental origins of health and disease. *J Endocrinol*, 242(1), T105-t119. <https://doi.org/10.1530/joe-19-0009>

- Han, S., Middleton, P., Shepherd, E., Van Ryswyk, E., & Crowther, C. A. (2017). Different types of dietary advice for women with gestational diabetes mellitus. *Cochrane Database Syst Rev*, 2(2), Cd009275. <https://doi.org/10.1002/14651858.CD009275.pub3>
- Haugen, G., Hanson, M., Kiserud, T., Crozier, S., Inskip, H., & Godfrey, K. M. (2005). Fetal liver-sparing cardiovascular adaptations linked to mother's slimness and diet. *Circ Res*, 96(1), 12-14. <https://doi.org/10.1161/01.Res.0000152391.45273.A2>
- Hayder, H., O'Brien, J., Nadeem, U., & Peng, C. (2018). MicroRNAs: crucial regulators of placental development. *Reproduction*, 155(6), R259-r271. <https://doi.org/10.1530/rep-17-0603>
- Heindel, J. J., & Vandenberg, L. N. (2015). Developmental origins of health and disease: a paradigm for understanding disease cause and prevention. *Curr Opin Pediatr*, 27(2), 248-253. <https://doi.org/10.1097/mop.0000000000000191>
- Hernández-Alvarez, M. I., Thabit, H., Burns, N., Shah, S., Brema, I., Hatunic, M., Finucane, F., Liesa, M., Chiellini, C., Naon, D., Zorzano, A., & Nolan, J. J. (2010). Subjects with early-onset type 2 diabetes show defective activation of the skeletal muscle PGC-1 α /Mitofusin-2 regulatory pathway in response to physical activity. *Diabetes Care*, 33(3), 645-651. <https://doi.org/10.2337/dc09-1305>
- Herrera, E., & Desoye, G. (2016). Maternal and fetal lipid metabolism under normal and gestational diabetic conditions. *Horm Mol Biol Clin Investig*, 26(2), 109-127. <https://doi.org/10.1515/hmbci-2015-0025>
- Hoeks, J., Hesselink, M., & Schrauwen, P. (2012). Mitochondrial Respiration. In F. C. Mooren (Ed.), *Encyclopedia of Exercise Medicine in Health and Disease* (pp. 587-590). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-29807-6_136
- Hoffman, D. J., Reynolds, R. M., & Hardy, D. B. (2017). Developmental origins of health and disease: current knowledge and potential mechanisms. *Nutr Rev*, 75(12), 951-970. <https://doi.org/10.1093/nutrit/nux053>

- Hu, Y., Du, G., Li, G., Peng, X., Zhang, Z., & Zhai, Y. (2019). The miR-122 inhibition alleviates lipid accumulation and inflammation in NAFLD cell model. *Arch Physiol Biochem*, 1-5. <https://doi.org/10.1080/13813455.2019.1640744>
- Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I. J., & Zannis, V. I. (2010). MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. *J Lipid Res*, 51(6), 1513-1523. <https://doi.org/10.1194/jlr.M004812>
- International Association of Diabetes and Pregnancy Study Groups Consensus Panel, Metzger, B. E., Gabbe, S. G., Persson, B., Buchanan, T. A., Catalano, P. A., Damm, P., Dyer, A. R., Leiva, A. d., Hod, M., Kitzmiller, J. L., Lowe, L. P., McIntyre, H. D., Oats, J. J., Omori, Y., & Schmidt, M. I. (2010). International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. *Diabetes care*, 33(3), 676–682. <https://doi.org/10.2337/dc09-1848>
- Jiang, S., Teague, A. M., Tryggestad, J. B., & Chernausek, S. D. (2017). Role of microRNA-130b in placental PGC-1 α /TFAM mitochondrial biogenesis pathway. *Biochemical and biophysical research communications*, 487(3), 607-612.
- Jiang, S., Teague, A. M., Tryggestad, J. B., Aston, C. E., Lyons, T., & Chernausek, S. D. (2017). Effects of maternal diabetes and fetal sex on human placenta mitochondrial biogenesis. *Placenta*, 57, 26-32. <https://doi.org/10.1016/j.placenta.2017.06.001>
- Jiang, S., Young, J. L., Wang, K., Qian, Y., & Cai, L. (2020). Diabetic-induced alterations in hepatic glucose and lipid metabolism: The role of type 1 and type 2 diabetes mellitus (Review). *Mol Med Rep*, 22(2), 603-611. <https://doi.org/10.3892/mmr.2020.11175>
- Jornayvaz, F. R., & Shulman, G. I. (2010). Regulation of mitochondrial biogenesis. *Essays Biochem*, 47, 69-84. <https://doi.org/10.1042/bse0470069>

- Kalaki-Jouybari, F., Shanaki, M., Delfan, M., Gorgani-Firouzjaee, S., & Khakdan, S. (2020). High-intensity interval training (HIIT) alleviated NAFLD feature via miR-122 induction in liver of high-fat high-fructose diet induced diabetic rats. *Arch Physiol Biochem*, 126(3), 242-249. <https://doi.org/10.1080/13813455.2018.1510968>
- Kalhan, S., & Parimi, P. (2000). Gluconeogenesis in the fetus and neonate. *Semin Perinatol*, 24(2), 94-106. <https://doi.org/10.1053/sp.2000.6360>
- Kamana, K., Shakya, S., & Zhang, H. (2015). Gestational diabetes mellitus and macrosomia: a literature review. *Annals of Nutrition and Metabolism*, 66(Suppl. 2), 14-20.
- Kamfar, S., Alavian, S. M., Houshmand, M., Yadegarazari, R., Seifi Zarei, B., Khalaj, A., Shabab, N., & Saidijam, M. (2016). Liver Mitochondrial DNA Copy Number and Deletion Levels May Contribute to Nonalcoholic Fatty Liver Disease Susceptibility. *Hepat Mon*, 16(12), e40774. <https://doi.org/10.5812/hepatmon.40774>
- Kc, K., Shakya, S., & Zhang, H. (2015). Gestational diabetes mellitus and macrosomia: a literature review. *Ann Nutr Metab*, 66 Suppl 2, 14-20. <https://doi.org/10.1159/000371628>
- Kim, D. W., Young, S. L., Grattan, D. R., & Jasoni, C. L. (2014). Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse. *Biol Reprod*, 90(6), 130. <https://doi.org/10.1095/biolreprod.113.117259>
- Kir, S., Beddow, S. A., Samuel, V. T., Miller, P., Previs, S. F., Suino-Powell, K., Xu, H. E., Shulman, G. I., Kliewer, S. A., & Mangelsdorf, D. J. (2011). FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science*, 331(6024), 1621-1624. <https://doi.org/10.1126/science.1198363>
- Klinge, C. M. (2020). Estrogenic control of mitochondrial function. *Redox Biol*, 31, 101435. <https://doi.org/10.1016/j.redox.2020.101435>
- Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., Schlensak, M., & Roden, M. (2015). Adaptation of hepatic mitochondrial function in humans

- with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab*, 21(5), 739-746. <https://doi.org/10.1016/j.cmet.2015.04.004>
- Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D., & Muoio, D. M. (2008). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*, 7(1), 45-56. <https://doi.org/10.1016/j.cmet.2007.10.013>
- Kwekel, J. C., Vijay, V., Han, T., Moland, C. L., Desai, V. G., & Fuscoe, J. C. (2017). Sex and age differences in the expression of liver microRNAs during the life span of F344 rats. *Biol Sex Differ*, 8, 6. <https://doi.org/10.1186/s13293-017-0127-9>
- Lain, K. Y., & Catalano, P. M. (2007). Metabolic changes in pregnancy. *Clin Obstet Gynecol*, 50(4), 938-948. <https://doi.org/10.1097/GRF.0b013e31815a5494>
- Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, 146(3), 726-735. <https://doi.org/10.1053/j.gastro.2013.11.049>
- Lavrentaki, A., Thomas, T., Subramanian, A., Valsamakis, G., Thomas, N., Toulis, K. A., Wang, J., Daly, B., Saravanan, P., Sumilo, D., Mastorakos, G., Tahrani, A. A., & Nirantharakumar, K. (2019). Increased risk of non-alcoholic fatty liver disease in women with gestational diabetes mellitus: A population-based cohort study, systematic review and meta-analysis. *J Diabetes Complications*, 33(10), 107401. <https://doi.org/10.1016/j.jdiacomp.2019.06.006>
- Lee, S., Libman, I., Hughan, K., Kuk, J. L., Jeong, J. H., Zhang, D., & Arslanian, S. (2019). Effects of Exercise Modality on Insulin Resistance and Ectopic Fat in Adolescents with Overweight and Obesity: A Randomized Clinical Trial. *J Pediatr*, 206, 91-98.e91. <https://doi.org/10.1016/j.jpeds.2018.10.059>
- Leng, J., Liu, G., Zhang, C., Xin, S., Chen, F., Li, B., Tian, H., Yu, Z., Tuomilehto, J., Hu, G., & Yang, X. (2016). Physical activity, sedentary behaviors and risk of gestational diabetes mellitus: a population-based cross-sectional study in

- Tianjin, China. *Eur J Endocrinol*, 174(6), 763-773. <https://doi.org/10.1530/eje-15-1103>
- Li, S., Chen, X., Zhang, H., Liang, X., Xiang, Y., Yu, C., Zen, K., Li, Y., & Zhang, C. Y. (2009). Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. *J Lipid Res*, 50(9), 1756-1765. <https://doi.org/10.1194/jlr.M800509-JLR200>
- Li, T., Chen, K., Liu, G., Huang, L. P., Chen, L., Wang, Q. W., Hu, C. L., & Hou, L. J. (2017). Calorie restriction prevents the development of insulin resistance and impaired lipid metabolism in gestational diabetes offspring. *Pediatr Res*, 81(4), 663-671. <https://doi.org/10.1038/pr.2016.273>
- Linden, A. G., Li, S., Choi, H. Y., Fang, F., Fukasawa, M., Uyeda, K., Hammer, R. E., Horton, J. D., Engelking, L. J., & Liang, G. (2018). Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice. *J Lipid Res*, 59(3), 475-487. <https://doi.org/10.1194/jlr.M081836>
- Linden, M. A., Fletcher, J. A., Morris, E. M., Meers, G. M., Laughlin, M. H., Booth, F. W., Sowers, J. R., Ibdah, J. A., Thyfault, J. P., & Rector, R. S. (2015). Treating NAFLD in OLETF rats with vigorous-intensity interval exercise training. *Med Sci Sports Exerc*, 47(3), 556-567. <https://doi.org/10.1249/mss.0000000000000430>
- Litvinova, L., Zatolokin, P., Vulf, M., Mazunin, I., & Skuratovskaia, D. (2019). The relationship between the mtDNA copy number in insulin-dependent tissues and markers of endothelial dysfunction and inflammation in obese patients. *BMC Med Genomics*, 12(Suppl 2), 41. <https://doi.org/10.1186/s12920-019-0486-7>
- Liu, Y. I., Wise, P. H., & Butte, A. J. (2009). The "etiome": identification and clustering of human disease etiological factors. *BMC Bioinformatics*, 10 Suppl 2(Suppl 2), S14. <https://doi.org/10.1186/1471-2105-10-s2-s14>
- Longo, M., Meroni, M., Paolini, E., Macchi, C., & Dongiovanni, P. (2021). Mitochondrial dynamics and nonalcoholic fatty liver disease (NAFLD): new perspectives for a fairy-tale ending? *Metabolism*, 117, 154708. <https://doi.org/10.1016/j.metabol.2021.154708>

- Luo, S., Valencia, C. A., Zhang, J., Lee, N. C., Slone, J., Gui, B., Wang, X., Li, Z., Dell, S., Brown, J., Chen, S. M., Chien, Y. H., Hwu, W. L., Fan, P. C., Wong, L. J., Atwal, P. S., & Huang, T. (2018). Biparental Inheritance of Mitochondrial DNA in Humans. *Proc Natl Acad Sci U S A*, 115(51), 13039-13044. <https://doi.org/10.1073/pnas.1810946115>
- Ma, S. G., Yu, W. N., Jin, Y., Hong, B., & Hu, W. (2012). Evaluation of serum ischemia-modified albumin levels in pregnant women with and without gestational diabetes mellitus. *Gynecol Endocrinol*, 28(11), 837-840. <https://doi.org/10.3109/09513590.2012.683069>
- Mai, M., Tönjes, A., Kovacs, P., Stumvoll, M., Fiedler, G. M., & Leichtle, A. B. (2013). Serum levels of acylcarnitines are altered in prediabetic conditions. *PLoS One*, 8(12), e82459. <https://doi.org/10.1371/journal.pone.0082459>
- Mandò, C., Calabrese, S., Mazzocco, M. I., Novielli, C., Anelli, G. M., Antonazzo, P., & Cetin, I. (2016). Sex specific adaptations in placental biometry of overweight and obese women. *Placenta*, 38, 1-7. <https://doi.org/10.1016/j.placenta.2015.12.008>
- Mansouri, A., Gattolliat, C. H., & Asselah, T. (2018). Mitochondrial Dysfunction and Signaling in Chronic Liver Diseases. *Gastroenterology*, 155(3), 629-647. <https://doi.org/10.1053/j.gastro.2018.06.083>
- Marson, E. C., Delevatti, R. S., Prado, A. K., Netto, N., & Kruehl, L. F. (2016). Effects of aerobic, resistance, and combined exercise training on insulin resistance markers in overweight or obese children and adolescents: A systematic review and meta-analysis. *Prev Med*, 93, 211-218. <https://doi.org/10.1016/j.ypmed.2016.10.020>
- Martinez-Lopez, N., & Singh, R. (2015). Autophagy and Lipid Droplets in the Liver. *Annu Rev Nutr*, 35, 215-237. <https://doi.org/10.1146/annurev-nutr-071813-105336>
- Matsuzaka, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Tomita, S., Sekiya, M., Hasty, A., Nakagawa, Y., Sone, H., Toyoshima, H., Ishibashi, S., Osuga, J., & Yamada, N. (2004). Insulin-independent induction of sterol regulatory

- element-binding protein-1c expression in the livers of streptozotocin-treated mice. *Diabetes*, 53(3), 560-569. <https://doi.org/10.2337/diabetes.53.3.560>
- McDaniel, K., Herrera, L., Zhou, T., Francis, H., Han, Y., Levine, P., Lin, E., Glaser, S., Alpini, G., & Meng, F. (2014). The functional role of microRNAs in alcoholic liver injury. *J Cell Mol Med*, 18(2), 197-207. <https://doi.org/10.1111/jcmm.12223>
- Michel, S., Wanet, A., De Pauw, A., Rommelaere, G., Arnould, T., & Renard, P. (2012). Crosstalk between mitochondrial (dys)function and mitochondrial abundance. *J Cell Physiol*, 227(6), 2297-2310. <https://doi.org/10.1002/jcp.23021>
- Ming, W. K., Ding, W., Zhang, C. J. P., Zhong, L., Long, Y., Li, Z., Sun, C., Wu, Y., Chen, H., Chen, H., & Wang, Z. (2018). The effect of exercise during pregnancy on gestational diabetes mellitus in normal-weight women: a systematic review and meta-analysis. *BMC Pregnancy Childbirth*, 18(1), 440. <https://doi.org/10.1186/s12884-018-2068-7>
- Miranda, R. A., De Almeida, M. M., Rocha, C., de Brito Fassarella, L., De Souza, L. L., Souza, A. F. P., Andrade, C. B. V., Fortunato, R. S., Pazos-Moura, C. C., & Trevenzoli, I. H. (2018). Maternal high-fat diet consumption induces sex-dependent alterations of the endocannabinoid system and redox homeostasis in liver of adult rat offspring. *Sci Rep*, 8(1), 14751. <https://doi.org/10.1038/s41598-018-32906-0>
- Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, 191, 144-148. <https://doi.org/10.1038/191144a0>
- Mittwoch, U. (2004). The elusive action of sex-determining genes: mitochondria to the rescue? *J Theor Biol*, 228(3), 359-365. <https://doi.org/10.1016/j.jtbi.2004.02.001>
- Miyaaki, H., Ichikawa, T., Kamo, Y., Taura, N., Honda, T., Shibata, H., Milazzo, M., Fornari, F., Gramantieri, L., Bolondi, L., & Nakao, K. (2014). Significance of serum and hepatic microRNA-122 levels in patients with non-alcoholic fatty liver disease. *Liver Int*, 34(7), e302-307. <https://doi.org/10.1111/liv.12429>

- Moore, M. C., Coate, K. C., Winnick, J. J., An, Z., & Cherrington, A. D. (2012). Regulation of hepatic glucose uptake and storage in vivo. *Adv Nutr*, 3(3), 286-294. <https://doi.org/10.3945/an.112.002089>
- Mootha, V. K., Lindgren, C. M., Eriksson, K. F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., Houstis, N., Daly, M. J., Patterson, N., Mesirov, J. P., Golub, T. R., Tamayo, P., Spiegelman, B., Lander, E. S., Hirschhorn, J. N., Altshuler, D., & Groop, L. C. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*, 34(3), 267-273. <https://doi.org/10.1038/ng1180>
- Morris, E. M., Jackman, M. R., Meers, G. M., Johnson, G. C., Lopez, J. L., MacLean, P. S., & Thyfault, J. P. (2013). Reduced hepatic mitochondrial respiration following acute high-fat diet is prevented by PGC-1 α overexpression. *Am J Physiol Gastrointest Liver Physiol*, 305(11), G868-880. <https://doi.org/10.1152/ajpgi.00179.2013>
- Morris, E. M., Meers, G. M., Booth, F. W., Fritsche, K. L., Hardin, C. D., Thyfault, J. P., & Ibdah, J. A. (2012). PGC-1 α overexpression results in increased hepatic fatty acid oxidation with reduced triacylglycerol accumulation and secretion. *Am J Physiol Gastrointest Liver Physiol*, 303(8), G979-992. <https://doi.org/10.1152/ajpgi.00169.2012>
- Motahari-Tabari, N., Ahmad Shirvani, M., Shirzad, E. A. M., Yousefi-Abdolmaleki, E., & Teimourzadeh, M. (2014). The effect of 8 weeks aerobic exercise on insulin resistance in type 2 diabetes: a randomized clinical trial. *Glob J Health Sci*, 7(1), 115-121. <https://doi.org/10.5539/gjhs.v7n1p115>
- Mouralidarane, A., Soeda, J., Visconti-Pugmire, C., Samuelsson, A. M., Pombo, J., Maragkoudaki, X., Butt, A., Saraswati, R., Novelli, M., Fusai, G., Poston, L., Taylor, P. D., & Oben, J. A. (2013). Maternal obesity programs offspring nonalcoholic fatty liver disease by innate immune dysfunction in mice. *Hepatology*, 58(1), 128-138. <https://doi.org/10.1002/hep.26248>
- Muoio, D. M., & Koves, T. R. (2007). Lipid-induced metabolic dysfunction in skeletal muscle. *Novartis Found Symp*, 286, 24-38; discussion 38-46, 162-163, 196-203. <https://doi.org/10.1002/9780470985571.ch4>

- Myers, M. G., Jr., & Olson, D. P. (2012). Central nervous system control of metabolism. *Nature*, 491(7424), 357-363. <https://doi.org/10.1038/nature11705>
- Nasrallah, C. M., & Horvath, T. L. (2014). Mitochondrial dynamics in the central regulation of metabolism. *Nat Rev Endocrinol*, 10(11), 650-658. <https://doi.org/10.1038/nrendo.2014.160>
- Nassir, F., Rector, R. S., Hammoud, G. M., & Ibdah, J. A. (2015). Pathogenesis and Prevention of Hepatic Steatosis. *Gastroenterol Hepatol (N Y)*, 11(3), 167-175.
- Noctor, E., & Dunne, F. P. (2015). Type 2 diabetes after gestational diabetes: The influence of changing diagnostic criteria. *World J Diabetes*, 6(2), 234-244. <https://doi.org/10.4239/wjd.v6.i2.234>
- O'Brien, J., Hayder, H., Zayed, Y., & Peng, C. (2018). Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne)*, 9, 402. <https://doi.org/10.3389/fendo.2018.00402>
- Oostvogels, A., Hof, M. H. P., Gademan, M. G. J., Roseboom, T. J., Stronks, K., & Vrijkotte, T. G. M. (2017). Does maternal pre-pregnancy overweight or obesity influence offspring's growth patterns from birth up to 7 years? The ABCD-study. *Early Hum Dev*, 113, 62-70. <https://doi.org/10.1016/j.earlhumdev.2017.06.002>
- Pankey, C. L., Walton, M. W., Odhiambo, J. F., Smith, A. M., Ghnenis, A. B., Nathanielsz, P. W., & Ford, S. P. (2017). Intergenerational impact of maternal overnutrition and obesity throughout pregnancy in sheep on metabolic syndrome in grandsons and granddaughters. *Domest Anim Endocrinol*, 60, 67-74. <https://doi.org/10.1016/j.domaniend.2017.04.002>
- Papa, S., Martino, P. L., Capitanio, G., Gaballo, A., De Rasmio, D., Signorile, A., & Petruzzella, V. (2012). The oxidative phosphorylation system in mammalian mitochondria. *Adv Exp Med Biol*, 942, 3-37. https://doi.org/10.1007/978-94-007-2869-1_1
- Parakatselaki, M. E., & Ladoukakis, E. D. (2021). mtDNA Heteroplasmy: Origin, Detection, Significance, and Evolutionary Consequences. *Life (Basel)*, 11(7). <https://doi.org/10.3390/life11070633>

- Parrettini, S., Caroli, A., & Torlone, E. (2020). Nutrition and Metabolic Adaptations in Physiological and Complicated Pregnancy: Focus on Obesity and Gestational Diabetes. *Front Endocrinol (Lausanne)*, 11, 611929. <https://doi.org/10.3389/fendo.2020.611929>
- Patel, S., Lawlor, D. A., Callaway, M., Macdonald-Wallis, C., Sattar, N., & Fraser, A. (2016). Association of maternal diabetes/glycosuria and pre-pregnancy body mass index with offspring indicators of non-alcoholic fatty liver disease. *BMC Pediatr*, 16, 47. <https://doi.org/10.1186/s12887-016-0585-y>
- Patterson, R. E., Kalavalapalli, S., Williams, C. M., Nautiyal, M., Mathew, J. T., Martinez, J., Reinhard, M. K., McDougall, D. J., Rocca, J. R., Yost, R. A., Cusi, K., Garrett, T. J., & Sunny, N. E. (2016). Lipotoxicity in steatohepatitis occurs despite an increase in tricarboxylic acid cycle activity. *Am J Physiol Endocrinol Metab*, 310(7), E484-494. <https://doi.org/10.1152/ajpendo.00492.2015>
- Patti, M. E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E. J., Goldfine, A. B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C. R., & Mandarino, L. J. (2003). Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A*, 100(14), 8466-8471. <https://doi.org/10.1073/pnas.1032913100>
- Pereira, T. J., Fonseca, M. A., Campbell, K. E., Moyce, B. L., Cole, L. K., Hatch, G. M., Doucette, C. A., Klein, J., Aliani, M., & Dolinsky, V. W. (2015). Maternal obesity characterized by gestational diabetes increases the susceptibility of rat offspring to hepatic steatosis via a disrupted liver metabolome. *J Physiol*, 593(14), 3181-3197. <https://doi.org/10.1113/jp270429>
- Pinti, M. V., Fink, G. K., Hathaway, Q. A., Durr, A. J., Kunovac, A., & Hollander, J. M. (2019). Mitochondrial dysfunction in type 2 diabetes mellitus: an organ-based analysis. *Am J Physiol Endocrinol Metab*, 316(2), E268-e285. <https://doi.org/10.1152/ajpendo.00314.2018>

- Polyzos, S. A., Kountouras, J., Tsatsoulis, A., Zafeiriadou, E., Katsiki, E., Patsiaoura, K., Zavos, C., Anastasiadou, V. V., & Slavakis, A. (2013). Sex steroids and sex hormone-binding globulin in postmenopausal women with nonalcoholic fatty liver disease. *Hormones (Athens)*, 12(3), 405-416. <https://doi.org/10.1007/bf03401306>
- Pons, R. S., Rockett, F. C., de Almeida Rubin, B., Oppermann, M. L. R., & Bosa, V. L. (2015). Risk factors for gestational diabetes mellitus in a sample of pregnant women diagnosed with the disease. *Diabetology & Metabolic Syndrome*, 7(1), A80. <https://doi.org/10.1186/1758-5996-7-S1-A80>
- Popov, L. D. (2020). Mitochondrial biogenesis: An update. *J Cell Mol Med*, 24(9), 4892-4899. <https://doi.org/10.1111/jcmm.15194>
- Puigserver, P., & Spiegelman, B. M. (2003). Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev*, 24(1), 78-90. <https://doi.org/10.1210/er.2002-0012>
- Radesky, J. S., Oken, E., Rifas-Shiman, S. L., Kleinman, K. P., Rich-Edwards, J. W., & Gillman, M. W. (2008). Diet during early pregnancy and development of gestational diabetes. *Paediatr Perinat Epidemiol*, 22(1), 47-59. <https://doi.org/10.1111/j.1365-3016.2007.00899.x>
- Rajia, S., Chen, H., & Morris, M. J. (2013). Voluntary post weaning exercise restores metabolic homeostasis in offspring of obese rats. *Nutr Metab Cardiovasc Dis*, 23(6), 574-581. <https://doi.org/10.1016/j.numecd.2011.12.009>
- Ramírez-Vélez, R. (2012). A 12-week exercise program performed during the second trimester does not prevent gestational diabetes in healthy pregnant women. *J Physiother*, 58(3), 198. [https://doi.org/10.1016/s1836-9553\(12\)70112-1](https://doi.org/10.1016/s1836-9553(12)70112-1)
- Regnault, N., Gillman, M. W., Rifas-Shiman, S. L., Eggleston, E., & Oken, E. (2013). Sex-specific associations of gestational glucose tolerance with childhood body composition. *Diabetes Care*, 36(10), 3045-3053. <https://doi.org/10.2337/dc13-0333>

- Rius, R., Cowley, M. J., Riley, L., Puttick, C., Thorburn, D. R., & Christodoulou, J. (2019). Biparental inheritance of mitochondrial DNA in humans is not a common phenomenon. *Genet Med*, 21(12), 2823-2826. <https://doi.org/10.1038/s41436-019-0568-0>
- Rui, L. (2014). Energy metabolism in the liver. *Compr Physiol*, 4(1), 177-197. <https://doi.org/10.1002/cphy.c130024>
- Saben, J., Zhong, Y., Gomez-Acevedo, H., Thakali, K. M., Borengasser, S. J., Andres, A., & Shankar, K. (2013). Early growth response protein-1 mediates lipotoxicity-associated placental inflammation: role in maternal obesity. *Am J Physiol Endocrinol Metab*, 305(1), E1-14. <https://doi.org/10.1152/ajpendo.00076.2013>
- Sadakierska-Chudy, A., Kotarska, A., Frankowska, M., Jastrzębska, J., Wydra, K., Miszkiel, J., Przegaliński, E., & Filip, M. (2017). The Alterations in Mitochondrial DNA Copy Number and Nuclear-Encoded Mitochondrial Genes in Rat Brain Structures after Cocaine Self-Administration. *Mol Neurobiol*, 54(9), 7460-7470. <https://doi.org/10.1007/s12035-016-0153-3>
- Sampath Kumar, A., Maiya, A. G., Shastri, B. A., Vaishali, K., Ravishankar, N., Hazari, A., Gundmi, S., & Jadhav, R. (2019). Exercise and insulin resistance in type 2 diabetes mellitus: A systematic review and meta-analysis. *Ann Phys Rehabil Med*, 62(2), 98-103. <https://doi.org/10.1016/j.rehab.2018.11.001>
- Santos-Alves, E., Marques-Aleixo, I., Coxito, P., Balça, M. M., Rizo-Roca, D., Rocha-Rodrigues, S., Martins, S., Torrella, J. R., Oliveira, P. J., Moreno, A. J., Magalhães, J., & Ascensão, A. (2014). Exercise mitigates diclofenac-induced liver mitochondrial dysfunction. *Eur J Clin Invest*, 44(7), 668-677. <https://doi.org/10.1111/eci.12285>
- Santos-Alves, E., Marques-Aleixo, I., Rizo-Roca, D., Torrella, J. R., Oliveira, P. J., Magalhães, J., & Ascensão, A. (2015). Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling. *Life Sci*, 135, 124-130. <https://doi.org/10.1016/j.lfs.2015.06.007>
- Satapati, S., Sunny, N. E., Kucejova, B., Fu, X., He, T. T., Méndez-Lucas, A., Shelton, J. M., Perales, J. C., Browning, J. D., & Burgess, S. C. (2012).

- Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *J Lipid Res*, 53(6), 1080-1092. <https://doi.org/10.1194/jlr.M023382>
- Schmid, A. I., Szendroedi, J., Chmelik, M., Krssák, M., Moser, E., & Roden, M. (2011). Liver ATP synthesis is lower and relates to insulin sensitivity in patients with type 2 diabetes. *Diabetes Care*, 34(2), 448-453. <https://doi.org/10.2337/dc10-1076>
- Schrepfer, E., & Scorrano, L. (2016). Mitofusins, from Mitochondria to Metabolism. *Mol Cell*, 61(5), 683-694. <https://doi.org/10.1016/j.molcel.2016.02.022>
- Seo, A. Y., Joseph, A. M., Dutta, D., Hwang, J. C., Aris, J. P., & Leeuwenburgh, C. (2010). New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *J Cell Sci*, 123(Pt 15), 2533-2542. <https://doi.org/10.1242/jcs.070490>
- Serviddio, G., Bellanti, F., Tamborra, R., Rollo, T., Capitanio, N., Romano, A. D., Sastre, J., Vendemiale, G., & Altomare, E. (2008). Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. *Gut*, 57(7), 957-965. <https://doi.org/10.1136/gut.2007.147496>
- Si-Tayeb, K., Lemaigre, F. P., & Duncan, S. A. (2010). Organogenesis and development of the liver. *Dev Cell*, 18(2), 175-189. <https://doi.org/10.1016/j.devcel.2010.01.011>
- Stewart, M. S., Heerwagen, M. J., & Friedman, J. E. (2013). Developmental programming of pediatric nonalcoholic fatty liver disease: redefining the "first hit". *Clin Obstet Gynecol*, 56(3), 577-590. <https://doi.org/10.1097/GRF.0b013e3182a09760>
- Strackowski, M., Kowalska, I., Dzienis-Strackowska, S., Kinalski, M., Górski, J., & Kinalski, I. (2001). The effect of exercise training on glucose tolerance and skeletal muscle triacylglycerol content in rats fed with a high-fat diet. *Diabetes Metab*, 27(1), 19-23.
- Strakovsky, R. S., Zhang, X., Zhou, D., & Pan, Y. X. (2011). Gestational high fat diet programs hepatic phosphoenolpyruvate carboxykinase gene

- expression and histone modification in neonatal offspring rats. *J Physiol*, 589(Pt 11), 2707-2717. <https://doi.org/10.1113/jphysiol.2010.203950>
- Sunny, N. E., Parks, E. J., Browning, J. D., & Burgess, S. C. (2011). Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab*, 14(6), 804-810. <https://doi.org/10.1016/j.cmet.2011.11.004>
- Surugihalli, C., Porter, T. E., Chan, A., Farley, L. S., Maguire, M., Zhang, C., Kattapuram, N., Muyyarikkandy, M. S., Liu, H. C., & Sunny, N. E. (2019). Hepatic Mitochondrial Oxidative Metabolism and Lipogenesis Synergistically Adapt to Mediate Healthy Embryonic-to-Neonatal Transition in Chicken. *Sci Rep*, 9(1), 20167. <https://doi.org/10.1038/s41598-019-56715-1>
- Symonds, M. E., Pope, M., Sharkey, D., & Budge, H. (2012). Adipose tissue and fetal programming. *Diabetologia*, 55(6), 1597-1606. <https://doi.org/10.1007/s00125-012-2505-5>
- Szendroedi, J., Chmelik, M., Schmid, A. I., Nowotny, P., Brehm, A., Krssak, M., Moser, E., & Roden, M. (2009). Abnormal hepatic energy homeostasis in type 2 diabetes. *Hepatology*, 50(4), 1079-1086. <https://doi.org/10.1002/hep.23093>
- Talton, O. O., Bates, K., Salazar, S. R., Ji, T., & Schulz, L. C. (2019). Lean maternal hyperglycemia alters offspring lipid metabolism and susceptibility to diet-induced obesity in mice†. *Biol Reprod*, 100(5), 1356-1369. <https://doi.org/10.1093/biolre/ioz009>
- Tan, C. M. J., & Lewandowski, A. J. (2020). The Transitional Heart: From Early Embryonic and Fetal Development to Neonatal Life. *Fetal Diagn Ther*, 47(5), 373-386. <https://doi.org/10.1159/000501906>
- Tower, J. (2006). Sex-specific regulation of aging and apoptosis. *Mech Ageing Dev*, 127(9), 705-718. <https://doi.org/10.1016/j.mad.2006.05.001>
- Tsamou, M., Martens, D. S., Winckelmans, E., Madhloum, N., Cox, B., Gyselaers, W., Nawrot, T. S., & Vrijens, K. (2017). Mother's Pre-pregnancy BMI and Placental Candidate miRNAs: Findings from the ENVIRONAGE Birth Cohort. *Sci Rep*, 7(1), 5548. <https://doi.org/10.1038/s41598-017-04026-8>

- Tüfekci, K. U., Oner, M. G., Meuwissen, R. L., & Genç, S. (2014). The role of microRNAs in human diseases. *Methods Mol Biol*, 1107, 33-50. https://doi.org/10.1007/978-1-62703-748-8_3
- Ventura-Clapier, R., Moulin, M., Piquereau, J., Lemaire, C., Mericskay, M., Veksler, V., & Garnier, A. (2017). Mitochondria: a central target for sex differences in pathologies. *Clin Sci (Lond)*, 131(9), 803-822. <https://doi.org/10.1042/cs20160485>
- Visiedo, F., Bugatto, F., Sánchez, V., Cózar-Castellano, I., Bartha, J. L., & Perdomo, G. (2013). High glucose levels reduce fatty acid oxidation and increase triglyceride accumulation in human placenta. *Am J Physiol Endocrinol Metab*, 305(2), E205-212. <https://doi.org/10.1152/ajpendo.00032.2013>
- Von Schulze, A., McCoin, C. S., Onyekere, C., Allen, J., Geiger, P., Dorn, G. W., 2nd, Morris, E. M., & Thyfault, J. P. (2018). Hepatic mitochondrial adaptations to physical activity: impact of sexual dimorphism, PGC1 α and BNIP3-mediated mitophagy. *J Physiol*, 596(24), 6157-6171. <https://doi.org/10.1113/jp276539>
- Wankhade, U. D., Zhong, Y., Kang, P., Alfaro, M., Chintapalli, S. V., Piccolo, B. D., Mercer, K. E., Andres, A., Thakali, K. M., & Shankar, K. (2018). Maternal High-Fat Diet Programs Offspring Liver Steatosis in a Sexually Dimorphic Manner in Association with Changes in Gut Microbial Ecology in Mice. *Sci Rep*, 8(1), 16502. <https://doi.org/10.1038/s41598-018-34453-0>
- Wankhade, U. D., Zhong, Y., Kang, P., Alfaro, M., Chintapalli, S. V., Thakali, K. M., & Shankar, K. (2017). Enhanced offspring predisposition to steatohepatitis with maternal high-fat diet is associated with epigenetic and microbiome alterations. *PLoS One*, 12(4), e0175675. <https://doi.org/10.1371/journal.pone.0175675>
- Webber, J., Charlton, M., & Johns, N. (2015). Diabetes in pregnancy: management of diabetes and its complications from preconception to the postnatal period (NG3). *British Journal of Diabetes*, 15(3), 107-111. <https://doi.org/10.15277/bjdv.2015.029>

- Wegler, C., Ölander, M., Wiśniewski, J. R., Lundquist, P., Zettl, K., Åsberg, A., Hjelmæsæth, J., Andersson, T. B., & Artursson, P. (2020). Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genom Bioinform*, 2(1), lqz010. <https://doi.org/10.1093/nargab/lqz010>
- Wei, Y., Rector, R. S., Thyfault, J. P., & Ibdah, J. A. (2008). Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World J Gastroenterol*, 14(2), 193-199. <https://doi.org/10.3748/wjg.14.193>
- Westbrook, R. H., Dusheiko, G., & Williamson, C. (2016). Pregnancy and liver disease. *J Hepatol*, 64(4), 933-945. <https://doi.org/10.1016/j.jhep.2015.11.030>
- Wiebe, H. W., Boulé, N. G., Chari, R., & Davenport, M. H. (2015). The effect of supervised prenatal exercise on fetal growth: a meta-analysis. *Obstet Gynecol*, 125(5), 1185-1194. <https://doi.org/10.1097/aog.0000000000000801>
- Winn, N. C., Jurrissen, T. J., Grunewald, Z. I., Cunningham, R. P., Woodford, M. L., Kanaley, J. A., Lubahn, D. B., Manrique-Acevedo, C., Rector, R. S., Vieira-Potter, V. J., & Padilla, J. (2019). Estrogen receptor- α signaling maintains immunometabolic function in males and is obligatory for exercise-induced amelioration of nonalcoholic fatty liver. *Am J Physiol Endocrinol Metab*, 316(2), E156-e167. <https://doi.org/10.1152/ajpendo.00259.2018>
- Wu, W. Y., Ding, X. Q., Gu, T. T., Guo, W. J., Jiao, R. Q., Song, L., Sun, Y., Pan, Y., & Kong, L. D. (2020). Pterostilbene Improves Hepatic Lipid Accumulation via the MiR-34a/Sirt1/SREBP-1 Pathway in Fructose-Fed Rats. *J Agric Food Chem*, 68(5), 1436-1446. <https://doi.org/10.1021/acs.jafc.9b04259>
- Yabaluri, N., & Bashyam, M. D. (2010). Hormonal regulation of gluconeogenic gene transcription in the liver. *J Biosci*, 35(3), 473-484. <https://doi.org/10.1007/s12038-010-0052-0>
- Yamamoto, H., Morino, K., Nishio, Y., Ugi, S., Yoshizaki, T., Kashiwagi, A., & Maegawa, H. (2012). MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead

- box j3. American Journal of Physiology-Endocrinology and Metabolism, 303(12), E1419-E1427.
- Yamamoto, T., Shimano, H., Nakagawa, Y., Ide, T., Yahagi, N., Matsuzaka, T., Nakakuki, M., Takahashi, A., Suzuki, H., Sone, H., Toyoshima, H., Sato, R., & Yamada, N. (2004). SREBP-1 interacts with hepatocyte nuclear factor-4 alpha and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *J Biol Chem*, 279(13), 12027-12035. <https://doi.org/10.1074/jbc.M310333200>
- Yamashita, H., Shao, J., Qiao, L., Pagliassotti, M., & Friedman, J. E. (2003). Effect of spontaneous gestational diabetes on fetal and postnatal hepatic insulin resistance in *Lepr(db/+)* mice. *Pediatr Res*, 53(3), 411-418. <https://doi.org/10.1203/01.Pdr.0000049667.58071.7d>
- Young, J. C., & Treadway, J. L. (1992). The effect of prior exercise on oral glucose tolerance in late gestational women. *Eur J Appl Physiol Occup Physiol*, 64(5), 430-433. <https://doi.org/10.1007/bf00625062>
- Yu, S. B., & Pekkurnaz, G. (2018). Mechanisms Orchestrating Mitochondrial Dynamics for Energy Homeostasis. *J Mol Biol*, 430(21), 3922-3941. <https://doi.org/10.1016/j.jmb.2018.07.027>
- Zabielski, P., Hady, H. R., Chacinska, M., Roszczyc, K., Gorski, J., & Blachnio-Zabielska, A. U. (2018). The effect of high fat diet and metformin treatment on liver lipids accumulation and their impact on insulin action. *Sci Rep*, 8(1), 7249. <https://doi.org/10.1038/s41598-018-25397-6>
- Zeng, Z., Liu, F., & Li, S. (2017). Metabolic Adaptations in Pregnancy: A Review. *Ann Nutr Metab*, 70(1), 59-65. <https://doi.org/10.1159/000459633>
- Zhang, C., Solomon, C. G., Manson, J. E., & Hu, F. B. (2006). A prospective study of pregravid physical activity and sedentary behaviors in relation to the risk for gestational diabetes mellitus. *Arch Intern Med*, 166(5), 543-548. <https://doi.org/10.1001/archinte.166.5.543>
- Zhang, H. J., He, J., Pan, L. L., Ma, Z. M., Han, C. K., Chen, C. S., Chen, Z., Han, H. W., Chen, S., Sun, Q., Zhang, J. F., Li, Z. B., Yang, S. Y., Li, X. J., & Li, X. Y. (2016). Effects of Moderate and Vigorous Exercise on Nonalcoholic

- Fatty Liver Disease: A Randomized Clinical Trial. *JAMA Intern Med*, 176(8), 1074-1082. <https://doi.org/10.1001/jamainternmed.2016.3202>
- Zhang, J., Jazii, F. R., Haghighi, M. M., Alvares, D., Liu, L., Khosraviani, N., & Adeli, K. (2020). miR-130b is a potent stimulator of hepatic very-low-density lipoprotein assembly and secretion via marked induction of microsomal triglyceride transfer protein. *American Journal of Physiology-Endocrinology and Metabolism*, 318(2), E262-E275.
- Zhang, Y., Lei, T., Huang, J. F., Wang, S. B., Zhou, L. L., Yang, Z. Q., & Chen, X. D. (2011). The link between fibroblast growth factor 21 and sterol regulatory element binding protein 1c during lipogenesis in hepatocytes. *Mol Cell Endocrinol*, 342(1-2), 41-47. <https://doi.org/10.1016/j.mce.2011.05.003>
- Zhang, Y., Zhang, Y., Ding, G. L., Liu, X. M., Ye, J., Sheng, J. Z., Fan, J., & Huang, H. F. (2017). Regulation of hepatic pyruvate dehydrogenase phosphorylation in offspring glucose intolerance induced by intrauterine hyperglycemia. *Oncotarget*, 8(9), 15205-15212. <https://doi.org/10.18632/oncotarget.14837>
- Zhang, Z. C., Liu, Y., Xiao, L. L., Li, S. F., Jiang, J. H., Zhao, Y., Qian, S. W., Tang, Q. Q., & Li, X. (2015). Upregulation of miR-125b by estrogen protects against non-alcoholic fatty liver in female mice. *J Hepatol*, 63(6), 1466-1475. <https://doi.org/10.1016/j.jhep.2015.07.037>
- Zhao, R. Z., Jiang, S., Zhang, L., & Yu, Z. B. (2019). Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int J Mol Med*, 44(1), 3-15. <https://doi.org/10.3892/ijmm.2019.4188>