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A MOTHER'S HEART: IMPACTS OF GESTATION HABITS ON MATERNAL CARDIAC MITOCHONDRIA METABOLISM

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pela Doutora Susana P. Pereira e pela Professora Doutora Anabela Rolo e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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A mother's heart: impacts of gestation habits on maternal cardiac mitochondrial metabolism

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Master's Dissertation in Cellular and Molecular Biology

Trabalho realizado com a orientação científica da Doutora Susana P. Pereira e da Professora Doutora Anabela Rolo

MitoXT CNC CENTER FOR NEUROSCIENCE AND CELL BIOLOGY CIBB CENTRE FOR INNOVATIVE BIOMEDICINE AND BIOTECHNOLOGY FUTURE U.PORTO CIAFEL

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"Don't shoot for the stars, we already know what's there… Shoot for the space in between because that's where the real mystery lies."
- Vera Rubin

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Abstract

Pregnancy composes a metabolic challenge for the maternal organism. The cardiovascular and other systems undergo adaptational processes to guarantee the energetic and nutritional demands of fetal development. Among other potential factors, the rising consumption of high caloric diets and sedentary lifestyles contribute to increased risk for metabolic-related pregnancy complications. Gestational diabetes mellitus (GDM) is the most common metabolic pregnancy disorder, affecting 14% of pregnancies. Postnatally, GDM mothers have an increased risk of developing type-2-diabetes (T2D) and cardiovascular diseases (CVD). Mitochondrial dysfunctions have been implicated in the CVD and T2D pathogenesis. Lifestyle modifications, which involve exercise practice, are used as GDM first-line treatment. However, it is still unknown if exercise practice during GDM can prevent the increased maternal risk for CVD by modulating cardiac mitochondria metabolism.

This study aimed to characterize the cardiac metabolic memory induced by GDM that prevails after pregnancy in the maternal heart and understand if exercise practice exclusively during GDM-pregnancy prevents the GDM-induced cardiometabolic effects that predispose to a higher risk of cardiac disease development.

We developed a Sprague-Dawley rat model of GDM, induced by a high-fat high sugar (HFHS) diet consumption, starting at 7-weeks of age (6 weeks before impregnation) through the whole experiment. The non-pregnant (NP) and pregnant (P) controls were fed a standard chow diet (C). We validated that the HFHS protocol leads to GDM phenotype. In the GDM exercise (E) intervention group, the exercise protocol began at 14-weeks of age, the time point in which mating protocols were implemented, and consisted of voluntary physical activity in a running wheel and 6-days per week endurance training in an adapted treadmill. The other female rats were considered sedentary (S). The exercise protocol lasted the 3 weeks of pregnancy. The female rats were euthanized at 25-weeks of age, after the nursing and weaning periods that finished at 20-weeks of age. Maternal morphologic characteristics and glucose tolerance were assessed during the experiment. The levels of plasma biochemical parameters were evaluated by MS/MS and the cardiac tissue morphological characterization was histologically assessed. Cardiac inflammation markers, insulin signaling, metabolic flexibility, and mitochondrial function, through evaluation of mitochondrial bioenergetics, dynamics, macroautophagy, and redox balance were determined using Clark-type and TPP+ electrodes, qPCR, and Western blot. Data were compared between P-C-S (n=7), NP-C-S (n=7), GDM-S (n=6), and GDM-E (n=6) using Mann-Whitney or t-student test according to Gaussian distribution, with $p \leq 0.05$ considered statistically significant.

Maternal GDM was confirmed through glucose intolerance at mid-pregnancy and resulted in increased gestational weight gain and enlarged litter size. In the maternal cardiac tissue, 8 weeks postpartum, the GDM groups revealed augmented levels of p-Akt (Ser473). However, GDM-S mothers inefficiently responded to the induced adaptation showing decreased p-GSK3. GDM's negative impact on cardiac metabolism was detected by impaired HIF-1α signaling and FA oxidation transcript levels of ACAA2 and PPARα in GDM-S, with the inability to metabolically adapt as opposed to P-C-S and GDM-E that showed increased AMPK activation. However, exercise practice during pregnancy and GDM-S decreased mitochondrial bioenergetics with decreased ADP/O and RCR values and requiring more time to complete ADP phosphorylation. Despite no considerable alterations in the OXPHOS protein levels analyzed, and in proteins and transcripts involved in mitochondrial dynamics, biogenesis, and mitochondrial mass indicators, physiologic adaptations observed in the P-C-S group (increased TFAM levels to decreased Tom20 and POLRMT) seem to fail to occur in the hearts of GDM dams. Moreover, the hearts of GDM-E mothers failed to respond to the increased PGC1α transcript levels despite the increased UQCRC2 and MTCO2 protein levels. Blockage of the autophagic flux was marked in the GDM-S group due to increased LC3II/LC3I ratio and p62 levels. The latter was increased in GDM-E concomitantly with an increased ratio of p-Bcl2 to total protein. An unbalanced redox state

marked the cardiac tissue in the postpartum period with increased Nrf2 and Gpx4 protein levels, which may compose an adaptational response exacerbated in the GDM-S group and possibly impaired in the GDM-E group, with normal Nrf2 levels.

In summary, although exercise practice during GDM gestation could counteract some of the impaired cardiac characteristics and mechanisms observed in a GDM-pregnancy in the postpartum period, mitochondrial function was highly affected. Though, the hearts of GDM mothers display stimulated adaptational mechanisms to answer to this metabolic challenge, the response efficacy was insufficient resulting in impaired cardiac metabolic flexibility and compromised mitochondrial function. Elucidation of the exercise type a GDM-mother should adopt and provide close monitoring of these activities during pregnancy and after delivery should be a priority among clinicians to guarantee the long-term health of mothers after a GDM-pregnancy to combat the CVD increased risk.

Keywords: Postpartum health, cardiovascular diseases, gestational diabetes mellitus, cardiometabolic dysfunction, cardiac mitochondrial impairment, exercise during gestation, pregnancy-induced heart remodeling

Resumo

A gravidez trata-se de um desafio metabólico para o organismo materno. O sistema cardiovascular e outros são submetidos a processos adaptativos para garantir as necessidades energéticas e nutricionais ao desenvolvimento fetal. O consumo de dietas calóricas e hábitos sedentários, entre outros fatores, contribuem para um risco acrescido ao desenvolvimento de complicações metabólicas durante a gravidez. A diabetes gestacional *mellitus* (GDM) é a doença metabólica mais comum da gravidez, afetando 14% das gestações. Mães que tiveram GDM têm risco acrescido de desenvolver diabetes e doenças cardiovasculares (CVD) no período pós-natal. Disfunções mitocondriais foram associadas a várias CVD e diabetes. Modificações no estilo de vida, como a prática de exercício, são utilizadas como primeira linha de tratamento da GDM. No entanto, é ainda desconhecido se a prática de exercício durante GDM pode prevenir o risco para o desenvolvimento de CVD por modulação do metabolismo cardíaco mitocondrial.

Este estudo teve como objetivo caracterizar a memória metabólica induzida por GDM mantida pós-gravidez no coração materno e compreender se a prática de exercício apenas durante uma gravidez GDM previne os efeitos cardiometabólicos induzidos por GDM que predispõem para um risco acrescido de desenvolver doenças cardíacas.

Desenvolvemos um modelo de GDM em Sprague-Dawley induzido por ingestão de uma dieta rica em gordura e açúcares (HFHS), iniciada às 7 semanas de idade (6 semanas antes do acasalamento) durante toda a experiência. Os controlos não-grávidos (NP) e grávidos (P) foram alimentados com dieta controlo (C). Validámos que o protocolo de dieta HFHS leva a um fenótipo GDM. No grupo com GDM exercitado (E), o protocolo de exercício começou às 14 semanas, mesma altura em que protocolos de acasalamento foram implementados, consistindo em atividade física voluntária numa roda e 6 dias por semana de treino de resistência numa passadeira. Os outros animais consideraram-se sedentários (S). O protocolo de exercício estendeu-se durante as 3 semanas da gravidez. Às 25 semanas os animais foram eutanasiados, após o período de amamentação e desmame que terminaram às 20 semanas de idade. As características maternas morfológicas e tolerância à glucose foram avaliadas durante a experiência. Os parâmetros bioquímicos no plasma foram determinados por MS/MS e a caracterização morfológica do tecido cardíaco avaliada por histologia. Marcadores de inflamação, sinalização da insulina, flexibilidade metabólica e função mitocondrial cardíaca, por avaliação da bioenergética, dinâmica mitocondrial, macroautofagia, e equilíbrio redox foram determinados utilizando elétrodos Clark e TPP^+, qPCR e Western blot. Os dados foram comparados entre P-C-S (n=7), NP-C-S (n=7), GDM-S (n=6) e GDM-E (n=6), utilizando Mann-Whitney ou t-student test de acordo com a distribuição Gaussiana, com $p \leq 0.05$ considerado estatisticamente significativo.

GDM confirmada pelo desenvolvimento de intolerância à glucose durante a gravidez resultou num aumento do ganho ponderal e do tamanho da ninhada. No tecido cardíaco materno, às 8 semanas pós-parto, os grupos GDM revelaram elevados níveis de p-Akt (Ser473). Contudo, mães GDM-S responderam ineficientemente à adaptação induzida exibindo p-GSK3 diminuído. O impacto negativo de GDM no metabolismo cardíaco foi detetado por sinalização HIF-1α e níveis de transcritos ACAA2 e PPARα da oxidação de FA comprometidos e GDM-S, associado a uma incapacidade de adaptação metabólica, contrariamente a P-C-S e GDM-E que mostraram maior ativação de AMPK. No entanto, a prática de exercício durante a gravidez e GDM-S resultou numa atividade bioenergética mitocondrial diminuída, com baixos valores de ADP/O e RCR e necessitando de mais tempo para a fosforilação completa de ADP. Embora poucas alterações nos níveis de proteínas OXPHOS analisados e de proteínas e transcritos envolvidos na dinâmica, biogénese e indicadores de massa mitocondriais, as adaptações induzidas pela gravidez observadas em P-C-S (elevados níveis de TFAM para Tom20 e POLRMT diminuídos) parecem não ocorrer em corações de mães GDM. Os corações de mães GDM-E falharam na resposta aos níveis elevados de transcrito PGC1α, embora com elevados níveis de UQCRC2 e MTCO2. O bloqueio do fluxo autofágico foi marcado em GDM-S devido a um elevado rácio LC3II/LC3I e

níveis de p62, também elevados em GDM-E concomitantemente a um rácio p-Bcl2/Bcl2 elevado. O período pós-parto foi ainda marcado no tecido cardíaco por um desequilíbrio redox com níveis Nrf2 e Gpx4 elevados, que podem consistir numa adaptação que é exacerbada em GDM-S e possivelmente comprometida em GDM-E, com níveis normais de Nrf2.

Assim, apesar da prática de exercício durante GDM ter conseguido contrabalançar algumas das características e mecanismos cardíacos comprometidos em resposta a uma gravidez GDM no período pós-parto, a função mitocondrial foi altamente afetada. Embora corações de mães GDM mostrem mecanismos adaptativos estimulados para responder a este desafio metabólico, a eficácia de resposta foi insuficiente, resultando numa flexibilidade metabólica e função mitocondrial comprometidas. Então, elucidar qual o tipo de exercício a adotar por uma mãe GDM, assim como fornecer a devida monitorização destas atividades durante e pós-gravidez devia ser uma prioridade na clínica para garantir a saúde materna a longo prazo para combater o risco acrescido de CVD.

Palavras-chave: Saúde pós-parto, doenças cardiovasculares, diabetes gestacional mellitus, disfunção cardiometabólica, comprometimento da função mitocondrial, exercício durante a gestação, remodelação cardíaca induzida pela gravidez

Statement of originality

This dissertation includes material from manuscripts and conference proceedings that described work completed during my registration as a graduate student at the University of Coimbra:

1. Luís F. Grilo, **Carolina Tocantins**, Mariana S. Diniz, Rodrigo Mello Gomes, Paulo J. Oliveira, Paulo Matafome, Susana P. Pereira (2021). Metabolic Disease Programming: from Mitochondria to Epigenetics, Glucocorticoid Signaling and Beyond. European Journal of Clinical Investigation. https://doi.org/10.1111/eci.13625

2. Luís Grilo, João D. Martins, Mariana S. Diniz, Chiara H. Cavallaro, **Carolina Tocantins,** Inês Baldeiras, Teresa Cunha-Oliveira, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira, Susana P. Pereira (2021). Maternal hepatic adaptations to obesity during pregnancy involve mitochondria dysfunction and oxidative stress and are lobe specific. Hepatology (submitted).

3. **Carolina Tocantins**, Mariana S. Diniz, Luís F. Grilo, Susana P. Pereira. (2022). The birth of cardiac disease: the contribution of maternal womb. WIRE Mechanisms of Disease (in preparation).

Part of this dissertation work has been presented in several national and international scientific meetings in the form of two oral communications and thirteen poster communication and its relevance prized.

Publications as conference proceedings

1. Susana P. Pereira, Luís F. Grilo, Mariana S. Diniz, **Carolina Tocantins**, João Martins, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira. Maternal obesity induces fetal hepatic oxidative stress (OS) and mitochondrial damage predominantly in the right lobe. Reproductive Sciences, Vol. 28, p.70. https://doi.org/10.1007/s43032-021-00654-8

2. Luís F. Grilo, João Martins, Mariana S. Diniz, **Carolina Tocantins**, Chiara H. Cavallaro, Inês Baldeiras, Teresa Cunha-Oliveira, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira, Susana P. Pereira. Maternal Obesity During Pregnancy Induces Oxidative Stress and Mitochondria Functional Alterations in Sheep Maternal Liver. Reproductive Sciences, Vol. 28, p.264. https://doi.org/10.1007/s43032-021-00654-8

3. Susana P. Pereira, Mariana S. Diniz, João D. Martins, Óscar M. Rodrigues, **Carolina Tocantins**, Luís F. Grilo, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães Paulo J. Oliveira. Moreno, A. Ascensão, J. Magalhães, P.J. Oliveira. Preventing cardiac disease: maternal gestational exercise to mitigate obesity-induced

cardiac metabolic impairment in the offspring. Society for Heart and Vascular Metabolism 2021: Metabolism at the crossroads of health and disease. Jena, Germany, September 23rd-24th, 2021. Abstract published in the abstract book of SHVM 2021.

4. **Carolina Tocantins**, Óscar M. Rodrigues, João D. Martins, Luís F. Grilo, Mariana S. Diniz, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães Paulo J. Oliveira, Susana P. Pereira. Maternal cardiac mitochondrial alterations induced by gestational diabetes mellitus prevail at 8 weeks postpartum. Society for Heart and Vascular Metabolism 2021: Metabolism at the crossroads of health and disease. Jena, Germany, September 23rd-24th, 2021. Abstract published in the abstract book of SHVM 2021.

5. Susana P. Pereira, Mariana S. Diniz, João D. Martins, Óscar M. Rodrigues, **Carolina Tocantins**, Luís F. Grilo, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães, Paulo J. Oliveira. Maternal physical exercise during obesogenic pregnancy to protect the offspring cardiac mitochondrial function. Nature Conferences, 28-30th June, 2021, Virtual edition.

6. Luís F. Grilo, Ivan Viegas, Mariana S. Diniz, **Carolina Tocantins**, João D. Martins, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira, Susana P. Pereira. Maternal obesity (MO) during pregnancy induces maternal liver damage and compromises fetal hepatic function. European Journal of Clinical Investigation, Vol. 51, p.22. https://doi.org/10.1111/eci.13567

7. **Carolina Tocantins**, Óscar M. Rodrigues, João D. Martins, Luís F. Grilo, Mariana S. Diniz, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães Paulo J. Oliveira, Susana P. Pereira. The metabolic challenge of pregnancy: A toll on the maternal heart aggravated by Gestational Diabetes Mellitus. European Journal of Clinical Investigation, Vol. 51, p.42. https://doi.org/10.1111/eci.13567

8. Susana P. Pereira, Mariana S. Diniz, **Carolina Tocantins**, Óscar M. Rodrigues, João D. Martins, Luís F. Grilo, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães Paulo J. Oliveira. Beating cardiovascular disease: Physical exercise performed during obesogenic pregnancy to improve offspring mitochondrial cardiac function. European Journal of Clinical Investigation, Vol. 51, p.43. https://doi.org/10.1111/eci.13567

9. Luís F. Grilo, **Carolina Tocantins**, Mariana S. Diniz, João D. Martins, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira, Susana P. Pereira. Obesidade Gestacional Causa Stress Oxidativo e Dano Mitocondrial Fetal Hepático Com Maior Impacto No Lóbulo Direito Do Fígado. Revista Portuguesa de Diabetes Vol. 16, p. 64. (ISSN: 1646-3994).

10. **Carolina Tocantins**, Óscar M. Rodrigues, João D. Martins, Luís F. Grilo, Mariana S. Diniz, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães

Paulo J. Oliveira, Susana P. Pereira. Prática de Exercício Físico Durante Diabetes Gestacional como Estratégia para a Modulação da Função Cardíaca da Gestante. Revista Portuguesa de Diabetes Vol. 16, p. 60. (ISSN: 1646-3994).

11. Mariana S. Diniz, Susana P. Pereira, **Carolina Tocantins**, Óscar M. Rodrigues, João D. Martins, Luís F. Grilo, Jelena Stevanovic-Silva, Jorge Beleza, Pedro Coxito, David Rizo-Roca, Estela Santos-Alves, Manoel Rios, António J. Moreno, António Ascensão, José Magalhães Paulo J. Oliveira. Poderá a Obesidade Materna Alterar a Bioenergética Mitocondrial Cardíaca dos Descendentes? Revista Portuguesa de Diabetes Vol. 16, p. 60. (ISSN: 1646-3994).

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List of abbreviations

4EBP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
ACAA2	acetyl-CoA Acyl Transferase 2
ACC	Acetyl-CoA Carboxylase
ACOG	American College of Obstetricians and Gynecologists
ACOX1	Acyl-CoA Oxidase 1
ADA	American Diabetes Association
ADP	Adenosine Diphosphate
AIP	Atherogenic Index of Plasma
Akt	Protein Kinase B
AMPK	AMP-activated Protein Kinase
ANT	Adenine Nucleotide Translocase
ARE	Antioxidant Response Element
AT	Adipose Tissue
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body Mass Index
BP	Blood Pressure
CACT	Carnitine/acylcarnitine Translocase
cGMP	Cyclic Guanosine Monophosphate
CIRKO	Cardiomyocyte-selective Insulin Receptor Knockout
CoA	Coenzyme A
CoQ	Ubiquinone
CPT	Carnitine Palmitoyl Translocase
CV	Cardiovascular
CVD	Cardiovascular disease
DAG	Diacylglycerols
DIO	Diet-induced Obesity
DM	Diabetes Mellitus
DRP1	Dynamin-related Protein 1
ECM	Extracellular Matrix
EHN	European Heart Network
eNOS	Endothelial Nitric Oxide Synthase
ETC	Electron Transport Chain
EU	European Union
FA	Fatty Acid
FAT/CD36	Fatty Acid Translocase/Cluster Differentiation 36
FATP	Fatty Acid Transport Protein
FBG	Fasting Blood Glucose
FIS1	Mitochondrial Fission 1 Protein
G/M	Glutamate and Malate
G6PD	Glucose-6-phosphate Dehydrogenase
GATA4	Gata Binding Protein 4
GDM	Gestational Diabetes Mellitus
GLUT	Glucose Transporters
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSK3	Glycogen Synthase Kinase 3
GSSG	Oxidized Glutathione
GWG	Gestational Weight Gain
H&E	Hematoxylin and Eosin

HAPO	Hyperglycemia and Adverse Pregnancy Outcomes
HbA1C	Glucose Glycosylated Hemoglobin A1C
HCD	High-carbohydrate Diet
HDL	High-density Lipoproteins
HFD	High-fat Diet
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IGF-1	Insulin-like Growth Factor 1
IGF1R	Insulin-like Growth Factor 1 Receptor
IHC	Immunohistochemistry
IHD	Ischemic Heart Disease
IL-6	Interleukine 6
IMM	Inner Mitochondrial Membrane
InsR	Insulin Receptor
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JNK	Jun N-terminal Kinase
LC3	Microtubule-associated Protein 1A/1B-light Chain 3
LCFA	Long-chain Fatty Acyl CoA
LDH	Lactate Dehydrogenase
LDL	Low-density Lipoproteins
MCD	Malonyl-CoA Decarboxylase
MFN	Mitofusin
MPC	Mitochondrial Pyruvate Carrier
mTOR	Akt/mammalian Target of Rapamycin
mTORC	Akt/mammalian Target of Rapamycin Complex
NCDs	Non-communicable Diseases
NFAT	Nuclear Factor of Activated T-cells
NHP	Non-human Primates
NIH	National Institutes of Health
NO	Nitric Oxide
Nrf2	Nuclear Factor Erythroid 2-related Factor 2
OAA	Oxaloacetate
OGTT	Oral Glucose Tolerance Test
OHA	Oral Hypoglycemic Agents
OMM	Outer Mitochondrial Membrane
OPA1	Dynamin-like 120 kDa Protein
OS	Oxidative Stress
OXPHOS	Oxidative Phosphorylation System
PDH	Pyruvate Dehydrogenase Complex
PDK	Pyruvate Dehydrogenase Kinase
PDK1	Phosphoinositide-dependent Kinase 1
PGC-1α	Peroxisome Proliferator-activator Receptor Coactivator 1 alpha
PINK1	PTEN-induced Putative Kinase Protein 1/ubiquitin Ligase
PKC	Protein Kinase C
POLRMT	Mitochondrial RNA Polymerase
PPAR	Peroxisome Proliferator-activator Receptor
qPCR	Quantitative Polymerase Chain Reaction
RBG	Random Blood Glucose
RCR	Respiratory Control Ratio
ROS	Reactive Oxygen Species
SCs	Supercomplexes
sGC	Soluble Guanylate Cyclase
SMFM	Society for Maternal-Fetal Medicine
SOD	Superoxide Dismutase

STZ	Streptozotocin
T2DM	Type-2 Diabetes Mellitus
TAG	Triacylglycerols
TCA	Tricarboxylic Acid
TFAM	Mitochondrial Transcription Factor A
TG	Triglyceride
TNF-α	Tumor Necrosis Factor α
TPP+	Tetraphenylphosphonium
US	United States
VDAC	Voltage-dependent Anion Channel
WHO	World Health Organization

Chapter 1 – Introduction

1.1 **Cardiovascular disease burden**

Cardiovascular disease (CDV) remains the leading cause of mortality and morbidity worldwide[1–4]. According to The World Health Organization (WHO), CVD consist of a group of disorders that can affect the heart and blood vessels. It is estimated that, in 2019, CVD led to 18.6 million deaths, being responsible for 32% of the total number of deaths around the globe, meaning that 1 in 5 people aged less than 70 years old will die due to CVD[4]. Despite the decreasing mortality rates of CVD in certain countries, especially in developed countries, due to raised awareness campaigns, and due to investments in more frequent monitoring of CVD's risk factors, as well as in the disease diagnosis and treatment protocols[2], the rate of decline is now deaccelerating[4]. For the first time in the last 50 years an increase in premature CVD death (< 65 years of age) was reported in some European Union (EU) countries[4], possibly due to the increased incidence of the risk factors[2,3], which is becoming a concern among society since these adverse effects affect not only adults but also young adults and children[3]. The rise in CVD premature death was noticed even before the COVID-19 outbreak, which dramatically aggravated the situation for more than 520 million people living with CVD[4].

According to the European Heart Network (EHN), CVD accounted for 45% of all deaths in Europe in 2017[5]. In 2015, nearly 85 million people were living with CVD in Europe. The study revealed that the major contributors to the risk of developing CVD in Europe include physiological risk factors, such as high systolic blood pressure (BP), and manageable risk factors (e.g. diet)[5]. In 2015, CVD costs represented 8% of the total health care expenditure across the EU, costing the health care systems nearly 111 billion euros in direct health care costs of a total of 210 billion euros per year (including productivity losses and the informal care of people with CVD)[5].

Cardiovascular diseases are a type of noncommunicable diseases (NCDs), which apart from CVDs, include cancer, diabetes and chronic respiratory disease[6,7]. NCDs are defined as diseases that cannot be transmitted from one person to the other[8], that can be caused by a combination of genetic, physiological, behavioral, and environmental factors[6]. NCDs kill 41 million people worldwide each year, contributing to 71% of the total number of deaths[6,7]. CVD has a major contribution to NCDs deaths, killing even more than cancer, especially in Europe and in the United States (US). Ischemic heart disease (IHD) and stroke are the two most common CVD that contribute to the total number deaths per year in Portugal, Europe, and the US (**Table 1.1**). In fact, in Europe, just IHD contributes almost as much (~19.7%) to all causes of death as cancer (~22.0%)[5].

The United Nations pointed NCDs as a major challenge in the 2030 Agenda for Sustainable Development, setting the goal to reduce premature mortality from NCDs by one-third, through prevention and treatment (goal 3 – "Ensure healthy lives and promote well-being for all at all ages", target 3.4, more information in https://sdgs.un.org/goals/goal3). As CVD is the major NCDs cause of death, it is essential to invest in the prevention of CVD and early diagnosis, which requires the identification of the main predisposing factors and a complete understanding of the CVD pathology.

Initially, genetic factors, often related to ethnicity, were known as the major contributors, for instance, the remarkable heritability of elevated BP[9]. Nevertheless, the synergetic risk observed between genetic, environmental, and lifestyle habits might explain the variable incidence of the disease across different countries[9]. For all the reasons mentioned above, to understand the causes behind CVD development, cardiovascular research has been focused on adult life, based on ancestrality or lifestyle.

To beat CVD, it is essential to understand the role of the heart and cardiovascular (CV) system in the organism and how nutrition, in particular cardiometabolic insults, may represent a risk factor promoting a premature development of CVD. Scrutinizing the cardiac mechanisms behind substrate utilization and metabolization, and possible interactions with other crucial cellular mechanisms related to overall metabolism are vital since energy production is critical to maintaining a normal and healthy cardiac function.

Table 1.1 – Number of deaths by cause per year in Portugal, Europe, and the United States.

Region	All Causes	Ischemic Heart Disease	Stroke	Other	All CVD	All CVD (%)	Cancer	Cancer (%)
Portugal (2014) (5)	105219	7456	11808	13022	32286	30.68	26742	25.42
Europe (2000-2014) (5)	8 846 296	1 739 435	988 573	1 212 657	3 940 665	44.50	1 942 755	21.96
United States (2017) (10)	2 686 301	365 987	146 051	347 087	859 125	32.0*	599 108	22.3*

*Percentages were calculated considering the US population estimate in 2017 (325 100 000), according to the U.S. Census Bureau.

1.2 **Cardiac energy metabolism**

In the adult heart, the major source of adenosine triphosphate (ATP) production is fatty acid (FA) oxidation, providing 60 to 90% of the energy, followed by carbohydrates (10 to 40%) such as glucose and lactate, and, in lower amounts, ketone bodies and amino acids[11–13]. So the heart can respond to the high energetic requirements of contractility, the metabolic pathways involved in substrate oxidation must be in equilibrium.

1.2.1 **Fatty acid oxidation**

Circulating free FAs, usually bound to albumin, or released from triacylglycerols (TAG), are transported to cardiomyocytes mainly through the FA transport protein (FATP) or the FA translocase/cluster differentiation protein 36 (FAT/CD36)[14–16]. Intracellularly, fatty acyl CoA synthase catalyzes the esterification of FAs to form long-chain fatty acyl coenzyme A (LCFA) by an ATP-dependent pathway. At this point, carnitine palmitoyl transferase (CPT) 1, a protein located at the outer mitochondrial membrane (OMM), facilitates LCFA's transport into the

mitochondria by converting these molecules to long-chain fatty acyl carnitine which, after being transported to the mitochondrial matrix by the carnitine/acylcarnitine translocase (CACT), are converted back to LCFA by CPT-2, which is located in the inner mitochondrial membrane (IMM) [14,15]. This process is known as the carnitine shuttle, as while coenzyme A (CoA) is released into the cytoplasm of the cardiomyocyte, carnitine is released into the cardiac mitochondrial matrix but shuttled back into the intermembrane space so it can be used again by CPT-1[16] (**Figure 1.1**). Once in the mitochondria, LCFA are oxidized through FA β-oxidation[14,16]. In this process, FAs are catabolized through a cycle of four main steps resulting in NADH, $FADH_2$, and acetyl-CoA[14].

In the myocardium of obese mice, CD36 deficiency was shown to prevent obesity-associated cardiac steatosis[17], and while rats fed a high-fat diet (HFD) demonstrated increased uptake of LCFA, the administration of a CD36 inhibitor reversed this effect[18]. In the human left ventricle myocardium of heart failure patients, the mRNA levels of CD36 were found to be significantly elevated[19]. However, CD36 deletion resulted in accelerated hypertrophy and heart failure in the hearts of mice subjected to pressure overload[20,21], which suggests that the benefit of limiting CD36 expression previously observed in the heart might be dependent on the underlying condition, for instance, the presence of elevated circulating FAs[21]. This denotes the importance of maintaining the balance between oxidative mechanisms for energy production.

The rate-limiting step of the whole process occurring in cardiomyocytes for the oxidation of free FAs is the reaction catalyzed by CPT-1[14,15]. Since malonyl-CoA is an allosteric inhibitor of CPT-1, it has a pivotal role in regulating FA oxidation[11]. The levels of malonyl CoA, on the other hand, are dependent on the levels of cytosolic acetyl CoA, and the equilibrium between the reactions catalyzed by acetyl CoA carboxylase (ACC), which produces malonyl CoA by carboxylation of acetyl CoA, and malonyl CoA decarboxylase (MCD), which catalyzes the opposite reaction is determinant for FA oxidation[14,15]. To date, several metabolic inhibitors of enzymes involved in FA oxidation have been used as adjuvant therapy to treat heart failure and other complications related to an unbalanced cardiac metabolism[22]. These compounds mainly include inhibitors of the CPT-1 and MCD enzymes[15,22], intending to reduce FA uptake into the mitochondria, subsequently limiting FA utilization and oxidation, thus promoting glucose metabolism.

1.2.2 **Glucose oxidation**

Glucose transport into cardiomyocytes mainly occurs through two specific glucose transporters (GLUT) isoforms: GLUT-1, which is insulin independent, and GLUT-4, an insulin dependent transporter[14,23]. However, while GLUT-1 is predominantly expressed in the heart during the embryonic and neonatal stages, GLUT-4 is the predominantly expressed isoform in the healthy adult heart since its expression is upregulated right after birth[23]. Once glucose enters cardiomyocytes, the molecule can be metabolized by several pathways, including glycogen synthesis, the pentose phosphate pathway, or the glycolytic pathway[14].

Glycolysis is the process by which glucose is converted to pyruvate yielding one NADH and two ATP molecules[14,16]. Pyruvate can further be converted to lactate by the enzyme lactate dehydrogenase (LDH) or transferred to the mitochondria through the mitochondrial pyruvate carrier (MPC) localized at the IMM[14] (**Figure 1.1**). Pyruvate must be converted to acetyl-CoA by the multi-subunit pyruvate dehydrogenase complex (PDH) for ATP production in the electron

transport chain. The protein PDH establishes the rate-limiting step of glucose oxidation, being susceptible to regulation by inhibition by PDH kinase (PDK)[14,15,23]. PDK activation and consequent PDH inhibition through phosphorylation in three serine residues (254, 271, and 203) can occur due to the levels of mitochondrial FA oxidation by-products, such as NADH and acetyl-CoA[15,23], while pyruvate's high levels can also contribute to PDH inhibition[23].

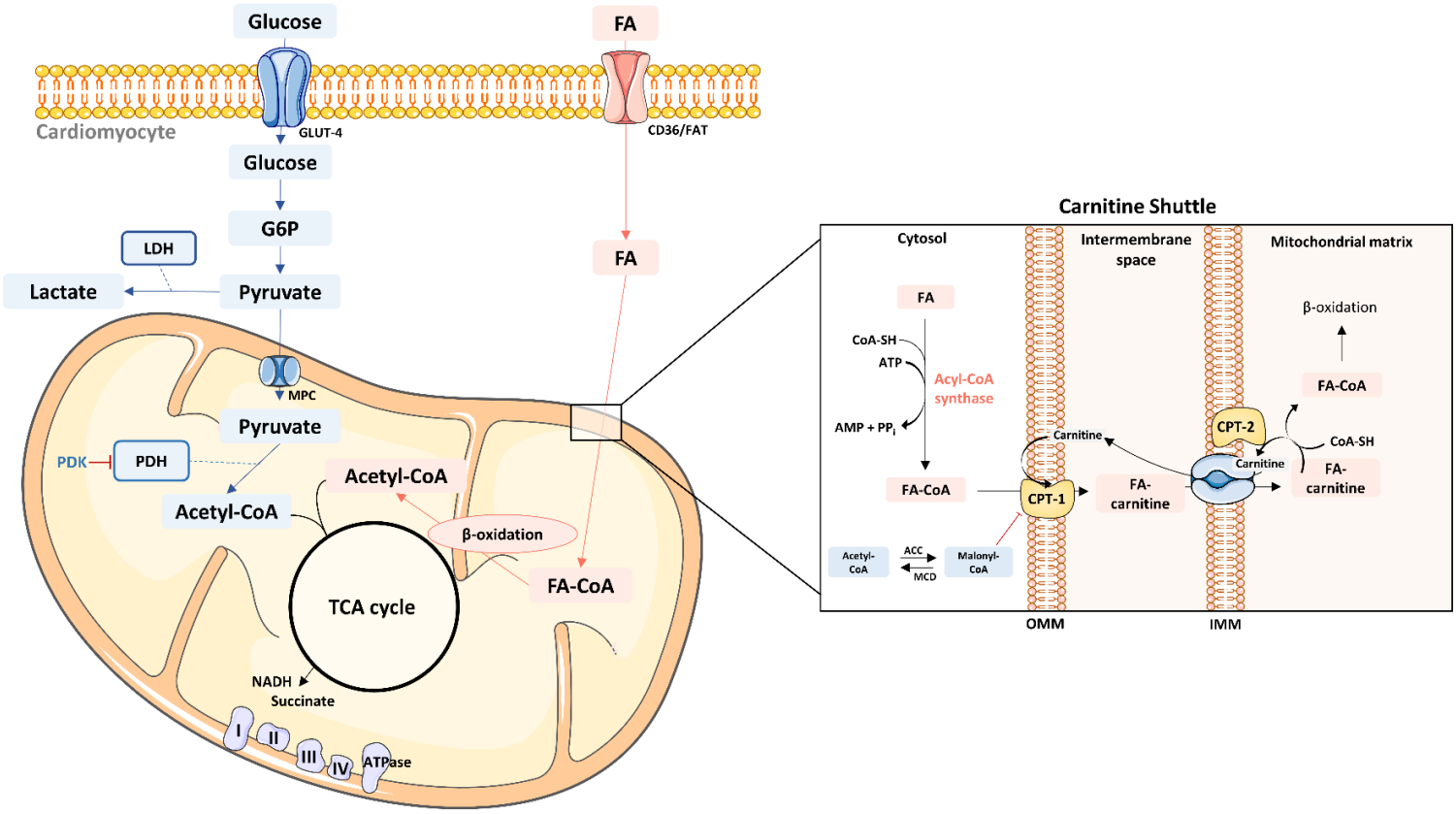

Figure 1.1 – The interplay between fatty acid and glucose metabolism in cardiomyocytes. Fatty acids are transported to mitochondria through the carnitine shuttle. The final product of fatty acid oxidation (through β-oxidation) and glycolysis, acetyl-CoA, converge in the tricarboxylic acid cycle. Dysregulation of these mechanisms can result in cardiac metabolic and mitochondrial dysfunction.

1.2.3 **The tricarboxylic acid cycle**

Since the final product of FA oxidation and glycolysis is acetyl-CoA, these two distinct metabolic pathways converge at some point. Acetyl-CoA is a two-carbon molecule that can fuel the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, in which acetyl-CoA and the four-carbon molecule oxaloacetate (OAA) generate citrate, a reaction catalyzed by citrate synthase[24]. Citrate synthase is often used as a marker of mitochondrial content and function and decreased activity of this enzyme in the heart has been related to cardiac dysfunction due to impaired mitochondrial function[25,26].

Aconitase catalyzes the following TCA cycle reaction by converting citrate into isocitrate, forming cis-aconitase as an intermediate metabolite[21]. To obtain the following molecule in the cycle, α-ketoglutarate, isocitrate is decarboxylated by isocitrate dehydrogenase[24]. Another decarboxylation takes place within the cycle, generating the four-carbon molecule succinyl-CoA accompanied by the release of two CO_2 and two NADH molecules[24,27]. Furthermore, succinate is formed by a GTP-releasing reaction catalyzed by succinyl-CoA synthase. Succinate is then oxidized to fumarate by succinate dehydrogenase, the complex-II corresponding enzyme in the electron transport chain, catalyzing a reaction in which two hydrogen atoms are transferred to FAD, generating $FADH_2$ as cofactor that feeds mitochondrial respiration[24,27]. In the final steps of the TCA cycle, fumarate is converted into malate and further into OAA, enabling the continuation of the cycle[24] (**Figure 1.1**).

1.2.4 **Oxidative phosphorylation as source of energy**

Cardiac mitochondria provide most of the necessary ATP for energy production in the myocardium through oxidative phosphorylation in the mitochondrial respiratory chain[28,29]. Located in the inner IMM, multi-subunit protein complexes constitute the electron transport chain (ETC)[28]. These unique subunits are encoded by nuclear and mitochondrial DNA and comprehend complex I to IV (complex II subunits are exclusively encoded by nuclear DNA)[29]. Together with ATP synthase, also known as complex V, they form the oxidative phosphorylation system (OXPHOS)[28,29]. NADH and succinate generated during the TCA cycle are oxidized in complex I (NADH ubiquinone reductase) and complex II (succinate dehydrogenase), respectively, donating electrons to electron acceptors within the ETC[28,30]. Further on, the electrons are delivered to complex III (cytochrome c reductase) through ubiquinone (CoQ) and then to complex IV (cytochrome c oxidase) through cytochrome c[27]. Oxygen is the final electron acceptor of ETC and is reduced to H_2O[27,30] (**Figure 1.2**). The electron transfer within the ETC is concurrent with proton pumping through the IMM by complex I, III, and IV into the intermembrane space. This generates a proton motive force that is used by ATP synthase for the phosphorylation of adenosine diphosphate (ADP), forming ATP[27,30].

Alterations in intervenient proteins of the ETC have been associated with cardiac dysfunction. Diabetic streptozotocin (STZ)-induced rats showed decreased levels of the complex I subunit encoded by NDUFB8 in the cardiac tissue and reduced complex I-dependent cardiac mitochondrial oxygen respiration[31]. Rats fed with a HFD demonstrated a severe decrease in the maximum activities of mitochondrial complexes I to III, accompanied by alterations in other mitochondrial parameters (e.g. decreased mtDNA copy number, increased AMP/ATP ratio) and morphological changes in cardiac mitochondria (reduced density and size, and disruption of the IMM cristae), which resulted in cardiac dysfunction, hypertrophy, and lipid accumulation[25].

Multiple studies have shown that the ETC complexes may assemble into higher-order structures called supercomplexes (SCs) and that this brings several advantages to the efficiency of mitochondrial respiratory chain[32,33]. The evidence demonstrated that SCs facilitate substrate channeling, improve electron transfer efficiency through the ETC, promote the stability and integrity of the ETC complexes individually, and contribute to tighter control of reactive oxygen species (ROS) production[32,33]. Furthermore, SCs have a defined stoichiometry, which means that the ETC complexes contribute disproportionately to the assembly of SCs[32]. The main SC, known as the respirasome, is composed of complexes I, III, and IV, that in conjunction with CoQ and

cytochrome c assemble and enable the transfer of electrons from NADH to oxygen[33]. The studies suggest a particular role of complex I for the assembly of SCs within cardiac mitochondria[32,34], along with the crucial function of cardiolipin[35], a phospholipid that particularly contributes to the stability of mitochondrial complexes and SCs[32].

Maintenance of the ETC's integrity is of extreme importance since electron leakage during oxidative phosphorylation leads to oxygen reduction and consequent generation of ROS. At physiological levels ROS play an important role in cellular homeostasis, functioning as signaling molecules[28,36]. However, when generated in exacerbated levels, ROS can primarily have a pathological role, causing oxidative damage to essential macromolecules[28,36].

1.2.5 **Cardiac metabolic flexibility**

In 1963, Randle et al. observed that FA preference is ensured in the presence of both FA and glucose by feedback mechanisms based on allosteric inhibitions[13,37]. Today, this mechanism is known as the Randle Cycle and describes that FA and glucose work together so the heart can adapt to different energy demands[14].

The heart's capacity, under physiological conditions, to shift between different substrates depending on its energy requirements is remarkable and has shown to be crucial and determining for the normal cardiac energy metabolism and function[13,14,38,39]. This metabolic flexibility of cardiomyocytes depends on the substrate compartment concentrations, closely controlled by a complex network of signaling cascades and the catalytic activity of rate-limiting enzymes, as well as metabolic transporters[11,13,14,39]. Furthermore, the adult heart's metabolic flexibility depends not only on substrate availability but also on the current physiological conditions (e.g. oxygen availability) and hormonal environment[12,13]

Taking this into consideration, it is not surprising that the heart's metabolism changes throughout life. After all, during development, in the womb, the fetus is exposed to a unique environment. In postnatal life, the conditions can be modulated by lifestyle, while during aging, the heart can reveal a limited ability to cope with the cumulative insults[40].

1.2.6 **The aged heart resembles fetal cardiac metabolism**

As the womb has a hypoxic environment, the low oxygen levels determine substrate utilization in the fetal heart[12,40]. Therefore, the oxidation of glucose and lactate enables higher energetic efficiency, sustaining cardiac growth, and development[12,40]. After birth, the adaptation to the extrauterine life (e.g. increased oxygen availability) involves a significant decrease in both glucose and lactate levels, and a shift towards FA metabolism, a transition that in humans occurs in the first two weeks of the newborn[12,40]. The extraordinary capacity of the myocardium's metabolic flexibility is clearly supported by the alterations that occur in the first weeks of life, but the adaptational capabilities of the aged heart and the consequences that might occur due to an impaired metabolic shift are still poorly understood.

In fact, throughout life, the natural aging process defies metabolic flexibility[41]. While FA oxidative metabolism is lessened, membrane phospholipids shelter higher amounts of saturated

FA, affecting membrane fluidity and transport[12,40]. Besides, the glycolysis rate is boosted, and the aged heart presents a metabolic profile leaning towards the "immature phenotype" of a fetal heart[40]. However, this resemblance to a more fetal myocardium is common to pathologic cardiac hypertrophy, a major risk factor for the development of CVDs, such as heart failure, in which this shift is also observed[40,42].

Due to the high energetic demands of the heart, mitochondria, known as the "powerhouses" of the cell, are extremely abundant in cardiomyocytes and play a crucial role in cardiac bioenergetics in the postnatal stages of life[43,44]. On that note, mitochondria may establish a solid bridge, linking changes in cardiac metabolism to an enhanced risk for CVD development or to an already established CVD. Therefore, the identification of the mitochondrial contribution to the CVD pathophysiology is crucial and may unveil important insights for early detection, diagnosis, or new therapeutics of the disease.

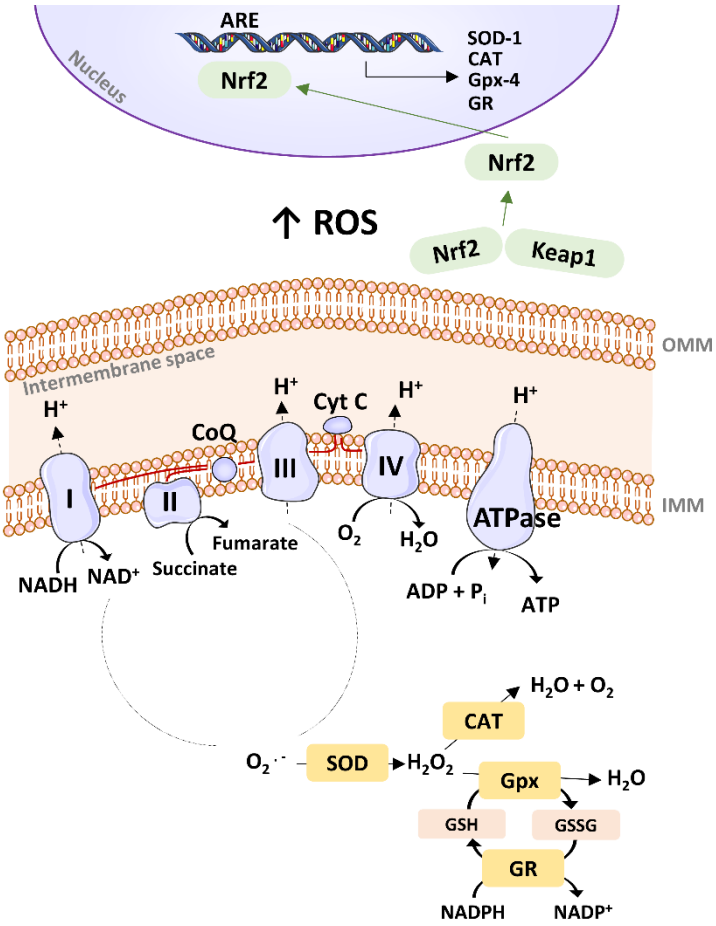

Figure 1.2 – The oxidative phosphorylation system (OXPHOS) and antioxidant defense systems. Increased reactive oxygen species (ROS) production and dysregulation of the antioxidant defense systems may lead to oxidative stress and concomitant cellular damage contributing to CVD development.

1.3 **Cardiac mitochondria: gates of life and death**

Mitochondria are the main energy suppliers of the heart, producing approximately 90% of the ATP to maintain normal cardiac performance[32]. Furthermore, mitochondria occupy around 30% of the total cardiomyocyte cell volume[44,45]. These motile organelles, which are in continuous motion and dynamically alter their shape, join forces to cope with the damaging processes that lead to cardiomyocyte homeostasis disruption, being involved in Ca^{2+} homeostasis, inflammation processes, and control of regulated cell death cascades[45,46]. Recent research supports mitochondria involvement in the pathogenesis of different CVD, affecting crucial pathways responsible for the correct response upon deleterious insults[30,44,46]. Cardiac mitochondrial dysfunction might result in augmented OS, decreased ATP synthesis, impaired autophagy, and , ultimately, enhanced apoptosis (**Figure 1.3**)[30,44,46].

1.3.1 **Mitochondrial dynamics and homeostasis**

Growth and division of pre-existent mitochondria to increase mitochondrial mass is termed mitochondrial biogenesis[47], in which mitochondrial mass and function are controlled by transcription factors that may be meticulously regulated in response to cellular stress[48]. The peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 alpha (PGC-1α), also known as the master regulator of mitochondrial biogenesis, has a crucial role in cardiomyocyte function[49]. Studies demonstrated that cardiac-specific deletion of PGC-1α leads to decreased OXPHOS activity and decreased cardiac contractility, despite normal mitochondrial content, contributing to an increased susceptibility to heart failure[49]. Mitochondrial transcription factor A (TFAM) is another important component in the process. Previously published data revealed that hearts from mice subjected to *Tfam* knockout showed increased levels of genes encoding glycolytic enzymes and concomitantly decreased levels of genes encoding for FA oxidation[50], similarly to the phenomenon observed in the aged heart. Furthermore, *Tfam* knockout mice showed depletion of mitochondrial DNA, cardiac hypertrophy, and reduced function of the respiratory chain activity[50].

To guarantee mitochondrial quality and, therefore correct function, mitochondrial biogenesis must be coordinated with mitophagy (**Figure 1.3**), the autophagic process through which mitochondria are engulfed by autophagosomes and delivered to lysosomes for degradation[30,45,51]. The highly dynamic mitochondrial network is controlled by fusion and fission processes[45]. In mammals, mitochondrial fusion is regulated by the proteins mitofusin-1 (MFN1), mitofusin-2 (MFN2), and dynamin-like 120 kDa protein (OPA1), while fission is orchestrated by GTPases dynamin-related protein 1 (DRP1), mitochondrial fission 1 protein (FIS1), mitochondrial fission factor (MFF), among other molecules[45].

In the heart, a known mechanism involved in the process of mitophagy is the PTEN-induced putative kinase protein 1/ubiquitin ligase Parkin (PINK1/Parkin) pathway. In 2013, Chen and Dorn demonstrated that Mfn2 is involved in the pathway as a downstream target of PINK1, responsible for its phosphorylation and consequently promoting Parkin translocation from the cytosol to the damaged mitochondria's membrane, where Parkin drives the targeting for mitochondrial degradation via ubiquitination of mitochondrial OMM targets[52]. The ablation of Mfn2 in embryonic cardiomyocytes caused dilated cardiomyopathy[52]. Nevertheless, in 2015, Kubli and colleagues demonstrated that Parkin's translocation occurs and promotes mitophagy in

PINK1-deficient mice, proposing the activity of a different kinase[53]. These results suggest that Mfn2 has an essential role in mitophagy. Its activation is required either through a PINK1 dependent or independent manner. Indeed, studies demonstrated that hearts deficient in Mfn2 are protected against myocardial infarction due to suppressed mitophagy, however, these hearts showed decreased mitochondrial respiratory function, fragmented mitochondria, cardiac hypertrophy, and impaired cardiac contractility[54,55]. Therefore, notable changes in mitochondria morphology and cardiac tissue denote the important role of a controlled and regulated mitophagy process for the heart's normal function.

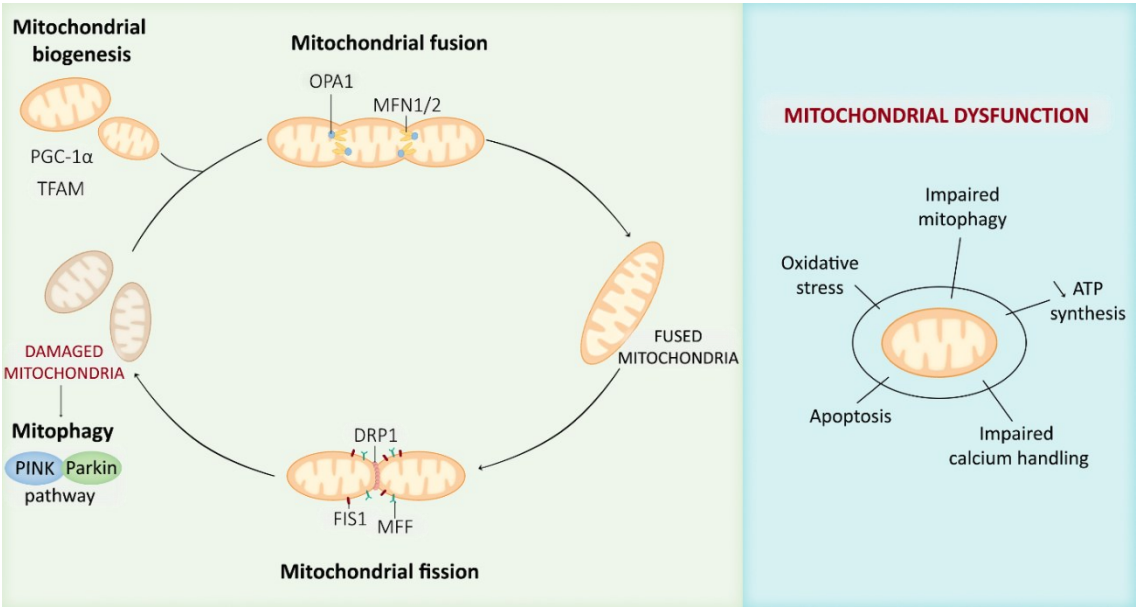

Figure 1.3 – The loss of equilibrium between mitochondrial biogenesis and mitophagy in cardiomyocytes leads to mitochondrial dysfunction. The latter might contribute to the pathogenesis of cardiovascular disease by dysregulation of genes and/or proteins involved in crucial mechanisms to maintain mitochondrial homeostasis.

1.3.2 **Oxidative stress and antioxidant defenses, a dynamic balance**

As previously mentioned, the OXPHOS is extremely important for energy production in cardiac cells, as in other types. During oxidative phosphorylation, a very small percentage of the electrons transferred within the ETC leaks mainly from complex I and complex III, causing the partial reduction of oxygen[36,56]. This results in the formation of a highly reactive species, the superoxide anion ($^{\bullet}O_2^-$), which is rapidly converted to H_2O_2 by superoxide dismutase (SOD)[30]. Within mitochondria, two isoforms of SOD have been described: SOD1, which is present in the cytoplasm and intermembrane space, and SOD2, located at the mitochondrial matrix[30]. Catalase and glutathione peroxidase (GPx) constitute two important antioxidant enzymes that catalyze the dismutation of H_2O_2 to H_2O and O_2 and eliminate H_2O_2 by forming H_2O, respectively[30]. In the heart, catalase is mainly expressed in peroxisomes and, to a lesser extent, in mitochondria[57]. Catalase's activity in the heart is lower than that detected in other tissues, such as the liver (~2% per gram of heart in rats and mice)[58]. However, augmented catalase levels have been shown to be involved in the protective cardiac response to a HFD[57]. Through the reaction of GPx, the oxidized

form of glutathione (GSSG) is generated and used by glutathione reductase (GR) to subsequently form reduced glutathione (GSH), at the expense of NADPH, which is oxidized (**Figure 1.3**). The GSH/GSSG ratio is a great indicator of the intracellular redox state and decreased ratio has been related to cardiac mitochondrial dysfunction in mice fed a high-fat, high-sucrose diet[59]. Hydrogen peroxide can further interact with intracellular transition ions, such as Fe^{2+} by the Fenton reaction, resulting in the formation of hydroxyl radicals ($^{\bullet}OH$), which causes extensive cellular damage[60,61]. Interestingly, the contribution of iron homeostasis to CVD development is complex and somehow a paradox. While HF patients show decreased iron levels, atherosclerosis is associated with iron overload[62].

Oxidative stress occurs when the balance between ROS production and the capacity of the antioxidant systems to buffer that exacerbated local production is impaired[56,60,61,63]. It is important to denote that ROS production is not exclusive to mitochondria[64]. Cellular enzymes such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenases, uncoupled nitric oxide synthases, between others, contribute to ROS production and have been related to CVD exacerbated ROS levels[64,65]. The antioxidant defense systems involve not only enzymatic antioxidants but also non-enzymatic antioxidants, including endogenous molecules (e.g. melatonin, uric acid) and substances obtained through diet (e.g. vitamin A, C, and E, polyphenol)[61]. Oxidative stress has been shown to be intimately involved in the progression of heart failure[66], in the development of diabetic cardiomyopathy[67], having a tremendous weight as a major contributor to cardiac dysfunction in individuals with metabolic syndrome-related disorders[68,69]. It is well established that ROS play an important role in the progression and development of CVD, therefore, maintaining redox balance is of extreme importance to sustain the cellular functions necessary for normal cardiac metabolism and performance[64].

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a protein highly involved in the regulation of cellular redox (im)balance[70–72]. This transcription factor acts as a major regulator in the transcription of endogenous antioxidant and detoxication genes containing the antioxidant response element (ARE) in their promoter regions[70–72]. These include SOD1, catalase, GPx, GR, between others[70,71] (**Figure 1.3**). The own gene sequence of Nrf2 contains an ARE-like element in its promoter region, exerting a positive feedback mechanism in its transcription and consequent expression[70]. Nevertheless, Nrf2 is involved in the cellular antioxidant response and a series of other important cellular functions, such as cell signaling, autophagy, cell proliferation, and organ development, among others[71]. Nrf2 has been seen as a cyto- and cardioprotective transcription factor, playing a central role in cellular defense[70,71]. Nrf2 knockout mice subjected to myocardial infarction rapidly developed heart failure, marked by distinct altered cardiovascular parameters, and had higher mortality rates than wild-type mice[73]. On the other hand, Nrf2 inducers have shown promising capacities in providing cardiac protection in the context of several cardiac diseases, including heart failure, related cardiac hypertrophy[74] and diabetes-associated cardiac complications[75].

Redox-related biomarkers can be key hallmarks for CVD detection and prevention. Mitochondrial aconitase, the enzyme responsible for the reversible isomerization of citrate to isocitrate in the TCA cycle, has a particular characteristic that enables the evaluation of cellular redox state. Aconitase has an iron-sulfur prosthetic group in which one of the iron ions is free (known as the Fe_{α}) and susceptible to oxidation by reaction with $^{\bullet}O_2^-$[76]. This leads to the reversible inactivation of the enzyme. The inactive protein can be reactivated by iron and reducing agents, therefore, the relation between the aconitase inactive and active forms can be used to determine the steady-state concentration of mitochondrial $^{\bullet}O_2^-$[76]. A study involving different tissues of a rat model revealed that, in the heart, mitochondrial respiration is significantly affected even with a

reduced percentage of inactivated aconitase (5%)[77], demonstrating the high sensibility of this protein and its relevance as a redox biomarker.

Conjointly, mitochondrial biogenesis and mitophagy promote cardiac homeostasis and regulate responses to harmful insults derived from the pathological onset of CVD, concomitantly with a balanced redox state. However, the heart's metabolic flexibility is also dependent on other pathways, such as the insulin signaling pathway, that plays an essential role in glucose and FA metabolism in the adult heart[78,79]. As previously mentioned, the process of glycolysis may represent a physiological response to CVD development in the elderly heart, it would be important to understand the underlying mechanisms of glucose homeostasis and identify the possible dysfunctions that arise in these conditions. Furthermore, a hyperglycemia state is highly related to elevated OS within cardiomyocytes due to increased flux in the ETC and consequent increased production of $^{\bullet}O_2^-$ [61,80], being considered one of the major contributors and high-risk factor for CVD development among obese and diabetic individuals.

1.4 **The essential role of insulin signaling in cardiac metabolism**

Belke et al. reported the importance of insulin signaling using cardiomyocyte-selective insulin receptor knockout (CIRKO) mice, whose hearts showed an abnormal size and metabolic resemblances to the fetal heart[81]. Since then, insulin signaling has been extensively studied and a relationship between its deregulation and the risk of CVD development established.

The postprandial state is characterized by increased insulin secretion, stimulating cardiac glucose uptake, through GLUT-4 membrane translocation, utilization, and the synthesis of macromolecules. At this stage, cardiac ATP production is mainly accomplished by glucose oxidation (60 to 70%)[79]. In the adult heart, glucose uptake into the intracellular compartment of cardiomyocytes is predominantly facilitated by GLUT-4, being its expression and action insulin-dependent[82]. The signaling cascade of insulin is represented in **Figure 1.4.A**. Briefly, for signal transduction, insulin binds to its receptor (InsR), ultimately leading to the activation of protein kinase B (Akt) and concomitant activation or inhibition of its downstream targets, which have an essential action for the control of cardiac growth, homeostasis, and survival[79].

The disruption of the insulin signaling cascade may result in impaired cardiac function and contribute to increased risk of CVD, especially in metabolic disorders such as obesity and diabetes mellitus (DM)[83]. Diabetes mellitus is characterized by a state of insulin resistance (IR), which refers to the hormone's incapacity to perform its normal biological function, resulting in irregular glucose uptake, glycogen synthesis, and glucose oxidation[84]. Regarding the insulin's signaling cascade, IR leads to diminished phosphorylation of insulin receptor substrate (IRS) and downstream targets PI3K and PDK, ultimately resulting in reduced Akt activation and consequently decreased translocation of GLUT-4 to the plasma membrane[82] (**Figure 1.4.B**). Furthermore, under stressful and impaired conditions, the heart's metabolic flexibility shifts from FA to glucose metabolism[13]. However, in an IR state, this adaptive characteristic is impaired and cardiac metabolism relies even more upon FAs as a source of energy[84].

Considering the emerging pandemic of obesity and diabetes, it becomes urgent and extremely pertinent to understand how obesity and diabetes contribute to the pathogenesis of CVD and how the heart copes with such metabolic changes upon IR-induced damage. The hearts of non-insulin resistant patients rely on glucose metabolism under damaging conditions due to the heart's

metabolic flexibility, suggesting that at a certain point, to avoid cardiac injury, it becomes beneficial[84,85]. However, insulin-resistant patients rely mostly on FA to produce ATP under stressful conditions, lacking the necessary levels of oxygen to maintain mitochondrial OXPHOS[86].

1.4.1 **Obesity and diabetes: is being alarmed enough?**

Diabetes mellitus is associated with high rates of microvascular complications, however, in the last decades, macrovascular complications have been observed to a greater extent among the type 2 diabetes mellitus (T2DM) population[87–89]. In fact, CVD is the most common cause of death in DM adult patients, who account for a 2-4 fold increased risk for CVD development[87–91]. Numerous common pathophysiological conditions between T2DM and CVD have already been identified, including hypertension, dyslipidemia, systemic inflammation, among others[92,93]. Moreover, two major risk factors for CVD development that occur in DM, especially in T2DM, include IR and hyperglycemia[44,48], common risk factors also observed in obesity, along with increased BP[94]. Indeed, obesity is a metabolic abnormal state that might precede T2DM[91].

The WHO defines obesity as a result of an abnormal fat accumulation which may ultimately jeopardize health[95]. Furthermore, obesity is considered a pandemic due to the great increase and its prevalence worldwide over the last 50 years[6,94,96]. The doubling rates of adult and childhood obesity, coupled to the tripling rates of adolescent obesity[96] raised serious concerns among society, and this continuous trend is estimated to reach alarming proportions by 2025, affecting 18% of men and over 21% of women[97]. Nevertheless, according to the WHO, in 2016 39% of adults already exhibited excessive weight (body mass index (BMI) $\geq$ 25 kg/m^2)[7].

Obesity is classified by different level categories according to the body mass index (BMI), calculated as an individual's body weight in kilograms (kg) divided by the height in square meters (m^2)[94,96], being considered obese individuals with a BMI $\geq$ 30 kg/m^2 [95]. As it has become more evident that obesity is closely related to CVD, several studies have been conducted, evaluating the association between BMI and increased CVD risk[98,99]. In a simplistic way an obese state occurs when dietary energy intake is higher than energy expenditure, resulting in an accumulative positive energy balance[6,94]. This excess energy is then converted and stored in triglyceride (TG) form in the subcutaneous adipose tissue (AT) which increases in size, leading to weight gain[94]. The progression of the energy imbalance may lead to the saturation of AT depots and the excess fat starts to accumulate in visceral and ectopic sites around tissues and organs, including the heart[100,101].

1.4.2 **Metabolic contribution of obesity and diabetes mellitus to cardiovascular disease**

In response to AT dysfunction during obesity and DM, macrophages accumulate in the stromal vascular fraction of the AT, promoting a pro-inflammatory response by the release of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) cytokines[102]. Increased levels of circulating IL-6 and TNF-α promote inflammation in the heart and may activate Jun N-terminal kinase (JNK) which has an inhibitory effect on IRS proteins through phosphorylation of the serine residue 307[102], contributing, therefore, to impaired insulin signaling.

On the other hand, hyperinsulinemia promotes increased FA uptake into cardiomyocytes by upregulation of CD36[82]. Intracellularly, increased concentrations of FA lead to their utilization for non-oxidative metabolic pathways and the overexpression of PPAR-α, resulting in the accumulation of toxic lipid metabolites[82]. Lipotoxicity might result in excess production of mitochondrial NADH and $FADH_2$, and promoting ROS generation by stimulation of the Krebs cycle[103], apoptosis, and impaired contractile function, while altering cellular signaling. For example, ceramides and diacylglycerols (DAG) activate atypical protein kinase C (PKC) and typical PKC, respectively, which inhibit Akt, disrupting the insulin signaling pathway[82,103]. Furthermore, decreased glucose utilization leads to the accumulation of glycolytic intermediates, inducing glucotoxicity[82,84]. Overall, these cardiac metabolic alterations and impairments in the insulin signaling cascade can ultimately result in cardiomyocyte death[84] (**Figure 1.4.B**) and have been related to several CVD, such as heart failure[104] and related cardiac hypertrophy, and diabetic cardiomyopathy[78].

Up to this point, cardiac metabolism has been discussed during CVD pathogenesis and other disorders that compose risk factors for the development of CVD. Furthermore, cardiac metabolism changes throughout life having the ability to adapt when facing challenges and in the presence of pathology. However, there is a critical period of life in which the heart of women in the reproductive age faces tremendous and incredibly unique challenges to cope with the complex demands of developing a new life. Now, we will focus on the ability of the maternal heart to sustain the high cardiac requirements during gestation, and clarify if pregnancy can act as a "stress-test" untangling previous cardiac dysfunctions and/or if pregnancy complications induce cardiac disease.

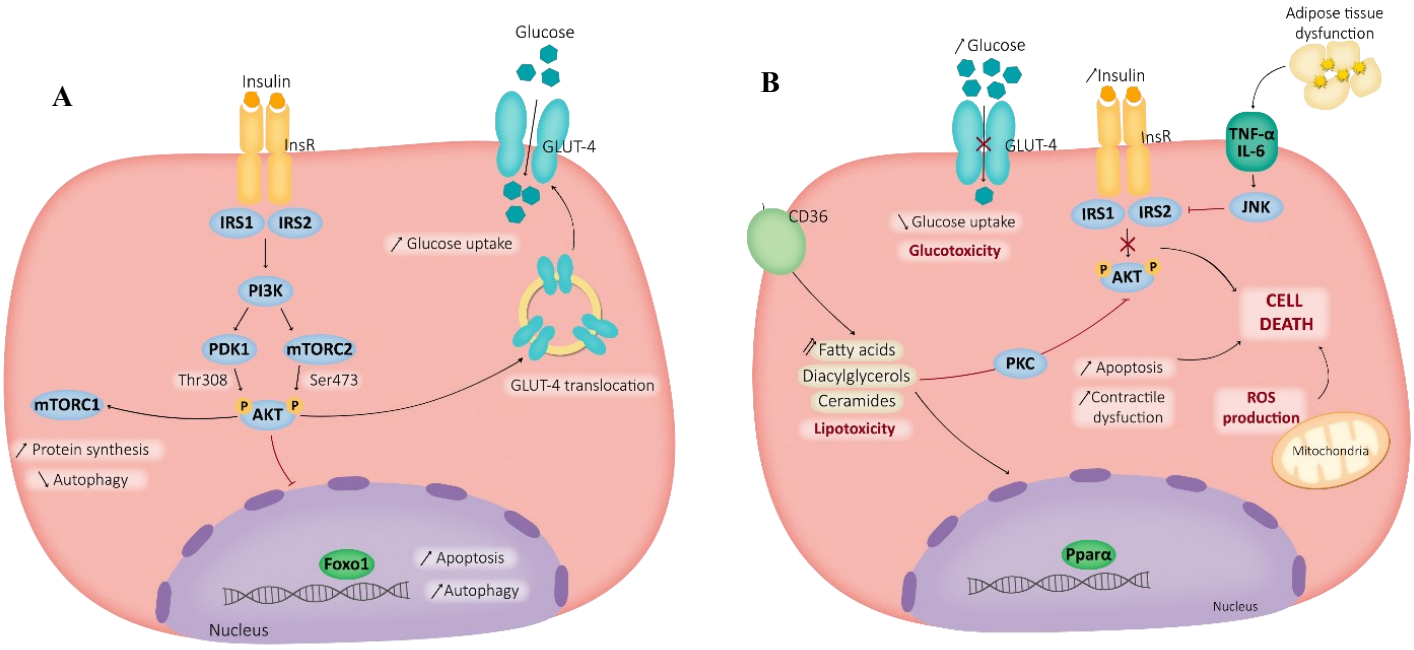

Figure 1.4 – Insulin signaling cascade and cellular function in a normal metabolic state (A) and in an impaired metabolic state, such as insulin resistance due to obesity or diabetes mellitus (B).

1.5 **Maternal cardiovascular adaptations during pregnancy**

A healthy pregnancy is a dynamic state accounting for several adaptational processes crucial for normal fetal growth and development. Therefore, the various organ systems of the maternal organism must go through several adaptational events during gestation[105]. The CV system along with metabolism undergoes drastic changes to facilitate the perfusion between the uterus and the placenta, so the necessary levels of oxygen and nutritional demands of the developing fetus can be achieved[106–108].

The evolution of pregnancy leads to different mechanical and structural changes of the maternal heart, for instance, due to compression of the diaphragm, is displaced from its normal position to the left and upwards[109]. Furthermore, mild and physiological hypertrophy is developed, in which ventricular muscle mass is reinforced to ensure a larger blood injection[110]. In the front line of adaptation, pregnant women are subjected to an increased cardiac output (~30 to 50%)[109], which would suggest an increase in BP. However, the raised concentration of circulating hormones secreted by the placenta, such as progesterone, estrogen, and prostaglandins, reduces systemic resistance and consequent increased arterial compliance, resulting in a BP decrease observed until mid-pregnancy, which is further re-established at term[106,107,109].

Early gestation is characterized by an anabolic stage with increased insulin sensitivity. However, between the 12th and 14th weeks of pregnancy, a decrease in peripheral insulin sensitivity is observed, with a gradual decline throughout the 2nd and 3rd trimester[111,112]. At this point, along with the development of IR, there is a concomitant increase in the release of diabetogenic hormones, including estrogen, progesterone, prolactin, between others[113]. The placenta is the major mediator of maternal-fetal interactions, facilitating the proper delivery of metabolic substrates to the fetus. Most of the changes occurring in the women's body during pregnancy are influenced by hormones produced by placenta[105,107]. Moreover, estrogen especially contributes to a physiologic upregulation of the lipoprotein fractions, resulting in hyperlipidemia and leaving pancreatic β-cells subjected to great stress[114].

Hence, pregnancy represents a challenging period for the maternal organism, in which the CV and other systems are pushed to their limit to accomplish the required physiological adaptations to support a new life. Therefore, it is an optimal opportunity window to assess the mother's health status since mild dysfunctions, previously unnoticed, may be exacerbated during this period. In fact, in the last decade, adverse pregnancy outcomes such as preeclampsia, pre-term birth, fetal growth restriction, and gestational diabetes mellitus (GDM) have been associated with the risk of maternal CVD development in the future[115]. The latter has raised special awareness in the past years due to the observed surge in obesity's prevalence and the increasingly common advanced maternal age[116].

1.5.1 **Gestational diabetes mellitus burden and complexity**

Gestational diabetes mellitus is the most common metabolic disorder of pregnancy and is increasing worldwide[116]. It is estimated to affect 14% of pregnancies globally[117], although recent data denote a prevalence discrepancy (1 to >30%) due to the lack of consistent diagnosis criteria across different regions and over the years[118]. While the state of glucose intolerance usually returns to a normal state after pregnancy, GDM mothers have a ~7-fold and ~2-fold increased risk

of developing T2DM[119] and CVD[120], respectively, in the first decade after pregnancy. Their offspring are also at greater risk of developing obesity, T2DM, and heart disease early in life or later in adult life[116,118]. The proportion of these statements has only started to be noticed in follow-up studies. For example, Blotsky et al. reported that offspring born to mothers with GDM had a ~2-fold higher incidence of pediatric diabetes (per 10 000 person-years) than those born to non-GDM mothers before the age of 22 years in a study comprising a total of 73 180 mothers (with and without GDM)[121]. Moreover, Yu et al., in a study with 2 432 000 children, that involved mothers with pre-GDM (T1DM and T2DM) and GDM showed that the latter was associated with an increased rate of CVD in offspring especially in the first 4 years of life but up until adulthood (hazard ratio 1.19 (95% confidence interval 1.07 to 1.32))[3].

One of the major drawbacks of GDM is the attached complexity concerning its diagnostic criteria. To date no diagnostic criteria have been universally accepted[118,122], creating difficulties in the conjugation of the data reunited in epidemiological studies. Currently, most entities, including the WHO and the American Diabetes Association (ADA), adopt the criteria that were established by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) in 2008[122–124], which emerged from the consideration of the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study[125]. This study, comprising more than 25 000 pregnant women, consisted of administering a 75 g oral glucose tolerance test (OGTT) between the 24th and 32nd week of gestation. Increased fasting, 1-hour, and 2-hour plasma glucose levels were observed among the pregnant women, and a link between increased plasma glucose levels and several compromised pregnancy outcomes was established[126]. The diagnosis for GDM based on IADPSG criteria is considered when one or more of the following tests have increased values after the OGTT: fasting glucose plasma ≥5.1 mmol/L; 1-hour plasma glucose ≥10.0 mmol/L; and 2-hour plasma glucose ≥8.5 mmol/L[122–126]. ADA has considered these last criteria as a "one-step approach" and has adopted a "second-step approach" based on the recommendation of the panel of the 2013 National Institutes of Health (NIH) conference in which the criteria passes through a first 1-hour, 50 g glucose load test in a non-fasting state and that, only if positive (≤7.2, 7.5 or 7.8 mmol/L), proceeds to a 3-hour, 100 g OGTT. If two of the four plasma glucose levels achieve or exceed the mentioned values, there is a diagnosis of GDM[79]. In Europe, most epidemiological studies use the IADPSG/WHO 2013 criteria[122,127].

One question that remains is whether GDM predisposes to CVD development independently of progression to T2DM or not. The relationship and interplay between both pathophysiologies is still poorly understood and emerging studies have stated controversial opinions on the matter[120,128]. Efforts to understand the metabolic changes in the maternal heart during pregnancy are scarce and it is necessary to establish a bridge between the metabolic impairments predisposing to CVD among women with GDM and the key cardiac molecular mechanisms involved in the physiologic process, as well as understand the capacity of the maternal heart to adapt after a GDM pregnancy.

1.5.2 **Contribution of gestational diabetes mellitus to cardiovascular disease development**

The two major hallmarks of GDM are systemic IR[83] and β-cell dysfunction, which are closely related in the context of the GDM pathophysiology. The rate of insulin-stimulated glucose uptake has been reduced by 54% in the skeletal muscle of GDM patients when compared to control

pregnant groups[130]. Furthermore, impairment in the insulin signaling cascade due to the altered expression of vital intervenient proteins, such as IRS-1, PI3K, and GLUT4 (represented in **Figure 1.2.A**), are observed in the GDM pathophysiology[130]. However, these results are based on studies performed in the skeletal muscle tissue of women with GDM, emphasizing the need to study the cardiometabolic alterations that take place in the maternal heart during GDM. On the other hand, IR is responsible for the exacerbated β-cell dysfunction observed in GDM[118]. The phenom of IR may be seen as a redundant cycle, in which the reduced glucose uptake leads to glucose accumulation in the serum, resulting in hyperglycemia. Upon increased levels of glucose in the blood, β-cells respond by secreting more insulin to which the InsR has developed lower sensitivity for[118]. However, β-cells continue to produce insulin and their progressive dysfunction lingers even after GDM, highly contributing to the future development of T2DM[118].

In addition to the maternal adaptational processes during pregnancy mentioned above, AT mass is also increased in the early phase, although fats from the AT are mobilized to fuel fetal growth later in pregnancy[131]. Therefore, defects in the AT are also thought to feature the GDM pathophysiology. Indeed, studies have indicated adipokine dysfunction and a low inflammatory grade, with the detection of decreased levels of adiponectin[114]. However, adiponectin has been more strongly associated with IR than adiposity[132]. Adiponectin has an important role in the promotion of insulin signaling and FA oxidation while inhibiting gluconeogenesis[132]. This is mediated by the activation of AMP-activated protein kinase (AMPK) within insulin-sensitive cells, facilitating the action of IRS-1[132]. Therefore, the levels of this adipokine are thought to be a better predictor for GDM pathophysiology since they may be independent of obesity, which is also in agreement with the observation that these metabolic risks are increased even when recommended gestational weight gain (GWG) is followed[118].

Overall, IR is the major contributor to GDM, being also responsible for β-cell dysfunction. Therefore, it is not surprising that GDM has also been associated with other damaging metabolic disturbances related to this non-responsive state to insulin, such as altered glucose metabolism[133], dyslipidemia, and elevated circulation of FA[74,86], between others. In fact, some of these impairments have already been reported to continue even after a GDM pregnancy[135].

1.5.3 **Gestational diabetes mellitus management and treatment**

The main goal of GDM interventions and treatment is to reduce blood glucose levels during pregnancy and prevent glucose toxicity and adverse pregnancy outcomes, including preeclampsia and gestational hypertension, and offspring complications, such as macrosomia, shoulder dystocia, preterm birth, between others[133]. The treatment of GDM can be seen as a multi-disciplinary approach[136]. According to ADA's criteria, the first line of intervention must achieve glycemic control by frequently self-monitor blood glucose, both in the fasting and postprandial state[137]. The subsequent intervention, which may even dismiss further pharmacological treatment, acts on individualized lifestyle changes, for instance dietary modifications based on medical nutritional advice and physical exercise with the appropriate monitoring, frequency of practice, intensity, and duration[136,137]. Therefore, women with GDM must fully commit to a healthier lifestyle to improve both maternal and offspring prognosis. However, when this approach fails, pharmacological treatment is required. Currently, two types of pharmacological approaches are

available: insulin and oral hypoglycemic agents (OHA)[118]. Nevertheless, it is still highly controversial which strategy should stand as the first-line pharmacological treatment.

Insulin has been used for several years as a first-line treatment during pregnancy. However, the rising concerns of its utilization on maternal and offspring outcomes have questioned its safety[138]. The introduction of OHA led to their substantial replacement over insulin. Metformin and glyburide compose the two most used OHA to treat GDM. Metformin's central mechanism consists in the inhibition of gluconeogenesis, decreasing glucose production and release in the liver[139,140]. This pharmacological therapy also contributes to higher glucose uptake in peripheral tissues and lower glucose absorption in the gastrointestinal tract. Glyburide acts in the pancreas by stimulating insulin secretion[139,140].

Several entities have stated their opinions on which constitutes the best pharmacological treatment for GDM. To this end, important concerns were taken into consideration: 1) the behavior of each drug in the organism during pregnancy (**Table 1.2**); As the controversy between treatments rose, several meta-analysis studies and systematic reviews comparing the different drugs were performed. With this, 2) the short-term effects of these drugs on maternal and offspring health were evaluated (**Table 1.2**).

Table 1.2 – Reported characteristics and short-term effects related to the use of pharmacological therapies in GDM.

	Insulin	Metformin	Glyburide
1)	Does not cross the placenta	Crosses the placenta Fetal concentration levels similar to maternal circulation[139]	Crosses the placenta Fetal levels lower than maternal circulation[138,140]
2)	↑ gestational age at delivery[141] ↑ macrosomia[141,144] ↑neonatal and ICU admission rates[141]	↓ cases of preeclampsia and gestational hypertension[141] ↓ maternal and neonatal hypoglycemia[141] ↓ GWG[144] ↓ neonatal admissions in the ICU[141] ↑ insulin (coadjutant therapy)[144]	↑ birth weight and low birth weight[142,143] ↑ macrosomia[142] ↑ neonatal hypoglycemia[142,143]

1) Behavior of the drug in the organism;
2) Short-term maternal and offspring effects.

These findings encouraged the Society for Maternal-Fetal Medicine (SMFM) to suggest the use of metformin as "a reasonable and safe first-line pharmacologic alternative to insulin"[140]; Nevertheless, the SMFM statement denoted the possible requirement of insulin administration to help control blood glucose levels and the need to establish 3) the long-term effects of these drugs - Barbour et al. promptly elaborated a "cautionary response" to SMFM's statement, in which they identified several metformin effects at a cellular level that may compromise fetal and childhood development[139]. They pointed out that metformin is being used in cancer research due to its

distinctive properties in growth inhibition and mitochondrial respiration suppression[139]. Furthermore, recent trial studies suggested that although this drug might have the intended effects on the mothers, it may not prevent the increased BMI values in their offspring. Increased BMI values were observed in offspring with 4 years of age[145] and in a 5-10 year follow-up study[146], suggesting predisposition for CVD development later in life.

Indeed, the effects these pharmacological therapies have on both the mother and the offspring are of extreme importance, however, the lack of consensus on which drug should be used to maintain euglycemia when dietary and lifestyle interventions fail leaves space for the interference of other factors with a heavy contribution on the choice of action. For instance, insulin treatment involves daily injections that are dependent on patient compliance. Besides, OHA compose a cheaper alternative to insulin. Langer's point of view on pharmacological treatment for GDM appealed to the physician's ability to recognize the best treatment[138], which can only be achieved if a woman with GDM-pregnancy receives the correct monitoring during gestation. What is clear is that more research is needed to understand the underlying pathological mechanisms that link GDM and increased incidence of T2DM and CVD for the mothers and also the link between the therapeutic approach and the modulation of the T2DM and CVD risk for the mother and offspring.

1.5.4 **How COVID-19 is affecting gestational diabetes mellitus diagnosis and management**

The COVID-19 pandemic hit in proportions no one thought it could, obligating the healthcare systems to adapt. Pregnancy by itself already requires frequent medical visits to guarantee a good progression in the health of the mother and the fetus, but in the specific case of GDM, close monitoring is vital to ensure maternal and fetal health and good pregnancy outcomes[147,148]. During this pandemic, several protocols and guidelines had to be revised and adapted to the conditions of social distancing and self-isolation to reduce the risk for the patients and healthcare professionals and to prevent further burden to the healthcare systems[147].

The guidelines for GDM diagnosis and management were reviewed and changed by several associations of different countries for the sake of safety during the pandemic[147–149]. In general, early pregnancy screening was only recommended to women at higher risk of overt diabetes, presenting one or more risk factors for GDM[147,148]. To select future mothers for increased risk, two main parameters were considered: the levels of glucose glycosylated hemoglobin A1C (HbA1c), and random blood glucose (RBG). Later, in the 24-28 weeks screening test, instead of the 2-h OGTT that would be performed to assess a state of GDM, HbA1c and fasting blood glucose (FBG) were used as alternatives[147,148]. It is important to refer that the entities that suggested these alterations defend OGTTs as the prime method for GDM diagnosis and that it should not be replaced at any point after the pandemic[147–149]. The parameters implemented by each society and the values established to consider a GDM diagnosis are shown in **Table 1.3**, as well as the plans considered for the post-partum follow-up.

Between a GDM diagnosis and postpartum follow-up, physicians and future mothers must consider a series of important factors. As mentioned before, the monitorization of blood glucose levels, attention to GWG, the adoption of a healthy lifestyle regarding dietary options and physical activity, and the correct use of the prescribed pharmacological treatment (if needed) are crucial to guarantee the best outcome after the diagnosis of GDM[136,137]. To facilitate GDM monitoring and management during the pandemic, that normally would occur face-to-face, pregnant women with GDM should be offered remote telehealth consultations, in which education on beneficial lifestyle adaptations should be provided[147–149].

Table 1.3 – Defined parameters by different societies of the world for the diagnosis of GDM during the COVID-19 pandemic.

	Society		
	RCOG[147,148]	**Australia**[147]	**SOGC**[147]
Early pregnancy screening	RPG or HbA1c	HbA1c	HbA1c
	Unanimous HbA1C: ≥ 5.9%		
24-28 weeks screening	FBG ≥ 5.3 mmol/L	OGTT when 4.7 mmol/L ≤ FBG ≤ 5 mmol/L	FBG ≥ 5.1 mmol/L
Post-partum follow-up	3-month delay and HbA1c screening	6-month delay for persistent diabetes	

RCOG (Royal College of Obstetricians and Gynecologists);
Australia comprehends different societies: ADIPS (Australasian Diabetes in Pregnancy Society), ADS (Australian Diabetes Society), RANZCOG (Royal Australian and New Zealand College of Obstetricians and Gynecologists and Queensland Health);
SOGC (Society of Obstetricians and Gynecologists of Canada).

A cohort study in the UK, which implemented the guidelines suggested by RCOG, revealed that when using these guidelines only 4.2% of women were diagnosed with GDM[150]. This percentage was significantly lower than that usually observed when performing normal OGTTs for GDM diagnosis outside the pandemic context or during the pandemic when facilitations enabled OGTT testing (7.7%, p=0.0003)[150]. This suggested that the new RCOG guidelines for GDM management during the pandemic were not effective. Further results confirmed it[150]. If possible and safe, women were reevaluated with a normal OGTT. According to the study, 20.4% of pregnant women were diagnosed with GDM after blood glucose levels reassessment[150]. These results show that, as supported by all entities when releasing the new guidelines for the COVID-19 pandemic, the best method for GDM diagnosis is the OGTT. However, these results are preoccupying since we are unaware of the percentage of women misdiagnosed and uncontrolled for GDM and the repercussions that will carry in the future.

A retrospective study of a single-center in Lille (France) evaluated the effects of the pandemic lockdown on GDM blood glucose control, comparing two distinct lockdown periods (2019 vs. 2020)[151]. Each patient was followed through telemedicine consultations for ten days to ensure the correct blood glucose monitoring. The study revealed that in the 2020 lockdown, pregnant women with GDM had worse glycemic control, leading to increased insulin use as therapy. The authors stated that home confinement during the lockdown impacted the activities and dietary habits of diabetic patients. Although they report that women engaged in a healthier lifestyle[151], possibly due to the increase in time spent at home and the worry caused by the COVID-19 situation,

pregnant women also showed signs of depression and anxiety during the lockdown[151], which could have had a heavier role in the observed loss of blood glucose control.

These are concerning data that support our view, which consists of understanding the mechanisms underlying long-term cardiometabolic defects on the mother and if they can be preventable through changes in lifestyle habits and consequently prevent GDM-induced CVD development. Moreover, it is also pertinent to assess if these changes may also prevent future development of CVD in the offspring.

1.5.5 **Maternal cardiometabolic health deserves more attention**

Considering the above-mentioned information, the metabolic impairments observed in GDM overlap with the more studied metabolic defects during the pathophysiology of obesity and DM (**Figure 1.5**). Since these impairments contribute to CVD development and, indeed, GDM mothers have an increased risk of developing CVD in the first decade after pregnancy, one can predict that these risks are also "imprinted" in the maternal heart, predisposing to metabolic impairments later in life[120]. Furthermore, a group of molecular mechanisms has been proposed in obesity and DM, involving specific studies concerning the dysfunction in the cardiac tissue[152,153]. Nevertheless, similar studies have not been performed in light of GDM.

As described, cardiac mitochondria are the major contributors to energy production within cardiomyocytes and may play an important role in the pathophysiology of GDM. Studies in the area are also scarce. Although it has not been fully elucidated whether GDM progresses to CVD dependently or independently of T2DM development after pregnancy[120,128], it is important to bear in mind that the pathophysiology observed for GDM shares several risk factors with DM and obesity, even when obesity is not a consequence of DM or vice-versa. Therefore, women that have been diagnosed with GDM should be monitored for CV complications, independently of having developed T2DM.

One important question that arises is whether the fetus is affected by these metabolic abnormalities during pregnancy and understanding if the attempts of the maternal organism to overcome the adaptational difficulties are sufficient, and the consequences of that. Studies on this matter have only emerged in recent years. However, a great amount of research has already been gathered. Multiple studies focused on placental impairments[154]. Placental mitochondrial dynamics were observed to be altered in GDM, suggesting the possible contribution to short-term and long-term implications for the offspring[155]. Interestingly, placentas from GDM mothers have shown to be more susceptible to nitrosative damage and the investigators suggest the role of AMPK in regulating placental mitochondrial biogenesis[156]. Epigenetics is also thought to play a crucial role in offspring disease predisposition[157], as well as maternal diet during pregnancy[158] contributing to increased cardiometabolic risk in the offspring.

On the other hand, studies that aim to prevent impaired cardiac health of the offspring and future CVD development are also being conducted, and exercise during pregnancy is often used as an intervention[159] since it shows beneficial effects over the course of gestation, pregnancy outcomes, and in the maternal short-term health[160]. However, how exercise impacts the cardiometabolic health of both the mother and the fetus and which molecular pathways are involved in the pathophysiology of future CVD remain to be unveiled.

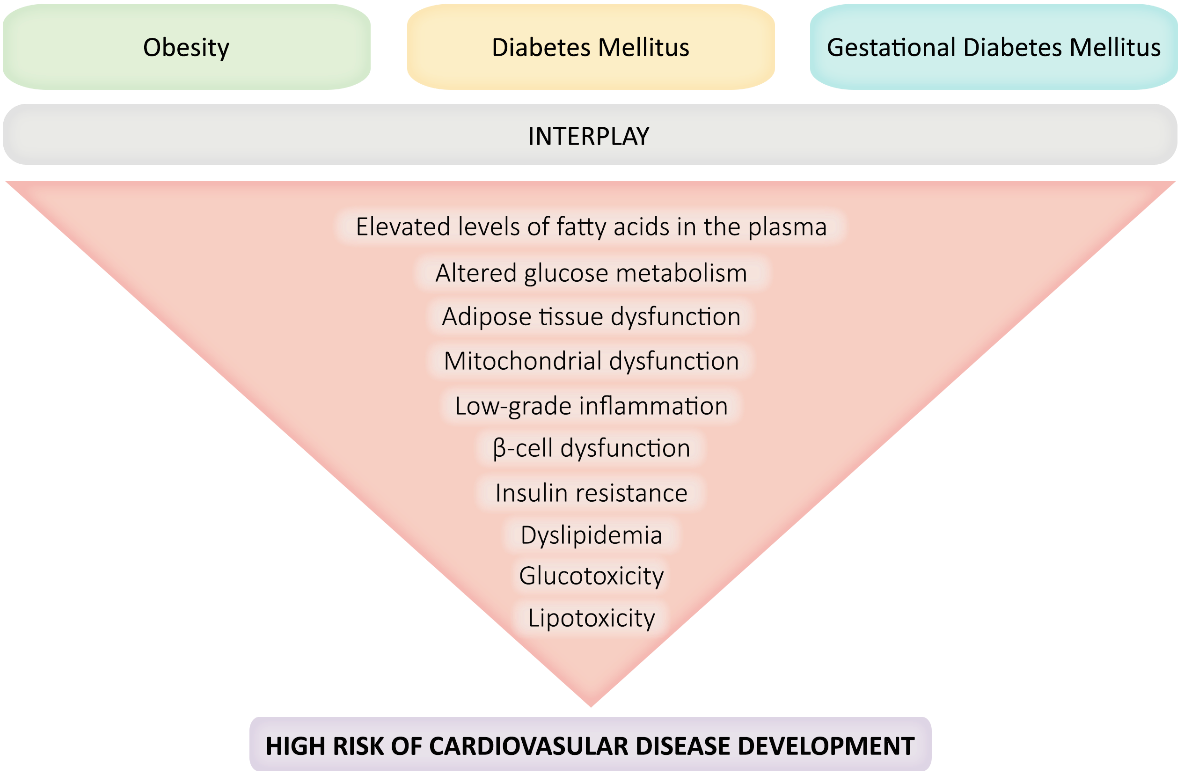

Figure 1.5 – Interplay of the common risk factors associated with obesity, diabetes mellitus and gestational diabetes mellitus that play a role for the future development of cardiovascular disease.

1.6 **Exercise as a novel non-pharmacological approach to improve cardiometabolic health**

1.6.1 **Sedentarism, a modern problematic**

The modern days have advanced in the direction of a predominant sedentary behavior that was not accompanied by the organism's adaptative evolution[161]. Nowadays, people spend more time sitting than ever due to modern daily leisure activities (e.g., television viewing, videogames) and work-related activities (e.g., computer work, home-work commutes)[162,163]. Unfortunately, this maladaptive response is evidenced by the increasing prevalence of NCDs[7,162,163]. The growing number of epidemiological studies that establish a relationship between a sedentary lifestyle or the lack of physical activity and the increased risk of chronic disease development is extremely preoccupying[164]. Therefore, a sedentary lifestyle represents a direct risk factor for adverse health outcomes.

With the increasing concern of sedentary behaviors for future disease development, lifestyle interventions that intend to increase physical activity levels have been considered of utmost importance. Furthermore, these interventions appear as attractive non-pharmacological therapeutic approaches to help control this rising epidemic.

1.6.2 **Exercise: a proxy for physical activity?**

When discussing the benefits of physical activity to combat the effects and repercussions of the modern sedentarism obstacle, one must consider that physical activity and exercise have different definitions. WHO defines physical activity as any movement performed by the body to execute an action that requires energy expenditure[165]. On the other hand, exercise includes elaborating a structured plan to perform specific movements in a repetitive manner to achieve the goal to improve or maintain physical fitness[161,165]. Given this, exercise may be considered a subcategory of physical activity and is believed to promote improved health outcomes in various pathophysiologies[166].

1.6.2.1 **Evidence of exercise's beneficial effect on metabolic health**

Exercise practice has long been associated with a general improvement in metabolic health. Over the years, studies have shown that exercise contributes to improvements in morphometric characteristics, such as body weight, body fat mass, BMI, and waist circumference[167,168], and in circulating biomarkers related to a higher risk of metabolic complications, including decreased levels of TGs, total cholesterol, and low-density lipoproteins (LDL) levels[167–169] with a concomitant increase in high-density lipoproteins (HDL) levels[168–170], and a decrease in HbAc1 blood concentration[169]. Furthermore, exercise has been related to improved systolic blood pressure, diastolic BP, mean BP[167], better cardiorespiratory fitness[169,171], decreased fasting insulin levels, and higher insulin sensitivity[169,171–173], as well as improved glucose homeostasis[171,172] and peripheral tissue oxygenation[174].

Despite the vast amount of evidence collected, there is a serious concern among physicians and researchers about the type of exercise that should be applied to a certain population, never undervaluing the health conditions, age, sex, and lifestyle habits. Nevertheless, the studies gathered above consider healthy individuals[173], subjects with CVD[169] or chronic heart failure[174], metabolic syndrome[167], men and women, adults, and older people[168]. Of note, a study that involved the evaluation of cardiorespiratory fitness and the blood parameters of interest in adults with CVD showed that exercise had higher beneficial effects for individuals who were over 50 years old and that had complications, such as T2DM, hypertension, and metabolic syndrome[169]. Furthermore, a 12-week study (commonly used period for exercise interventions among studies, as well as 16-weeks) revealed that the observed improvements in cardiorespiratory fitness and insulin sensitivity were better linked to a more intense exercise program, while better glucose homeostasis was related to a moderate exercise program[171].

Altogether these data denote the importance of having a more active lifestyle and that, independently of age, sex, and related health conditions, exercise practice contributes to improve metabolic health. Nevertheless, it is essential to continue to search for specific and adapted exercise training programs depending on the individual, their associated conditions, compliance, and taken in consideration the peculiar phases of pregnancy. With this, the need to unravel and understand the mechanisms behind exercise's beneficial effects on metabolic health has become evident.

1.6.3 **Exercise during pregnancy: yes or no?**

Although exercise during pregnancy is still a controversial topic in modern society, the scientific evidence gathered for over 25 years[175,176] has indicated that exercise during pregnancy is safe and contributes to a healthier pregnancy and better pregnancy outcomes[177]. Currently, the American College of Obstetricians and Gynecologists (ACOG) advocates that exercise programs prescribed to pregnant women should not differ from those directed to the rest of the population, recommending a goal of at least 20-30 minutes of daily moderate-intensity exercise on most if not all days of the week[177]. The US Department of Health and Human Services suggests no less than 150 minutes of moderate-intensity aerobic activity per week during pregnancy and the postpartum period[178]. Both the ACOG and the US Department of Health and Human Services highlight the possible need to implement modifications to the exercise program according to medical advice[177,178]. There are no established European guidelines for exercise during pregnancy, despite some European countries having national guidelines. Pregnancy-related physical activity guidelines that exist around the world highly contrast, and some even include indications to stop exercise during pregnancy[179]. Efforts must be made to develop uniform worldwide guidelines.

Observational studies show that exercise during pregnancy reduces the risk of excessive weight gain[180,181], hypertension[180], and contributes to decreased levels of circulating blood glucose[182]. These improvements seem to be especially observed among women who are overweight, obese, or diabetic[181,182]. Furthermore, a study that implemented an aerobic exercise program for most of the pregnancy period (27-29 weeks) revealed that non-exercised women are 2.5-fold more prone to deliver macrosomic babies[180]. On the other hand, a study in which mothers were subjected to a resistance training program, suggested that exercise contributes to a lower risk of preterm birth and low birth weight outcomes[183]. However, the great concern that prevailed throughout these years of research was the possible long-term effects in the offspring of exercised mothers during pregnancy. On this note, several animal studies are being developed to evaluate long-term offspring outcomes.

Female rats subjected to a prenatal obesogenic diet demonstrated increased levels of blood parameters associated with metabolic syndrome, fat mass, and OS[184]. Although maternal exercise prevented the increase in TGs levels, it contributed to a slight increase in glucose, cholesterol, and insulin levels and to an increase in OS[184]. This model of maternal obesity led to elevated levels of leptin, TGs, and fat mass among 36-days male offspring, which were partially prevented by maternal exercise[184]. The 3-month old offspring of swim-trained female mice after being subjected to a HFD in adulthood gained less weight and fat mass and showed decreased leptin levels and increased insulin sensitivity[185]. Although exercise during pregnancy in a mouse model did not improve the fetal-growth restriction induced by maternal obesity[186], the 8-week-old offspring from obese mothers observed cardiac hypertrophy and cardiac dysfunction, but not hypertension, were prevented by maternal exercise practice[187]. The last-mentioned study may indicate that some of the observed characteristics in newborns may not correlate to long-term health consequences[186].

Considering this, the main question comes to when and how, which goes by the generic concerns about the effect of the exercise type and duration on healthy and unhealthy individuals. Ultimately, the answer is yes most of the time.

1.6.3.1 **Exercise and gestational diabetes mellitus**

Nowadays, it is well established and accepted that exercise during pregnancy reduces the risk of GDM development, especially among overweight and obese women[188–190]. In fact, Davenport and colleagues reported that several studies showed that interventions solemnly based on exercise reduced the risk of developing GDM but also gestational hypertension and preeclampsia[190]. However, the specific effects of exercise during gestation on women already diagnosed with GDM are highly limited. A study revealed that women with GDM who performed vigorous-intensity exercise reduced the odds of excessive GWG, while no improvements were observed when women performed moderate-intensity exercise[191]. In a systematic review, Harrison et al. reported that exercise practice during pregnancy contributed to an improved postprandial glycemic control and reduced FBG, which were associated with different types of exercise (aerobic and resistance training), at a moderate or more vigorous intensity, and with different duration and frequency (20-30 minutes, 3-4 times per week)[192]. However, these glycemic improvements were not reflected in the possibility to discard insulin injections[192].

Animal models that recapitulate the GDM pathophysiology have incremented among the literature throughout the years however several fail to fully recapitulate this pregnancy complication since in some animal model presented as GDM the mothers already had glucose intolerance before pregnancy[193] or may have been subjected to a STZ treatment which is toxic for pancreatic β-cells and exerts extreme effects on maternal glucose and insulin control[194]. Therefore, the use of animal models to study the effects of exercise during GDM is extremely scarce. An interesting study used a Wistar-Kyoto rat model to generate uteroplacental insufficiency restricted offspring that were subjected either to a chow or HF diet that begin after weaning[195], recapitulating the women "at-risk" of GDM development. Using two different exercise programs (only during pregnancy or prior and during pregnancy) the authors concluded that both exercise types increased basal metabolic rate but only the latter contributed to reduced relative fat weights[195]. Exercise before and during pregnancy prevented the development of GDM in restricted rats fed a control diet and in rats fed a HFD, highly suggesting that previous acclimatization to exercise is beneficial to prevent the exacerbation of pregnancy-related metabolic adaptations[195].

Moreover, the number of studies that aim to evaluate the effects of a pregnancy complicated by GDM on short- and long-term offspring metabolic health is rapidly increasing, and the attention to understand the postnatal metabolic effects of a GDM pregnancy on the mothers has been sidetracked[196]. As previously highlighted, mothers who had GDM have an increased risk of developing T2DM and CVD after pregnancy[119,120]. Therefore, attention needs to be drawn again to the metabolic mechanisms induced by GDM *per se* and understand if those effects can be prevented or not by exercise.

1.6.4 **The cardioprotective effect of exercise**

As cardiomyocytes reach the adult stage, their proliferative capacity is lost as cellular turnover lowers down to 0.3-1% per year[197]. Therefore, upon injury, the heart does not have enough regenerative ability to counteract the consequences of the lesion that might have been caused. Given this, the adult heart responds to an insult through an increase in cardiomyocyte size[198]. This adaptational capacity of the heart to meet the body's requirements at a cellular and molecular

level is called cardiac remodeling[197–199]. A known type of cardiac remodeling is cardiac hypertrophy which occurs when the heart is subjected to elevated workload[199].

1.6.4.1 **Pathologic *versus* physiologic cardiac hypertrophy**

Cardiac hypertrophy can be defined as the enlargement of the cardiac chambers in response to a certain stimulus, which is then reflected in the increase of cardiomyocyte size[198]. A "malign" stimulus, including hypertension or other complications related to the CV system, can induce a hypertrophic response in the heart. However, the heart can also develop a hypertrophic response to a "benign" stimulus, such as exercise practice, or pregnancy[197–199]. Pathologic hypertrophy is characterized by increased width or length of the cardiac walls, with disrupted contractile function. This pathologic growth occurs when there is a maladaptive remodeling of the cardiac tissue. On the other hand, physiological hypertrophy is described as the overall proportional enlargement of the cardiac chambers[197] (**Figure 1.6**).

As previously mentioned in section 1.1.2, cardiac hypertrophy is associated with a fetal-like cardiomyocyte metabolic profile[44]. This change refers to pathological growth and occurs due to the reactivation of the fetal gene expression, which, in contrast, is not observed in physiologic cardiac hypertrophy[197,199]. Another distinguishable characteristic between these two forms of cardiac hypertrophy is the heart's capacity to reduce the physiologic enlargement observed due to exercise after long-term detraining[197], similar to the myocardial remodeling that occurs after pregnancy[200]. Considering this and the data gathered among observational studies, it becomes crucial to understand the mechanisms behind the cardioprotective effects of exercise.

1.6.4.2 **Mechanisms behind exercise cardioprotection**

The most well-known and better-characterized pathway involved in the exercise-induced cellular response in physiologic growth is the PI3K/Akt pathway[197–199,201–206]. PI3K was revealed as a key player in physiologic but not pathological hypertrophy, using dominant-negative PI3K(p110α) transgenic mice with exercise training and pressure overload as stimuli, respectively[201]. The PI3K/Akt pathway is mainly activated by insulin-like growth factor 1 (IGF-1), which increased levels have been related to cardiac physiologic enlargement in athletes[202]. IGF-1 treatment was shown to attenuate the adverse cardiac consequences induced by isoproterenol in rats[203], suggesting that IGF-1 might constitute a potential therapeutic approach.

Besides Akt's crucial role in the insulin signaling pathway (discussed in section 1.4), this protein plays a critical role in numerous cellular functions in the heart. Akt is one of the downstream effectors of PI3K and was also shown to be activated[202] and required for physiologic enlargement since the hearts of Akt-1 deficient mice adversely responded to swimming exercise, resulting in pathological cardiac growth[204]. The further activation of the Akt/mammalian target of rapamycin (mTOR) signaling has been implicated in the exercise-induced cardiac hypertrophic response in diet-induced obese rats with concomitantly reduced insulin resistance[205]. The mTOR is an important component of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)[198]. Interestingly, Chatterjee et al. reported that a detraining period and an exercise protocol altered the activation of PKC's different isoforms (α and δ) in IGF1 targeted and IGF1 receptor (IGF1R) knock-down mice, respectively[206]. The authors had previously correlated the switch between PKC

isoform α to δ with reduced cardiac function in detrained mice[206]. Furthermore, both experimental conditions resulted in decreased mTORC2 protein expression, which was folowing the observed results of impaired cardiac function in mTORC2 knock-down mice[206]. These results suggest the specific implication of mTORC2 and its interactions with both PKC isoforms in the physiologic cardiac adaptation to exercise. The physiologic cardiac enlargement is further mediated by S6 kinase (also known as p70) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), downstream targets of mTOR[198]. These proteins have an active role in the regulation of processes such as protein biosynthesis and cardiac growth[197,198].

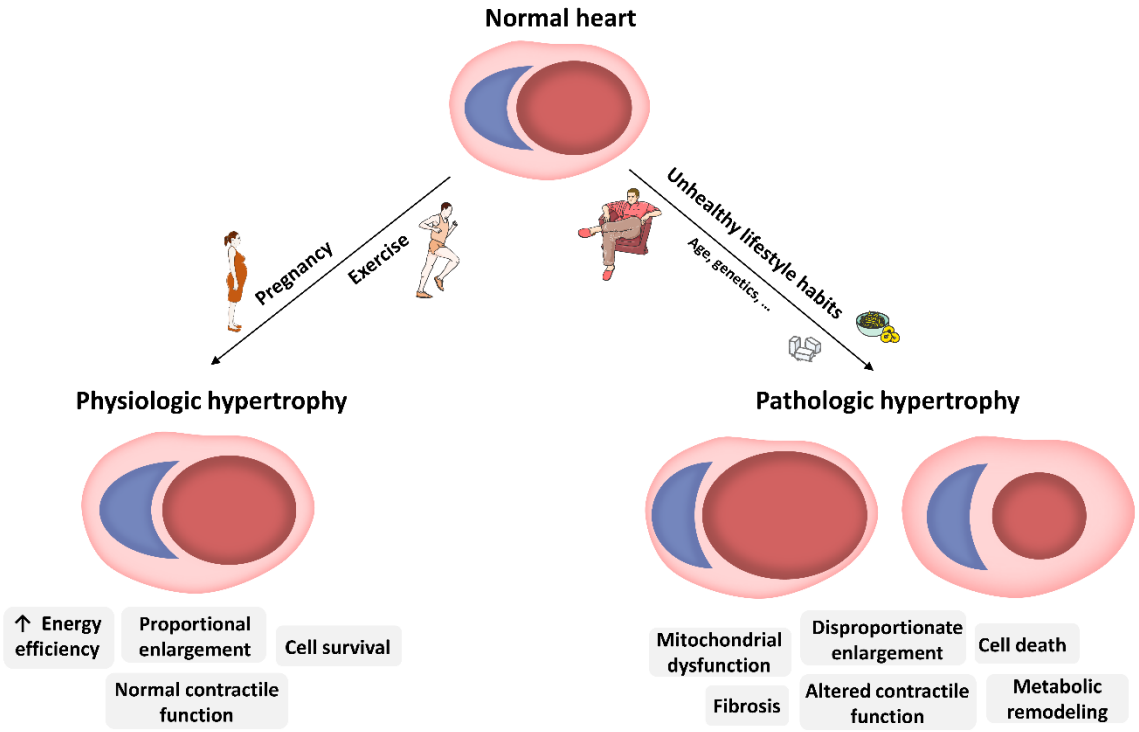

Figure 1.6 – Cardiac physiologic hypertrophy vs cardiac pathologic hypertrophy, represented by ventricular wall width and length alterations. Exercise and pregnancy exert positive effects on the heart that lead to a positive adaptative response, resulting in overall enlargement of the ventricular walls. Other stimuli such as unhealthy lifestyle habits, age, genetics, among others induce a pathologic response by a disproportionate increase in length and width, usually favoring width.

Akt activation and signaling lead to the inhibition, by phosphorylation, of its downstream target, glycogen synthase kinase 3 (GSK3), a protein involved in glycogen synthesis and storage[207,208]. GSK3 (when active) functions as an inhibitor of glycogen synthase, therefore, preventing glycogen synthesis[207,208]. Specifically, GSK3β is responsible for diminished cardiac hypertrophy given its capacity to negatively regulate transcriptional factors involved in the hypertrophic response, such as a nuclear factor of activated T-cells (NFAT) and gata binding protein 4 (GATA4)[207]. It is well known that hypertrophic stimuli lead to the inhibition of GSK3β in the heart and the multiple benefits of this inhibitory regulation in cardiac adaptative hypertrophy (e.g. downregulation of apoptosis, suppression of the mitochondrial permeability transition pore) are still being unraveled[207]. Sharma and colleagues showed the cardioprotective

effects of exercise through GSK3 phosphorylation in rat I/R hearts, with main positive outcomes related to reduced OS and inflammatory response[209].

AMPK is a central regulator of energy homeostasis, an important player in energy metabolism, responsible for the regulation of energy supply and catabolic programs[210]. Li et al. demonstrated that induced long-term activation of AMPK in rats subjected to transaortic constriction attenuated the effects of maladaptive hypertrophy through decreased protein synthesis and low NFAT and NF-kB signaling[211]. Later, Ma and colleagues revealed that the beneficial effects observed in the attenuation of cardiac fibrosis are AMPK-dependent, by detecting decreased ROS production and a greater inhibition of NADPH oxidase in swim-trained isoproterenol-induced mice[212]. To this, Ma et al. added that exercise improves cardiac (dys)function in rats subjected to pressure overload by autophagy activation in an AMPK-dependent way[213].

This dive into the main pathway responsible for exercise's beneficial role in the adaptative hypertrophic cardiac response already elucidated some of the molecular mechanisms occurring within cardiomyocytes. Mitochondria are essential key players in regulating exercise-induced cardiac hypertrophy as these organelles are intimately involved in the energetic and metabolic control of cardiac cells.

1.6.4.3 **Contribution of mitochondria to exercise's success**

A side effect of the metabolic gene pattern reprogramming to a fetal-like profile in the failing and aged heart is mitochondrial dysfunction, which is extensively observed in pathologic cardiac hypertrophy[44,214,215]. On the other hand, exercise has shown to play a preventive or reversive role in developing mitochondrial dysfunction[216,217]. While the expression of genes involved in the mitochondrial FA oxidation is downregulated in pathologic cardiac growth, exercise contributes to their upregulation as an adaptative hypertrophic response[213,214]. For example, CD36 was found to be upregulated in response to exercise but not to maladaptive cardiac hypertrophy[218]. Furthermore, exercise has been shown to counteract the metabolic shift from FA oxidation to glucose utilization observed in heart failure since palmitate oxidation rates were increased in female Sprague-Dawley rats subjected to treadmill running and I/R injury[219]. Riehle et al. reported, in mice, that swimming exercise contributed to increased mitochondrial capacity, through increased citrate synthase activity, mtDNA content, and mitochondrial proteins involved in FA oxidation and OXPHOS expression[220]. Contrarily, these effects were not observed in IRS-deficient mice, which supports previous studies showing that not only the IGF1R is necessary for an adaptive hypertrophic response but that this response can also be mediated by the insulin receptor[220]. In addition, the authors observed an increase in PGC-1α protein levels[220].

Given the role of PGC-1α in controlling mitochondria biogenesis, and therefore, the efficiency of ATP production within cardiomyocytes, this master regulator of mitochondrial biogenesis was a primary target to evaluate the effects of exercise in cardiac mitochondrial and metabolic adaptative enlargement[221]. A few animal studies have reported an increase in the levels of cardiac PGC-1α protein expression and mRNA transcripts following exercise training[216,220,222], while decreased levels are observed in failing hearts[219]. PGC-1α has an active role in regulating FA import, storage, and oxidation in the heart as it has been recognized as a co-transcriptional factor of PPARs, including PPAR-α, a key element in the regulation of FA metabolism[222]. A study that evaluated the effect of exercise before myocardial infarction in female rats, revealed that exercise

had a preventive effect on the observed increased PPAR-α protein levels in infarcted areas[223]. Furthermore, the authors established a relation between cardiac PPAR-α increased levels and diminished cardiac expression of inflammatory factors (TNF-α and NF-kB) and lower apoptosis/area rate[223]. However, Santos et al. failed to relate the increase observed in PPAR-α levels with a concomitant increase in PGC-1α levels[223].

Due to the adult myocardium's low proliferative capacity, it is crucial to preserve mature cardiomyocytes to guarantee the healthy function of the organism throughout life[197]. Kavazis et al. demonstrated that mitochondria isolated from hearts of rats subjected to an exercise protocol exhibit a particular phenotype with increased levels of proteins involved in the antioxidant response (SOD1, SOD2, GPx, and catalase) and antiapoptotic proteins[224]. Furthermore, a study revealed that exercise practice for 12-weeks in old rats was associated with the attenuation of the Bax/Bcl-2 protein ratio[225]. Interestingly, although the protein levels of Bcl-2 and Bax were found to be increased and decreased in response to exercise, respectively, and vice-versa with aging, the levels of these proteins remained unchanged among young groups (control and exercised)[225]. Given this, it would be interesting to understand if the Bax/Bcl-2 ratio is altered in response to cardiac pathological maladaptation and if exercise exerts the same cardioprotective effect observed in the aged hearts.

As previously mentioned, Nrf2 is highly involved in the regulation of cellular redox balance[71]. As OS is one of the major drawbacks observed in CVD, the relation between the response activated by Nrf2 signaling in the heart and exercise has already been proposed[226]. A study evaluated the role of Nrf2 in response to acute exercise in WT and $Nrf2^{-/-}$ mice. The results point to a cardioprotective effect of acute exercise through an increase in ROS capable of enhancing Nrf2 function and promoting an antioxidant defense response[226]. On the other hand, in Nrf2 deficient mice exercise leads to exacerbated ROS[226]. Another study showed that moderate-intensity exercise in isoproterenol-treated mice stabilized the activity of the Nrf2 promoter, leading to an increase in the expression of antioxidant genes crucial to guarantee an antioxidant response that attenuated the ROS-induced cardiac damage[227]. Both these studies suggest the potential role of exercise as a non-pharmacological approach to protect against OS-related CV complications through promoting Nrf2 signaling.

Nitric oxide (NO) has also shown to be implicated in the adaptative cardiac response to exercise. Briefly, NO is generated by endothelial nitric oxide synthase (eNOS) activity and functions through activation of soluble guanylate cyclase (sGC). This results in increased levels of cyclic guanosine monophosphate (cGMP) and concomitant activation of PKG, critical for normal muscle contraction. eNOS was shown to be activated through phosphorylation mediated by Akt and shown to confer a cardioprotective effect in rat hearts subjected to I/R injury and early preconditioning[228]. Voluntary exercise showed to reduce myocardial injury in mice due to an increase in the phosphorylation of eNOS in the residue Ser1177 (believed to be mediated by Akt[228]) and also an increase in NO levels and in NO-related metabolites storage[229]. Interestingly, a study using primary mouse cardiomyocytes showed that stimulation with NO donors resulted in activated mitochondrial biogenesis (increased PGC-1α and TFAM levels) and increased mtDNA content and citrate synthase activity[217]. Moreover, while swim-trained WT mice showed increased eNOS expression, improved mitochondrial biogenesis, and mitochondrial number and density, eNOS deficient mice were unable to demonstrate the same adaptative response[217].

The study of interplaying pathways involved in exercise response at a cardiometabolic level (the ones discussed summarized in **Figure 1.7**) is highly facilitated by the development of animal models, which becomes an important tool for applying different interventions and the characterization of the effects of specific variables.

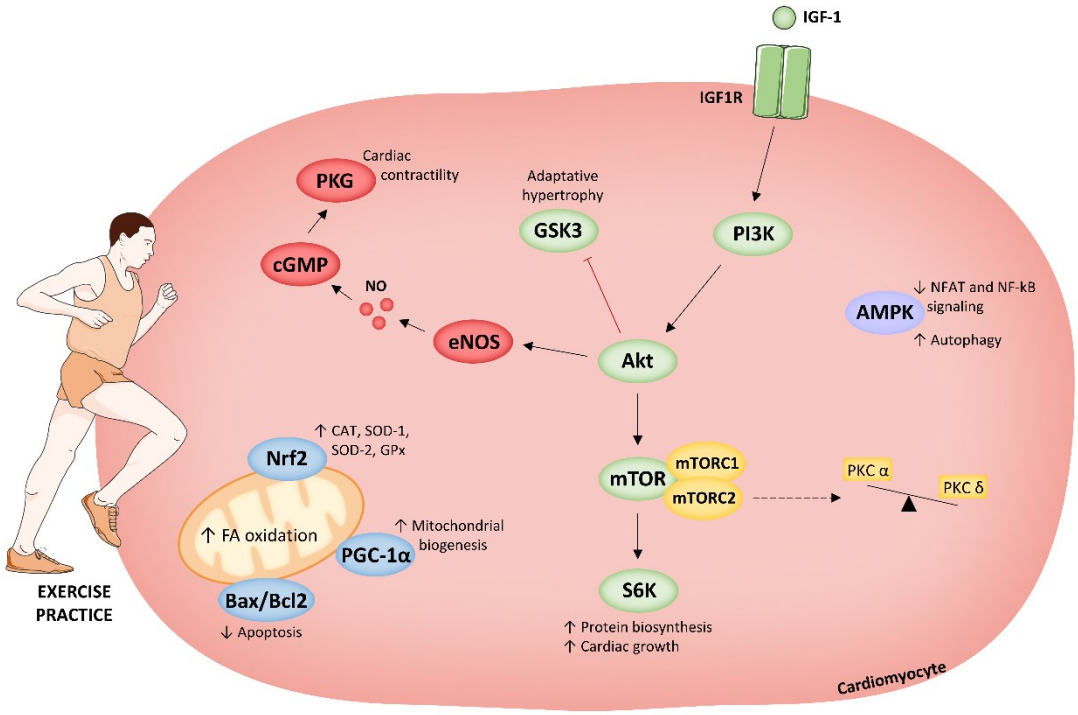

Figure 1.7 – Exercise and mitochondrial metabolism contribution to cardioprotection in cardiomyocytes. The PI3K pathway is the best-known mechanism involved in the cardiac metabolic response to exercise. Protein kinase B (Akt) activation leads to alterations in the signaling response of many downstream targets, contributing to a positive adaptative physiologic response.

1.7 **Animal models used in metabolism, cardiovascular and pregnancy research**

Initial research on a particular disease usually begins with epidemiologic studies, in which the distribution of health complications is studied in a population, or at a global level. For instance, it considers the incidence of the disease or its mortality. The further analysis comes with cohort studies, which unravel hints of the putative causes by comparing two groups of individuals according to their exposure or non-exposure to a certain factor[230]. Therefore, it is not surprising that there are numerous epidemiological and cohort studies of CVD or other chronic diseases[231–233]. Nevertheless, human studies are limited since it is impossible to control important variables that can skew the results. Several variables are not considered or controlled, making it extremely hard to obtain individuals who share the same quotidian life and activities, to exclude or exploit environmental variables. There is also the risk of ignoring genetic factors that might affect the outcomes[152]. Age, weight, BMI, among other factors, but also type of diet, exercise, and stress may influence the results of human studies[230].

The use of animal models becomes necessary to investigate further since variables can be tightly controlled and samples easier to obtain. Samples may be obtained at different time points

allowing tissue collection and the specific description of the mechanisms implicated. Nevertheless, it is fundamental to establish a bridge between animal studies and human health. The results obtained in an animal model experiment must successfully translate to humans[234]. To guarantee translational success, investigators must (1) fully understand their scientific question and its underlying mechanisms, so (2) all the criteria to choose the right species are considered; (3) study the physiological characteristics of the species and know (4) their advantages and disadvantages[234]; (5) gather all the knowledge and draw the most appropriate experimental design, englobing the considered criteria and the interplay between the different species chosen to achieve human translation.

Several animal models have been developed to study the mechanisms behind obesity and DM, especially T2DM, for some decades. The first animal model used in the field of metabolic research was the dog, at the beginning of 1920[235]. In 1923 Frederick Banting and Charles Best were awarded a Nobel Prize for discovering insulin in 1921[236]. Although the dog's genome presents a homology level of approximately 90% to the human genome[237], carnivore dogs are resistant to high lipid content diets, the phenomenon of atherosclerosis, and, when compared to humans, significant differences concerning their CV system are established[238].

Other mammalian animals with higher similarities to humans at a metabolic and CV level have been used in this field of research. Small rodents, pigs, sheep, and non-human primates (NHP), compose a great part of the mammalian systems used for the study of metabolic disorders (**Table 1.4**). Mice are small animals with a short life cycle that can have a tremendous advantage since they can be easily manipulated. Usually, mice reach sexual maturity after 4 to 8 weeks of being born and have large reproductive outputs (~6-12 pups) out of a 19-20 days gestation[104,108]. The physiology and metabolism of rats are also similar to that of humans[238] and one advantage, when compared to mice, is their bigger size, which makes them easier to handle[239]. However, both the mice and the rat rely on a HDL, as opposed to humans that mostly rely on LDL metabolism, making them more resistant to the development of atherosclerosis, which has been seen as a disadvantage for the study of CVD[238]. Nevertheless, when it comes to the study of pregnancy these models have shown to be of great importance since they resemble most of the CV adaptations seen in pregnant women, such as the increase in vasodilatation, cardiac output, systemic resistance, and arterial compliance[240]. Moreover, the pregnancy in these animals is longer (~22 days) and the fetuses are larger compared with mice[108].

Large animal models compose an exceptional advantage in cardiovascular research due to higher similarity to the human heart, regarding size, anatomy, and physiology[241]. The pig is a well-known model in cardiovascular research, given its particular resemblance to the human myocardium that, in comparison with other large animal models, extends further to cardiac function and hemodynamics[242]. Besides, the pig also has other similarities to humans beyond the CV system, including its biochemistry and lifestyle[241]. Therefore, the pig would represent an optimal model to study metabolic alterations with high translational success. The pig is prone to the development of pregnancy-related metabolic disorders[243]. Despite being a polytocous species, pigs are diurnal animals and have a more prolonged gestation period[241,243], which composes a tremendous advantage compared to rodents. Nevertheless, the maintenance of these animals is laborious and more expensive[244].

Since the study of nutrient exchange and vascular development is significant for the study of maternal-fetal interactions, other animals, such as sheep, also show advantages for their use as pregnancy models due to their similar fetal placental vascular structure compared to humans[245].

Evidently, from a metabolic and physiological perspective, the most similar animal models to humans are the NHP. In common with humans is the part of the organism where *de novo* lipogenesis occurs and the physiology of insulin and glucose utilization[236], making NHP ideal models for CVD study. Studies in NHP have special strengths concerning the production of translational data[246]. Nevertheless, this model comes with several disadvantages in terms of logistics and economic costs due to its long lifespan. Another limitation of this model is the associated ethical issues[238].

Table 1.4 – Gestational and metabolic characteristics of animal models used in cardiovascular and pregnancy-related disorders research.

Animal model	Sexual maturity	Reproductive output	Length of gestation	Main advantages	Main disadvantages
Mouse	4-8 weeks	6-12	19-20 days	Small animals, short life cycle, easily manipulated, cost-effective	HDL metabolism reliance, polytocous species
Rat	~6 weeks	~9	~22 days	Larger fetuses, easily manipulated, cost-effective	HDL metabolism reliance, polytocous species
Sheep	4-6 moths	1-2	148-153 days	High vascular structure similarity of the fetal placenta to humans, long gestation	Anatomically different placenta comparing to humans, high-cost maintenance
Pig	5-6 moths	5-14	112-114 days	High similarities to the human heart, long gestation, diurnal animals	High-cost maintenance, polytocous species
Non-human primates	~3 years	Non-litter bearing	173-180 days	Highest similarity to human physiology and anatomy	Ethically less accepted, high-cost maintenance

1.7.1 **Diet-induced metabolic disorders**

One of the most common methodologies to study obesity and diabetes in these animal models is the use of high caloric diets, so the injuries in metabolism and, for instance, the CV system observed in humans can be mimicked and the mechanisms behind those harmful effects elucidated. On this note, diet-induced obesity (DIO) has been widely used as a non-pharmacological or non-genetic approach to induce the features of obesity and, in some cases, T2DM[236]. Although these may compose expensive and time-consuming studies[236], it becomes an extremely important method due to the increasing consumption of Western diets, rich in sugar and fat[152]. This is also a good way to naturally understand the occurring events during pregnancy when women have free access to diets abundant in fat and sugars and are not familiar with the

underlying effects that this excessive dietary intake behavior may impose on themselves and the fetus.

A HFD leads to increased FFA levels and consequent formation of DAG in various tissues, including the heart[152]. The latter may activate proteins of the PKC family[152]. This ultimately culminates in the impairment of the phosphorylation responsible for activating the IRS of the insulin signaling pathway. Besides, the low-grade inflammation levels caused by the FFA increase also lead to the inhibition of IRS-1, with concomitant development of chronic IR[152]. A diet rich in carbohydrates can be also used. However, in animal models it does not contribute as heavily to obesity development. Nevertheless, a diet rich in carbohydrates leads to a faster progression to IR and hyperglycemia than HFD[152]. These two diets are usually used together to obtain noticeable results within a short period.

Known mouse models in which these types of diet are normally used are the inbred C57BL/6J or C57BL/6N strains. These distinct models demonstrate different metabolic outcomes, such as obesity, adiposity, glucose intolerance, IR, and hepatic steatosis, hyperglycemia, and hyperinsulinemia, respectively[236]. Studies using C57BL/6J mice as a model have reported impairments in leptin sensitivity[247], insulin sensitivity, and a state of chronic inflammation[248] when fed a HFD. Furthermore, it has been previously shown that feeding this model with a HFD negatively impacts glucose metabolism independent of the caloric intake, suggesting that the content of the diet has a greater influence on the outcomes than its caloric density[249]. Moreover, a study using C57BL/6N mice showed rapid development of impaired lipid metabolism and obesity when fed a HFD, and changes at a hematological level, showing a certain predisposition to the development of CVD[250].

Sprague-Dawley and Wistar rats are the commonly used rat strains for studies using a HFD[236,239]. It has long been established that Wistar rats develop diabetes with concomitant IR even in the absence of genetic factors[251]. The plasma glucose disappearance rate has also been studied in this model and was shown to be decreased in rats subjected to a HFD when compared to a control group[251]. Changes in the levels of transcripts related to glucose metabolism were also observed in the liver, fat, and skeletal muscle[252]. Lozano et al. developed a Wistar rat model of induced T2DM involving the combined HFD and high-carbohydrate diet (HCD) (HFHC). The researchers reported low-grade inflammation and exacerbated hepatic ROS production, which contributed to endothelial dysfunction and consequently resulted in CV complications[153]. A study involving Sprague-Dawley rats demonstrated the importance of susceptibility for obesity and the increase in several components of lipid metabolism when fed a HFD, compared to those that were resistant or fed a low-fat diet. The study also revealed that the rats fed a HFD had a higher energy balance coupled to a low rate of energy substrate utilization and, therefore, fat storage was promoted[253].

Regarding pregnancy, fetal development, and susceptibility for the development of obesity and GDM during pregnancy, Pereira and colleagues raised an important question concerning the fact that subjecting rats to a HFD during pregnancy only mimics obesity but not diabetes. However, they also highlighted the previous confirmation that dams subjected to the diet before pregnancy would develop obesity pre-pregnancy, which would further lead to a state of hyperglycemia, hyperinsulinemia, and hyperlipidemia at mid-gestation[194]. Having this into account, the authors later developed a rat model for GDM using Sprague-Dawley rats subjected to a HFHC diet *ad libitum*, having provided that in Sprague-Dawley rats pre-pregnancy weight has an important influence on the development of GDM, and how it may further contribute to

offspring complications, such as the development of obesity, fatty liver, and IR[254]. Accordingly, a study using C57BL/6 mice demonstrated that subjecting obese females to a low-fat diet before pregnancy resulted in less critical metabolic effects during pregnancy, as compared to the ones subjected to a HFD[255].

Animal models compose great tools for the study of specific disorders and enable the observation of different outcomes by adapting the animal diet or lifestyle habits, in between other genetic and drug known methodologies allowing the collection and analysis of tissues that would be inaccessible in humans, as the heart.

1.8 **Hypothesis and objectives of the work**

Pregnancy composes a metabolic challenge in which the maternal organism suffers crucial adaptational processes to guarantee normal fetal growth, the normal energy requirements to support gestation, and ensure maternal health and survival. The cardiovascular system is no exception, and the heart goes through mechanical, structural, and metabolic adjustments. Any inability to overcome these challenging demands may contribute to the development of pathophysiological disorders. Gestational diabetes mellitus is the most common metabolic disorder of pregnancy and puts both the mother and the offspring at increased risk for long-term diabetes and cardiovascular disease development.

In a GDM pregnancy, modifications in the mother's lifestyle habits are the first-line treatment. However, the success of this treatment is highly dependent on medical orientation and maternal compliance. These include alterations in the type of diet and the practice of exercise. Therefore, it is of extreme importance to understand how maternal habits during pregnancy can impact maternal health after delivery and the incidence of long-term penalties of a GDM-pregnancy. Furthermore, since mothers might only adopt a healthier lifestyle during the period of gestation, a better understanding of the implications of exercise practice exclusively during pregnancy becomes crucial.

Considering this, we hypothesized that a GDM-pregnancy induces alterations in the maternal cardiac metabolism that prevail postpartum, modulating the cardiac energetic function and potentiating maternal cardiovascular disease development. We also questioned if exercise exclusively during a GDM-pregnancy can prevent the induced memory for higher risk of cardiovascular disease development or if the detraining after pregnancy will exacerbate the GDM predisposition to CVD. Furthermore, we want to clarify if pregnancy per se leads to a postpartum cardiac metabolic memory and the energetic implications of that acquired memory.

To test our hypothesis we aim to 1) identify the players for the maternal GDM cardiac imprint that remains after pregnancy and predispose to CVD, 2) characterize the impact of exercise practice exclusively during GDM-pregnancy in the maternal cardiac metabolism and myocardial remodeling after pregnancy, and 3) depict the physiologic memory led by the pregnancy challenge in the maternal heart, namely at the cardiac bioenergetic level since limited data exist.

This information can generate fresh insights into disease etiology that, in turn, could be useful to develop targeted interventions aimed to reverse this obstetric outcome and reduce CVD deaths, promoting maternal well-being and ensuring healthy lives.

Chapter 2 – Materials and methods

2.1 **Reagents**

All the reagents used throughout this work were of the highest grade of purity available. Aqueous solutions were prepared in ultrapure water (type I, Milli-Q Biocel A10 with pre-treatment via Elix 5, Millipore), while non-aqueous solutions were either prepared in ethanol (99.5%, Sigma-Aldrich, Barcelona, Spain) or dimethyl sulfoxide (DMSO, Sigma-Aldrich).

The full list of reagents used in this work, with the respective CAS number, reference and the supplier company is presented in **Table 2.1**.

Table 2.1 – List of all the reagents used in the present work, with the indication of the respective CAS number, reference, and supplier company.

Reagent	CAS number	Reference	Company
40% Acrylamide/Bis Solution	79-06-1	1610148	Bio-Rad
Acetic acid glacial	64-19-7	A/0400/PB15	fisher bioreagents
APS (Ammonium Persulphate)	7727-54-0	1708.0020	GERBU
Bromophenol Blue sodium	62625-28-	092K3720	Sigma-Aldrich
BSA (Bovine Serum Albumin)	9048-46-8	A7906	Sigma-Aldrich
Clarity Western ECL substrate	-	170-5061	Bio-Rad
DTT (DL-1,4-Dithiotheitol)	03/12/3483	D9779	Sigma-Aldrich
EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate)	381-92-6	ED2SS	Sigma-Aldrich
Eosin Y	15086-94-9	E4009	Sigma-Aldrich
Glycerol	56-81-5	G5516	Sigma-Aldrich
Glycine	56-40-6	120070010	Acros Organics
Guanidine Hydrochloride	50-01-1	BP178	ThermoFisher Scientific
HCl (Hydrochloric Acid)	7647-01-0	131020.1212	PanReact AppliChem
Hematoxylin solution	-	1.05175	Merck Millipore
HEPES	7365-45-9	H4034	Sigma
Isopropanol	67-63-0	190764	Sigma
L-Glutamic Acid	56-86-0	G8415	Sigma

L-Malic Acid	97-67-6	M7397	Sigma
Methanol	67-56-1	M/4000/17	fisher bioreagents
Na_2HPO_4 (Sodium phosphate dibasic)	7558-79-4	S5136	Sigma-Aldrich
NaCl (Sodium Chloride)	7647-14-5	BP152-1	fisher bioreagents
NAF (Sodium Fluoride)	7681-49-4	27859.293	VWR
NaH_2PO_4 (Sodium phosphate monobasic)	7558-80-7	S5011	Sigma-Aldrich
NAM (Nicotinamide)	98-92-0	N0636	Sigma-Aldrich
Oligomycin from Streptomyces diastochronogenes	1404-19-9	O4876	Sigma-Aldrich
Pierce™ BCA Protein Assay Kit	-	23227	ThermoFisher Scientific
PMSF (Phenylmethylsulfonyl fluoride)	329-98-6	P7626	Sigma-Aldrich
Ponceau S	6226-79-5	J60744	Alfa Aesar
Precision Plus Protein TM Standard Dual Color	-	161-0374	Bio-Rad
Protease Inhibitor Cocktail (PIC)	-	P8340	Sigma-Aldrich
Rotenone	83-79-4	R8875	Sigma-Aldrich
SDS (Sodium Dodecyl Sulphate)	151-21-3	MB01501	nzytech
Sodium Orthovanadate	13721-39-6	S6508	Sigma-Aldrich
SsoFast™ EvaGreen® Supermix	-	172-5204	Bio-Rad
TEMED (1,2-Bis(dimethylamino)ethane)	110-18-9	MB03501	NZY tech
Tris BASE	77-86-1	BP152-1	fisher bioreagents
Triton X-100	9002-93-1	327371000	Acros Organics
Trizma® hydrochloride	1185-53-1	T3253	Sigma-Aldrich
Tween20	77-86-1	BP152-1	ThermoFisher Scientific

2.2 **Animal treatment and experimental design**

2.2.1 **Animals and ethical disclosure**

All animal procedures were approved by the Ethics Committee of the Faculty of Sport of the University of Porto, and the Portuguese National Government Authority (No

0421/000/000/2018), were performed according to the guidelines for care and use of laboratory animals in research, recommended by the Federation for European Laboratory Animal Science Associations (FELASA) and conducted in animal care-approved facilities.

Six-week-old female Sprague-Dawley rats (CD-SIFE05SS - R/SPF CD) and male rats (CDSIMA05SS - R/SPF CD), were purchased from Charles River Laboratories (L'Arbresle, France). After a 1-week acclimatization period, the rats were assigned to the experimental room with appropriate bedding and environmental enrichment. The rats were kept on artificial lighting for 12-h during the night and in a dark environment during the day and the room conditions were adapted to maintain the temperature at 21-22ºC, humidity at 50-60%, and noise level below 55dB. The rats had *ad libitum* access to food (D12450K, Ssniff, Germany) and water. To test our hypothesis, we implemented a novel diet-induced GDM model, that mimics crucial features of the disease (e.g.: gestational glucose intolerance, hyperglycemia, and supra-physiological levels of systemic insulin resistance) based on the model previously described by Pereira et al.[254]. All the animals were weighed weekly. Behavioral assessment, exercise protocols, and animal manipulation were performed during the active period of the animals (dark phase) by certificated operators. **Figure 2.1** summarizes the study design.

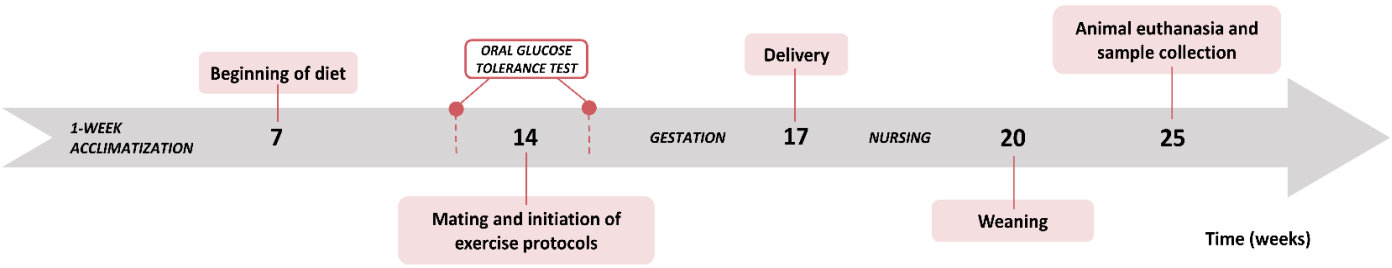

Figure 2.1 – Timeline of the experimental design used in the present study, with important experimental interventions denoted.

2.2.2. **Diet treatment**

In the first weeks, the animals were fed a standard rodent control diet (ssniff® DIO – 10 kJ% fat, no sucrose addition D12450K, Ssniff, Germany). At 7 weeks of age, 14 animals were randomly selected to continue the control diet while the remaining 12 females were subjected to a high-fat-high-sugar (HFHS) diet (ssniff® EF R/M acc. D12451 II) to induce the GDM phenotype. All diets were supplemented with the essential minerals, vitamins, and trace elements and were maintained until euthanasia, resulting in two experimental groups based on the type of diet consumption: rats fed a control diet (C), and rats fed a HFHS. The composition of the diets is detailed in **Figure 2.2** and **Table 2.2**.

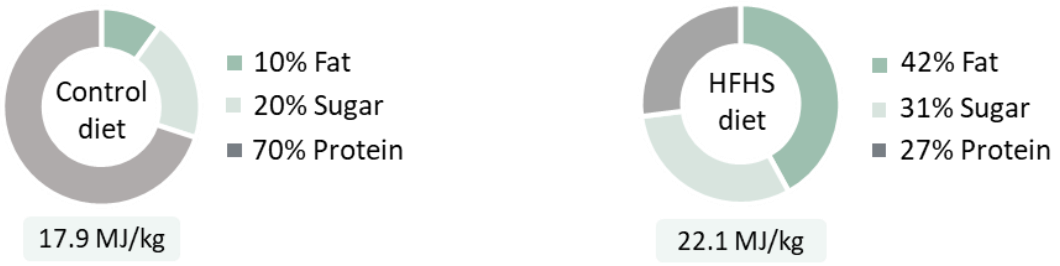

Figure 2.2 – Control diet (ssniff ® DIO 10 kJ% fat, no sucrose addition D12450K); high-fat-high-sugar (HFHS) diet ssniff ® EF R/M acc. D12451 II), *ad libitum*. *Adapted from ssniff ®*.

Table 2.2 – Composition of the control diet (A) and HFHS diet (B) fed to the female rats during the experimental study.

A

Control Diet

Crude Nutrients	**(%)**
Crude Protein (N x 6.25)	18.2
Crude Fat	4.1
Crude fibre	5.0
Crude ash	5.3
Starch	47.0
Sugar	1.0
N free extracts	62.9

B

HFHS Diet

Crude Nutrients	**(%)**
Dry matter	93.9
Crude Protein (N x 6.25)	22.5
Crude Fat	23.1
Crude fibre	5.7
Crude ash	5.9
Starch	6.6
Sugar	20.4
Dextrin	11.1
N free extracts	36.8

2.2.3 **Mating protocol**

A fertile male was introduced to each breeding cage to induce pregnancy in the 14-week-old females. Pregnancy was confirmed by observation of the vaginal mating plug formation and once confirmed, the female rats were considered pregnant (P, n=19), and the remaining were considered non-pregnant (NP, n=7).

2.2.4 **Exercise intervention**

With the aim of evaluating the impact of exercise during GDM-pregnancy on postnatal maternal cardiometabolic health, the dams were submitted to an exercise protocol during the 3 weeks of pregnancy, 6 days a week. The group of female rats subjected to the exercise program consisted of the exercised experimental group (E), while the remaining females were considered sedentary (S). The exercise program consisted of two types of exercise: 1) voluntary physical activity (VPA), in which dams were offered free access to a running wheel (circumference = 103.73 cm, Type 304 Stainless steel, Tecniplast, Casale Litta, Italy) connected to a counter (ECO

701 Hengstler, Lancashire, UK). Since each cage was shared by 2 females, the final travel distance recorder by the wheel revolutions counter was divided by two; and 2) endurance exercise with an initial 2-day adaptation period (before the initiation of the mating protocols), followed by a 6-day gradual intensity increase training on a motor-driven treadmill (LE8700, Panlab, Harvard, USA). After the first week, the exercise intensity achieved was maintained until the end of gestation (**Figure 2.3**).

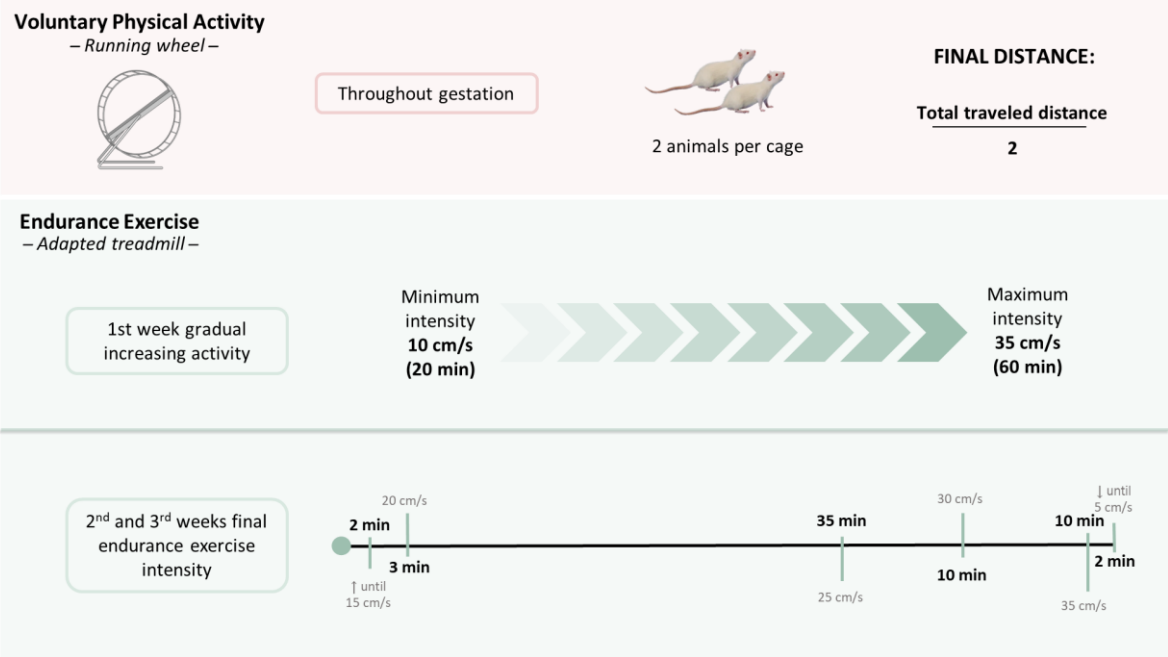

Figure 2.3 – Schematic representation of the exercise protocols used.

2.2.5 **Experimental size**

Seven-week-old female Sprague-Dawley rats with similar morphometric characteristics were randomly subjected to a control diet or a HFHS-diet. At 14 weeks of age the pregnant dams were assigned to an exercise program or considered sedentary whilst on a HFHS-diet, while rats subjected to the control diet were considered sedentary. The non-pregnant group was subjected to a control diet and a sedentary lifestyle. This study involved the evaluation of the effects of a dietary intervention capable of inducing GDM, the practice of exercise during the 3-weeks of pregnancy, and the impact of pregnancy *per se*.

Therefore, 4 different experimental groups (represented in **Figure 2.4**) were considered in the present work: pregnant rats subjected to a control diet and a sedentary (S) lifestyle, and their non-pregnant counterparts (P-C-S, n=7 & NP-C-S, n=7); pregnant rats subjected to a HFHS GDM-inducing diet and a sedentary lifestyle (GDM-S, n=6); as well as pregnant rats also subjected to the HFHS GDM-inducing diet but subjected to an exercise (E) program (GDM-E, n=6).

2.3 **Animal procedures**

2.3.1 **Oral glucose tolerance tests**

The glycemic control of the female rats was evaluated using oral glucose tolerance tests (OGTT) before mating and on the 16th day of pregnancy (mid-pregnancy). In an OGTT the individual is orally given an amount of glucose, and, after administration, blood is collected at specific time points to evaluate glucose metabolization. As described in Chapter 1, women get a positive diagnosis for GDM when their blood glucose levels during an OGTT achieve certain values at different time points (see section 1.5.1). Although these levels have not yet been defined for Sprague-Dawley rats, glucose intolerance was considered by comparing the treatment groups with the respective control group since the latter should represent a physiologic response to glucose administration. Given that most of the used methods to administer glucose in rodents require intensive handling and restraining, a method developed by Zhang et al.[256] that involves drug administration through artificially sweetened and flavored jelly was implemented. In this case, a non-flavored jelly (Globo Gelatina Neutra, A Colmeia do Minho, Portugal) containing a glucose dose of 2 g per kg of body weight was administered. The use of this protocol avoids the stress levels induced by the other methods, adding to the fact that pregnancy is *per se* capable of inducing stress, preventing the interference it would have on the study's parameters.

To perform OGTT the animals were fasted for 6-18 h. During the test, blood was removed from the tail vein, at 15, 30, 60, 90 and 120 minutes after glucose consumption. The OGTT was performed 1 week before mating and at mid-pregnancy (15 weeks of age), to confirm that pregnancy was the catalyzing challenge to develop glucose intolerance during gestation, inducing GDM, and reinsure that the animals were not pre-diabetic.

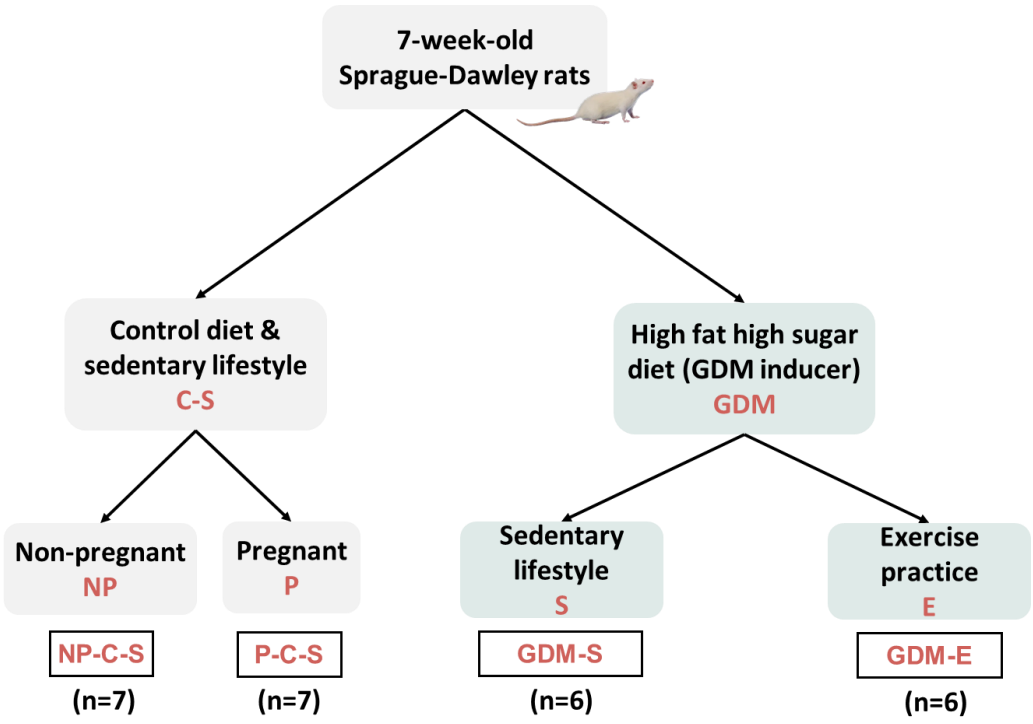

Figure 2.4 – Schematic representation of the experimental groups of the present study.

2.3.2. **Animal euthanasia and tissue collection**

The female rats were euthanized 8 weeks post-partum, at 25 weeks of age. To consider the long-lasting cardiac implications of the treatment on maternal health and to minimize the interference of the weaning period in this study, the reason why the experiment was extended 5 weeks after the nursing period. The animals were fasted for 12 h and just before the euthanasia the glycemic level and bodyweight were determined. Animal euthanasia was performed between 8-10 a.m. under anesthetic conditions. Firstly, the animals were placed in an induction camera at 1.5 L/m O_2 5% isoflurane and kept in a deep anesthetic state with 0.8 L/m O_2 4% isoflurane. The abdominal cavity was opened to elicit blood from the inferior vena cava, followed by the opening of the thoracic cavity to remove the heart. After removal, the heart was weighed, rinsed with ice-cold PBS (NaCl, KCl, Na_2HPO_4, and KH_2PO_4) and segmented to be used in different experiences.

2.3.2.1. **Cardiac tissue for mitochondrial isolation**

A segment of the heart was immersed in ice-cold Heart Isolation Buffer (HIB), prepared with 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES, pH=7.4 with KOH, supplemented with 0.1% fatty acid free Bovine Serum Albumin (A4503, Sigma; Saint Louis, USA) (HIB+) and finely minced to facilitate blood removal and mitochondrial isolation.

2.3.2.2. **Cardiac tissue for biochemical analysis**

To posteriorly perform the biochemical analysis of the cardiac tissue, a segment of the heart was snap-frozen in liquid nitrogen and stored at -80ºC until further use.

2.3.2.3. **Cardiac tissue for optical microscopy**

The segment of the heart kept for optical microscopy was fixed by immersion in 4% paraformaldehyde (pH 7.4), dehydrated in a graded series of alcohol percentages (70–100), and embedded in a paraffin block for histological and immunohistochemical analyses.

2.3.2.4. **Blood tissue processing**

Blood was collected from the vena cava and placed in EDTA-treated tubes for centrifugation at 2005 G (L500 tabletop low-speed centrifuge, Cence®, China) to separate and collect the plasma fraction (light-yellow supernatant) for subsequent determination of biochemical parameters.

2.4 **Blood plasma biochemical characterization**

The blood plasma levels of glucose, triglycerides, cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), alanine aminotransferase (ALT), aspartate transaminase (AST), urea, creatinine, and creatine kinase were assessed using tandem mass spectrometry (MS/MS).

2.5 **Optical Microscopy**

The images collected from the different techniques used for the optical microscopy of the rat heart tissue were acquired in a Nikon ECLIPSE Ci microscope with a Nikon Ds-Fi 3 camera (Nikon Europe BV, Amsterdam, Netherlands).

2.5.1 **Tissue cut**

The paraffin blocks in which the heart tissue was embedded were carefully trimmed at 10 μm using a LEICA RM2255 microtome to obtain the whole extension of the tissue samples. Afterwards, the paraffin sections were trimmed at a final thickness of 3 μm and used for further analysis.

2.5.2 **Hematoxylin-Eosin staining**

The Hematoxylin and Eosin (H&E) staining is widely used in histopathology studies since it enables a detailed visualization of the tissue of interest, demonstrating cytoplasmic, nuclear, and extracellular matrix features. The histological H&E staining was performed in heart tissue samples using Tissue-Tek DRS 2000. The samples were dewaxed and hydrated through graded alcohols to water and stained in Gill Hematoxylin for 1 min. After thoroughly washing the samples for 4 min, they were stained in 1% Eosin Y for 1 min and washed with water for 2 min. In the end, the samples were dehydrated through alcohols and cleared with xylene. The last step was the mounting of the histological slides performed by Sakura Tissue-Tek GLS.

2.5.3 **Masson's Trichrome staining**

Masson's Trichrome staining method detects collagen fibers of paraffin-embedded tissues by staining the collagen fibers blue and the nuclei black, while the background is stained with a red color. First, the section of the cut heart tissue was deparaffinized, hydrated, and washed with ddH_2O. The samples were then treated with Bouin's solution for 30 min at 60ºC and after cooling, the sections were rinsed in running tap water to remove the yellow color. After staining with Gill hematoxylin working solution for 5 min, the samples were washed for 10 min. Subsequently, the heart sections were stained with Ponceau-fuchsin solution for 2 min and washed with ddH_2O. For differentiation, a phosphomolybdic-phosphotungstic acid solution was used for 15 min. Finally, the samples were stained with Aniline blue solution for 5 min and rapidly washed in distilled water without washing. In the end, the samples were dehydrated through alcohols and cleared with xylene. The last step was the mounting of the histological slides performed by Sakura Tissue-Tek GLS.

2.5.4 **Immunohistochemistry**

Immunohistochemistry (IHC) is a technique used to assess the presence of a specific protein marker in a certain tissue. The immunohistochemical study was performed in heart tissue using Bond Polymer Refine Detection™ (DS9800 Leica Biosystems, Newcastle, United Kingdom) on BondMax, according to the manufacturer's instructions. After performing the antigen retrieval

for 20 min using the Bond Epitope Retrieval Solution 1 (Leica Biosystems, Newcastle, United Kingdom), Peroxide Block was used in the sections for 5 min. Afterwards, a primary antibody against Vimentin (clone V9, PA0640, LEICA Biosystems) was applied to the heart tissue sections and incubated at RT for 15 min. Further steps involved the sequential use of Post Primary (8 min), Bond Wash Solution (6 min), Polymer (8 min), Bond Wash Solution (4 min), the Mixed DAB Refine brown chromogen (10 min), and finally, Hematoxylin (5 min).

2.6. **Determination of cardiac mitochondria bioenergetics**

2.6.1 **Cardiac mitochondria isolation**

Cardiac mitochondria were isolated according to the protocol described by Pereira et al.[257] and adapted from Silva and Oliveira[258]. All procedures for mitochondria isolation were performed at 4ºC. The minced heart was washed with HIB+ to remove the remaining blood completely. The minced blood-free tissue was resuspended in HIB+ and homogenized with a tightly fitted Potter-Elvehjem homogenizer. After 3-4 homogenization steps, the HIB+ was supplemented with 0.2 mL/g wet tissue of protease (subtilisin fraction type VIII, Sigma; Saint Louis, USA) and incubated for 1 min. Afterwards, the lysate was re-homogenized and the protease removed by centrifugation at 13 000 G for 10 min (2-16KC centrifuge with rotor 12139-H, Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was discarded, and the pellet gently resuspended in HIB+ and transferred to another Potter-Elvehjem for manual homogenization. The resultant suspension was centrifuged at 800 G for 10 min and the supernatant (containing the mitochondrial fraction) collected for a new centrifugation at 10 000 G for 10 min. The resultant supernatant was discarded, and the mitochondria pellet resuspended with a smooth brush and washed in Heart Washing Buffer (HWB – 250 mM sucrose, 10 mM HEPES, KOH pH=7.4). The suspension was centrifuged at 10 000 G for 10 min and the supernatant discarded. Finally, the pellet (containing the isolated mitochondria) was resuspended in HWB. Between isolation and utilization for bioenergetic assays (max. 3 h), the isolated mitochondria were kept on ice.

2.6.2. **Mitochondrial protein quantification**

The colorimetric biuret method was used to quantify mitochondrial protein[259]. By observing the color development resultant from the reaction of copper sulfate with biuret in alkaline conditions spectrophotometrically, this method allows the quantification of polypeptide chains. To perform the assay, 96 multi-well plates were incubated for 15 min at 22ºC after reaction initiation. Absorbance was measured at a wavelength of 540 nm in Multiskan GO 1500-10 device with SkanItTM Software 3.2 (Thermo Fisher Scientific, Massachusetts, USA). To obtain the final protein concentration, a standard curve of BSA (0 - 0.2 mg/mL) was used.

2.6.3. **Mitochondrial bioenergetics**

2.6.3.1. **Mitochondrial oxygen consumption rates**

The evaluation of mitochondrial respiration allows inferring mitochondria's oxygen consumption rates. Oxygen is the final electron acceptor of the ETC, where at complex IV

(cytochrome c oxidase) this molecule is reduced to H_2O and protons are pumped from the mitochondrial matrix to the intermembrane space.

To assess the cardiac mitochondrial oxygen consumption rates in this work, an Oxygraph (Hansatech Instruments, Norfolk, England) was used. Oxygraph is a Clark-Type oxygen electrode constituted by an anode made of platinum and a cathode, the silver reference electrode. A membrane permeable to oxygen is used to avoid contamination of the platinum electrode. Since the electrolytic oxygen reduction in the platinum electrode is proportional to the oxygen concentration in the medium, the potential generated between the two electrodes may be transferred to a computer that registers the oxygen concentration in the medium. This accelerated oxygen consumption requires frequent re-oxygenation, so the medium needs to be stirred throughout the experiment.

The mitochondrial oxygen consumption rate (OCR) assays were performed using a final mitochondrial protein concentration of 0.5 mg/mL suspended in Heart Reaction Buffer (HRB – 130 mM Sucrose, 10 mM HEPES, 65 mM KCl, 2.5 mM KH_2PO_4, 20 µM EGTA pH=7.4). At this point, the evaluation of state 2 is possible since it represents the basal state in which only endogenous substrates influence O_2 consumption (e.g. ADP, proton leakage). Complex I (NADH dehydrogenase-ubiquinone oxidoreductase) supported O_2 consumption was assessed using glutamate and malate (G/M) as substrates at a concentration of 0.5 mM and 1 mM, respectively. Succinate 1 mM was used as the substrate for complex II (succinate dehydrogenase-ubiquinone oxidoreductase) and Rotenone 1.5 µM was used to inhibit Complex I. To induce a mitochondrial phosphorylative state (state 3), ADP (30 mM) was added to the chamber. State 3 leads to an increase in ETC's activity, stimulated by the injection of ADP and consequent ATP production. After observing the re-establishment of mitochondrial respiration due to the full phosphorylation of the ADP previously added to the chamber (state 4), the maximum mitochondrial O_2 consumption rate was evaluated with the addition of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) at 0.1 mM. FCCP acts as an uncoupler, enabling the passage of protons across the mitochondrial inner membrane and disrupting membrane potential, which stimulates the ECT to reach its maximum electron transport capacity, corresponding to the uncoupled state.

To evaluate the mitochondria's efficiency, two other criteria were taken into consideration in this work: the respiratory control ratio (RCR), which may be calculated by the ratio between state 3 and state 4 and reflects the coupling between substrate oxidation and ADP phosphorylation, giving a notion of mitochondrial coupling efficiency; and the ADP/O ratio, which reflects the amount of oxygen consumed until all the ADP added to the suspension in the chamber is phosphorylated, an important measure to evaluate the mitochondrial phosphorylative system efficiency.

2.6.3.2. **Mitochondrial membrane potential**

Mitochondrial membrane potential was indirectly assessed by measuring the amount of tetraphenylphosphonium (TPP^+) in the medium. Since the mitochondrial matrix becomes negative due to the proton pumping required for ATP synthesis, cationic compounds may be attracted into the matrix. TPP^+ is a lipophilic cation composed of 4 aromatic rings surrounding the phosphor positive center, allowing it to freely diffuse across the membranes. A TPP^+-sensitive electrode containing the ion changer tetraphenylboron and a saturated calomel electrode (SCE) was used as the reference. The electrodes were connected to a potentiometer and the

measurements were registered in a paper chart recorder. Mitochondrial membrane potential assays were performed using a mitochondrial suspension in HRB (0.5 mg/mL). For complex I (NADH dehydrogenase-ubiquinone oxidoreductase) assays, G/M (0.5/1.0 mM, respectively) were used as substrate. Succinate 1 mM was used as the substrate for complex II (succinate dehydrogenase-ubiquinone oxidoreductase) assays, as well as rotenone 1.5 µM. To induce a mitochondrial phosphorylative state ADP (30 mM) was added to the chamber.

A higher mitochondrial membrane potential will lead to the accumulation of TPP^+, allowing the establishment of the mitochondrial maximum membrane potential ($\Delta\Psi$ max). The addition of ADP stimulates ATP production, consequently stimulating the passage of protons through the ATP synthase. This results in a depolarization in mitochondrial membrane potential, observed by increased TPP^+ levels in the medium. To reestablish membrane potential, the ETC increases its activity, and when ADP is fully consumed, repolarization occurs and the mitochondrial membrane potential returns to a basal level, leading to decreased TPP^+ levels in the medium. The time for total ADP phosphorylation is termed lag phase and can compose an indicator of mitochondrial efficiency and coupling.

2.7 **Quantitative polymerase chain reaction**

The expression of transcripts of target genes was assessed using quantitative polymerase chain reaction (qPCR). The cDNA samples previously obtained through reverse-transcription PCR were diluted in DNase/RNase-free water to a final concentration of 10 ng/µL. A pool of all samples was also prepared by mixing equal amounts of each sample, and dilutions of 1:10 and 1:100 were prepared using DNase/RNase-free water. A negative control (Non-Template Control - NTC) in which the cDNA was replaced by RNase-free water was also prepared. The reaction was performed in hard-shell PCR 96-well plates and each well was loaded with 7.5 µL of reaction mix and 2.5 µL of sample. The reaction mix consisted of SsoFast™ EvaGreen Supermix (600 µL), forward primer (6 µL) and reverse primer (6 µL), and DNase/RNase-free water (288 µL).

The plates were incubated in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, California, U.S.A.) and the reaction was set with the software CFX Manager™ 3.1 (Bio-Rad, California, U.S.A.), following the protocol provided at the SsoFast™ EvaGreen Supermix datasheet. The program consisted of 30 sec at 95ºC for polymerase activation, followed by 40 cycles of amplification - 5 sec at 95ºC for denaturation, followed by 5 sec at 55-65ºC for annealing (annealing temperature is variable according to the optimum annealing temperature of each primers pair – **Table 2.3**). A melting curve program for quality control was immediately performed after the cycling program. Fluorescence was detected from each well during the annealing step of each cycle, as well for housekeeping genes, internal controls, controls for efficiency of reverse transcription, PCR and the absence of contaminating genomic DNA. The values were exported to an Excel template file for analysis. Data were normalized for 18S, Beta-Actin and GAPDH housekeeping genes and analyzed with the $\Delta\Delta$Ct method, where Ct is the threshold cycle.

Table 2.3 – List of primers used in the study with the respective sequences and optimal extension temperature.

Gene		Reference	Forward primer	Reverse primer	Optimal Extension ºC
ACAA2	Acetyl-Coa acyltransferase 2	NM_130433	CCTCAGTTCTTGG CTGTTCA	CCACCTCGACGC CTTAAC	63.7
ACOX1	Acyl-CoA oxidase 1	NM_017340.2	GCAGACAGCCAG GTTCTTGATG	ACTCGGCAGGTC ATTCAGGTAT	62.0
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008.4	GTCATCCCAGAG CTGAACGG	ACTTGGCAGGTTT CTCCAGG	62.2
HIF-1α	Hypoxia inducible factor subunit alpha	NM_017008.4	CAAGCAGCAGGA ATTGGAACG	CTCATCCATTGAC TGCCCCA	60.9
POLRMT	Mitochondrial RNA polymerase	NM_001106766.1	GAGACAGGTACC TTCGATCTGG	GGTGGGTTTGTGT GTAGCCA	61.6
PGC-1α	PPAR-γ coactivator 1 alpha	NM_031347.1	GGGACGAATACC GCAGAGAG	CGGCGCTCTTCAA TTGCTTT	61.7
PPAR-α	Peroxisome proliferator activator receptor alpha	NM_013196	AGACTAGCAACA ATCCGCCTTT	TGGCAGCAGTGG AAGAATCG	62.4
RNA18S	18S Ribosomal RNA	NR_046237.1	ACTCAACACGGGAAA CCTC	ACCAGACAAATCGCT CCAC	62.3
VDAC2	Voltage-dependent Anion Channel 2	NM_031354.1	GACACCCGCAGATCA CCTTT	ACATCCAGCTTTACCA ACCCAAA	62.7
β-actin	Beta-actin	NM_031144.3	AGATCAAGATCATTG CTCCTCCT	ACGCAGCTCAGTAAC AGTCC	63.2

2.8 **Protein quantification**

To determine the protein concentration of the tissue lysates obtained after homogenization, the Pierce™ BCA Protein Assay Kit from Thermo Fisher Scientific (Illinois, USA) was used, according to the manufacturer's instructions. This colorimetric assay allows protein detection at a wavelength of 562 nm by a two-step reaction with amino acids of the polypeptide chain (mainly cysteine, cystine, tyrosine and tryptophan). While the first step consists of a normal biuret assay, in which in an alkaline medium Cu^{2+} is reduced, the second consists of a chelation of the BCA agent with the resultant cuprous cation, developing an intense purple color proportional to protein concentration. After a 30 min incubation at 37ºC, the absorbance was measured using a Cytation 3 multi-mode microplate reader (BioTek Instruments, Inc.). To determine protein concentration, a standard curve of BSA (0 to 2 mg/mL) was prepared in the same buffer the heart's tissue was homogenized and both the standards and samples were quantified using duplicates.

2.9 **Western blot**

A piece of the heart tissue with approximately the same mass for every sample was homogenized in 2 cycles of 30 sec using an Ultra-Turrax homogenizer from IKA (Staufen, Germany) at speed 5 using RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 1% Triton X-100) supplemented with 0.5 mM PMSF, 20 mM NaF, 10 mM NAM, 5 mM Sodium Butyrate, 100 mM Orthovanadate, 0.5% DOC, and 2.5% protease inhibitor cocktail (containing 104 mM AEBSF, 80 µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin and 1.5 mM Pepstatin). The lysates were centrifuged at maximum speed for 20 min at 4ºC. The supernatant was collected, and the pellet discarded. After protein quantification, the samples were diluted to a concentration of 1.5 mg/mL with the supplemented RIPA buffer and diluted to a final concentration of 1.25 mg/mL after the addition of 6x Laemmli buffer (18,8% 1 M Tris-HCl pH 6.8, 218 mM SDS, 79% glycerol, 0.44 mM Bromophenol blue and 10% 3 M DTT). The samples were subsequently boiled at 95ºC for 5 min and stored at -20ºC until further use.

Samples (20 µg of protein) were loaded in 10% or 12% acrylamide gels with a Precision Plus Protein Dual Color Standard running in parallel with the samples. For protein separation, electrophoresis was performed at room temperature (RT) with running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS), at a constant current intensity (30 mA per gel) in a Mini-PROTEAN tetra Cell (Bio-Rad) for approximately 90 min. The separated proteins were then transferred to PVDF membranes in a Trans-Blot Cell (Bio-rad). To that end, the PVDF membranes were activated for 1 min in methanol, followed by 5 min in ddH_2O and 15 min in 4ºC transfer buffer (190 mM glycine, 25 mM Tris, 20% methanol and 0.001% SDS). The transfer process occurred for 100 min at a constant current intensity of 90 V. The transfer quality was assessed using a Ponceau solution (0.1% Ponceau, 5% acetic acid) for protein staining.

Afterwards, the membranes were blocked in 5% BSA in TBS-T buffer (Tris 20 mM pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 hour at RT. Following 3 washing steps with TBS-T for 5 minutes, the membranes were incubated with the primary antibody (**Table 2.4**) overnight, on a roller at 4ºC. The antibodies were diluted in TBS-T and used according to the starting dilution recommended in the antibody's datasheet and adjusted if needed. The membranes were washed 3 times again and subsequently incubated with the respective secondary antibody (**Table 2.5**) prepared in TBS-T buffer for 2 hours on a roller at RT.

Table 2.4 – List of primary antibodies used in the present study.

Protein		Company	Reference	Host	MW (kDa)	Dilution
Akt	Protein kinase B	Cell Signaling	4691	Rabbit	60	1:1000
AMPKα	AMP-activated protein kinase	Cell Signaling	2603	Rabbit	62	1:1000
ANT	Adenine nucleotide translocator	Santa Cruz	9300	Goat	33	1:1000
Bad	Bcl2 associated agonist	Cell Signaling	9292	Rabbit	23	1:500

Bcl2	β-cell lymphoma 2	Cell Signaling	2870	Rabbit	26	1:1000
Cardiac Troponin T	-	Cell Signaling	5593	Rabbit	40	1:1000
CD36	Platelet glycoprotein 4	Santa Cruz	7309	Mouse	88	1:1000
DRP1	Dynamin 1 like protein	BD Bio Sciences	611113	Mouse	79-84	1:1000
Fis1	Mitochondrial fission 1 protein	Santa Cruz	B76447	Mouse	17	1:1000
GLUT-4	Glucose transporter 4	Santa Cruz	53566	Mouse	50-63	1:1000
GPx4	Glutathione peroxidase 4	Santa Cruz	166570	Mouse	21	1:500
GSK3 α/β	Glycogen Synthase Kinase 3 β	Santa Cruz	7291	Mouse	47, 51	1:1000
IL-6	Interleukin-6	Santa Cruz	28343	Mouse	21	1:1000
IRS-1	Insulin receptor substrate 1	Santa Cruz	80038	Mouse	170-185	1:1000
JNK	c-Jun N-terminal kinase	Santa Cruz	7345	Mouse	46, 54	1:1000
LC-3	Microtubule-associated protein 1A/1B-light chain 3	MBL International	PD014	Rabbit	16, 18	1:500
Nrf2	Nuclear factor erythroid 2-related factor 2	Santa Cruz	365949	-	61	1:1000
OPA1	Dynamin-like 120 kDa protein	Santa Cruz	30573	Goat	120	1:1000
OXPHOS	Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, Complex IV subunit II, and ATP synthase subunit alpha	Abcam	110411	Mouse	18, 22, 29, 48, 54	1:500
p70	Ribosomal protein S6 kinase beta-1	Santa Cruz	8418	-	70	1:1000
p-Akt (Ser473)	Protein kinase B	Cell Signaling	2965	Rabbit	60	1:500

p-Akt (Thr308)	Protein kinase B	Cell Signaling	4060	Rabbit	60	1:500
p-AMPKα (Thr172)	AMP-activated protein kinase	Cell Signaling	2531	Rabbit	62	1:1000
p-Bad (Ser112)	Bcl2 associated agonist	Cell Signaling	9296	Mouse	23	1:1000
p-Bcl2 (Ser70)	Bcl2 associated agonist	Cell Signaling	2827S	Rabbit	28	1:1000
PDK	Pyruvate dehydrogenase kinase	Santa Cruz	28783	Rabbit	60	1:1000
p-GSK3 α/β (Ser21/9)	Glycogen Synthase Kinase 3 β	Cell Signaling	9327	Rabbit	46, 51	1:500
p-JNK (Thr183, Tyr185)	c-Jun N-terminal kinase	Santa Cruz	6254	Mouse	46, 54	1:1000
p-p70 (Thr389)	Ribosomal protein S6 kinase beta-1	Cell Signaling	9205S	Rabbit	70	1:1000
SOD2	Superoxide dismutase 2	Abcam	16956	Mouse	25	1:1000
TFAM	Mitochondrial transcription factor A	Santa Cruz	23588	Goat	25	1:500
TNF-α	Tumor necrosis factor alpha	Santa Cruz	12744	Mouse	17, 26	1:1000
Tom20	Mitochondrial import receptor Tom20	Santa Cruz	11415	Rabbit	20	1:1000
β-catenin	-	Cell Signaling	9582	Rabbit	92	1:1000

Table 2.5 – List of secondary antibodies used in the present study.

Antibody	Company	Description	Reference	Dilution
Anti-Goat	Santa Cruz	mouse@goat	2354	1:2000
Anti-Mouse	Cell Signaling	horse@mouse	7076S	1:2000
Anti-Rabbit	Cell Signaling	goat@rabbit	7074S	1:2000

After incubation with the secondary antibody, the membranes were washed 3 times with TBS-T. For band detection, the membranes were incubated with Clarity Western ECL Substrate (Bio-Rad) for 5 min. ECL is a chemiluminescent substrate that reacts with the horseradish peroxidase

conjugated to the secondary or primary antibody and oxidizes, generating luminescence. The images were collected with a Gel Documentation System Imager (VWR) and analyzed with the TotalLab software (Nonlinear Dynamics, Newcastle, UK), using the background subtraction method "Rolling Ball" with a radius of 50. Band density was used to compare the protein levels between the different lanes and the results were normalized by Ponceau staining and are reported relative to the mean of the P-C-S group normalized to 1.

2.10 **Data and statistical analysis**

This work focused on understanding the impact of exercise practice exclusively during pregnancy on the cardiac effects induced by a GDM-pregnancy in maternal mitochondria metabolism. Furthermore, the role of pregnancy *per se* on different cardiac parameters was also assessed. All data were analyzed using GraphPad Prism 8.0.2 (GraphPad Software, Irvine, CA, USA) and the results are expressed as median, 1st quartile (Q1) and 3rd quartile (Q3), with four significant digits. Some results may be expressed as mean and standard deviation (SD).

Gestational diabetes mellitus-induced alterations and the implication of exercise were measured by comparison of P-C-S (Pregnant Control Sedentary), GDM-S (Gestational Diabetes Mellitus Sedentary), and GDM-E (Gestational Diabetes Mellitus Exercised). The physiologic role of pregnancy on the maternal heart was evaluated by comparison of NP-C-S (Non-Pregnant Control Sedentary) and P-C-S. Comparisons between all the groups were also performed, allowing to understand the mechanisms that may trigger cardiac dysfunction not only by pregnancy itself but also by the combination with other interventions compared to the control group (NP-C-S group). The data's distribution was assessed using the Shapiro-Wilk normality test and presented a normal distribution when $\alpha \leq 0.05$. If data had a normal distribution the parametric unpaired t-test was performed. However, the non-parametric Mann-Whitney test was performed if data were not normally distributed.

In all statistical tests, values of $p \leq 0.05$ were considered statistically significant, with the symbol * representing different statistical relevance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

Data regarding the animal model morphological characterization and plasma biochemical parameters have already been published in a study by Stevanović-Silva et. al, 2021[260] that evaluated the effects of maternal HFHS-diet consumption and the practice of exercise on hepatic fat accumulation and liver mitochondrial respiratory capacity in the mothers and male offspring in the same animal study. Here, data will be analyzed in the context and according to the aim of the work to enable a better discussion of the obtained results. Furthermore, as this study is the continuation of previous work, some of the molecular pathways involved in the impact of exercise during a GDM pregnancy have been presented in Rodrigues, 2020[261]. When previously obtained data is used it clearly is mentioned in the respective figure legend. In this work, an integrative analysis of all data was performed to build up evidence and accomplish a better answer to our hypothesis.

Chapter 3 – Results

This chapter includes material from an original paper that was previously published in the journal Metabolism: Clinical and Experimental and is referred below:

Jelena Stevanović Silva, Jorge Beleza, Pedro Coxito, Susana Pereira, Hugo Rocha, Tiago Bordeira Gaspar, Fátima Gärtner, Rossana Correia, Maria João Martins, Tiago Guimarães, Sandra Martins, Paulo Oliveira, António Ascensão, José Magalhães (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: Clinical and Experimental. https://doi.org/10.1016/j.metabol.2021.154704

3.1 **Establishment of a gestational diabetes mellitus model**

Currently, the OGTT is the chosen method used to diagnose GDM. By evaluating the organism's capacity to metabolize glucose, this screening test helps to infer a diabetic state or even an already acquired risk for diabetes development. This method presents a great advantage compared to the well-known finger prick test, which also allows the determination of blood glucose levels but lacks sensitivity. The area under the curve (AUC) can be calculated to measure the total increase in blood glucose during the whole screening test, being often used alongside the OGTT.

In this study, OGTTs were performed before mating protocols were implemented (pre-pregnancy), and at mid-pregnancy (**Figure 3.1**). No alterations in glucose metabolization were observed between the groups fed a normal chow diet (C) or a HFHS diet (HFHS) before mating (**Figure 3.1A**), as specified by the AUC values ($p = 0.4305$) (**Figure 3.1B**). In the mid-pregnancy period, the OGTTs revealed that female rats fed a HFHS diet and subjected either to a sedentary lifestyle (HFHS-S) or to an exercise plan (HFHS-E) had impaired glucose metabolization, being the impairment more pronounced in the HFHS-S group as shown by the AUC values (HFHS-S vs C-S: 13991 ± 435 vs 12132 ± 395, $p = 0.0089$ and HFHS-E vs C-S: 13571 ± 330 vs 12132 ± 395, $p = 0.0193$) (**Figure 3.1D**). In the HFHS-S group, glucose utilization was impaired during the first 60 minutes (HFHS-S vs C-S: 15 min, $p < 0.0001$; 30 min, $p = 0.0009$; 60 min, $p = 0.0048$), while in the HFHS-E group only at minute 15 a significant difference was observed (HFHS-E vs C-S: $p < 0.0001$) (**Figure 3.1C**).

3.1.1 **Impact of HFHS diet consumption in female rats' body and tissues mass**

The body weights of the female rats were measured throughout the whole experiment. After implementing the mating protocols (15-weeks of age) the pregnant rats' weight increased in comparison with the NP-C-S group, and this difference persisted until the end of the nursing period (19-weeks of age) (**Figure 3.2**). The major differences in body mass were observed before delivery (16-weeks of age), when the weights of all the pregnant groups were significantly increased in comparison with the NP-C-S group (P-C-S vs NP-C-S, GDM-S vs NP-C-S, and GDM-E vs NP-C-S $p < 0.0001$), and also at 18-weeks of age, during the nursing period, for the P-C-S group (P-C-S vs NP-C-S $p = 0.0014$) (**Figure 3.2A**). Furthermore, no differences were observed in body weight between the groups at euthanasia, neither between the pregnant groups

(GDM-S vs P-C-S p = 0.8297, GDM-E vs P-C-S p = 0.3979, GDM-E vs GDM-S p = 0.2773) nor between the NP-C-S and P-C-S groups (p = 0.6073) (**Figure 3.2B**).

It has been described that an increased gestational weight gain (GWG) is associated with a higher risk of GDM development. In our animal model, the GWG of the diabetic female dams with a sedentary lifestyle was substantially higher than the GWG observed in the control dams (GDM-S vs P-C-S: median = 156.5, Q1 = 141.0, Q3 = 169.3 vs median = 127.0, Q1 = 78.0, Q3 = 130.0; p = 0.006). Exercise practice during pregnancy contributed to the prevention of the excess GWG induced by GDM (GDM-E vs GDM-S: median = 120.5, Q1 = 116.8, Q3 = 129.3 vs median = 156.5, Q1 = 141.0, Q3 = 169.3, p = 0.0042), culminating in similar GWG values when comparing to the P-C-S group (GDM-E vs P-C-S: p = 0.3314) (**Figure 3.2C**)

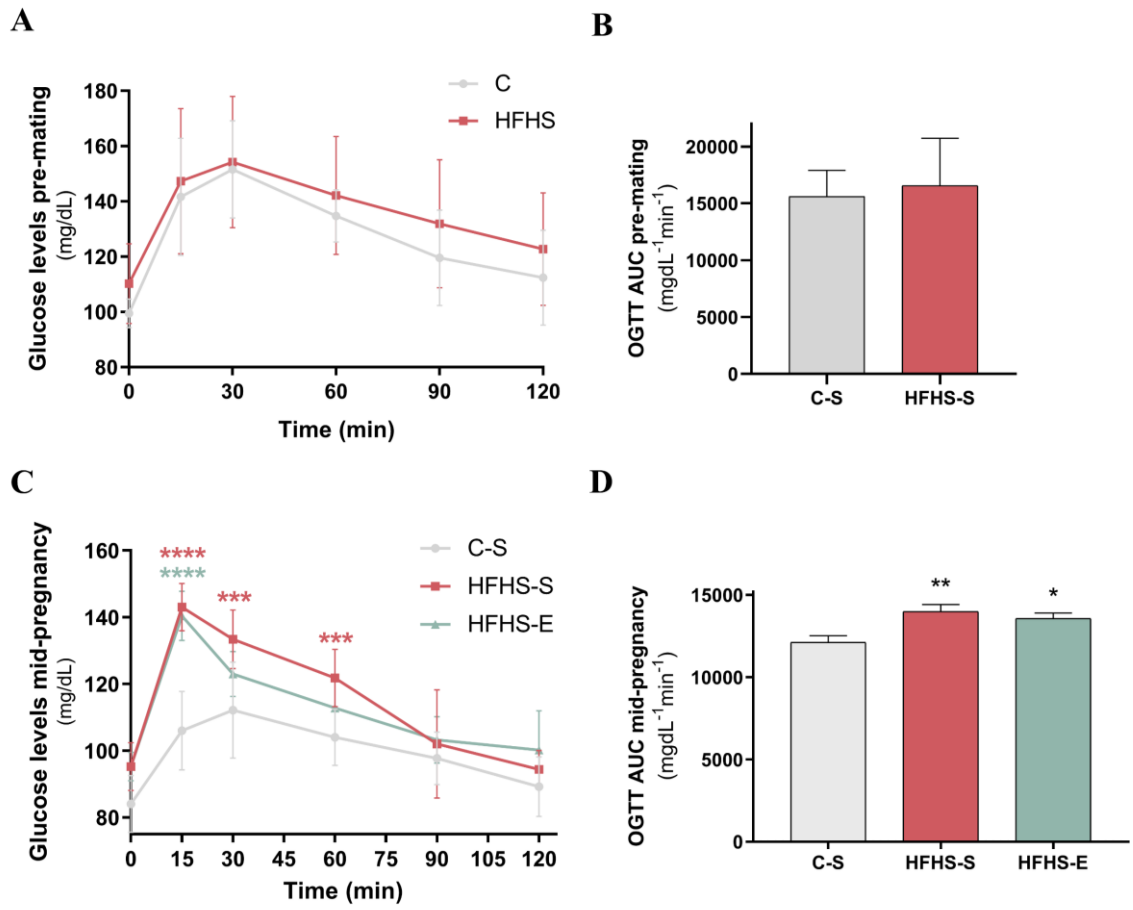

Figure 3.1 – Modulation of glucose metabolization capacity in response to a high-fat-high-sugar diet. Oral glucose tolerance test (OGTT) performed at A: pre-mating in the female rats fed a control (C-S) and high-fat-high-sugar (HFHS-S) diet, and respective B: area under the curve (AUC) and OGTT performed at C: mid-pregnancy in the female rats fed a control (C-S) and high-fat-high-sugar diet sedentary (HFHS-S) or subjected to exercise (HFHS-E) intervention during pregnancy, and respective D: area under the curve (AUC).
Statistical analysis: Comparison between the experimental groups was performed using multiple t tests corrected for multiple comparisons with the Holm-Sidak method for A and C and using unpaired t test in B and D. p-values lower than 0.05 were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$ vs C-S, *** $p \leq 0.001$ HFHS-S vs C-S, **** $p \leq 0.0001$ HFHS-S vs C-S, **** $p \leq 0.0001$ HFHS-E vs C-S). Grey line or bar, C-S; red line, HFHS; red bar HFHS-S, green line or bar, HFHS-E. Data are represented as mean and standard deviation. *Results previously published in Stevanović-Silva, 2021.*

Since some alterations may not be overall morphologically or morphometrically perceptible but can induce alterations in specific tissues, the weights of the heart, brain, and liver were assessed in the different experimental groups. No major differences were observed between the groups, although mothers that had GDM and lived a sedentary lifestyle tendentially had lower heart weights (GDM-S vs P-C-S: median = 0.945, Q1 = 0.911, Q3 = 1.016 vs median = 1.005, Q1 = 0.971, Q3 = 1.114, p = 0.0775) (**Figure 3.3A**). However, this was not reflected in the heart-to-brain ratio (**Figure 3.3C**) even though brain weight was similar between the groups (**Figure 3.3B**). Exercise practice during pregnancy led to increased liver weight comparing to the other experimental groups, especially to the non-pregnant female rats (NP-C-S) and the pregnant control group (P-C-S) (GDM-E vs NP-C-S: median = 10.55, Q1 = 9.725, Q3 = 11.30 vs median = 9.464, Q1 = 8.965, Q3 = 10.34, p = 0.0358 | GDM-E vs P-C-S: median = 9.563, Q1 = 8.763, Q3 = 10.21 vs median = 9.464, Q1 = 8.965, Q3 = 10.34, p = 0.0624) (**Figure 3.3D**).

3.1.2 **Immediate impacts of maternal habits in the offspring**

Maternal metabolic alterations throughout pregnancy may induce adaptations during fetal development that may affect the offspring. The immediate effects of maternal GDM and exercise practice during GDM pregnancy were assessed.

The pregnancy complicated by GDM led to an increase in litter size that was not totally prevented by the exercise intervention used during pregnancy (GDM-S vs P-C-S: median = 15.50, Q1 = 13.00, Q3 = 13.75 vs median = 12.00, Q1 = 11.00, Q3 = 15.00; p = 0.0291; and GDM-E vs P-C-S: median = 15.00, Q1 = 13.75, Q3 = 16.00 vs median = 12.00, Q1 = 11.00, Q3 = 15.00; p = 0.0314) (**Figure 3.4A**). Regarding the offspring's sex distribution, GDM-S mothers delivered more male pups comparing to the P-C-S, an effect that was prevented by exercise practice during pregnancy (GDM-S vs P-C-S (♂): median = 8.50, Q1 = 8.00, Q3 = 9.25 vs median = 6.00, Q1 = 2.00, Q3 = 8.00; p = 0.0321 | GDM-E vs GDM-S (♂): p = 0.5047) (**Figure 3.4B**).

3.2 **Effects of GDM and exercise practice on biochemical parameters**

Pregnancy is already accompanied by physiological dyslipidemia required for normal fetal growth and development, but GDM is accompanied by enlarged lipid profiles and exacerbated dyslipidemia. The effect of exercise during pregnancy on maternal lipid profiles is scarce, and the traits left by GDM in the postpartum period. The plasma levels of lipids (TGs, HDL, LDL, total cholesterol) of all groups were assessed, and capillary and plasma glucose levels and other biochemical parameters (see **Figure 3.5** and **Table 3.1**).

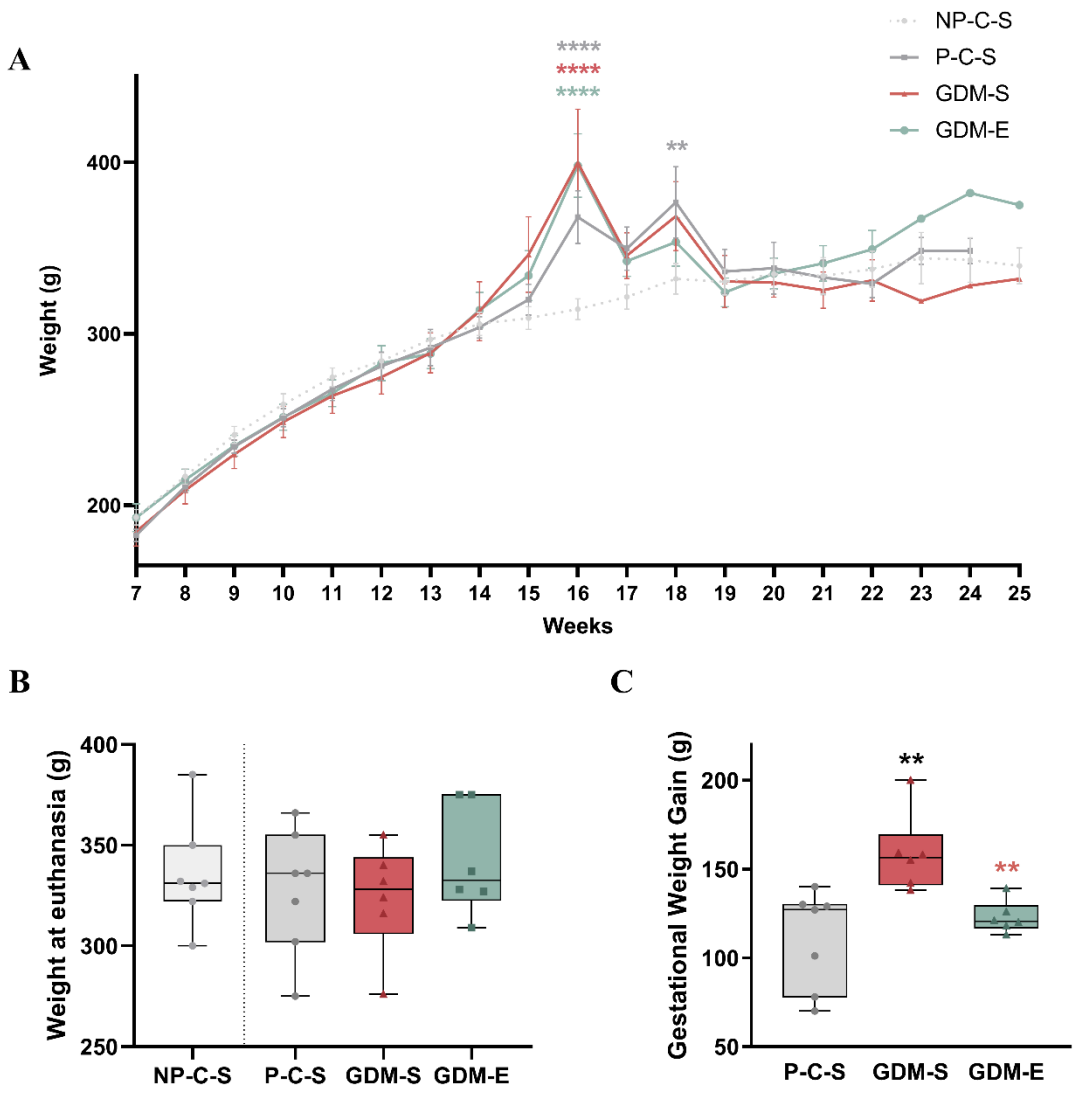

Figure 3.2 – Morphological characteristics of the female rats in response to the different interventions: non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy and sedentary lifestyle (GDM-S), and GDM-pregnancy and exercise intervention (GDM-E) groups. A: Body weight of the female rats throughout the whole experiment. B: Body weight of the female rats at euthanasia. C: gestational weight gain from the first day of pregnancy until delivery.
Statistical analysis: Comparison between the experimental groups was performed using multiple t tests corrected for multiple comparisons with the Holm-Sidak method for A and using unpaired t test (after performing Shapiro-Wilk normality test) in B and C. p-values lower than 0.05 were considered significant (** $p \leq 0.01$ P-C-S vs NP-C-S, ** $p \leq 0.01$ GDM-S vs P-C-S, ** $p \leq 0.01$ GDM-E vs GDM-S, **** $p \leq 0.0001$ P-C-S vs NP-C-S, **** $p \leq 0.0001$ GDM-S vs NP-C-S, **** $p \leq 0.0001$ GDM-E vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green line or bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Results previously published in Stevanović-Silva, 2021.*

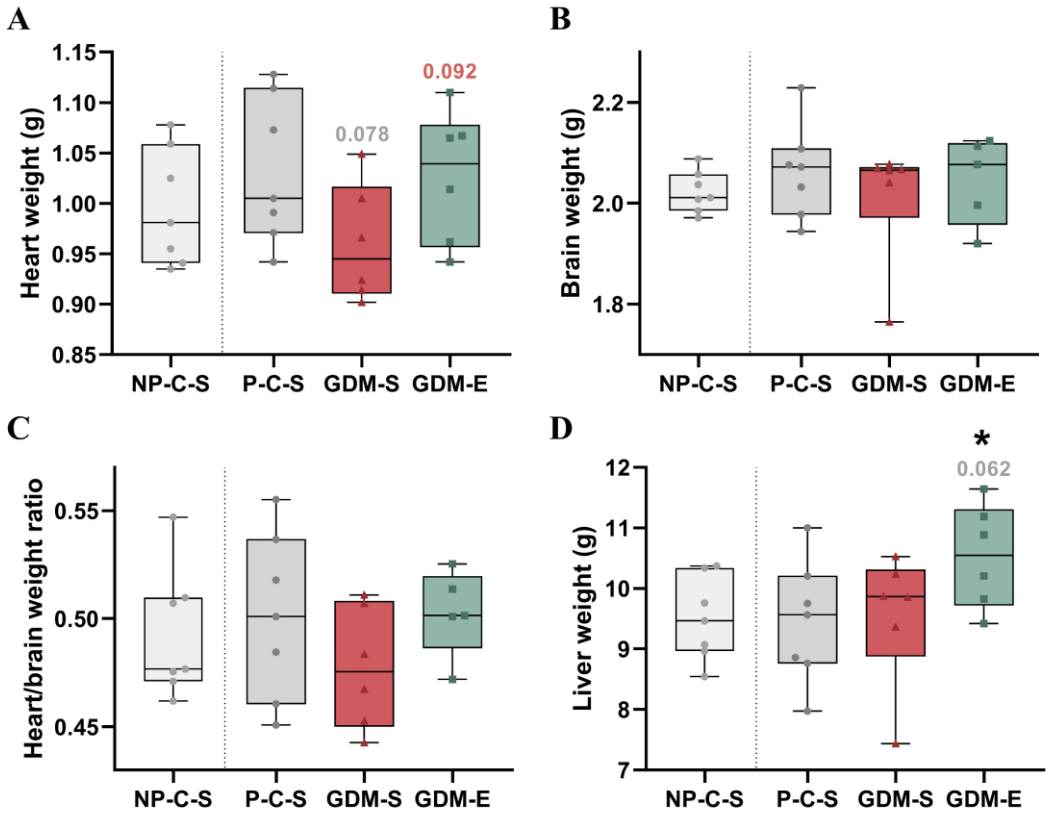

Figure 3.3 – Tissue weights of the non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. A: heart weight. B: brain weight. C: heart-to-brain weight ratio. D: liver weight.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, C, and D, and using Mann-Whitney test in B. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-E vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum.

The capillary glucose levels evaluated using the tail prick test show that the pregnant control group has tendentially decreased glucose levels in comparison with its non-pregnant counterpart (P-C-S vs NP-C-S: median = 100.0, Q1 = 96.0, Q3 = 107.0 vs median = 106.0, Q1 = 103.8, Q3 = 116.5, p = 0.0793), and that mothers subjected to an exercise plan during a GDM pregnancy reach high capillary blood glucose levels comparing to its control group (GDM-E vs P-C-S: median = 111.5, Q1 = 104.0, Q3 = 121.3 vs median = 100.0, Q1 = 96.0, Q3 = 107.0, p = 0.0306) (**Figure 3.5A**) On the other hand, the levels of glucose in the plasma evaluated by MS/MS revealed dissimilar results. Plasma glucose levels of the GDM-S group were increased comparing to the P-C-S group (GDM-S vs P-C-S: median = 234.7, Q1 = 217.3, Q3 = 244.5 vs median = 191.0, Q1 = 175.0, Q3 = 209.0; p = 0.0045) and exercise contributed to the prevention of this increase, presenting glucose levels close to the P-C-S (GDM-E vs GDM-S: median = 208.0, Q1 = 195.3, Q3 = 217.3 vs median = 234.7, Q1 = 217.3, Q3 = 244.5; p = 0.0095 | GDM-E vs GDM-S: p = 0.2358). However, the physiological pregnancy induced decreased glucose levels that remained when compared with its non-pregnant counterpart (P-C-S vs NP-C-S: median = 191.0, Q1 = 175.0, Q3 = 209.0 vs median = 226.0, Q1 = 208.0, Q3 = 232.0; p = 0.0181) (**Figure 3.5B**).

Regarding the levels of circulating lipids in the plasma, no significant differences were observed between groups for the different parameters evaluated (**Figure 3.5**), except for the increased TGs levels (**Figure 3.5G**) in GDM-S mothers concerning the TGs levels detected in the P-C-S group (GDM-S vs P-C-S: median = 81.40, Q1 = 75.0, Q3 = 89.0 vs median = 65.5, Q1 = 64.0, Q3 = 73.4; p = 0.0022). The increase of TGs levels observed in GDM-S was prevented by the practice of exercise during GDM pregnancy (**Figure 3.5G**). The atherogenic index of plasma (AIP), calculated by the ratio of TGs and HDL levels and considered a strong predictor of CVD risk development, was also determined although no differences were observed between the experimental groups (**Figure 3.5F**). The levels of other biochemical parameters were measured to assess the general state of health, providing information on how certain organs, such as the heart, liver, and muscle are working (**Table 3.1**).

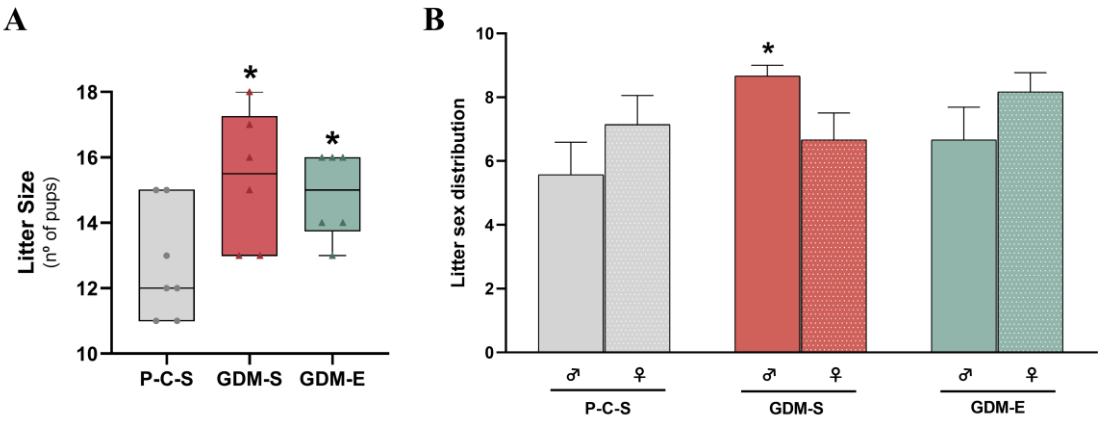

Figure 3.4 – Characteristics of the offspring. A: litter size. B: sex distribution of the litter. Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, and Mann-Whitney test in B. p-values lower than 0.05 were considered significant (***** $p \leq 0.05$ GDM-S vs P-C-S). Grey bar, offspring from P-C-S; red bar offspring from GDM-S, green bar, offspring from GDM-E. In B plain bars represent male and dotted bars represent female. Data are represented as median, interquartile distance, minimum and maximum in A and as mean and SD in B. *Adapted from Rodrigues, 2020.*

Table 3.1 – Plasma biochemical characterization of the experimental groups. Comparison between groups was performed using unpaired t test (after performing Shapiro-Wilk normality test). p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-E vs P-C-S, ** $p \leq 0.01$ GDM-E vs NP-C-S, ** $p \leq 0.01$ GDM-S vs P-C-S, *** $p \leq 0.001$ GDM-S vs NP-C-S). Data are represented as median (Q1, Q3). *AST and ALT previously published in Stevanović-Silva, 2021.*

	NP-C-S	P-C-S	GDM-S	GDM-E
AST (U/L)	72.20 (67.00, 83.00)	84.00 (57.00, 91.00)	77.50 (63.50, 101.00)***, **	82.25 (58.75, 104.50)**, *
ALT (U/L)	22.83 (21.00, 24.00)	23.40 (20.00, 27.00)	29.00 (27.00, 31.00)	28.50 (26.00, 32.00)
Urea (mg/mL)	0.32 (0.29, 0.36)	0.32 (0.28, 0.34)	0.32 (0.29, 0.35)	0.30 (0.27, 0.35)
Creatinine (mg/mL)	1.00 (0.90, 1.12)	1.00 (0.87, 1.06)	0.98 (0.89, 1,08)	0.92 (0.84, 1.08)
Creatine kinase (U/L)	0.98 (0.71, 1.20)	1.15 (0.62, 1.32)	1.03 (0.65, 1.48)	0.95 (0.65, 1.30)

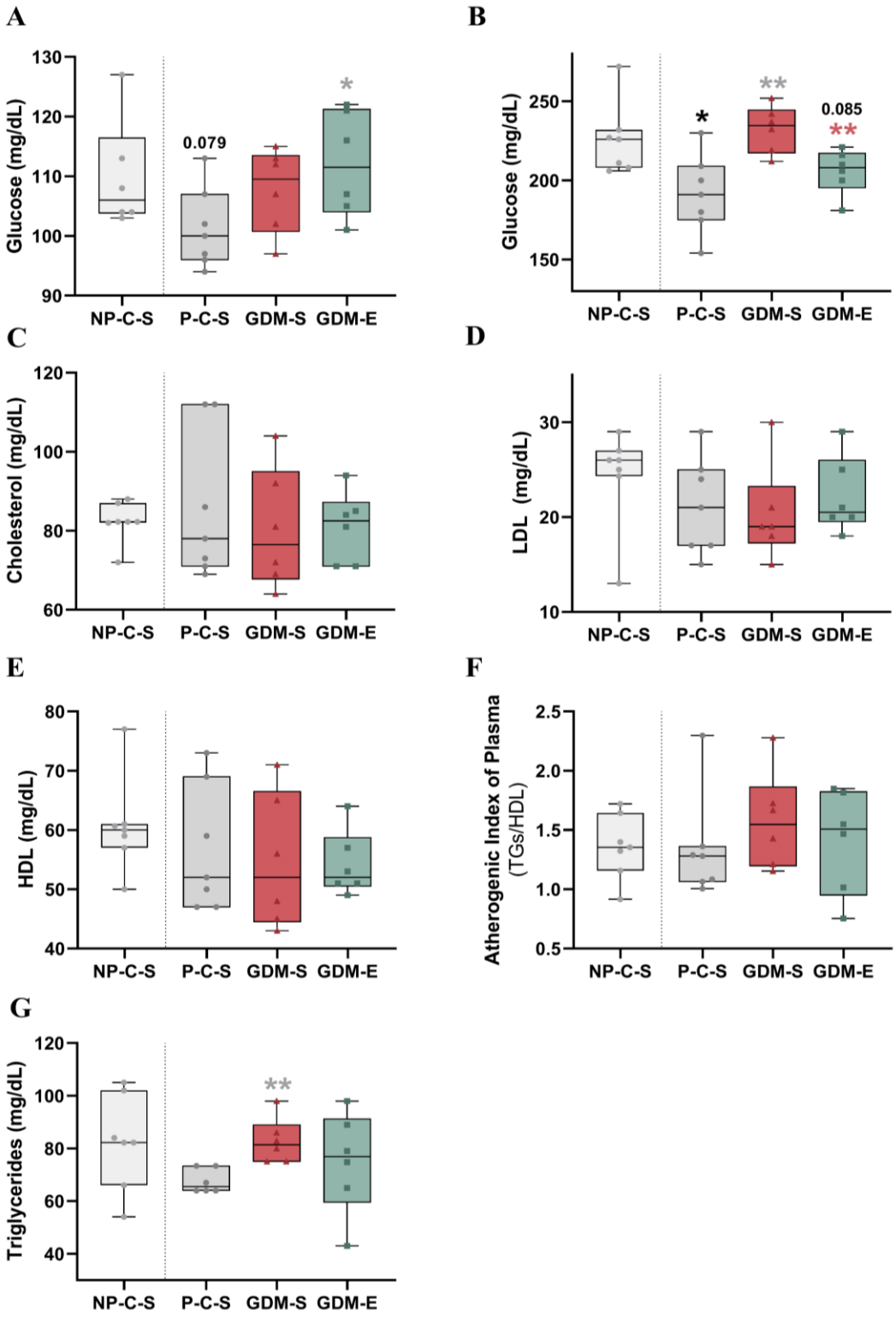

Figure 3.5 – Blood biochemical characterization of the experimental groups. A: capillary blood glucose levels. B: plasma glucose levels. C: plasma cholesterol levels. D: plasma low-density lipoprotein (LDL) levels. E: plasma high-density lipoprotein (HDL) levels. F: atherogenic index of plasma (AIP). G: plasma triglycerides levels.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, B, and E and Mann-Whitney test in C, D, F, and G. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S vs NP-C-S, * $p \leq 0.05$ GDM-E vs P-C-S, ** $p \leq 0.01$ GDM-S vs P-C-S, ** $p \leq 0.01$ GDM-E vs GDM-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *B, C, D, E, G adapted from Stevanović-Silva, 2021.*

3.3 **The impact of GDM in the cardiac perivascular matrix**

The histological analysis of the cardiac tissue allows the morphological characterization of the heart and the evaluation of a series of parameters essential to assess possible fibrosis and hypertrophy development. The perivascular matrix surrounding the cardiac blood vessels is essential for cardiac function and loss of its integrity is associated with atherosclerotic processes, a common cardiovascular complication.

In this study, the H&E staining of the tissue revealed no differences in heart morphology between the experimental groups. The cardiac cells present similar shapes and sizes (**Figure 3.6**). Although H&E allows the visualization of the perivascular matrix (empty blank space surrounding the vessels) it does not stain its components which allow its evaluation. One of the main components of the perivascular matrix is collagen. In order to detect collagen deposition in the cardiac tissue of the female rats, the Masson's Trichrome staining was used, and mild enlargement of the cardiac perivascular matrix was observed in the GDM-S group compared to both controls (NP-C-S and P-C-S), perceived by the intense blue color surrounding the blood vessels of the myocardium. This effect did not seem to be prevented by the practice of exercise during pregnancy (GDM-E) (**Figure 3.6**). The results were corroborated by the vimentin immunohistochemistry analysis, which also resulted in more intense staining, marked by the brown color surrounding the vessels, especially in the GDM groups (**Figure 3.6**). Vimentin is an interfilamentous protein of the cardiac tissue, which increased expression is frequently associated with cardiac fibrosis.

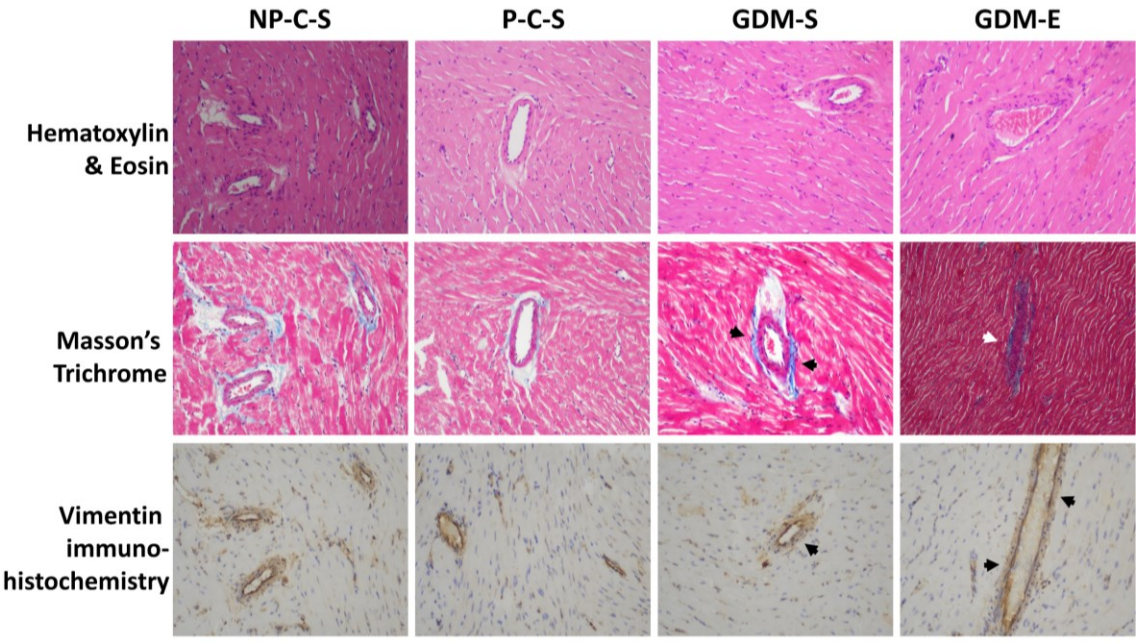

Figure 3.6 – Cardiac morphological characterization by histological analysis of the tissue of the non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups (in the columns). Technique used in the lines: first line – hematoxylin and eosin staining (H&E), second line – Masson's trichrome staining, third line – immunohistochemical staining using vimentin. Black and white arrows point to stronger stains of the perivascular matrix.
Amplification (x200). Assessment performed by a certified pathologist without quantification.

3.4 **Effect of pregnancy and GDM on cardiac inflammation and injury markers**

A diabetic state is normally accompanied by a low-grade systemic inflammatory state that consequently promotes inflammatory processes in the heart. This is also observed in GDM. The levels of different proteins used as markers of cardiac damage (cardiac troponin T, **Figure 3.7A**) and inflammation processes were evaluated (**Figure 3.7**). No differences were observed between the experimental groups for cardiac troponin T levels (**Figure 3.7A**). The protein levels of the inflammatory markers IL-6 and TNF-α were decreased in the exercised GDM group (GDM-E) comparing to the GDM-S group (GDM-E vs GDM-S (IL-6): median = 0.8642, Q1 = 0.5878, Q3 = 0.9861 vs median = 1.031, Q1 = 0.959, Q3 = 1.337, p = 0.0315 | GDM-E vs GDM-S (TNF-α): median = 0.787, Q1 = 0.154, Q3 = 0.989 vs median = 0.1.233, Q1 = 0.962, Q3 = 1.329, p = 0.0149) (**Figure 3.7C and 3.7B**). Furthermore, the physiologic process of pregnancy contributed to increased levels of JNK isoform 54 comparing with its non-pregnant counterpart group (P-C-S vs NP-C-S: median = 0.992, Q1 = 0.552, Q3 = 1.461 vs median = 0.433, Q1 = 0.290, Q3 = 0.695, p = 0.0236) (**Figure 3.7D**). No differences were observed between the experimental groups for the isoform 46 of the JNK protein, both in the total protein (**Figure 3.7E**) and in the ratio between its phosphorylated (activated) form and total protein levels (**Figure 3.7F**).

3.5 **Implications of GDM on the insulin signaling pathway**

Insulin resistance is one of the hallmarks of GDM and is also a physiological response observed during pregnancy, noting that insulin sensitivity should improve after delivery, reaching a physiologic response. Therefore, the levels of proteins involved in the insulin signaling pathway and the effects of pregnancy *per se*, GDM, and exercise during GDM pregnancy were evaluated in the present study.

The experimental interventions applied to the female rats did not exert any effect in the cardiac levels of the IRS-1 protein (**Figure 3.8A**). Furthermore, the levels of cardiac total Akt were increased due to pregnancy (P-C-S) comparing to its non-pregnant counterpart group (P-C-S vs NP-C-S: median = 1.026, Q1 = 0.864, Q3 = 1.123 vs median = 0.606, Q1 = 0.468, Q3 = 0.845; p = 0.0070) and decreased in the GDM-S group comparing to the P-C-S (GDM-S vs P-C-S: median = 0.461, Q1 = 0.375, Q3 = 0.727 vs median = 1.026, Q1 = 0.864, Q3 = 1.123; p = 0.0026). The practice of exercise during GDM pregnancy also contributed to tendentially decreased levels of cardiac Akt (GDM-E vs P-C-S: median = 0.577, Q1 = 0.412, Q3 = 1.031 vs median = 1.026, Q1 = 0.864, Q3 = 1.123; p = 0.0518) (**Figure 3.8B**). Increased values of the ratio between Akt phosphorylated form in residue Ser473 and total Akt (p-Akt/Akt) were observed for the GDM groups (GDM-S vs P-C-S: median = 2.160, Q1 = 1.484, Q3 = 3.157 vs median = 1.106, Q1 = 0.692, Q3 = 1.486; p = 0.0270 | GDM-E vs P-C-S: median = 1.807, Q1 = 1.576, Q3 = 3.472 vs. median = 1.106, Q1 = 0.692, Q3 = 1.486; p = 0.0296) (**Figure 3.8C**). That behavior was not observed for the ratio between phosphorylated Akt on residue Thr308, although the GDM-S group showed tendential increased levels in comparison with the P-C-S group (GDM-S vs P-C-S: median = 1.790, Q1 = 1.225, Q3 = 2.691 vs median = 1.087, Q1 = 0.620, Q3 = 1.533; p = 0.0788) (**Figure 3.8D**).

Additional analysis revealed that the levels of cardiac p70 protein and the ratio between its phosphorylated form and total protein were unaltered between the groups (**Figure 3.8E and 3.8F**), which was also observed for the levels of the GLUT-4 protein (**Figure 3.8G**).

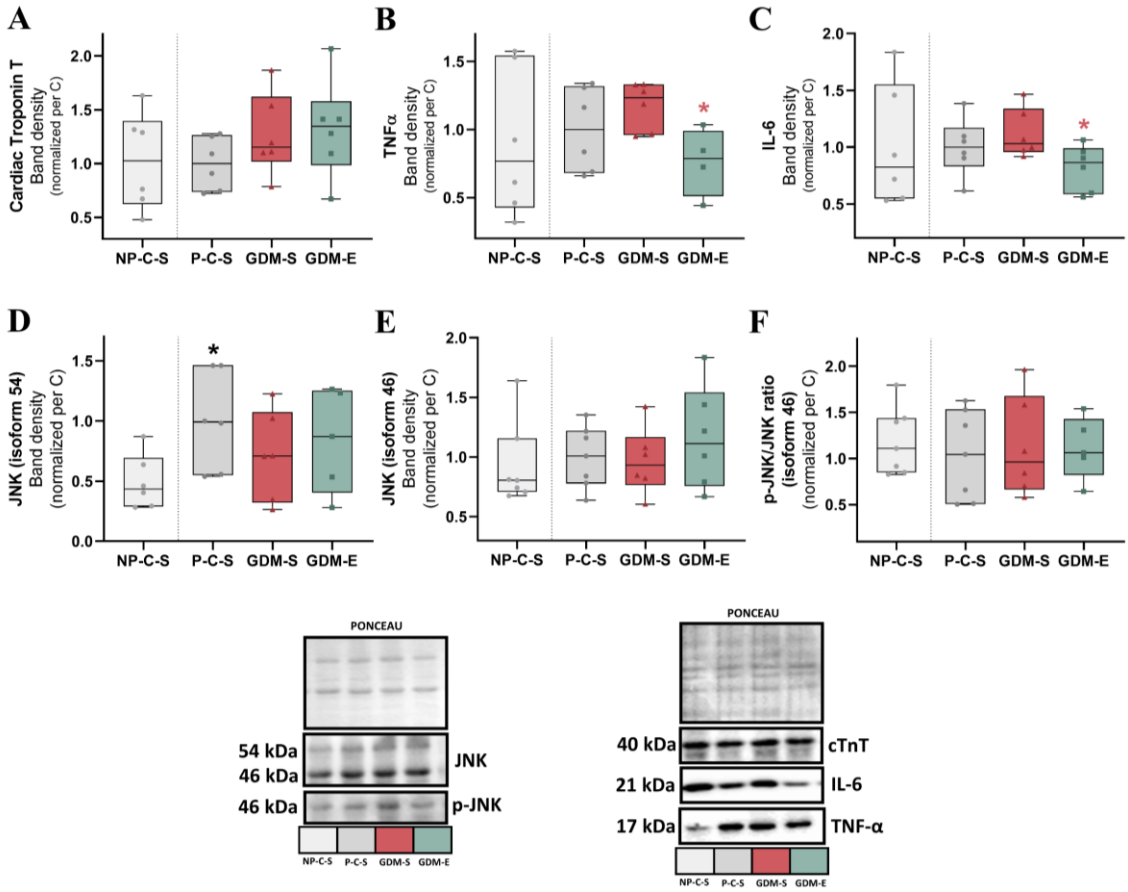

Figure 3.7 – Protein levels of damage and inflammation markers in the cardiac tissue of non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. A: cardiac troponin T. B: tumor necrosis factor alpha (TNF-α). C: interleukin six (IL-6). D: c-Jun N-terminal kinase (JNK) isoform 54. E: JNK isoform 46. F: ratio of phosphorylated JNK (p-JNK) at residues Thr183 and Tyr185 to total protein JNK. Data were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test). p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S vs NP-C-S, * $p \leq 0.05$ GDM-E vs GDM-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *A, B, C adapted from Rodrigues, 2020.*

3.5.1 **Effects of GDM pregnancy and exercise during GDM on Akt-downstream anabolic program**

Besides Akt's role on insulin signaling, this protein has a central role in other cellular mechanisms, being involved in cell proliferation, growth, survival, metabolism, among other critical cellular functions[262]. A known downstream target of Akt is one of the proteins involved in the cellular anabolic program, GSK3. This protein has two isoforms, GSK3-α and GSK3-β. The levels of both isoforms were evaluated (**Figure 3.9**).

The cardiac levels of GSK3-α isoform were decreased in response to exercise practice during GDM pregnancy, comparing both to the pregnant control and to the GDM-S groups (GDM-E vs P-C-S: median = 0.560, Q1 = 0.364, Q3 = 0.832 vs median = 1.000, Q1 = 0.923, Q3 = 1.077, p =

0.0091; and GDM-E vs GDM-S: median = 0.560, Q1 = 0.364, Q3 = 0.832 vs median = 0.966, Q1 = 0.935, Q3 = 1.457, p = 0.0412) (**Figure 3.9A**), however the ratio between the levels of phosphorylated GSK3-α and total GSK3-α showed no differences between the experimental conditions (**Figure 3.9B**). Decreased levels of GSK3-β were observed in response to exercised GDM comparing to the P-C-S group (GDM-E vs P-C-S: median = 0.670, Q1 = 0.550, Q3 = 0.902 vs median = 1.000, Q1= 0.944, Q3 = 1.056, p = 0.0211) (**Figure 3.9C**). Interestingly, on the other hand, the levels of phosphorylated GSK3-β (inhibited form) showed to be decreased after a GDM pregnancy characterized by a sedentary lifestyle (GDM-S vs P-C-S: median = 0.530, Q1 = 0.266, Q3 = 0.935 vs median = 1.002, Q1 = 0.900, Q3 = 1.092, p = 0.0230) (**Figure 3.9D**).

3.6 **Cardiac metabolic flexibility**

HIF-1α is intimately involved in the response to hypoxia, which might be induced due to hyperglycemia within several tissues, including the heart. Pregnancy *per se* led to an increase in HIF-1α transcript levels (P-C-S vs NP-C-S: median = 1.070, Q1 = 0.580, Q3 = 1.409 vs median = 0.447, Q1 = 0.164, Q3 = 0.866, p = 0.0491), that were slightly exacerbated in response to a GDM pregnancy with sedentary lifestyle (GDM-S vs NP-C-S: median = 1.103, Q1 = 0.710, Q3 = 1.550 vs median = 0.447, Q1 = 0.164, Q3 = 0.866, p = 0.0425). Exercise practice during GDM pregnancy prevented the observed increase, maintaining HIF-1α transcript levels close to the NP-C-S group (GDM-E vs NP-C-S: p = 0.5109) (**Figure 3.10A**).

Cardiac alterations in HIF-1α transcript levels were accompanied by variations in the levels of transcripts involved in FA metabolism. Although the levels of acyl-CoA oxidase 1 (ACOX1) were unchanged between the experimental groups (**Figure 3.10B**), increased levels of the acetyl-CoA acyltransferase 2 (ACAA2) were observed for the hearts from the GDM-S group (GDM-S vs P-C-S: median = 2.071, Q1 = 1.472, Q3 = 2.658 vs median = 0.988, Q1 = 0.790, Q3 = 1.043, p = 0.0062).

Exercise during GDM pregnancy prevented the substantial increase in the cardiac levels of the ACAA2 transcript (GDM-E vs GDM-S: median = 1.281, Q1 = 0.686, Q3 = 1.763 vs median = 2.071, Q1 = 1.472, Q3 = 2.658, p = 0.0807) (**Figure 3.10C**). On the other hand, the cardiac transcript levels of PPARα were significantly decreased due to pregnancy *per se* and to a GDM pregnancy with a sedentary lifestyle (P-C-S vs NP-C-S: median = 0.489, Q1 = 0.428, Q3 = 1.118 vs 1.360, Q1 = 0.963, Q3 = 1.741, p = 0.0422 | GDM -S vs NP-C-S: median = 0.314, Q1 = 0.068, Q3 = 0.845 vs 1.360, Q1 = 0.963, Q3 = 1.741, p = 0.0175) (**Figure 3.10D**).

Maintenance of cardiac metabolic flexibility is essential for cardiac homeostasis. Alterations in substrate utilization in the heart are related to cardiac dysfunction. AMPK is a protein involved in energy balance. The levels of AMPK isoform α were evaluated in the cardiac tissue and a decrease in its levels was observed due to exercise practice during GDM pregnancy (GDM-E vs P-C-S: median = 0.488, Q1 = 0.320, Q3 = 0.867 vs median = 1.000, Q1 = 0.720, Q3 = 1.280, p = 0.0383) (**Figure 3.11A**). The cardiac levels of the phosphorylated (activated) form of AMPKα were also assessed and an increase in the ratio of the phosphorylated form by total protein was observed for all the pregnant groups, especially denoted in the control and GDM-exercised groups (P-C-S vs NP-C-S: median = 1.066, Q1 = 0.670, Q3 = 1.445 vs median = 0.631, Q1 = 0.333, Q3 = 0.821, p = 0.0482 | GDM-E vs NP-C-S: median = 0.957, Q1 = 0.735, Q3 = 1.473 vs median = 0.631, Q1 = 0.333, Q3 = 0.821, p = 0.0675) (**Figure 3.11B**).

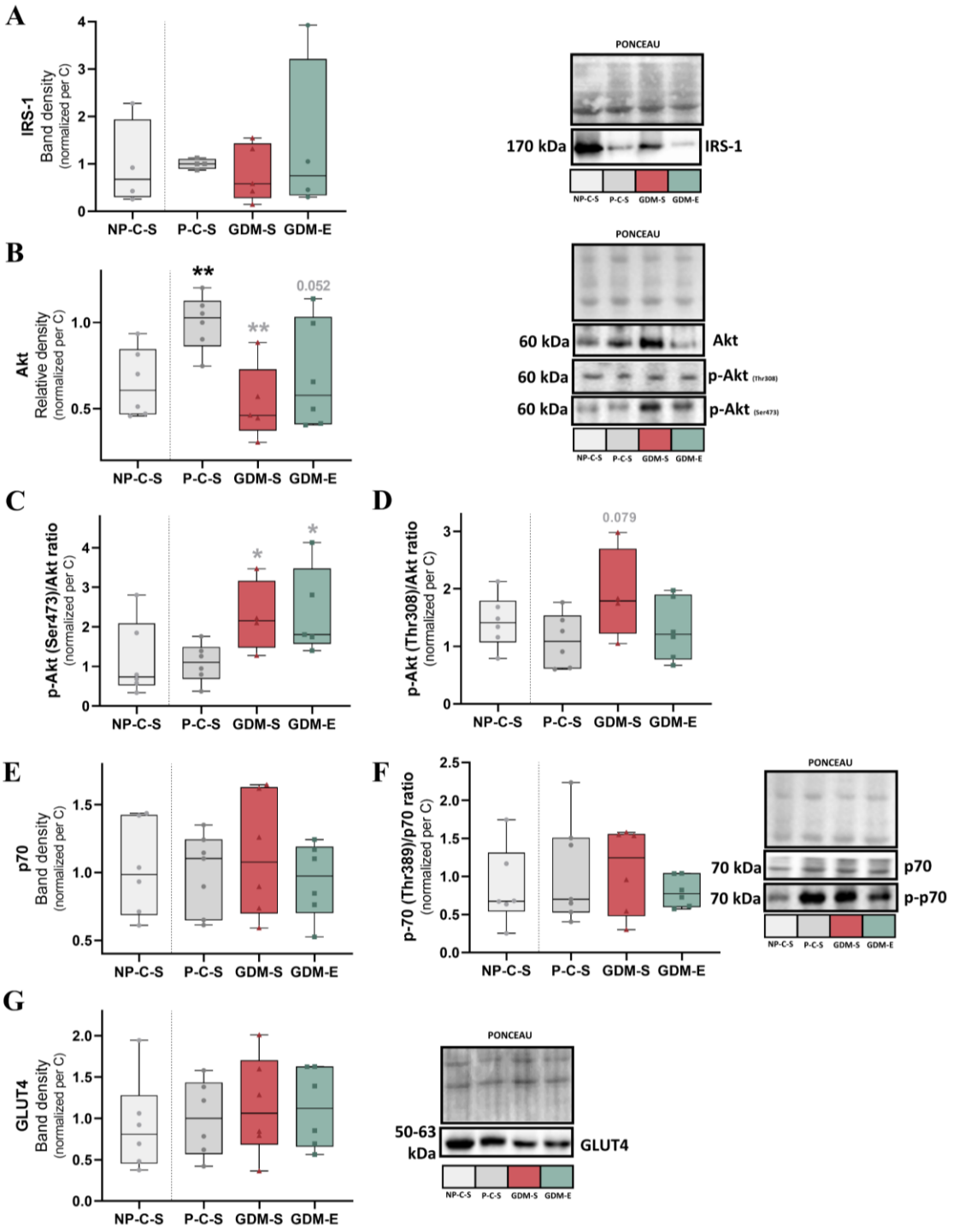

Figure 3.8 – Levels of insulin signaling-related protein in the hearts of non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. A: insulin receptor substrate one (IRS-1). B: protein kinase B (Akt). C: ratio of phosphorylated Akt form (p-Akt) at residue Ser473 to total protein Akt. D: ratio of p-Akt at residue Thr308 to total protein Akt. E: p70. F: ratio of phosphorylated p70 (p-70) to total protein p70. G: glucose transporter four (GLUT4). Data were normalized by ponceau staining and band density is represented relative to the P-C-S group.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test). p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-S or GDM-E vs P-C-S, ** $p \leq 0.01$ GDM-S vs P-C-S, ** $p \leq 0.01$ P-C-S vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *A, G adapted from Rodrigues, 2020.*

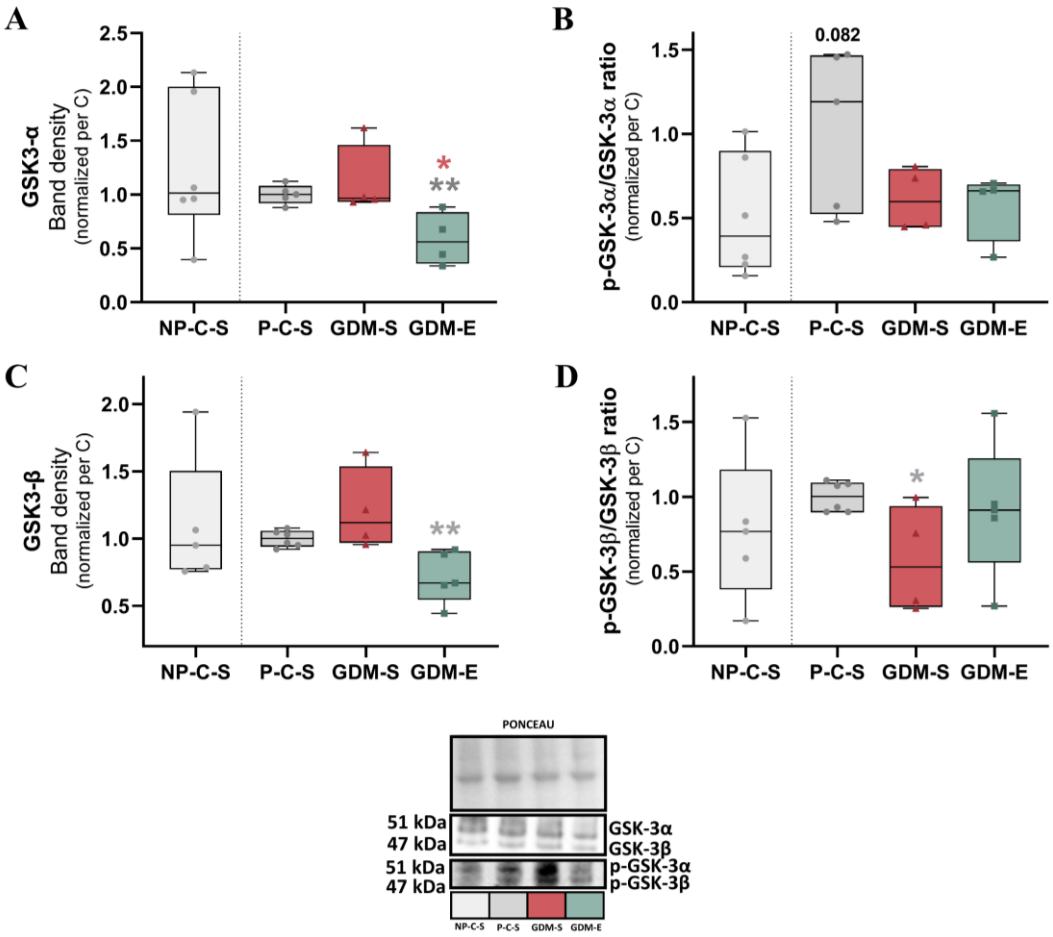

Figure 3.9 – Regulation of Akt-downstream anabolic program by glycogen synthase kinase three (GSK-3) in non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. A: GSK-3 isoform alpha (GSK-3α). B: ratio of phosphorylated GSK-3α form (p-GSK-3α) at residue Ser21 to total protein GSK-3. C: GSK-3 isoform beta (GSK-3β). D: ratio of phosphorylated GSK-3β form (p-GSK-3β) at residue Ser21 to total protein GSK-3. Data were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, C, and D and Mann-Whitney test in B. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-S vs P-C-S, * $p \leq 0.05$ GDM-E vs GDM-S, ** $p \leq 0.01$ GDM-S vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum.

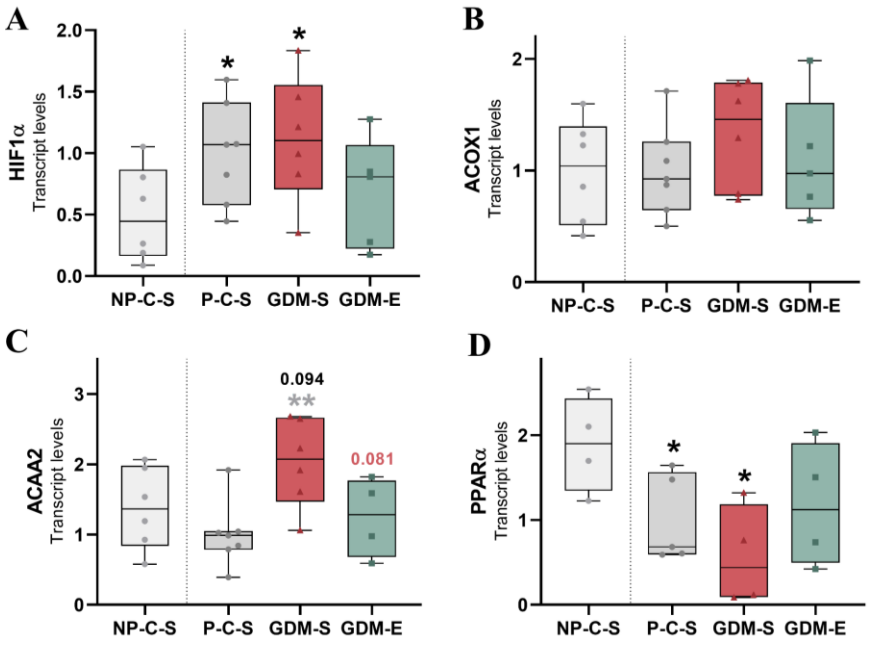

Figure 3.10 – Regulation of fatty acid (FA) metabolism. Transcript levels of genes involved in FA metabolism of non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. A: hypoxia inducible factor subunit alpha (HIF-1α). B: acyl-CoA oxidase 1 (ACOX1). C: acetyl-CoA acyltransferase 2 (ACAA2). D: Peroxisome proliferator activator receptor alpha (PPARα). Data were normalized for 18S, β-actin, and GAPDH housekeeping genes and transcript levels are represented relative to the P-C-S group.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test). p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S or GDM-S vs NP-C-S, ** $p \leq 0.01$ GDM-S vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Adapted from Rodrigues, 2020.*

3.7 **Effect on cardiac mitochondrial function**

Mitochondrial dysfunction is often associated with several diseases. The heart is highly dependent on ATP production within mitochondria, therefore maintaining the balance between mitochondrial processes and physiognomy is vital for heart function. The evaluation of mitochondrial oxygen consumption rates and membrane potential is prime to characterize and understand the impact of GDM and GDM with exercise on cardiac mitochondrial function and efficiency. Complex I supported cardiac mitochondrial bioenergetics revealed that although basal respiration (**Figure 3.12A**) is not affected by any of the experimental conditions, GDM contributes to decreased cardiac mitochondrial respiratory control ratio (RCR) in comparison with the non-pregnant group, with a significant effect on mothers that practiced exercise during GDM pregnancy (GDM-S vs NP-C-S: median = 4.231, Q1 = 3.670, Q3 = 4.417 vs median = 4.286, Q1 = 4.286, Q3 = 5.861, p = 0.0816; and GDM-E vs NP-C-S: median = 3.582, Q1 = 3.621, Q3 = 4.192 vs median = 4.286, Q1 = 4.286, Q3 = 5.861, p = 0.0338), that also showed decreased values in relation to the pregnant control group (P-C-S) (GDM-E vs P-C-S: median = 3.582, Q1 = 3.621, Q3 = 4.192 vs median = 4.605, Q1 = 4.167, Q3 = 4.998, p = 0.0321) (**Figure 3.12D**). State 3 showed to strongly contribute to the observed effects in the RCR of the GDM-E group (GDM-E

vs P-C-S: median = 37.01, Q1 = 33.53, Q3 = 39.52 vs median = 43.52, Q1 = 37.33, Q3 = 46.22; p = 0.0580) (**Figure 3.12B**).

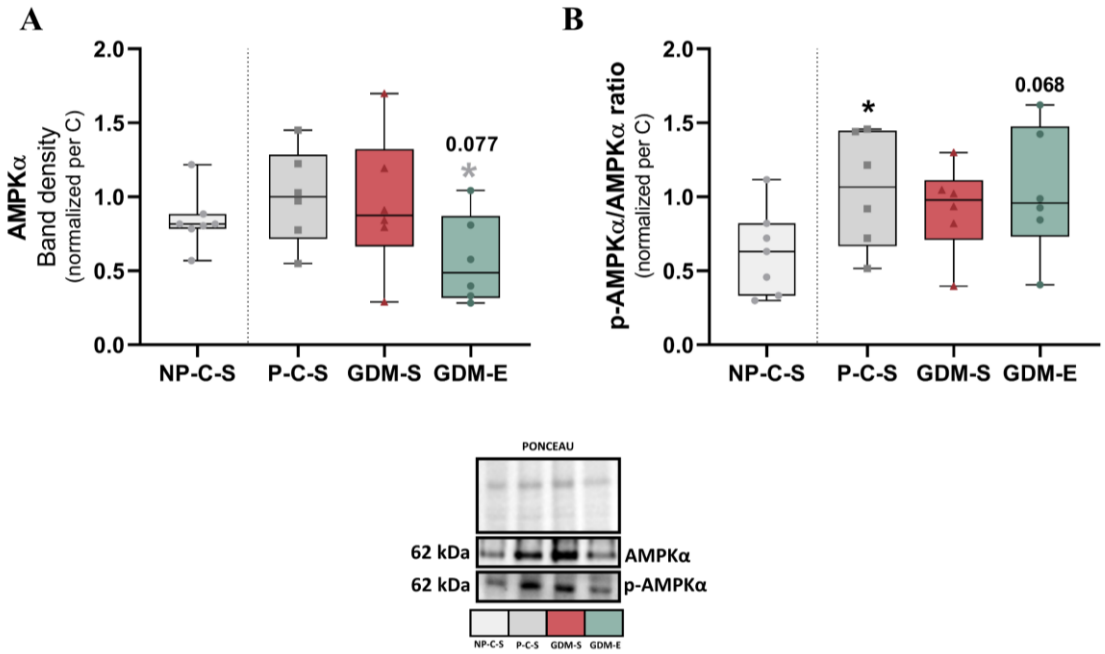

Figure 3.11 – AMP-activated protein kinase (AMPK)-mediated energy balance in the cardiac tissue of female rats in the non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. Protein levels of A: AMPK alpha subunit (AMPKα). B: ratio of phosphorylated AMPKα form (p-AMPKα) at residue Thr172 to total protein AMPKα. Data were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test). p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S vs NP-C-S, * $p \leq 0.05$ GDM-E vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum.

Cardiac mitochondrial complex I supported coupling efficiency showed to also be affected, taking in consideration the determined ADP/O for the different groups. Once again, the cardiac mitochondria from the GDM groups are particularly affected, presenting decreased values comparing to the NP-C-S group (GDM-S vs NP-C-S: median = 3.735, Q1 = 3.148, Q3 = 4.689 vs median = 4.940, Q1 = 4.014, Q3 = 7.269, p = 0.0350 | GDM-E vs NP-C-S: median = 3.877, Q1 = 3.710, Q3 = 4.833 vs median = 4.940, Q1 = 4.014, Q3 = 7.269, p = 0.0734) (**Figure 3.12E**).

Cardiac mitochondrial oxygen consumption rates were also assessed when supported by complex II substrates, however, no differences between the experimental groups were detected (**Figure 3.13**).

Hearts from mothers belonging to the control group (P-C-S) showed higher mitochondrial membrane depolarization induced by ADP addition, comparing to the non-pregnant control group (NP-C-S) when using complex I substrates (P-C-S vs NP-C-S: median = 15.25, Q1 = 14.67, Q3 = 16.03 vs median = 13.85, Q1 = 12.00, Q3 = 14.98, p = 0.0262). Nevertheless, exercise practice

during GDM pregnancy exacerbated this effect (GDM-E vs NP-C-S: median = 16.51, Q1 = 15.33, Q3 = 18.41 vs median = 13.85, Q1 = 12.00, Q3 = 14.98, p = 0.0012) (**Figure 3.14A**). Moreover, the cardiac mitochondria from GDM mothers hearts take longer to completely phosphorylate ADP than pregnant and non-pregnant females, an effect that is exacerbated by exercise lifestyle during GDM pregnancy (GDM-S vs NP-C-S: median = 46.75, Q1 = 40.50, Q3 = 46.75 vs median = 40.50, Q1 = 37.50, Q3 = 49.50, p = 0.098 | GDM-E vs NP-C-S: median = 52.13, Q1 = 47.06, Q3 = 63.25 vs median = 40.50, Q1 = 37.50, Q3 = 49.50, p = 0.0169 | GDM-S vs P-C-S: median = 46.75, Q1 = 40.50, Q3 = 46.75 vs median = 37.75, Q1 = 37.00, Q3 = 38.81, p = 0.0112 | GDM-E vs P-C-S: median = 52.13, Q1 = 47.06, Q3 = 63.25 vs median = 37.75, Q1 = 37.00, Q3 = 38.81, p = 0.0030) (**Figure 3.14C**).

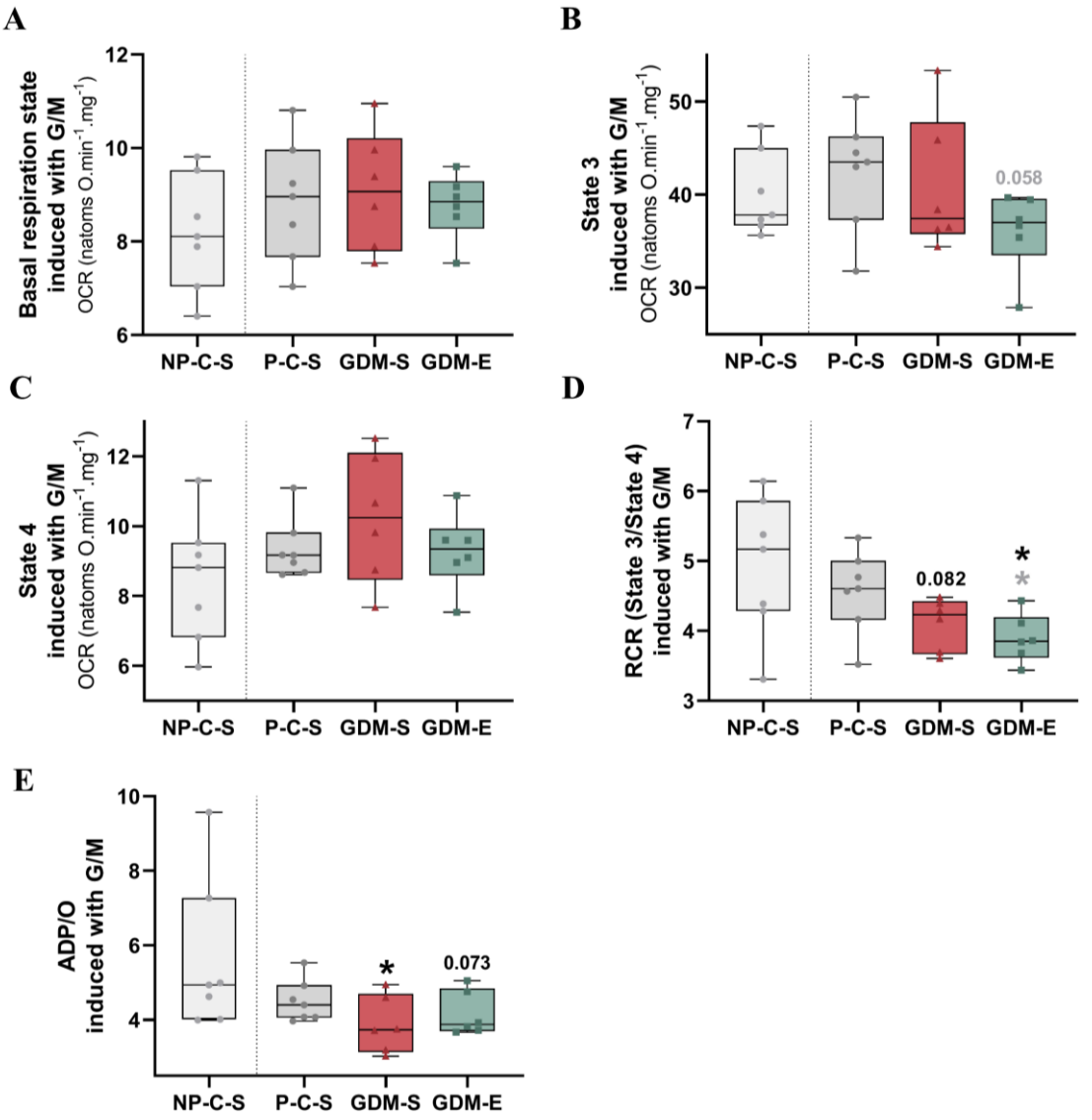

Figure 3.12 – Cardiac mitochondrial oxygen consumption rates using complex-I substrates glutamate/malate (G/M). A: basal respiration state (state 2). D: respiratory control ratio (RCR) determined by the ratio between B: state 3 and C: state 4. E: amount of oxygen necessary to phosphorylate ADP (ADP/O ratio).
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, B, C, and D and Mann-Whitney test in E. p-values lower than 0.05 were considered significant (***** $p \leq 0.05$ GDM-S or GDM-E vs NP-C-S, * $p \leq 0.05$ GDM-E vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Adapted from Rodrigues, 2020.*

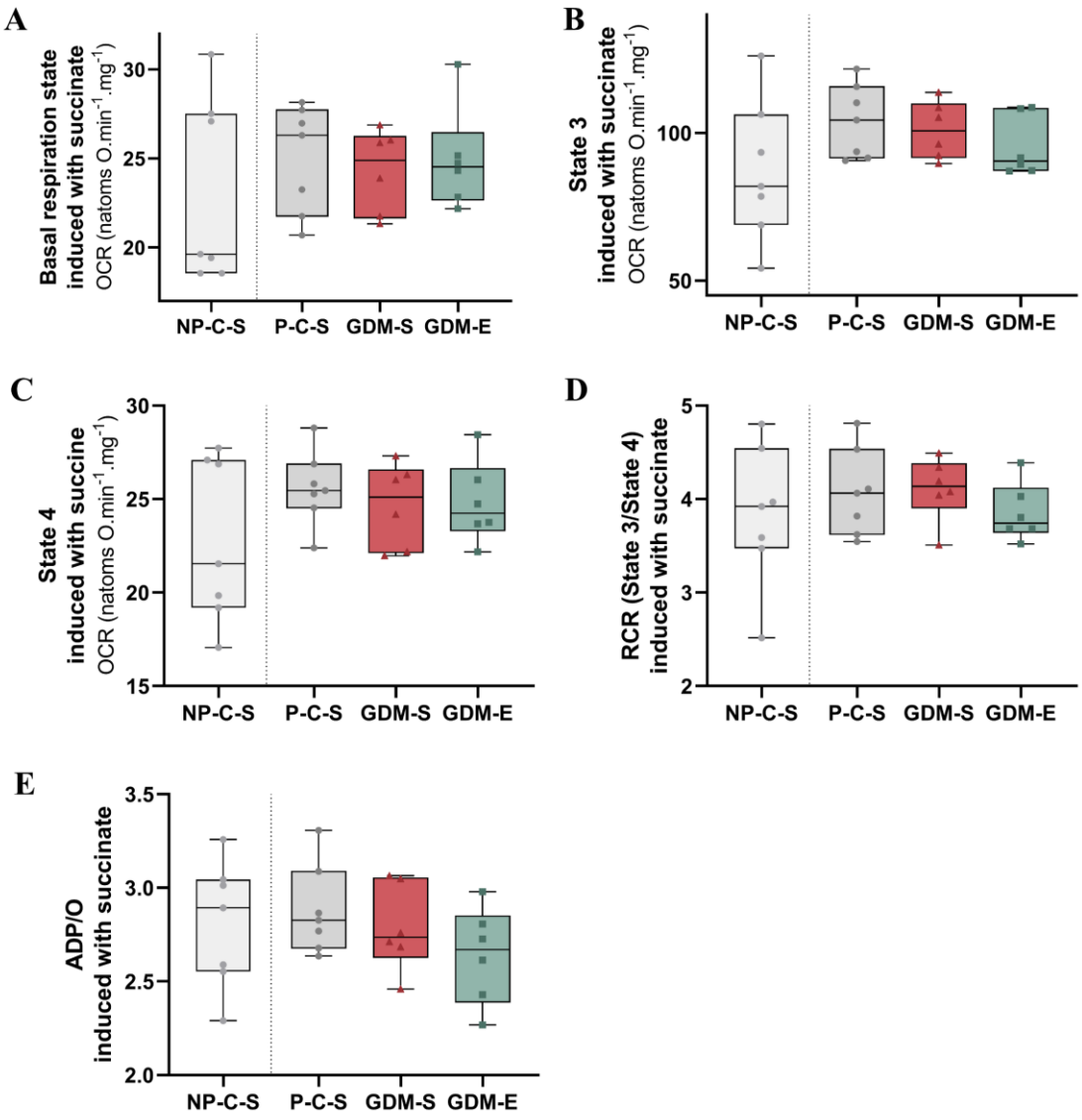

Figure 3.13 – Cardiac mitochondrial oxygen consumption rates using complex-II substrates, succinate. A: basal respiration state (state 2). D: respiratory control ratio (RCR) determined by the ratio between B: state 3 and C: state 4. E: amount of oxygen necessary to phosphorylate ADP (ADP/O ratio).
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in C, D, and E and Mann-Whitney test in A, and B. Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Adapted from Rodrigues, 2020.*

Concerning cardiac mitochondrial membrane potential when using complex II substrates, pregnancy predisposed cardiac mitochondria to higher mitochondrial membrane depolarization upon ADP administration, which is further exacerbated in GDM-complicated pregnancies (P-C-S vs NP-C-S: median = 12.87, Q1 = 10.27, Q3 = 14.82 vs median = 10.04, Q1 = 9.27, Q3 = 10.45, p = 0.0199 | GDM-S vs NP-C-S: median = 12.23, Q1 = 10.52, Q3 = 13.18 vs median = 10.04, Q1 = 9.27, Q3 = 10.45, p = 0.0061 | GDM-E vs NP-C-S: median = 12.40, Q1 = 10.70, Q3 = 14.14 vs median = 10.04, Q1 = 9.27, Q3 = 10.45, p = 0.0048) (**Figure 3.15A**). Nevertheless, no differences

were identified for the other evaluated mitochondrial parameters when supported by complex II substrates.

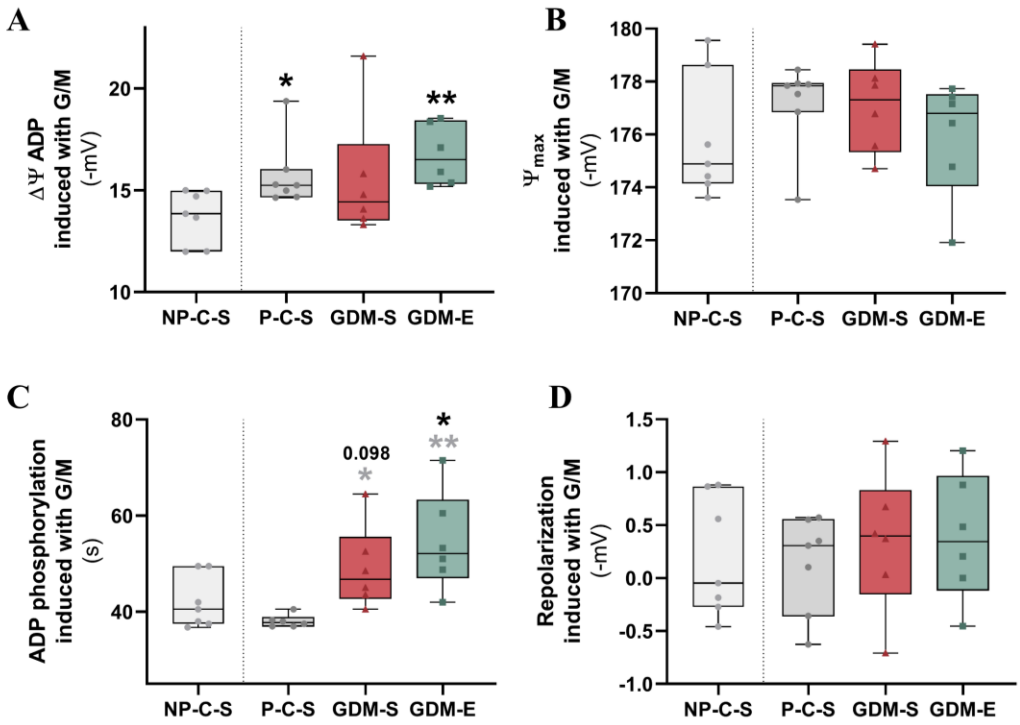

Figure 3.14 – Cardiac mitochondrial membrane potential using complex-I substrates, glutamate/malate (G/M). A: ADP-induced membrane depolarization (ΔΨ ADP). B: maximum mitochondrial membrane potential (Ψ max). C: time for total ADP phosphorylation (or lag phase). D: membrane potential repolarization.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in C, and D and Mann-Whitney test in A, and B. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S or GDM-E vs NP-C-S, * $p \leq 0.05$ GDM-S vs P-C-S, ** $p \leq 0.01$ GDM-E vs NP-C-S, ** $p \leq 0.01$ GDM-E vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Adapted from Rodrigues, 2020.*

Mitochondria endure constant fusion and fission processes crucial to maintain their characteristics and consequently their function. Together with mitochondrial biogenesis and mitophagy, these processes enable the maintenance of mitochondrial quality. Mothers that had GDM and practiced exercise during pregnancy (GDM-E) had higher cardiac levels of the PGC-1α transcript, the master regulator of mitochondrial biogenesis and tremendously important in response to exercise (GDM-E vs P-C-S: median = 2.698, Q1 = 1.593, Q3 = 3.412 vs median = 0.997, Q1 = 0.506, Q3 = 1.449, p = 0.0056) (**Figure 3.16B**). Nevertheless, no alterations were observed for the other evaluated proteins involved in the processes of mitochondrial biogenesis (TFAM, **Figure 3.16A**) and mitochondrial dynamics (Fis1, DRP1, and OPA1, **Figure 3.16C, 3.16D, and 3.16E**).

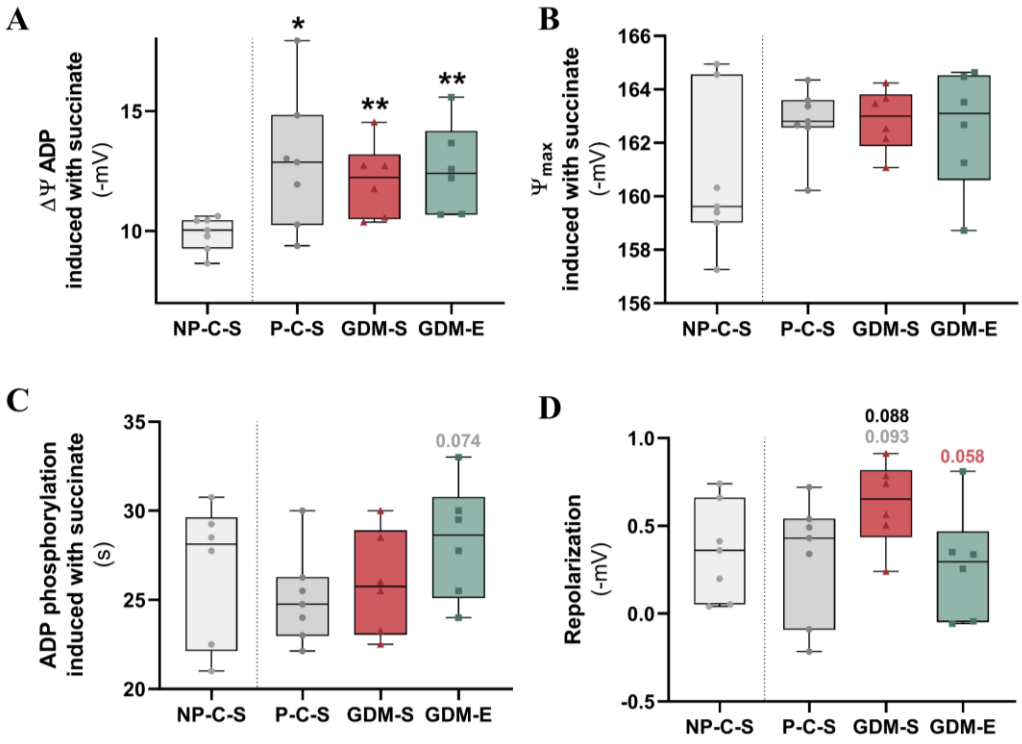

Figure 3.15 – Cardiac mitochondrial membrane potential using complex-II substrates, succinate. A: ADP-induced membrane depolarization (ΔΨ ADP). B: mitochondrial membrane potential (Ψ max). C: time for total ADP phosphorylation (or lag phase). D: membrane potential repolarization.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in A, C, and D and Mann-Whitney test in B. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S vs NP-C-S, ** $p \leq 0.01$ GDM-S or GDM-E vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *Adapted from Rodrigues, 2020.*

To further understand the induced alterations in cardiac mitochondrial bioenergetics, the protein levels of OXPHOS subunits were evaluated (**Figure 3.17A**). The levels of one subunit of each ETC complex and ATP synthase were assessed. No differences were observed for the complex I NDUFB8 subunit cardiac levels between the groups nor for the levels of the ATP synthase ATP5A subunit. However, the levels of the SDHB subunit, which is part of complex II, were increased in the P-C-S group, when comparing to its non-pregnant counterpart (P-C-S vs NP-C-S: median = 1.000, Q1 = 0.903, Q2 = 1.097 vs median = 0.728, Q1 = 0.523, Q3 = 0.928, p = 0.0255). A pregnancy complicated by GDM, and adopting a sedentary lifestyle leads to increased levels of the subunits tested for complex III (UQCRC2) and complex IV (MTCO2). While the GDM-S group shows increased levels of the UQCRC2 levels comparing to the non-pregnant control group (GDM-S vs NP-C-S: median = 1.156, Q1 = 0.925, Q3 = 1.852 vs median = 0.859, Q1 = 0.561, Q3 = 0.983, p = 0.0362), the cardiac postpartum effects of a GDM pregnancy coupled to a sedentary behavior result in increased levels of MTCO2 in relation to its control group (P-C-S) (GDM-S vs P-C-S: median = 1.878, Q1 = 0.997, Q3 = 2.402 vs median = 1.378, Q1 = 1.085, Q3 = 2.028, p = 0.0451).

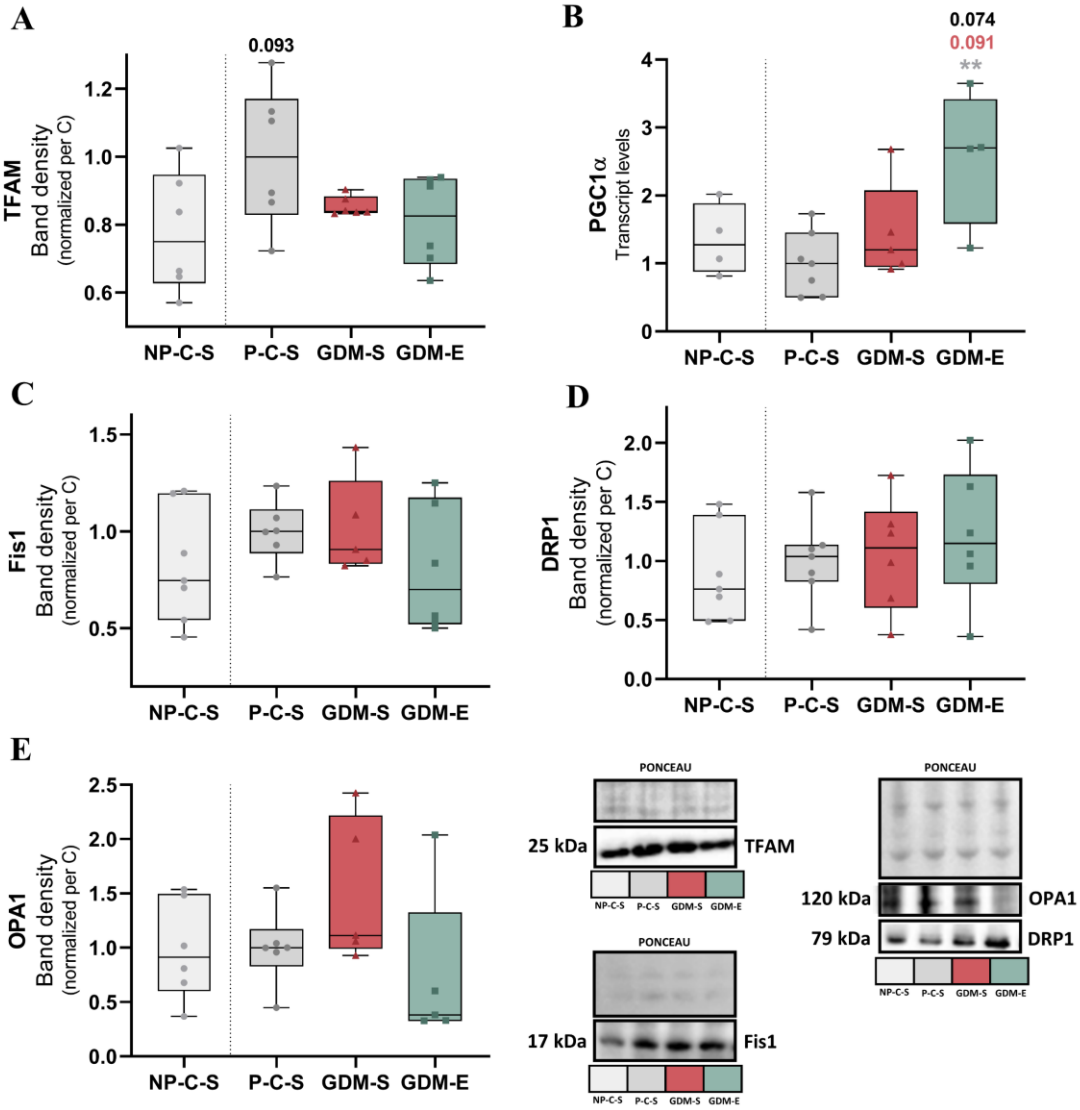

Figure 3.16 – Impact of GDM and lifestyle habits on mitochondrial dynamics and biogenesis in the cardiac tissue of female rats in the non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. Protein levels of A: mitochondrial transcription factor A (TFAM). C: mitochondrial fission 1 protein (Fis1). D: dynamin 1 like protein (DRP1) and E: dynamin like 120 Kda protein (OPA1). Transcript levels of B: PPARγ coactivator 1 alpha (PGC1α). Data regarding protein levels were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1. Data regarding transcript levels were normalized for 18S, β-actin, and GAPDH housekeeping genes and are represented relative to the P-C-S group.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in B, and C and Mann-Whitney test in A, and D. p-values lower than 0.05 were considered significant (** $p \leq 0.01$ GDM-E vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *A, B adapted from Rodrigues, 2020.*

The evaluation of the Tom20 protein levels, a well-known indicator of mitochondrial mass, revealed that pregnancy *per se* led to decreased expression of this protein in the cardiac tissue (P-C-S vs NP-C-S: median = 1.000, Q1 = 0.682, Q3 = 1.318 vs median = 1.814, Q1 = 1.117, Q3 =

2.127, p = 0.0369) (**Figure 3.17B**). The protein encoded by the RNA polymerase mitochondrial (POLRMT) gene is responsible for the transcription of mitochondrial DNA, being involved in mitochondrial DNA expression. Furthermore, POLRMT provides essential RNA primers for the initiation of mitochondrial DNA replication[263]. Pregnancy shows to lead to decreased levels of the POLRMT transcript in the heart postpartum, especially in the control group (P-C-S vs GDM: median = 0.948, Q1 = 0.626, Q3 = 1.318 vs median = 2.668, Q1 = 0.935, Q3 = 4.095, p = 0.0444) but also among exercised GDM female rats (GDM-E vs NP-C-S: median = 0.567, Q1 = 0.253, Q3 = 1.399 vs median = 2.668, Q1 = 0.935, Q3 = 4.095, p = 0.0770) (**Figure 3.17C**).

The mitochondrial membrane composes an important barrier between the mitochondrial matrix, the mitochondrial intermembrane space and the rest of the cell. The OMM permeability to cellular metabolites, ions, between other components is controlled by dedicated channels, such as the voltage-dependent anion channel (VDAC). Within the IMM, this exchange is often mediated by the mitochondrial carrier adenine nucleotide translocase (ANT). The cardiac levels of the VDAC2 transcript and the protein levels of ANT 1/2 were evaluated. Increased protein levels of ANT 1/2 were observed in the hearts of dams that had a GDM-pregnancy comparing to the non-pregnant control (GDM-S vs NP-C-S: median = 1.207, Q1 = 0.910, Q3 = 1.392 vs median = 0.783, Q1 = 0.483, Q3 = 1.005, p = 0.0234 | GDM-E vs NP-C-S: median = 1.014, Q1 = 0.887, Q3 = 1.258, p = 0.0522) (**Figure 3.17D**). Regarding the levels of the VDAC2 transcript, a pregnancy complicated by GDM and with a sedentary lifestyle resulted in increased cardiac levels of VDAC2 transcript comparing to the pregnant control group (GDM-S vs P-C-S: median = 1.691, Q1 = 1.016, Q3 = 2.641 vs median = 0.810, Q1 = 0.657, Q3 = 1.478, p = 0.0442). This cardiac VDAC2 transcript increase was prevented by the practice of exercise during a GDM-pregnancy (GDM-E vs GDM-S: median = 0.871, Q1 = 0.475, Q3 = 1.464 vs median = 1.691, Q1 = 1.016, Q3 = 2.641, p = 0.0811 | GDM-E vs P-C-S: p = 0.936) (**Figure 3.17E**).

Mitophagy is part of the quality control mechanisms. Dysfunctional mitophagy process leads to the accumulation of malfunctioning organelles that ultimately cause cellular and tissue damage. Microtubule-associated protein 1A/1B-light chain 3 (LC3) and p62 are two proteins usually involved in the complex interactions occurring in macroautophagy, being related with dysfunctional mitochondria elimination among other proteins. The levels of the isoforms I and II of LC3 were assessed in the cardiac tissues. The levels of LC3-I were decreased in the hearts of all the pregnant groups, especially in the control group (P-C-S vs NP-C-S: median = 1.000, Q1 = 0.686, Q3 = 1.314 vs median = 1.578, Q1 = 1.395, Q3 = 2.407, p = 0.0152 | GDM-S vs NP-C-S: median = 0.896, Q1 = 0.680, Q3 = 1.549 vs median = 1.578, Q1 = 1.395, Q3 = 2.407, p = 0.0649 | GDM-E vs NP-C-S: median = 0.684, Q1 = 0.467, Q3 = 1.671 vs median = 1.578, Q1 = 1.395, Q3 = 2.407, p = 0.0649) (**Figure 3.18A**). The coupled effects of GDM and a sedentary behavior resulted in increased LC-II/LC-I ratio comparing to the pregnant control (GDM-S vs P-C-S: median = 1.770, Q1 = 1.393, Q3 = 3.447 vs median = 0.940, Q1 = 0.549, Q3 = 1.846, p = 0.0874) (**Figure 3.18C**), mainly due to the increased levels of LC3-II observed for the same group (GDM-S) (GDM-S vs P-C-S: p = 0.0936) (**Figure 3.18B**). GDM highly contributed to increased levels of the p62 protein in the cardiac tissue of the female rats, comparing to their pregnant control group (GDM-S vs P-C-S: median = 1.617, Q1 = 1.374, Q3 = 1.732 vs median = 1.000, Q1 = 0.638, Q3 = 1.362, p = 0.0117 | GDM-E vs P-C-S: median = 1.606, Q1 = 1.331, Q3 = 1.933 vs median = 1.000, Q1 = 0.638, Q3 = 1.362, p = 0.0159) (**Figure 3.18D**).

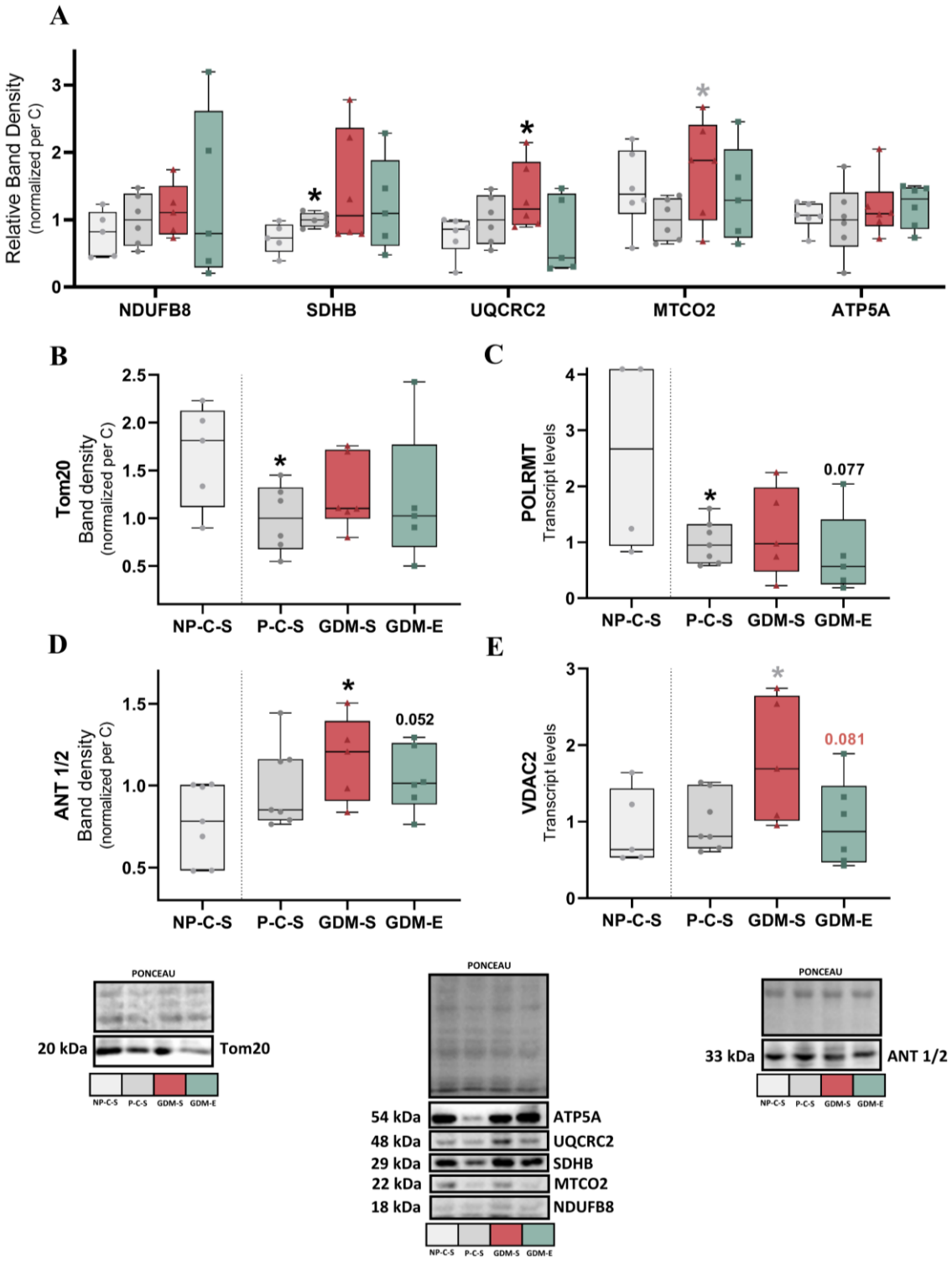

Figure 3.17 – Impact of GDM and lifestyle habits on cardiac abundance of oxidative phosphorylation system complexes subunits, mitochondrial mass indicators, and membrane channels and carriers. Protein levels of A: complex I to IV (NDUFB8, SDHB, UQCRC2, and MTCO2) and ATP synthase (ATP5A) subunits, B: mitochondrial import receptor (Tom20), and D: adenosine nucleotide translocator (ANT 1/2). Transcript levels of C: mitochondrial RNA polymerase (POLRMT), and E: voltage dependent anion-selective channel (VDAC2). Data regarding protein levels were normalized by ponceau staining and band density is represented relative to the P-C-S group. Data regarding transcript levels were normalized for 18S, β-actin, and GAPDH housekeeping genes and are represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in B, and C and Mann-Whitney test in A, and D. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-S vs P-C-S, ***** $p \leq 0.05$ P-C-S or GDM-S vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *A, B, C, E adapted from Rodrigues, 2020.*

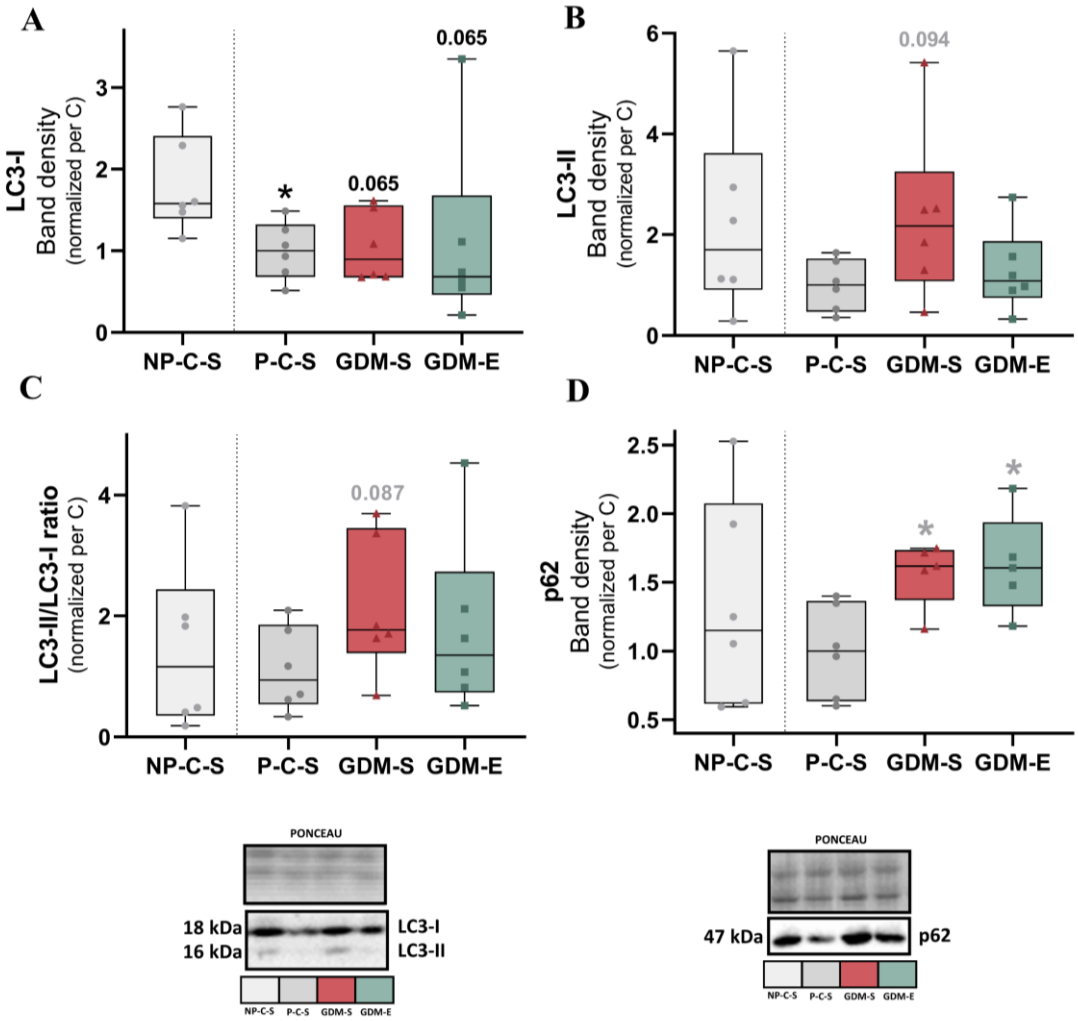

Figure 3.18 – Role of autophagy in the regulation of GDM-induced effects and maternal habits during pregnancy in the heart. Protein levels of A: microtubule-associated protein 1A/1B-light chain 3 (LC3) isoform I (LC3-I), B: LC3-II, C: ratio of LC3-II to LC3-I, and D: p62. Data were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in B, C, and D and Mann-Whitney test in A. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ GDM-S or GDM-E vs P-C-S, ***** $p \leq 0.05$ P-C-S vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. A*dapted from Rodrigues, 2020.*

The levels of proteins involved in the cellular death by apoptosis were also evaluated. Decreased levels of the anti-apoptotic protein Bcl-2 were observed in the heart of female rats that had a GDM-pregnancy and practice exercise during gestation in relation to the pregnant control group (GDM-E vs P-C-S: median = 0.777, Q1 = 0.629, Q3 = 0.8362 vs median = 0.990, Q1 = 0.940, Q3 = 1.095, p = 0.0241) (**Figure 3.19A**). However, the ratio between the phosphorylated form of Bcl-2 at the residue Ser70 and the total protein is increased in the GDM-E group (GDM-E vs P-C-S: median = 1.531, Q1 = 1.038, Q3 = 1.887 vs median = 1.044, Q1 = 0.623, Q3 = 1.320, p = 0.0647) (**Figure 3.19C**). The levels of the pro-apoptotic protein Bad also shows to be decreased in response to a GDM-pregnancy and an exercised lifestyle, comparing to its pregnant control group (GDM-E vs P-C-S: median = 0.588, Q1 = 0.464, Q3 = 0.727 vs median = 1.000, Q1 = 0.817, Q3 = 1.184, p = 0.0061). However, pregnancy *per se* contributed to increased levels

of this protein in the postpartum period in the mothers' hearts (P-C-S vs NP-C-S: median = 1.000, Q1 = 0.817, Q3 = 1.184 vs median = 0.574, Q1 = 0.502, Q3 = 0.882, p = 0.0400) (**Figure 3.19B**). Nevertheless, the observed alterations in the total protein did not reflect in the ratio of Bad phosphorylated form at residue Ser112 to Bad (**Figure 3.19D**).

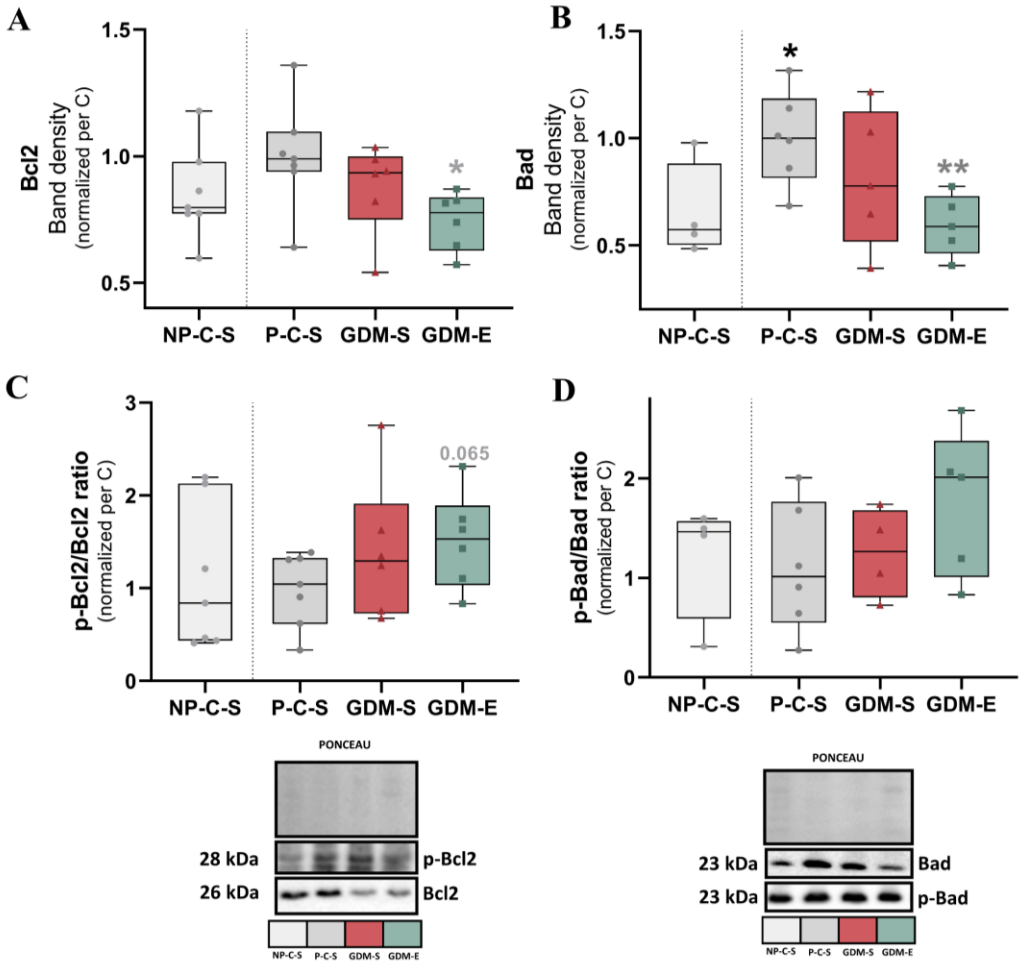

Figure 3.19 – Role of apoptosis in the regulation of GDM-induced effects and maternal habits during pregnancy in the cardiac tissue. Protein levels of A: β-cell lymphoma 2 (Bcl2), B: Bad, C: ratio of phosphorylated form of Bcl2 at the residue Ser70 (p-Bcl2) to total protein Bcl2, and D: phosphorylated form of Bad at the residue Ser112 (p-Bad) to total protein Bad. Data were normalized by ponceau staining and band density is represented relative to the P-C-S group = 1.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in B, C, and D and Mann-Whitney test in A. p-values lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S vs NP-C-S, ** $p \leq 0.01$ GDM-E vs P-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum.

Although mitochondria are involved in ROS production, processes that target excess ROS decomposition also occur in mitochondria, highly contributing to the maintenance of cellular redox balance and preventing oxidative stress. Nrf2 plays an important role in this regulation (see section 1.3.3) and the levels of this protein were evaluated in the cardiac tissue. Pregnancy, independently of GDM complications and exercise or sedentary lifestyle interventions contributes to elevated levels of this protein (P-C-S vs NP-C-S: median = 1.011, Q1 = 0.719, Q3 = 1.235 vs

median = 0.634, Q1 = 0.475, Q3 = 0.830, p = 0.0109), an effect that is further aggravated in GDM mothers that had a sedentary behavior (GDM -S vs NP-C-S: median = 1.343, Q1 = 0.8921, Q3 = 1.343 vs median = 0.634, Q1 = 0.475, Q3 = 0.830, p = 0.0031). On the other hand, GDM mothers that practiced exercise during pregnancy did not show significant increases in the levels of this protein (GDM-E vs NP-C-S: p = 0.1039) (**Figure 3.20A**).

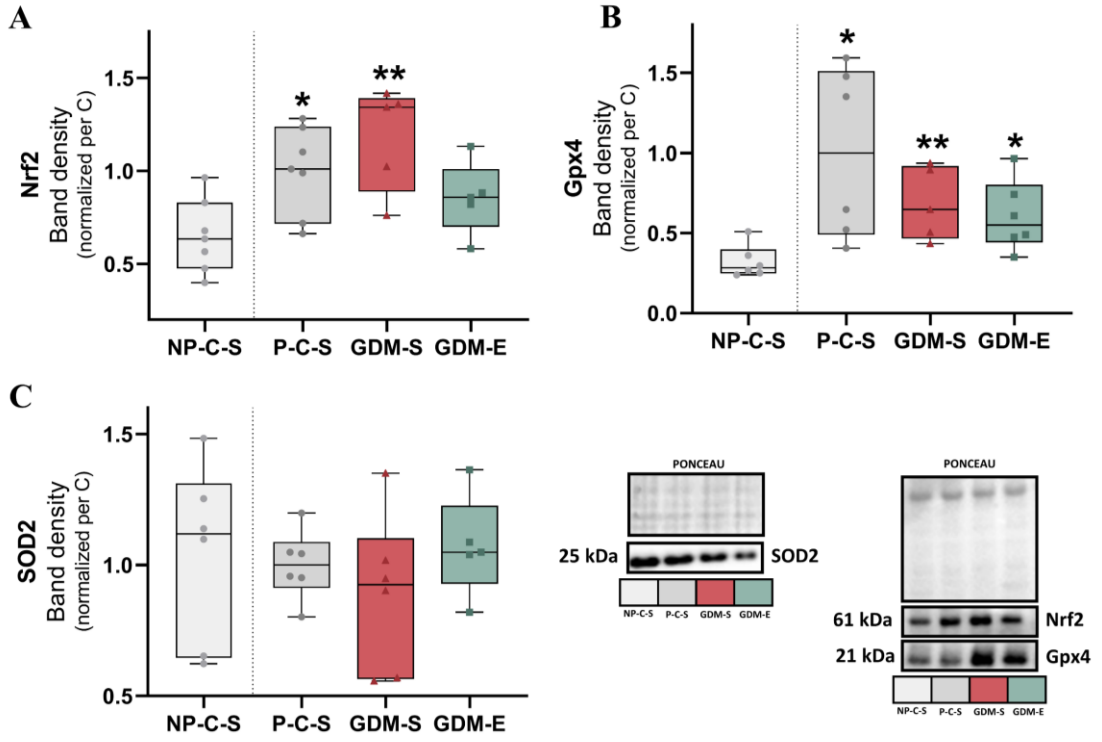

Figure 3.20 – Cellular redox balance and oxidative stress in the hearts of female rats in the non-pregnant control (NP-C-S), pregnant control (P-C-S), GDM-pregnancy with sedentary lifestyle (GDM-S), and GDM-pregnancy with exercise intervention (GDM-E) groups. Protein levels of A: Nuclear factor erythroid 2-related factor 2 (Nrf2), B: glutathione peroxidase 4 (Gpx4), and C: superoxide dismutase 2 (SOD2). Data were normalized by ponceau staining and band density is represented relative to the P-C-S group.
Statistical analysis: Comparison between the experimental groups was performed using unpaired t test (after performing Shapiro-Wilk normality test) in B, C, and D and Mann-Whitney test in A. p-value lower than 0.05 were considered significant (* $p \leq 0.05$ P-C-S or GDM-E vs NP-C-S, ** $p \leq 0.01$ GDM-S vs NP-C-S). Light grey bar, NP-C-S; grey bar, P-C-S; red bar GDM-S, green bar, GDM-E. Data are represented as median, interquartile distance, minimum and maximum. *C adapted from Rodrigues, 2020.*

The alterations in the levels of Nrf2 were accompanied by increased Gpx4 levels within all the pregnant groups, comparing to the non-pregnant control group (P-C-S vs NP-C-S: median = 1.000, Q1 = 0.492, Q3 = 1.508 vs median = 0.282, Q1 = 0.248, Q3 = 0.3998, p = 0.0117 | GDM-E vs NP-C-S: median = 0.549, Q1 = 0.444, Q3 = 0.798 vs median = 0.282, Q1 = 0.248, Q3 = 0.3998, p = 0.0167). However, the alterations in the levels of Gpx4 were more denoted in the GDM-S group (GDM-S vs NP-C-S: median = 0.647, Q1 = 0.469, Q3 = 1.508 vs median = 0.282, Q1 = 0.248, Q3 = 0.3998, p = 0.0062) (**Figure 3.20B**). Concerning the levels of other proteins involved in enzymatic antioxidant defense, no changes between the experimental interventions were observed for SOD2 (**Figure 3.20C**).

Chapter 4 – Discussion

Nowadays, the general population is adopting risk behaviors that may seriously contribute to metabolic disease development. The healthy organism can adapt to new challenges until the induced impairments outdo the necessary metabolic flexibility. During pregnancy, the woman's body needs to adapt to the physiological changes and nutritional and energetic requirements to maintain a healthy fetal development[106–108]. Pregnancy itself composes a metabolic challenge that may further evolve to a pathologic state due to metabolic disease development during pregnancy. Between the organism systems that go through these changes, the heart stands out. Cardiovascular diseases contemplate the number one cause of death in the world and, although medicine is advancing and new treatments arise, the necessity of prevention is evident. Gestational diabetes mellitus, whose prevalence is rapidly increasing and already constitutes the most common metabolic disorder during pregnancy, predisposes the mothers to develop metabolic disease after gestation[116]. The offspring are also at risk and numerous studies focus on GDM's impact on the offspring's health throughout life[116,118]. Nevertheless, few studies direct their research to the GDM's consequences to the mother's future health. Here, we dive into the cardiac molecular pathways that may contribute to metabolic disease development after a pregnancy complicated by GDM and investigate the role of exercise exclusively during GDM gestation in the postnatal modulation of cardiac effects.

4.1 **High-fat high-sugar diet-induced gestational diabetes mellitus**

The consumption of high-caloric diets is increasing, especially in westernized countries[152]. This is reflected in the rising epidemic of obesity and diabetes among adults, adolescents, and children[3]. The increasing adoption of a sedentary lifestyle and low physical activity rates also contribute to this epidemic. Women with inadequate lifestyle habits are at risk of developing GDM[136]. In this work, an adapted HFHS-diet-induced GDM rat model from Pereira et al.[254] was implemented.

Glucose intolerance with onset during pregnancy is the main characteristic of GDM[264,265]. This further results in a mild-hyperglycemic state that IR often accompanies during pregnancy[266]. In the present work, the female rats subjected to the HFHS-diet developed glucose intolerance only at mid-pregnancy, independently of exercise practice, which means that the female rodents were not diabetic before pregnancy and that this model is able to mimic the main feature of GDM. Importantly, exercise practice during pregnancy did not revert the GDM phenotype, but improved some immediate physiological parameters, as observed in humans. This is of particular importance since, in some of the few studies that advocate the use of a GDM model, the animal already had developed diabetes before pregnancy, which by definition is not a GDM case and may not recapitulate the pathophysiology of GDM[267]. Some studies also use drugs to induce diabetes, such as STZ, which composes a toxic compound for pancreatic β-cells, leading to their destruction[268]. This results in severe effects in insulin production and glycemic control that are not in accordance with the clinical definition of GDM.

Weight gain monitorization during a GDM-pregnancy is crucial to guarantee diabetic control and the best pregnancy outcomes[118]. Although the weights of the pregnant female rats throughout

the experiment varied similarly between the groups, GDM mothers with a sedentary lifestyle had excessive GWG. In fact, GWG composes a risk factor for GDM development and is also highly associated with the risk of fetal macrosomia[269,270]. Exercise had a beneficial impact on this parameter, preventing excessive GWG induced by GDM. This effect is similar to that previously observed in numerous human studies[180,191], demonstrating a good translational potential of the model used in this study.

Obesity, cardiac lipid accumulation, and cardiac hypertrophy are often associated with cardiac dysfunction and increased heart weight[271]. Pregnancy also has associated cardiac physiological hypertrophy that leads to increased heart weight during pregnancy, and one would assume that metabolic dysfunction, especially due to increased nutrient intake would lead to a more significant increase in heart weight during pregnancy. However, studies within the literature providing this information are scarce. Furthermore, although it is assumed that heart weight increases during pregnancy, it is unknown how this cardiac parameter progresses in the postpartum period. In the present study, the absolute heart weight of the GDM-dams 8 weeks after gestation was tendentially decreased and this effect was counteracted by exercise during GDM. The heart-to-brain weight was also evaluated since while there might be changes in body weight due to numerous factors, usually brain weight remains constant[272]. Although statistical significance was lost after measuring the heart-to-brain weight ratio, the behavior between the groups was similar and remained an interesting result since it seems that mothers that had GDM have a decreased capacity to return to normal heart weight, which could indicate an impaired myocardial remodeling after pregnancy and possibly be reflected in functional, physiological, and even molecular parameters. On the other hand, exercise during GDM seems to exert a beneficial role by reverting the heart-to-brain weight ratio GDM phenotype. Of course, these data are not sufficient to support this hypothesis, however, attention to the evaluation of these parameters in future GDM and cardiovascular studies must be drawn.

Maternal nutrition can lead to alterations in the offspring's development and growth. Some of these altered characteristics may be evaluated after delivery (e.g. offspring's morphology, parameters of the litter), constituting a primary factor that may further represent an easily assessed risk factor if observed among different studies. In this study, the effects of GDM itself and combined with exercise practice during pregnancy on litter size and litter sex distribution were evaluated. Data on litter size are inconsistent among studies and most find no differences in litter size[254,273] or do not reveal this parameter. In our study, the GDM sedentary female rats delivered more pups than the control group. Interestingly, researchers studied the effects of maternal body condition on *in vivo* production of zygotes and oocyte quality. Their study revealed that mice females with slightly increased body fat but physiological body weight showed an increase in spontaneously ovulated oocytes and a greater fertilization index[274]. Although this was not reflected in average litter size, these results suggest that increased availability of nutrients (not surpassing a certain threshold) may reunite optimal conditions to generate greater litter sizes. These results may also explain the observed lower litter size in a study that used STZ as a diabetic inducer and that produced dams with increased body weights[275]. However, to sustain this assumption the body fat mass should constitute an evaluated parameter in both studies. The observed increased litter size in our GDM model may represent one alternative avenue for the described human GDM-induced macrosomic babies in a polytocous species.

The influence of the mother on the sex allocation of the offspring is still controversial in the field of evolutionary biology. However, a great number of studies have observed that the

consumption of HFDs by rodent females and ewes results in litters with significantly more male offspring[276]. In this study, the GDM induced by the consumption of a HFHS diet resulted in increased male offspring in comparison with the control group. It is a widely held view that increased maternal glucose levels may influence embryo development, however, whether these effects take place pre-conception or post-conception is still not understood[276]. Interestingly, maternal exercise during GDM pregnancy only prevented the alterations in litter sex distribution favoring male offspring, which suggests that the changes occurring at a molecular level that may affect fetal sex distribution are attenuated by exercise.

4.2 **The postpartum period is marked by altered glucose levels**

After pregnancy, the lactation period is of extreme importance for the offspring's postnatal development and the changes occurring in the mother's organism. Lactation mobilizes an incredible amount of energy to respond to the growing requirements of the fetus[277]. Lactation is also known as the period of "weight loss after pregnancy". Given that, changes in the blood biochemical parameters of the mothers in the postpartum period are normal, and the most observed effect is on circulating levels of fast glucose. Lactating mothers have better glucose tolerance and insulin homeostasis than non-lactating mothers[277]. In the current study, the levels of capillary glucose using a glucometer and in the blood plasma by MS/MS detection differ. Both techniques have different sensitivities, being the sensitivity of the MS/MS method substantially higher[278]. That explains the generally increased values observed in glucose levels evaluated in the plasma by MS/MS, which are quite high compared to the usually normal glucose values observed in rodents that, in turn, are similar to those generally observed in humans[279].

One observation concerning the differences between the groups and the different used techniques, is that decreased glucose levels mark the postpartum period. This may occur due to the high energetic and caloric demands after the delivery that may persist in the further weeks after lactation. However, the glucose levels of the GDM-S group are significantly increased compared to the pregnant control group when assessing with MS/MS, but this is not observed for the results obtained with capillary glucose. Considering that MS/MS is a more sensitive technique, the results will be discussed from that perspective. Although glucose levels were not evaluated since mid-pregnancy until 8-weeks postpartum, these results suggest that mothers that had a sedentary GDM-pregnancy exhibit augmented increased glucose levels faster than mothers that had a normal pregnancy and mothers that had GDM but were submitted to exercise during pregnancy, which means that GDM-S mothers reach abnormal glucose levels that might be implicated in the GDM-induced memory for diabetes development, while GDM-E mothers have the metabolic flexibility to return to the normal glucose levels after the lactation period.

Although no major differences were observed in the levels of lipids in the plasma at the 8th postnatal week, pregnancies complicated by GDM and a sedentary lifestyle led to increased TGs levels in the postpartum period. Commonly, the levels of LDL were used as a parameter of casual risk for the development of atherosclerotic cardiovascular diseases, however, more recent studies have found TGs to be significantly associated with the risk of atherosclerosis[280,281], which explains why the AIP is now measured as the ratio between TGs and HDL levels[282].

Alterations in the extracellular matrix (ECM) within the myocardium and dysregulation of its homeostasis leads to cardiac fibrosis and consequent cardiac dysfunction, observed among aged

and diabetic hearts[283,284]. The primary component of the ECM is collagen[283]. In this study, the histological analysis of the cardiac tissue of the GDM-S dams revealed a slight increase in the staining of collagen fibers within the perivascular matrix surrounding the myocardium's blood vessels. Another process contributing to increased ECM and that is familiar to T2DM and GDM is a cardiac inflammatory state. The production of proinflammatory cytokines contributes to the upregulation of metalloproteinases that play a crucial role in ECM maintenance[283], possibly being involved in the progression of cardiac hypertrophy. While the levels of proinflammatory cytokines evaluated in the cardiac tissue (TNF-α and IL-6) of GDM dams are similar compared to the control groups, the practice of exercise during a GDM-pregnancy resulted in decreased values of these cytokines compared to GDM-S group at 8-weeks postpartum. However, increased collagen fibers were also detected among hearts from the exercised dams. Decreases in metalloproteinase activity due to exercise in hypertensive rats have been observed, contributing to the improvement of cardiac remodeling, however, there was no reflection in collagen accumulation within the cardiac tissue. This is following other studies[283]. Nevertheless, the preliminary histological results presented in this work need further research.

4.3 **Influence of GDM and exercise on cardiac insulin signaling**

An inflammatory state is characteristic of the obesogenic and diabetic pathophysiology[102,114]. A physiological low-grade inflammatory state occurs during pregnancy, and an exacerbated inflammatory process is related to GDM and the usually associated insulin resistance. Circulating cytokines, especially TNF-α contribute to insulin signaling impairment by activating signaling pathways that lead to phosphorylation of IRS-1 mediated by JNK[285]. Although the protein levels of total Akt in mothers that had GDM were decreased (independently of maintaining a sedentary behavior or adopting an exercised lifestyle), the ratio of phosphorylated Akt at the residue Ser473 to total Akt was increased in these groups. A state of insulin resistance would be characterized by impaired insulin signaling which would most likely result in lower levels of activated (phosphorylated) Akt. Akt overactivation can denote a mild physiological state of cardiac adaptation as a feedback response to a previous IR state and may represent a mechanism of pathology origin. It is relevant to consider that the cardiac tissue of the female rats in the present study was evaluated 8 weeks after delivery, a period in which GDM-associated insulin resistance usually disappears. Nevertheless, since GDM predisposes to further T2DM and CVD in the first decade after pregnancy, the evaluation of the alterations in the insulin signaling that remain in the postpartum period becomes very relevant.

Studies in the post pregnancy time-window already exist[286,287], but to our knowledge, none have evaluated insulin signaling in the cardiac tissue of dams. Nonetheless, studies performed in different maternal tissues (excluding the heart) at late-pregnancy revealed that the observed effects are tissue-dependent[288], a characteristic that may persist postpartum. The effects of exercise during pregnancy were also found to be tissue-dependent[186] so to prevent GDM-induced CVD it is mandatory to study the heart in GDM models.

Within the heart, the post-translational modification of Akt in the residue Ser473 is mediated by mTORC2, while Akt's phosphorylation at the residue Thr308 accrues from phosphoinositide-dependent kinase 1 (PDK1)[82]. In the present study, the ratio of p-Akt (Ser473)/Akt was increased in the hearts of GDM mothers, however, no alterations in the levels of other proteins involved in the insulin signaling pathway, such as the p70, were observed. An increase in phosphorylated Akt

would suggest an increased activation of this important kinase and even though full activation for optimal activity requires that both residues are phosphorylated, the levels of the phosphorylated to total GSK3β protein ratio, a downstream target of Akt, was decreased in the hearts of GDM-S mothers. This may be due to the fact that GSK3β might be phosphorylated by kinases other than Akt[289], which may include p70, PKC, between others. Since the levels of p70 and concomitant p-p70 to p70 ratio showed no alterations in the present study, the possibility that p70 might mediate the phosphorylation of GSK3β in the hearts of these mothers is excluded. It is established that GSK3β acts as a regulator of hypertrophy due to its role in cardiomyocyte proliferation in the adult myocardium[290], besides its ever-known role of inhibiting glycogen synthase activity. Phosphorylation of GSK3β at the residue Ser9 inhibits GSK3β kinase activity, leading to increased glycogen synthesis, which has proven to improve glucose tolerance among diabetic patients and prevented diabetes-induced cardiomyopathy in mice[291]. This suggests that the hearts of GDM-S mothers fail to regulate these mechanisms and that exercise during a GDM-pregnancy counteracts these effects since exercise helped to maintain the observed levels in physiologic pregnancy. Assessment of other kinases or other potential regulators of GSK3β phosphorylation will be required to fully comprehend the mechanism behind GSK3β despite increased Akt activity.

4.4 **Cardiac metabolic flexibility**

As highlighted in Chapter 1, maintenance of cardiac metabolic flexibility is crucial to guarantee cardiac function and homeostasis (section 1.2.3), and it may be regulated by physiological conditions depending on the organism's environment. In various tissues, including the heart, the hyperglycemia resultant from a diabetic state induces hypoxia[292]. The low oxygen availability obligates cardiomyocytes to adapt by shifting metabolism to a lower oxygen tension. This hypoxic state induces upregulation of HIF-1α to increase glycolytic activity[293]. The transcript levels of HIF-1α have shown to be elevated in the hearts of diabetic rats[294]. Moreover, the relationship between HIF-1α increased expression and decreased PPARα expression has been established and linked to the return to a "fetal-like" phenotype usually observed in the failing heart[197,199]. In this work, the transcript levels of HIF-1α of the GDM-S group were indeed increased, but as a surprising result pregnancy *per se* also contributed to increased levels of this transcript. Accordingly, decreased PPARα levels in the hearts of mothers belonging to the same groups was observed comparing to a non-pregnant female heart. Moreover, increased levels of the ACAA2 transcript were observed in the cardiac tissue from the GDM-S group in comparison to P-C-S, which seems contrary to the decreased PPARα levels since it is responsible for the regulation of the expression of genes involved in FA oxidation. Nevertheless, these results suggest that HIF-1α signaling may be compromised both in response to pregnancy and to a pregnancy complicated by GDM in which mothers had a sedentary lifestyle, and that it results in alterations in the levels of other transcripts involved in FA oxidation, showing signs of dysregulation. Indeed, alterations in the levels of PPARα have been related to loss of cardiac metabolic flexibility[295], but to further comprehend the adaptations occurring in the hearts of mothers in the postpartum period after a normal pregnancy or a pregnancy complicated by GDM, the evaluation of glycolytic pathways is suggested. On the other hand, the levels of HIF-1α and PPARα transcripts in the hearts of exercised GDM mothers did not significantly differ from basal levels of the NP-C-S group. Upregulated levels of HIF-1α have been related to exercise-related cardiac protection through regulation of the shift between aerobic metabolism to anaerobic glycolysis[296]. Although

this is not observed at 8 weeks postpartum, the practice of exercise during pregnancy might have induced an adaptive response that further allowed a faster response in the hearts of exercised mothers and a quicker return to basal levels of these transcripts.

Cardiac metabolic flexibility is governed by the energy state of cardiomyocytes. The protein AMPK is responsible for the coordination of a general metabolic regulation to respond to the altered cardiac energy status, stimulating catabolic processes for ATP production and switching off anabolic ATP-consuming pathways[211]. In this work, pregnancy, in general, contributed to an increased ratio of phosphorylated AMPK to total protein, which suggests that pregnancy-induced metabolic alterations in the postpartum period led to a consequent stimulation of AMPK functions so the heart can adapt to the new environment. However, this effect is not marked in the hearts of dams that had a GDM-pregnancy and kept a sedentary lifestyle which may indicate a higher difficulty in stimulating the heart's adaptational processes contributing to the maintenance of cardiac metabolic flexibility. Nonetheless, the previously suggested evaluation of glucose utilization in the heart in these conditions will better elucidate the results discussed in this section. Furthermore, it will help to enlighten the effects of exercise practice during pregnancy on cardiac metabolism.

Mitochondria are extremely important organelles that sustain cardiac contractility and normal function in cardiomyocytes. Mitochondrial dysfunction is associated with a series of diseases, including CVD and diabetes[44,215]. In the heart, mitochondria play a critical role in the maintenance of cardiac metabolic flexibility since this process molecularly relies on the interplay of metabolic pathways regulated by specific enzymes and transcription factors that interact with mitochondria.

4.5 **Implications for cardiac mitochondrial function**

Assessment of mitochondrial bioenergetics allows the evaluation of mitochondrial efficiency in response to physiological and pathological conditions. Regarding complex-I substrate-driven ATP synthesis, isolated cardiac mitochondria from mothers that had a pregnancy complicated by GDM showed worse coupling between substrate oxidation and ADP phosphorylation, indicated by decreased RCR values. This effect was further aggravated by exercise due to the decreased values in the maximum ADP-stimulated phosphorylation rate (state 3). Indeed, decreased state 3 respiratory rates and reduced RCR values were reported in the context of heart failure and western-diet consumption[297,298]. However, the models used in these studies are very different from the model in the present work. Studies that involve maternal diet-induced obesity or diabetes, solemnly evaluate the offspring's mitochondrial function. Once again, it is worth denoting the lack of studies on maternal health after complicated pregnancies that may further contribute to disease development.

Furthermore, mitochondria of GDM-S and GDM-E mothers need more oxygen to phosphorylate the same amount of ADP, presenting decreased ADP/O ratio values, which goes in accordance with the registered increased time that cardiac mitochondria from these two groups took to completely phosphorylate ADP, meaning that these mitochondria phosphorylate ADP at a lower rate. The cardiac ADP phosphorylation rate is more affected in mothers who practiced exercise during a GDM-pregnancy than in the ones that had a sedentary lifestyle. Mitochondrial complex-II driven respiration was also evaluated but no alterations were observed between the evaluated parameters, suggesting that complex-I has a strong contribution in the deleterious

cardiac memory led by GDM pregnancy. Studies evaluating mitochondrial respiration rates after exercise practice in the heart are also scarce. While some report a mild beneficial effect of exercise[299], others find protective compounds to the deleterious effect of exhaustive exercise[300]. Moreover, many of these studies are performed exclusively in male animals making it impossible to parallelize between other studies and the current study performed in females.

In summary, these results suggest that in the 8-weeks postpartum period, maternal cardiac mitochondria could not efficiently overcome the GDM-induced alterations during pregnancy and that the practice of exercise exclusively during GDM might even hamper that response. Understanding the mitochondrial-related molecular mechanisms behind these deleterious effects might uncover possible explanations for the adverse role of exclusive exercise during pregnancy on postnatal cardiac mitochondrial OCR and membrane potential and understand if these mitochondrial impairments are conditioning the effective myocardial remodeling.

Despite the observed decreased mitochondrial efficiency by evaluation of mitochondrial bioenergetics, the protein levels of the complex-III UQCRC2 subunit, but especially of the mtDNA encoded complex-IV MTCO2 subunit were increased in the cardiac tissue of dams that had a GDM pregnancy and were sedentary. Post-translational modifications of cardiac mitochondrial proteins that alter their activity and may consequently interfere in mitochondrial metabolism have already been observed in the context of heart failure and diabetes[301,302]. In particular, acetylation or O-GlcNAcylation of OXPHOS subunits has been shown to decrease the complex's activity[302], possibly resulting in altered mitochondrial function. Nevertheless, only a subunit of each complex was assessed in the current work. Research on these post-translational modifications in OXPHOS subunits is still recent, so before jumping into this hypothesis, the enzymatic activity of each OXPHOS complex should be assessed. Furthermore, although the levels of Tom20, a mitochondrial mass indicator, are decreased in the pregnant control group in relation to its non-pregnant counterpart, this decreased expression did not influence on mitochondrial bioenergetics. Mitochondrial RNA polymerase (POLRMT) is responsible for the transcription of the circular mammalian mtDNA and is indispensable for mtDNA replication[263]. The cardiac levels of this transcript showed to be decreased in the postpartum period of control dams and of mothers that had a GDM pregnancy and practiced exercise. Although conditional knock-out of this gene leads to severe mitochondrial dysfunction in mice[263], the exact cardiac repercussions of the alterations in its regulation are unknown.

TFAM is also required for mtDNA transcription, which together with POLRMT and other transcription factors form a complex to initiate mitochondrial RNA synthesis[263]. The interactions between TFAM and POLRMT are still undetermined but the absence of POLRMT results in increased free-TFAM pool[263]. On the other hand, overexpression of TFAM contributes to increased abundance of mtDNA copy number[303]. In the current study, TFAM protein levels were slightly increased in the group of dams that had a normal pregnancy, which was not observed for the group of GDM-mothers that practiced exercise during pregnancy. This may suggest that an increase in TFAM levels might constitute an adaptational process of cardiomyocytes to respond to an impaired condition and overcome induced mitochondrial dysfunction, and possibly oxidative stress. Taken that, this tendential increase in the control mothers' TFAM levels may have contributed to the observed normal mitochondrial bioenergetics, while GDM-mothers that had an exercised lifestyle did not have the same adapting capability, showing normal TFAM levels. Nevertheless, evaluating other mitochondrial mass markers such as the mtDNA copy number would be pertinent in further studies.

The proteins located in the IMM and OMM are essential to regulate mitochondrial functions (e.g. energy production, fusion and fission processes, calcium handling)[304]. The VDAC and ANT constitute two of the most abundant proteins in the outer and inner mitochondrial membrane, respectively. While VDAC enables the passage of metabolites, peptides and ions[304,305], and is particularly involved in apoptosis[306], ANT is central to ATP/ADP exchange between the mitochondrial matrix and intermembrane space[307]. In this study, the ANT1/2 protein and VDAC2 transcript show increased levels in the hearts of mothers that had GDM. This might suggest that mitochondria from GDM-S mothers are more prone to induced apoptosis, however, further studies and a better understanding of the interaction between VDAC and ANT in the heart and regulating mechanisms, for instance, the implications of Bax and Bcl2 interactions[306], is necessary to elucidate their role in the hearts of GDM mothers.

Mitochondrial biogenesis, together with mitochondrial dynamics and mitophagy play a crucial role in maintaining mitochondrial function and quality. PGC-1α is the master regulator of mitochondrial biogenesis by acting as a cofactor of important transcription factors involved in this process[273]. PGC-1α transcript and protein levels in the hearts of db/db mice were increased in response to exercise training, compared to sedentary db/db mice[221]. PGC-1α increased expression was accompanied by concomitant increased Akt activation (via phosphorylation at the Ser473 residue). Increased TFAM, mtDNA content, and Nrf1 levels were also observed in the hearts of exercised diabetic mice. Furthermore, exercise training prevented the accumulation of collagen fibers in the cardiac tissue of diabetic trained mice[221]. In the current study, although PGC-1α transcript levels are substantially increased in response to exercise practice during a GDM-pregnancy and p-Akt (Ser473)/Akt levels are also increased, PGC-1α downstream targets, which include TFAM and Nrf2 show similar or decreased levels compared to the remaining groups. Although it seems that the heart of mothers that had a pregnancy complicated by GDM and practiced exercise during pregnancy is activating defense mechanisms to counteract the induced deleterious effects, the myocardium itself cannot respond to that stimulation and adapt to the new conditions. Moreover, the histological analysis of the hearts of GDM-E mothers revealed the accumulation of collagen surrounding the cardiac blood vessels, sustaining this hypothesis even further.

The levels of proteins involved in the constant fusion and fissions processes (OPA1, and Fis1 and DRP1, respectively) that occur in the highly dynamic network of mitochondria are similar between all the experimental groups, which would suggest that mitochondrial fusion and fission are in equilibrium, as well as the process responsible for removal of damaged mitochondria. However, macroautophagy, responsible for the degradation of damaged cytosolic components, including mitochondria, is dysregulated in the hearts of GDM-mothers. During this process, LC3-I is converted to LC3-II by lipidation. LC3-II then associates with autophagic membranes and recognizes adaptor proteins, such as p62, responsible for polyubiquitinated proteins recognition[308,309]. Therefore, an increased ratio of LC3-II/LC3-I protein levels and p62 accumulation may be related to the blockade of autophagic flux[310]. In this study, a tendential increase in LC3-II/LC3-I ratio was observed in the hearts of mothers that had a GDM-pregnancy and were sedentary. Moreover, the levels of p62 were also increased in this group, and in the cardiac tissue of GDM-E mothers, suggesting an impairment in macroautophagy and an accumulation of autophagosomes. Furthermore, an increased autophagy in the heart is also associated with the dissociation of the Bcl2-Beclin1 complex. This occurs due to Bcl2 phosphorylation at the residue Ser70 in the cardiac tissue[311]. Hearts of dams subjected to exercise during a GDM-pregnancy revealed decreased levels of total Bcl2 protein accompanied by

increased p-Bcl2 (Ser70)/Bcl2 ratio, suggesting an increased autophagic process in the hearts of these dams. It has been demonstrated, in vitro, that the adaptor protein p62 stimulated autophagy through direct interaction with Bcl2[312]. This interaction would promote Bcl2 phosphorylation and dissociation with Beclin1. Indeed, the levels of p62 are significantly increased in the GDM-E group, suggesting an increased autophagic flux. Nevertheless, this does not seem to contribute to an improved mitochondrial respiration but might, on the other hand, compose a consequence of decreased mitochondrial respiration efficiency.

Another protein that has been implicated in the regulation of autophagy[313] and apoptosis[314] is JNK, through interaction with BH3-only members of the Bcl2-family. Stimulated autophagy and induced apoptosis mediated by JNK have been related to Bcl2 phosphorylation at the Ser70 residue[314], however, the levels of JNK detected in this study do not support this pathway. In the present study, the major result concerning JNK protein levels indicate a significant increase in JNK isoform 54 in the hearts of dams that had a normal pregnancy. Unfortunately the levels of the phosphorylated protein were not possible to detect. The JNK family is composed of 3 known isoforms: 2 ubiquitously expressed, JNK1 (isoform 46) and JNK2 (isoform 54); and 1 tissue-specific, JNK3[315]. Studies have supported distinct functions of both ubiquitously expressed isoforms[316], and the majority points to the exclusive implication of JNK1 in these processes[313,315]. Another BH3-only protein targeted by JNK to promote apoptosis is Bad, by activation mediated by phosphorylation at the residue Ser128 and, on the other hand, JNK can inhibit Bad proapoptotic activity through phosphorylation at the residue Ser112[314]. Although changes were observed in the levels of Bad in the P-C-S and GDM-E groups, no alterations were observed for the ratio between p-Bad (Ser112) and Bad.

Mitochondrial homeostasis dysregulation and damage result or are resultant of increased oxidative stress. Whenever ROS production is augmented, the antioxidant defense systems take action to prevent further damage. Nrf2 is involved in the transcription of several genes that encode proteins involved in antioxidant defense, playing a crucial role in redox balance[317]. In the current work, the levels of Nrf2 were increased in the cardiac homogenates of mothers that had a normal pregnancy and even exacerbated in mothers that had a pregnancy complicated by GDM and were subjected to a sedentary lifestyle. These results may suggest that redox balance is impaired in the hearts of these dams but, at the same time, an adaptive response to an impaired antioxidant status within the cardiomyocyte is taking place. Nrf2 directly regulates the expression of Gpx4[70] and the levels of this protein were also evaluated. Gpx4 expression is enhanced in all the groups in the postpartum period compared to a non-pregnant heart, including the exercised group, although no alterations were observed regarding Nrf2 levels in this specific group. This may indicate an unbalanced redox status in which Gpx4 expression is regulated by other signals, possibly mediated by exercise or that Gpx4 expression is stimulated only upon Nrf2 activation and translocation into the nucleus. Considering this, assessing phosphorylated Nrf2 and Nrf2 levels in the nuclear fraction would be an interesting evaluation, further complementing and helping enlighten these results. Furthermore, no alterations were observed in SOD2 protein levels, Nrf2 does not directly induce[70].

Chapter 5 – Conclusions and future perspectives

The last decades have been marked by increased prevalence of metabolic diseases among not adults, adolescents, and children. Obesity and diabetes are now recognized as epidemics and their rising incidence is highly associated with lifestyle habits, such as (but not limited to) diet consumption and physical activity. Both obesity and diabetes contribute to increased risk of cardiovascular disease development, the leading cause of mortality worldwide. Gestational diabetes mellitus constitutes the most common metabolic disease of pregnancy, and following the pattern, its incidence is increasing worldwide. This is extremely alarming since mothers who had a pregnancy complicated by gestational diabetes mellitus have an increased risk of developing cardiovascular disease between the first 5 to 10 years after delivery. In addition to this, offspring's metabolic health might be impaired due to maternal behavior.

In this work, we developed a novel GDM model in Sprague-Dawley rats that intended to study the post-pregnancy effects of a GDM-pregnancy on cardiac mitochondria metabolism and understand if exercise exerted a beneficial modulation by preventing these effects. Our model mimicked features of GDM that were reflected by glucose intolerance developed at mid-pregnancy and increased gestational weight gain. Alterations in the offspring's characteristics were also induced by GDM. Furthermore, the postpartum period of GDM mothers with a sedentary lifestyle is marked by increased blood glucose levels that counteracting mechanisms fail to regulate. Post-pregnancy effects observed in the cardiac tissue of dams that had a GDM-pregnancy and a sedentary lifestyle include dysregulated cardiac metabolic flexibility due to a shift among glucose and fatty acid metabolism and mitochondrial dysfunction by a less efficient mitochondrial bioenergetics, possibly impaired macroautophagy, and a potential stimulation of oxidative stress. Although cardiac metabolic flexibility seems to be somehow impaired in the hearts of mothers that had a normal pregnancy, compensatory mechanisms were found to be regulated in the hearts of these dams. Surprisingly, exercise practice during pregnancy showed to negatively contribute to cardiac mitochondrial function, which was found to be due to the incapacity of these exercised hearts of GDM mothers to cope with another challenge (**Figure 5.1**).

Taking this into consideration, the practice of exercise exclusively during pregnancy leads to impaired mitochondrial function and fails to upregulate compensatory mechanisms to prevent the memory induced by GDM, despite improvement of some of the evaluated parameters, especially physiologic parameters. These findings denote the need to closely monitor GDM pregnancies and of a careful implementation of healthy lifestyle habits in the clinical management of this pathophysiology. Exercise implicates that a series of adaptational mechanisms within the organism takes place to positively respond to the impaired environment it is challenged with. Implementing a training protocol that is only applied during pregnancy and ceased after delivery may result in the loss of the cardioprotective effects induced by exercise, subsequently leading to severe maladaptations due to the continuous metabolic challenge that occurs in the postpartum period.

To fill in the gaps of the present study, the evaluation of several enzymatic activities will be performed (**Figure 5.2**). Firstly, to better comprehend how cardiac metabolic inflexibility progressed in the hearts of the dams subjected to each experimental intervention, namely related to glucose metabolism, the enzymatic activities of hexokinase, glucose-6-phosphate dehydrogenase (G6PD), and lactate dehydrogenase (LDH) will be assessed. Hexokinase composes the initial enzyme of glycolysis which function is to phosphorylate glucose and produce

glucose-6-phosphate. This last molecule can further undergo different pathways. G6PD catalyzes its conversion to yield pyruvate that can further enter the TCA cycle or be converted to lactate by LDH. Moreover, since both fatty acid and glucose metabolism may culminate in the TCA cycle, the bridge between fatty acid and glucose oxidation and mitochondria will be established through pyruvate dehydrogenase (PDH). This enzyme is involved in the conversion of pyruvate to acetyl-CoA, regulating the entry of carbons into the TCA cycle. The enzymatic activity of PDH will also be complemented with the levels of pyruvate dehydrogenase kinase 1 (PDK1), a protein that was found involved in the suppression of PDH, already assessed in cardiac homogenates. Within the TCA cycle, citrate synthase, aconitase and fumarase activities will be evaluated in cardiac mitochondria. Due to aconitase's susceptible oxidation with superoxide anion, measuring its activity will give further insights into the cardiac redox state in this model. To further understand the extensiveness of oxidative damage in the hearts of these GDM-mothers and the role of exercise practice during pregnancy, the activities of catalase and glutathione peroxidase, and glutathione reductase will be measured, as well as the GSH/GSSG (reduced/oxidized glutathione) and NADP/NADPH ratios. Finally, the activity of creatine kinase, the primary enzyme involved in ATP flux in the myocardium and essential for cardiac contraction, will be evaluated (**Figure 5.2**).

By performing these further studies we hope to contribute to more in-depth elucidation of the molecular mechanisms contributing to impaired cardiac function and the metabolic state after a GDM pregnancy, which may be related to increased cardiac disease risk and confirm if exercise practice exclusively during pregnancy results in impaired cardiac function postpartum and post-cessation of exercising activities.

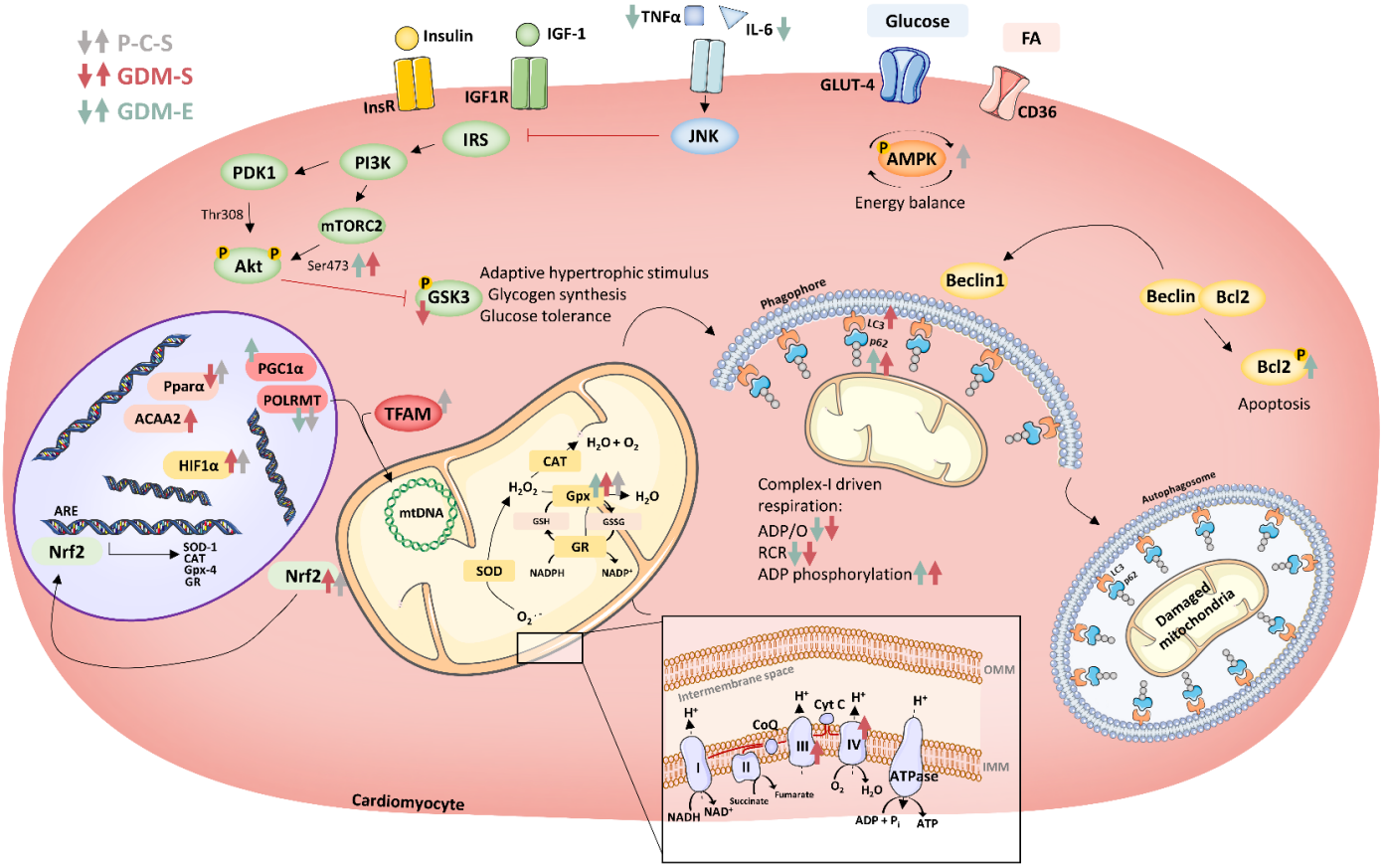

Figure 5.1 – Schematic representation of the postpartum effects of gestational diabetes mellitus and exercise practice during a pregnancy complicated by gestational diabetes mellitus on cardiac mitochondria metabolism.

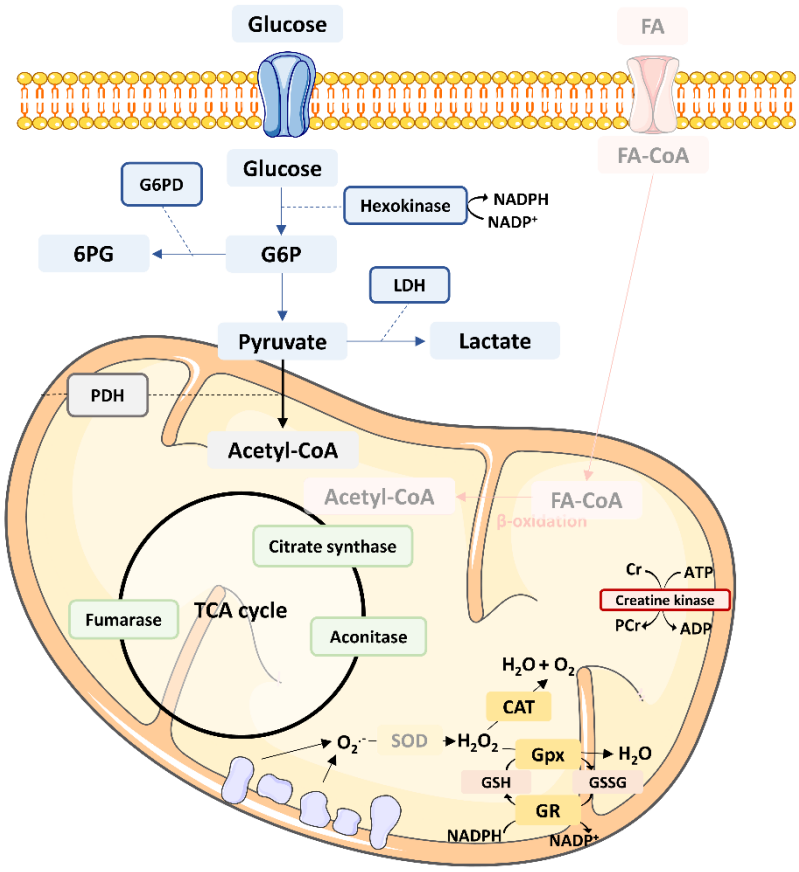

Figure 5.2 – Schematic representation of the future work suggested.

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