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COIMBRA

Mariana Filipa Simões Diniz

MADE IN THE WOMB

MATERNAL PROGRAMMING OF OFFSPRING CARDIOVASCULAR FUNCTION

Dissertação no âmbito do Mestrado em Bioquímica orientada pela Doutora Susana P. Pereira e pelo Professor Doutor Carlos Palmeira e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2021

Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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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:

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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. 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

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6. 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).

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10. **Mariana Diniz,** Luís F. Grilo, João D. Martins, Stephen Ford, Peter W. Nathanielsz, Paulo J. Oliveira, Susana P. Pereira. Maternal obesity (MO) during pregnancy: are there implications for maternal hepatic mitochondrial activity? European Journal of Clinical Investigation, Vol. 50, p.32. http://dx.doi.org/10.1111/eci.13369

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Abstract

Obesity incidence has been increasing at an alarming rate, especially in women of reproductive age. It is estimated that 50% of the total number of pregnancies occur in overweight or obese women. Maternal obesity (MO) predisposes the offspring to an increased risk of developing many chronic diseases in an early stage of life, including obesity, type 2 diabetes, and cardiovascular disease (CVD). CVD is the main cause of death worldwide among men and women. Despite this, CVD risk exhibits sexual dimorphism. Maternal diet and MO during gestation could prompt the offspring for CVD development through adaptations of the offspring's cardiovascular system in the womb. This could lead to cardiac epigenetic and persistent metabolic programming of signalling pathways, including mitochondrial metabolic function, culminating in offspring's increased predisposition to CVD development. Currently, despite diet supplementation alternatives being provided, effective therapeutical solutions to prevent the deleterious cardiac offspring function programming by obesogenic womb are lacking. This innovative work involves a novel approach to unravel how the offspring's cardiovascular system reacts to maternal physical exercise practice during an obesogenic pregnancy, which was induced with a high fat/high sugar (HFHS) diet, and whether exercising during an obesogenic pregnancy (MOEx) can modulate the offspring cardiac function programming. Thirty-two-week-old offspring Sprague-Dawley rats exposed to MO and MOEx were used. The long-term therapeutic effect of maternal physical exercise during pregnancy in reversing the MO-induced effects on the cardiac function of young-adult offspring was evaluated by measuring hallmarks of cardiac metabolic impairment and mitochondrial dysfunction. Although the innate sex-specific response in the offspring's cardiovascular physiology, MOEx induced biochemical modulation in the offspring, as indicated by the altered circulating levels of relevant molecules such as triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), despite the evident sexual dimorphism. These alterations were accompanied by cardiac metabolic remodelling, which was evaluated by measuring key-proteins involved in the insulin signalling pathway, along with alterations in the short-chain fatty acid transporter, CD36. This could indicate that MOEx affected cardiomyocyte fatty-acid uptake, and, along with the observed altered lipid metabolites levels, the MOEx offspring's cardiac mitochondrial function could be modulated. Indeed, the offspring's cardiac mitochondrial function seemed to be improved by MOEx comparing to MO, exhibiting a positive modulation of mitochondrial bioenergetics, along with possible mitochondrial dynamics modulation, which was observed through altered mitochondrial fusion- (MFN-1 and OPA1) and mitochondrial biogenesis-related proteins (PGC1α and TFAM). In addition, preliminary data indicated that MOEx prevents MO-induced nitrosative stress.

Overall, maternal physical exercise practice during an obesogenic pregnancy leads to the modulation of the offspring's biochemical, cardiac metabolic parameters and cardiac mitochondrial function, in a sex-specific way. These alterations may be favorable enough for the MO offspring's cardiovascular health, which might result in the attenuation or even prevention of the development of CVD in MOEx offspring early life.

Keywords: Maternal obesity; obesogenic pregnancy; offspring cardiac remodelling; maternal programming; cardiovascular disease; gestational exercise; cardiometabolic modulation; cardiac mitochondria; sexual dimorphism.

Resumo

O constante aumento da incidência de obesidade, especialmente em mulheres em idade fértil, tem sido um forte motivo de alarme. É estimado que 50% das grávidas têm excesso de peso ou são obesas. A obesidade materna predispõe o descendente para um risco acrescido de desenvolver inúmeras doenças crónicas, tais como obesidade, diabetes tipo 2 ou doença cardiovascular. A doença cardiovascular é, globalmente, a principal causa de morte para homens e mulheres. Não obstante, o risco de desenvolvimento de doença cardiovascular é diferente de acordo com o sexo. A obesidade e nutrição materna durante a gestação podem desencadear o desenvolvimento de doença cardiovascular na descendência, através de adaptações do sistema cardiovascular no útero. Isto pode levar à programação de mecanismos epigenéticos e de vias metabólicas, incluindo a função mitocondrial cardíaca. Atualmente, apesar de alternativas de suplementação serem indicadas nestes casos clínicos de modo a prevenir os efeitos deletérios da obesidade materna na função cardíaca dos descendentes, a falta de estratégias terapêuticas eficazes é notória.

Este trabalho engloba uma estratégia inovadora propondo investigar de que forma é que o sistema cardiovascular de um descendente reage à prática materna de exercício físico durante uma gravidez obesogénica, a qual foi induzida através de uma dieta rica em açúcares e gorduras, em específico, como é que a prática de exercício físico materno durante uma gravidez obesogénica pode modular a função cardíaca dos descendentes. Ratos machos e fêmeas com 32 semanas de idade, descendentes de fêmeas rato da estirpe *Sprague-Dawley*, foram utilizados neste trabalho. Foi avaliado o efeito, a longo termo, da prática de exercício físico materno durante uma gravidez obesogénica na prevenção dos efeitos deletérios induzidos pela obesidade materna durante a gravidez na função cardíaca, em importantes vias metabólicas e na função mitocondrial de descendentes jovens-adultos. Apesar da evidente resposta dimórfica na fisiologia inata dos descendentes, a prática de exercício físico materno durante uma gravidez obesogénica induziu a modulação de parâmetros bioquímicos nos descendentes, traduzida em níveis alterados dos seguintes metabolitos no sangue: triglicerídeos, HDL e LDL, tendo esta resposta um evidente dimorfismo sexual. Estas alterações foram acompanhadas por uma remodelação metabólica do tecido cardíaco, que foi apreciada pelos níveis de proteínas-chave na via de sinalização da insulina, assim como por alteração nos níveis do transportador de ácidos gordos de cadeia curta, o CD36. Isto pode indicar que o transporte de ácidos gordos para o cardiomiócito encontrasse comprometido e, em conjunto com alterados níveis de metabolitos de lípidos, pode modular a função cardíaca mitocondrial de descendentes de mães obesas e exercitadas. De facto, a função cardíaca mitocondrial destes descendentes foi melhorada pela prática de exercício físico durante uma gestação obesogénica, com uma modulação positiva da bioenergética mitocondrial, em conjunto com uma possível modulação da dinâmica mitocondrial, através da observação de níveis alterados de proteínas envolvidas em eventos de fusão (MFN-1 e OPA1) e biogénese (PGC1α e

TFAM) mitocondrial. Para além disso, resultados preliminares indicaram que o exercício materno praticado durante uma gravidez obesogénica pode prevenir o *stress* nitrosativo no tecido cardíaco. Concluindo, a prática de exercício físico durante uma gravidez obesogénica levou a uma modulação dos parâmetros bioquímicos, cardiometabólicos e da função mitocondrial cardíaca dos descendentes em idade jovem, com uma resposta específica de acordo com o sexo do descendente. Estas alterações podem ser benéficas o suficiente para melhorar a saúde cardiovascular dos descendentes, o que poderá levar à atenuação ou até mesmo à prevenção do desenvolvimento de doença cardiovascular numa fase mais tardia da vida.

Palavras-chave: obesidade materna; gravidez obesogénica; remodulação cardíaca da descendência; programação materna; doença cardiovascular; exercício gestacional; modulação cardiometabólica; mitocôndria cardíaca; dimorfismo sexual.

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List of Acronyms and Abbreviations

4-HNE	4-Hydroxynonemal
AA	Amino acid
ADP	Adenosine diphosphate
AMPK	AMP-activated kinase
ATP	Adenosine triphosphate
BMI	Body mass index
BNP	Brain natriuretic peptide
BW	Birth weight
CAT-1	Cationic amino acid transporter 1
CoA	Coenzyme A
CREB-1	Cyclic AMP-responsive-element-binding protein
CVD	Cardiovascular disease
Cx43	Connexin-43
DALY	Disability-adjusted life-years
DHA	Docosahexaenoic acid
DNA	Desoxyribonucleic acid
DNMT	DNA methyltransferase
DoHaD	Developmental Origins of Health and Disease
DRP1	Dynamin related protein 1
E_2	17β-estradiol
ETC	Electron transport chain
FA	Fatty acid
FABP	Fatty acid binding protein
FABPpm	Plasma membrane fatty acid binding protein
FAT/CD36	Fatty acid translocase/scavenger receptor cluster of differentiation 36
FATP	Fatty acid transport protein
Fis-1	Fission 1 protein
G/M	Glutamate/malate
GDB	Global disease burden
GDM	Gestational diabetes mellitus
GI	Glycaemic index
GLUT	Glucose transporters family
GMP	3',5' - cyclic guanosine monophosphate
GPx	Glutathione peroxidase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HDL	High-density lipoprotein
HFD	High-fat diet
HFHS	High fat-high sugar
IGF2	Insulin growth-factor 2

IL-6	Interleucin-6
IMM	Inner mitochondrial membrane
IMS	Innner mitochondrial membrane space
IR	Insulin receptor
IRS	Insulin receptor substrate
LCFA	Long-chain fatty acid
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LV	Left ventricle
MDA	Malondialdehyde
MEF2	Class IIa HDAC - myocyte enhancer factor 2D
MFF	Mitchondrial fission factor
MFN	Mitofusion
miRNA	Micro-RNA
MO	Maternal obesity
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotine adenine dinucleotide phosphate hydrogen
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
NFATc4	Nuclear factor of activated T-cells 4
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF	Nuclear respiratory factor
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation system
PDH	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PGC-1α	Peroxisome-proliferator-activated receptor γ co-activator-1α
PI3K	Lipid kinase phosphoinositol-3-kinase
PKG	Protein kinase G
RNA	Ribonucleic acid
RNS	Reactive nitrosative species
ROS	Reactive oxygen species
SAM	S-adenosyl methionine
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
SSM	Subsarcolemmal mitochondria
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TET	Ten-eleven translocase
TFAM	Mitochondrial transcription factor A
TG	Triglyceride
TNF	Tumor necrosis factor
TOM	Outer membrane translocase

tRNA	Transference RNA
UCP	Uncoupling protein
WHO	World Health Organization

Chapter 1 – Introduction

1.1. Obesity: the evolution of an ongoing pandemic.

"Are those people to do to whom their obesity is not only disagreeable, but also dangerous, especially if there be a predisposition to apoplexy or paralysis? They should eat less and take more exercise but at the same time adhere to their mixed diet."

– Charles Munde, 1865.

Obesity is defined as an abnormal or excessive fat accumulation in the body, affecting one's health. The most used anthropometric metrics to classify obesity is the body mass index (BMI). This index is calculated through the ratio between weight (kg) and the square of the height (m) (kg/m^2). Obesity in adults is considered as a BMI $\geq 30\ kg/m^2$ [1].

The oldest finding in the Pubmed database concerning obesity dates from 1865, which was when obesity started to be gradually recognised as a problem instead of a health symbol [2]. In 1980, it was estimated that in 70 countries, the obesity prevalence doubled [3]. Seventeen years later, the World Health Organization declared obesity a global epidemic [1].

Nowadays, the prevalence of obesity has been increasing alarmingly. In 2018, almost 30% of the worldwide population was obese or overweight [3], affecting both children and adults heterogeneously across regions. In 2017, the American population accounted for almost 30% of obese people, while Southeast Asia registered the lowest percentage, around 5%. By 2025, the WHO estimates that one in five adults will be obese [4], increasing the burden in health care systems. The growth in the prevalence of obesity is mainly attributed to an unbalance between lower energy expenditure and higher poor-quality food intake. This is partially caused by the "*westernization*" of lifestyles, where sedentary behaviours among the worldwide population and lower prices of high caloric and industrialized fast food are verified [5]. Obesity is a risk factor for several metabolic and noncommunicable diseases such as type 2 diabetes mellitus, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) [5].

Epidemic non-communicable diseases (NCD) are one of the major health challenges of the 21st century. NCDs are the leading cause of death, killing 41 million people globally each year (71% of all deaths), with CVD contributing for the majority. The United Nations 2030 Agenda proposed to combat NCDs with the adoption of the action plan to reduce by 1/3 premature mortality by NCDs through prevention and treatment by 2030 , thus new management strategies and/or prevention of CVD are demanded [6].

1.1.1. The Challenge of Maternal Obesity.

The human body composition differs slightly between males and females. The abdominal fat accumulation and adipose tissue morphology and function are distinct among sex [7]. For example, the femoral adipocyte size is higher for pre-menopausal lean women than lean men [8]. The prevalence of obesity in 2017 in regions such as Africa, Eastern Mediterranean, and Southeast Asia was twice higher in women [4].

The increasing incidence of obesity among women of childbearing age (18-39 years) has made it one of the most common and severe obstetric conditions. Between 2013 and 2014, 37% of women of reproductive age were obese in the United States [9]. For the same period, as expected, this rate varied according to the ethnic group being 10% for Asian women, 33% for non-Hispanic white women, 43% for Hispanic women, and 57% for non-Hispanic black women [10]. In 2016, according to the WHO, the incidence rate of obesity among women in this age group was 24.5% in Europe. In Portugal, this represented a rate of 21.2%, 3-fold increase since 1975, when only 6.8% of Portuguese women of reproductive age were obese [11].

Maternal Obesity (MO) results in short and long-term adverse outcomes for both the mother and offspring [12]. At the short-term, being obese during pregnancy increases the mother's risk of developin gestational diabetes mellitus (GDM), preeclampsia, sleep apnea, liver, kidney and lung disorders, heavier placental weight, and represents an increased risk of miscarriage [13,14]. MO has been associated with the long-term development of maternal chronic hypertension, increased insulin resistance and T2DM [12]. These adverse outcomes have led to the creation of specific guidelines of total recommended weight gain during pregnancy, according to the pre-gestational BMI (**Table 1.1**).

Table 1.1 | *Recommended total gestational weight gain according to prepregnancy BMI. Adapted from Gaillard, 2015* [15].

Prepregnancy condition	**BMI (kg/m^2)**	**Recommended amount of total gestational weight gain (kg)**
Underweight	< 18.5	12.5 - 18.0
Normal range	18.5 - 24.9	11.5 - 16.0
Overweight	≥ 25.0	-
Pre-obese	25.0-29.9	7 - 11.5
Obese class I	30.0 - 34.9	5.0 - 9.0
Obese class II	35.0-39.9	
Obese class III	≥ 40.0	

1.1.2. Maternal Obesity: a threat to the offspring's health.

Increasing evidence suggests MO as a key determinant of offspring's health not only in the womb but throughout an entire lifetime. Neonates born to obese mothers have an increased predisposition to overgrow, resulting in macrosomia (birth weight (BW) > 4000 g) [14]. Moreover, MO offspring have a higher risk of developing congenital anomalies, such as heart defects and neural tube defects, increasing the risk of injury during birth, stillbirth, and perinatal death. Later in life, offspring from obese mothers are more prone to develop childhood obesity and chronic diseases. According to a meta-analysis study performed in 2013 [16], excessive gestational weight gain increases the risk for childhood obesity by 33%. Additionally, studies have shown stronger associations between maternal weight gain status, obesity, and increased offspring cardiometabolic risk [17]. A cohort study showed increased systolic blood pressure in 6-year-old children of overweight or obese mothers, which is a primer for higher cardiometabolic risk, highlighting a solid link between MO and offspring early CVD development [15]. In fact, in an Australian cohort study, abnormal gestational weight gain was associated with higher systolic blood pressure in 21-year-old offspring [18]. These observations make it reasonable to associate MO with impairments in the offspring's cardiovascular health, enhancing the risk to develop CVD later in life [19,20], raising the need to revise the concept that cardiovascular risk is uniquely determined by genetic predisposition and postnatal lifestyle.

1.2. The Worldwide Burden of Cardiovascular Disease.

Disease burden refers to the number of premature deaths, expressed as years of life lost and years lived with disability, according to the Global Disease Burden (GBD). Disability-adjusted life-years (DALY) is one of the most used metrics to measure disease burden, referring to the sum of years of life lost prematurely and years lived with disability [21]. According to the WHO, CVD is a group of heart and blood vessels disorders (**Table 1.2.**). In the 2019 GBD study, ischemic heart disease registered the highest DALY numbers [22]. CVD is the number one cause of mortality worldwide. In 2019, 17.9 million people died from CVD. This represents a massive 32% of the global population. By 2030, it is estimated that more than 23.6 million people will die of CVD. Due to the high populational density, South and East Asia reported the highest absolute number of deaths in 2017 [23]. In Europe, CVD had a prevalence death rate of 45% in 2017 [24]. In the same year, Portugal registered 32,286 CVD-related deaths (14,574 males; 17,712 females), accounting for almost 31% of all deaths [25].

Table 1.2. | Cardiovascular death proportion according to cause. Adapted from Roth et al., 2020 [21].

CVD death cause	Percentage of CVD deaths (%)
Ischemic Heart Disease	49.2
Ischemic Stroke	17.7
Intracerebral hemorrhage	15.5
Hypertensive Heart Disease	6.2
Subarachnoid Hemorrhage	2.0
Cardiomyopathy and Myocarditis	1.8
Rheumatic Heart Disease	1.6
Atrial Fribillation	1.7
Aortic Aneurysm	0.9
Others	3.4

1.2.1. The Cardiometabolic Risk is Sex-Specific.

CVD burden and mortality rates are different among men and women and affect differently across age groups. The age-standardized CVD mortality rates are higher for men than for women, accounting for 35% and 32% in 2013, respectively [24]. The sex differences in cardiometabolic risk have been severely documented. These differences are partly due to the distinct physiology, such as the size of the arteries, and to the levels and action of sex steroid hormones (*section 1.7.2*), which is different throughout one's lifetime, varying with several factors such as pregnancy, hormonal contraceptives or even sex hormone treatment in the case of transgender individuals [26,27]. Despite of this, the causes for these sex-specific responses demand further investigation. Further insights into the molecular mechanisms that might be in the origin of sexual dimorphism in CVD are fully disclosed in *section 1.7*.

1.2.2. The Non-Modifiable and Modifiable CVD Risk Factors.

In 1948, the well-known and on-going Framingham Heart Study took place. This is considered the most important epidemiological study in medical history since this study's main goal is to accurately determine the main risk factors of CVD. The Framingham Heart Study started in Framingham, Massachusetts, where men and women aged between 30 and 62 years old were monitored. Currently, this study englobes 3 generations (approximately 15,000 individuals), providing valuable data regarding major CVD risk factors[28]. These are divided into two sub-groups: modifiable and non-modifiable. The modifiable risk factors relate behaviours and/or factors that an individual can control and reduce. These include hypertension, higher blood levels of total cholesterol and triglycerides (TG), tobacco use, T2DM, sedentarism, unhealthy dietary patterns, and obesity. The non-modifiable risk factors englobe age, sex, and family history [28,29]. Throughout recent years, research has suggested that the intra-uterine environment, modulated by maternal

behaviours such as MO, severely influences the offspring's cardiometabolic risk to develop CVD in adulthood (*section 1.2.*).

1.2.3. Cardiovascular Disease on the Rise in Young-Adults.

According to the WHO, one in three CVD deaths occurs in people underaged 70 years old. Over the past few years, the incidence rates of CVD among the young-adult age group (18 – 45 years) seems to have risen abruptly [28]. In 2017, approximately 10% of all strokes occurred in young adults. Despite better health care systems, CVD increased by approximately 50% in children and young adults in recent decades. Thus, it is reasonable to link the abrupt rise in MO and an increased number of registered premature deaths caused by CVD. Therefore, it has become urgent to understand the mechanisms underlying MO and offspring's development of CVD earlier in life.

1.3. The Developmental Origins of Health and Disease Hypothesis.

The early-life period is a critical time-window for organ development and function. The fetal programming concept states that an insult in the intrauterine environment should produce alterations in fetal formation and development. These alterations can be sufficient to induce developmental compensating mechanisms that can program the offspring for later chronic disease [30]. Adult health is influenced, in part, by the maternal health state before and during gestation. The Developmental Origins of Health and Disease (DOHaD) hypothesis is derived from the fetal programming concept. The DOHaD hypothesis proposed that human beings are exposed to a specific environment during early life, being a sensitive, limited and critical period to program life health and disease. This introduced the general phenomenon of developmental plasticity, which for most organs and systems occurs *in utero* [31]. Thus, the concept of developmental plasticity can be defined as the possibility that a single genotype may origin a range of different physiological or morphological states in response to the environmental conditions during development. In the scope of this theory, Gluckman and Hanson projected the Predictive Adaptive Response Theory. It is hypothesized that the fetus actively responds to the nutritional environment in preparation for its postnatal nutritional environment, and it could act as the trigger of disease development [32]. If a mismatch between the prenatal and postnatal environments occurs, the risk of metabolic disease increases [32]. Epidemiological studies have validated the DOHaD hypothesis. The most known one is the epidemiological study that took date after the Dutch famine. The cohort study was based on human male and female adult offspring born before, during and after the Dutch Hunger Winter, during World War II, when nutrient supply was scarce. The conclusions of the study showed that offspring born to undernourished mothers had lower blood glucose intolerance [33]. Similar studies in China suggested that maternal undernourishment during pregnancy leads to offspring development of hypertension [34] and T2DM [35].

1.4. Tracing the Origins of Fetal Cardiac Programming.

Fetal CVD programming results from biochemical, structural, morphological, and metabolic function adaptations of the cardiovascular system in response to induced stress *in utero* regulated by maternal habits, imprinting a memory for later cardiac dysfunction [36]. Thus, the influence of maternal lifestyle on the offspring's cardiac function is an interesting starting point to explore fetal programming of health and disease. During the embryonic stage, fetuses display high sensitivity to environmental changes due to placental function, leading to cardiac structure and function alterations [37]. Nevertheless, the fetal stage itself is also a critical period in cardiac development, being a vulnerable stage for a prompted onset of CVD in adulthood with risk of heart failure [38]. Given the fact that maternal and postnatal nutrition modulate the metabolism involved in energy homeostasis, a proper and equilibrated maternal diet is advised in order to maintain a healthy progeny cardiac structure and function, especially in these critical developmental stages, optimizing the ability of the heart to respond to metabolic challenges [37].

1.4.1. The Placental Function in Fetal Growth.

The first organ to be developed in mammals pregnancy is the placenta. This temporary-endocrine organ represents the interface between the circulatory systems of both the mother and the fetus, being critical for a successful pregnancy. Besides the placenta's transport function, many other functions are associated with this organ: digestive, excretory, respiratory, endocrine, and immune. This organ is responsible for the fetal protection from the mother's immune system, excreting the waste products, producing proteins, steroid hormones, and neurotransmitters [39]. The placenta is composed of various types of cells that play different roles in maintaining a transport interface of macronutrients, metabolites, and gases that assure the fetus' bioenergetic needs, allowing its protection and growth *in utero*. The dominant cell type present in the placenta is the trophoblast cell, located in the outer layer of the placenta [40], which is responsible for vascular remodelling in the first trimester of a human pregnancy [41,42].

1.4.1.1. Nutrient Transport Through the Placenta.

Fetal growth is highly dependent on nutrient transport through the placenta, guaranteeing the normal development of the fetus. Nutrients are hydrophilic, thus the transport through the plasma membrane is facilitated by hormones (glucocorticoids, insulin, leptin), growth factors, and cytokines (TNF, interleukin 6 (IL-6)) [43].

1.4.1.1.1. Glucose and Fatty-Acid Placental Transport.

The gluconeogenesis utilization by the fetus is low, thus circulating glucose concentrations are highly dependent on maternal glucose supply. The glucose transporters family (GLUT) are responsible for the facilitated diffusion of glucose on the placenta. It seems that dysregulation in placental glucose transport might be in the origin of pregnancy and adverse fetal outcomes. Fatty acids (FA) play an essential role in fetal brain development and fuel their energetic demands. Specifically, arachidonic acid and docosahexaenoic acid (DHA) are the precursors for vasoactive substances, playing a key role in fetal growth. DHA fetal levels have been highly associated with insulin sensitivity [44,45]. Non-esterified fatty acids (NEFA) enter the placenta linked to albumin, which is disassociated when NEFAs enter the plasma membrane. Esterified lipids such as TGs, cholesterol and phospholipids are the most abundant in maternal circulation. These lipids do not cross the plasma membrane, thus are hydrolysed by placental lipases into free FAs which are then transported by fatty acid transporters such as fatty acid transport proteins (FATP), fatty acid translocase/scavenger receptor cluster of differentiation 36 (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), and fatty acid binding proteins (FABP). It has been suggested that maternal behaviours have an influence over placental FA transport and lipid metabolism (*section 1.5.1)* [44,45].

1.4.1.1.2. Amino Acid Placental Transport.

Amino acids (AA) are important macronutrients responsible for developing fetal tissue, protein synthesis, and energy supply. AAs are transported through the placenta through active transport, enabling their transport against the concentration gradient. The transporter systems can be Na^+-dependent (system A), glycoprotein-associated (system L) or even cationic (System y^+). System A transports small and neutral amino acids being stimulated by insulin, leptin, IGF-1, IL-6, and TNF-α. In contrast, the system L transports large neutral amino acids, branched-chain amino acids and tryptophan, being stimulated by glucose, insulin, and the mammalian target of rapamycin (mTOR) signalling pathway. The cationic amino acids are transported through the y^+ family member, CAT1-4. Specifically, L-arginine, a cationic amino acid is transported mainly by the cationic amino acid transporter 1 (CAT-1) [46]. This Na^+-independent transporter is considered the most abundant cationic transporter in the human placenta [45,47].

1.4.2. Cardiogenesis – an overview.

After the placenta, the heart is the first organ to acquire function during fetal development. In the eighth week of the human developmental stage, the blood begins to circulate in the embryo, after which the heart continues to develop, having its maturation term in the postnatal stage [48]. Cardiogenesis relates to the process and mechanisms by which the heart tissue is formed *de novo*. During the early-life stage *in utero*, cardiogenesis occurs in the embryonic stage, resulting in a four-

chambered heart [49]. During cardiogenesis, the mesodermal progenitor cells specify and differentiate into non-muscular and muscular cells, forming the cardiac layers such as the epicardium, endocardium, the internal lining of the heart, and the myocardium [49,50]. The myocardium is composed of cardiomyocytes, cardiac muscle cells, that occupy the major volume of the heart and are responsible for the propagation of electrical impulses, allowing the heart to contract incessantly [51–53]. During the human embryonic stage, the mononuclear cardiomyocytes continue to grow, which corresponds to the period of hyperplasia or proliferative growth. This process continues until the perinatal/neonatal stages, after which the extracellular matrix suffers alterations, where the transition from mononuclear to binuclear cardiomyocyte occurs and cardiomyocyte renewal rates became low [52]. The heart loses the regenerative capacity, and this process is called postnatal hypertrophy. During adulthood, cardiomyocytes' hypertrophy also occurs and is sub-divided into two different types: physiological and pathological. Both types serve as a cardiac adaptative response to a determined stimulus, but the mechanisms by which each occurs, and phenotypes are significantly different [54]. Physiological hypertrophy ensures the heart's normal function and maintains or even increases muscle contractility capacity in response to, for example, high-intensity physical exercise practice, or even pregnancy [55]. However, pathological hypertrophy, which is characteristic in CVD patients, is responsible for a gradual loss of cardiac contractility and cardiomyocyte death, where increased collagen levels are verified. Pathological cardiac hypertrophy mechanisms are regulated, in part, by the nuclear factor of activated T-cells 4 (NFATc4). This protein is responsible for cardiac hypertrophy by regulating the expression of hypertrophic genes. Its dephosphorylation promotes cytoplasmatic translocation to the nucleus, regulating the expression of one of the hypertrophy biomarkers, brain natriuretic peptide (BNP), inducing cardiac hypertrophy [56]. The cardiac antihypertrophic response is mainly mediated through the nitric oxide (NO) signalling pathway. The activation of the soluble guanylyl cyclase (sGC) enzyme by NO catalysing the conversion of guanosine triphosphate (GTP) into 3',5' - cyclic guanosine monophosphate (GMP), which stimulates the activation of protein kinase G (PKG) [57]. On the one hand, PKG activates the cyclic AMP-responsive-element-binding protein (CREB), responsible for anti-apoptotic mechanisms, regressing cardiac hypertrophy [58]. On the other, PKG is responsible for inhibiting the Ca^{2+}-sensitive protein phosphatase calcineurin, inhibiting the translocation of NFATc4 to the nucleus, avoiding the development of cardiac pathological hypertrophy [56].

1.4.3. Epigenetic Mechanisms During Fetal Development.

Epigenetics is defined as genetic expression alterations due to changes in the genome that do not result from differences in the nucleotides' sequence of desoxyribonucleic acid (DNA). Besides genetics, physiologic, behavioral factors, the external environment such as the intra-uterine

environment also can modulate the epigenome. The four major types of registered epigenetics alterations are histone modifications, DNA methylation, chromatin condensation state alterations, and non-coding micro-ribonucleic acids (RNAs) (miRNA) [42]. Micro-RNAs are responsible for the negative regulation of target genes, playing an essential role in gene expression. The miRNAs present in the myocardium are important in the cardiogenesis process and heart function, either by regulating proteins responsible for heart contraction and electrical signal propagation [59]. Histone modifications such as phosphorylation, ubiquitination, methylation, and acetylation affect the access of the transcriptional machinery to the DNA, since these alter the chromatin's condensation state. Specifically, acetylation is regulated by histone deacetyltransferases (HDAC) and histone acetyltransferases (HAT). The activity of these enzymes is dependent on the availability of cellular acetyl-coenzyme A (CoA) and its nuclear pool. Higher nuclear acetyl-CoA levels induce acetylation [60]. DNA methylation occurs mainly in CpG islands, in cytosine carbon 5, which is covalently modified. This process is regulated by the enzymes ten-eleven translocases (TET), and DNA methyltransferases (DNMT) [61,62]. TETs activity is dependent on α-ketoglutarate, Fe(II), and oxygen, and is inhibited by succinate, fumarate, and 2-hydroxiglutarate. DNMTs require S-adenosyl methionine (SAM) as methyl donors. DNMT1 is responsible for DNA methylation maintenance during cell division. DNMT3A, DNMT3B are responsible for the *de novo* DNA methylation during embryogenesis and in differentiated cells. These epigenetic mechanisms could first start during gametogenesis and continue through the prenatal stage, influencing the fetal phenotype. During gametogenesis, epigenetic mechanisms mark the imprinted genes, that are only expressed from the maternal and paternal alleles in the offspring. It has been suggested that epigenetics also regulate tissue-specific gene expression, resulting in differential tissue and organ development. Epigenetics contribute to the process of developmental plasticity [63].

1.4.4. A Closer Look Into the Offspring's Cardiac Fuels and Oxidative Pathways.

1.4.4.1. The Role of Insulin Signalling Pathway, Glucose and Fatty-Acid Oxidation.

Insulin is an anabolic hormone with a pivotal role in regulating glucose and lipid metabolism in the heart, being essential for substrate preference and modulating cardiomyocyte size. Evidencing this, the specific knockout of insulin receptor (IR) in the hearts of mice led to cardiomyocyte size reduction resulting in heart size diminution by 20 to 30% [64]. These adult mice presented a higher preference for glucose utilization than TGs to obtain energy, typical in neonatal hearts, although not in adults'. This suggests that insulin is essential in cardiomyocyte substrate preference shift at the end of the first neonatal week [65]. Cardiac insulin regulation is dependent on the insulin signalling cascade. This cascade starts with insulin binding to the IR. The IR structure is hetero-tetrameric, constituted by two α subunits and two transmembrane β subunits. The β

subunits have tyrosine kinase catalytic activity, promoting autophosphorylation, thus, activation, when insulin binds [66].

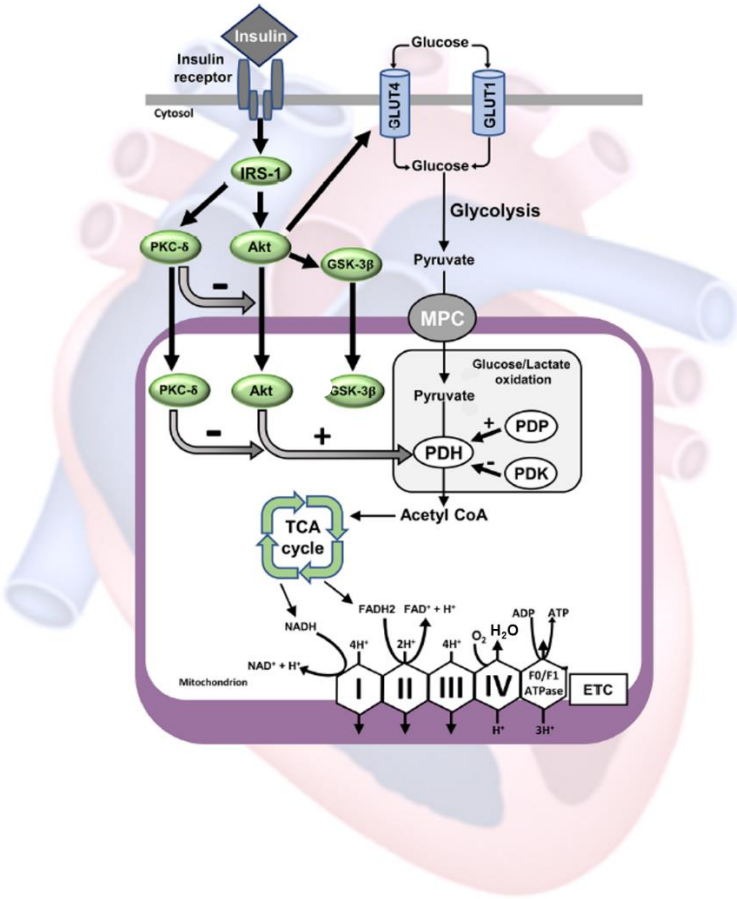


Fig. 1.1 | *Representative scheme of the role insulin in glucose oxidation in the heart, until substrate utilization in mitochondria. Adapted from Karwi et al.* [67].

Although the mechanisms by which insulin regulates the pyruvate dehydrogenase (PDH) complex are not fully elucidated, Karwi et al. demonstrated in mouse hearts that insulin-stimulated mitochondrial Akt is an essential protein for the propagation of the insulin signal, being a key regulator of cardiac glucose oxidation [67]. During the developmental stage, glucose uptake in the heart occurs in an insulin-independent manner, being gradually replaced for insulin-dependent transport after birth [68]. Glucose uptake in the heart is regulated by GLUT1 and GLUT4. While in the adult cardiomyocyte, the dominant isoform is GLUT4, in the fetal heart the dominant isoform is GLUT1 [68]. This is because, in early life, glucose transport occurs through GLUT1 which is then gradually replaced by GLUT4 later on in life [68]. Inside the cell, glucose can either be stored as glycogen or continue to the glycolytic pathway, being converted to pyruvate and the majority is then converted acetyl-CoA by the PDH complex and entering the tricarboxylic acid (TCA) cycle for adenosine triphosphate (ATP) generation, by oxidation [69]. Glucose storage as glycogen is regulated by glycogen synthase (GS), which is then inhibited by the activation of glycogen synthase kinase 3 β (GSK3β) through phosphorylation. IR activation promotes interaction with insulin

receptor substrates 1 and 2 (IRS1/2). A network of pathways is then activated, starting with lipid kinase phosphoinositol-3-kinase (PI3K), the posterior activation of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates Akt in the Ser473 residue, starting the protein kinase B (PKB)/Akt pathway which then inhibits GSK3β, thus, controlling glucose storage in the heart [66].

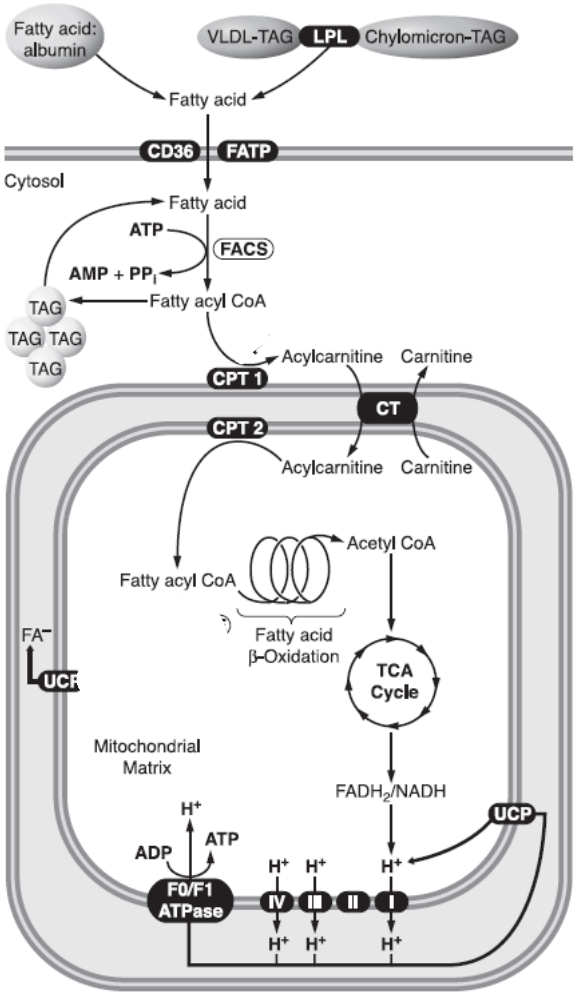


Fig. 1.2 | *Representative scheme of fatty acid transport and β-oxidation in the cardiac cells, until substrate utilization in mitochondria. Adapted from Lopachuck et al.* [70].

Although the heart also utilizes glucose as fuel during adulthood, fatty acid oxidation through β-oxidation is the main energy source in healthy adult cardiomyocytes. Long-chain fatty acids (LCFA) oxidation is responsible for 65-80% of ATP production in the heart [70]. In blood circulation, most LCFAs circulate as triacylglycerol (TAG), but can also circulate bound to albumin, or even as phospholipid components of lipoproteins. FA uptake in cardiomyocytes is mediated through transporters such as FATP FAT/CD36, FABPpm, and FABP. Cardiac β-oxidation rates are dependent on the TCA cycle, electron transport chain (ETC), and energetic demands [70]. β-oxidation starts with the cytoplasmatic conversion of FAs into long-chain acyl CoA

esters by fatty acyl-CoA synthetase. The role of insulin in the oxidation of fatty acids still remains little explored. However, studies have suggested that insulin is responsible for inhibiting lipoprotein lipase (LPL) activation [71]. LPL is responsible for the hydrolysis of TAGs, thus being very important for the regulation of β-oxidation rates.

Both glucose oxidation and β-oxidation final product inside the mitochondria is acetyl-CoA (**Fig. 1.1.; 1.2.**), which then can enter the TCA cycle in the mitochondrial matrix, with the final ATP production. The human heart generates and utilizes between 3.5-5 kg of ATP a day to support the energetic demands and constant pumping activity [72]. Thus, constant and uninterrupted substrate fuel is needed for the heart to maintain its functioning.

1.4.5. A Deeper Insight into Cardiac Mitochondria.

The heart has high energetic demands, which are mainly powered by cardiac mitochondria, responsible for almost 95% of cardiac ATP production [73,74]. Mitochondria occupy around 30% of the cardiomyocyte volume [53]. Cardiac mitochondria have been characterized by the subcellular arrangement, being classified as subsarcolemmal mitochondria (SSM), which are present beneath the cellular membrane, as interfibrillar mitochondria, located between the cardiac muscle fibrils and the perinuclear mitochondria, located at the nuclear poles [75]. In addition, mitochondria also act as localized cytosolic calcium buffering organelles, regulating cytosolic calcium concentration, providing signals to vital events, such as muscle contraction. Furthermore, mitochondria are pivot organelles of convergence and integration of both survival and death signalling pathways, participating in the production of hormones, required for cholesterol metabolism, and are major players in reactive oxygen species (ROS) generation (*section 1.4.6*). Mitochondria are perceptive organelles that easily respond to alterations in womb environment [73]. Given that internal and external factors can modulate mitochondrial function, it seems unquestionable that mitochondria are essential in maintaining an adequate balance between the cardiac metabolic function and myocardial contractility, which together are responsible for a normal cardiac function.

1.4.5.1. Mitochondrial Structure and Function.

Mitochondria originated from an aerobic eukaryotic ancestor and are separated by two membranes: the outer (OMM), and the inner (IMM), the separation of which defines the intermembrane space (IMS). The IMM space is called the matrix [76]. Proteins are translocated to the mitochondria by outer membrane translocases (TOM) and inner membrane translocases (TIM). Mitochondria have their own genome, the mitochondrial DNA (mtDNA), which is circular and encodes for 13 proteins that are components of the oxidative phosphorylation (OXPHOS) system, and small peptides [77] (**Fig. 1.3**).

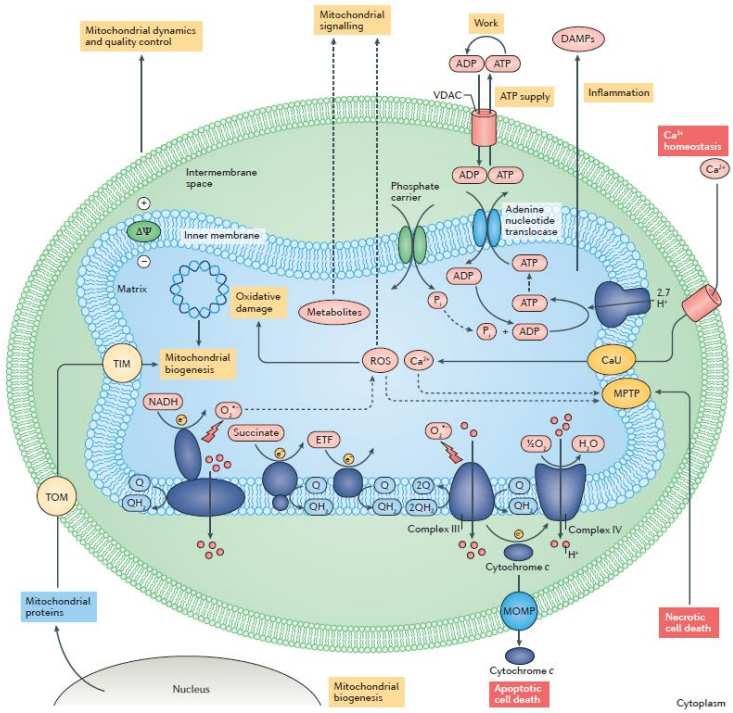


Fig. 1.3 | *Representative scheme of mitochondrial structure and functions. Adapted from Murphy et al.*[77].

The products of glycolysis, β-oxidation, and, mainly, TCA cycle are coupled to the OXPHOS system. The TCA cycle occurs in the mitochondrial matrix and oxidizes acetyl-CoA resultant from carbohydrate, lipid and amino acid metabolism. Succinate, a TCA cycle metabolite is the substrate of ETC complex II (succinate dehydrogenase), also part of the TCA cycle. Mitochondrial function highly relies on the ETC system. The substrate oxidation is coupled to ATP production. ETC is composed of four enzymatic complexes (I-IV). The products of cellular metabolism, nicotinamide adenine dinucleotide hydrogen (NADH) and succinate, fuel this system through complex I (NADH dehydrogenase) and II. Complex I and II transfer the electrons to ubiquinone, reducing to ubiquinol. Ubiquinol, a mobile electron carrier, is oxidized by complex III (cytochrome *c* reductase), which passes electrons to complex IV (cytochrome *c* oxidase) through the oxidation-reduction of cytochrome *c* (Cyt *c*). Each complex enzymatic reaction is described below (Equations 1-4). This electron transfer system is coupled to proton ejection from the mitochondrial matrix to the intermembrane space in every complex except for complex II [78].

Complex I (NADH dehydrogenase)

$$NADH + H^+ + \text{Coenzyme Q} \rightarrow NAD^+ + \text{Coenzyme Q} \cdot H_2 \quad (1)$$

Complex II (succinate dehydrogenase)

$$\text{Succinate} + \text{Coenzyme Q} \rightarrow \text{Fumarate} + \text{Coenzyme Q} \cdot H_2 \quad (2)$$

Complex III (cytochrome c reductase) (3)

Coenzyme Q $\cdot H_2$ + Cyt *c* (oxidized) → Coenzyme Q + Cyt *c* (reduced)

Complex IV (cytochrome c oxidase)
Cyt *c* (reduced) + O_2 → Cyt *c* (oxidized) + H_2O (4)

Proton ejection generates an electrochemical gradient, creating a proton-motive force that is characterized by increased concentrations of H^+ in the IMS and an electric gradient (membrane potential, $\Delta\Psi$, ~ 150-160 mV). This force is used to phosphorylate adenosine diphosphate (ADP) to ATP by ATP synthase (F_oF_1 – ATPsynthase). ATP synthase is constituted by two dimers: the F_o, in which protons are translocated across the IM and F_1, where ADP is phosphorylated [77,79].

1.4.5.2. Mitochondrial Biogenesis and Dynamics.

The mtDNA is a double-stranded circular molecule of approximately 16.5 kb, 37 genes that encodes for complex I, III, IV and ATP synthase, transference RNA (tRNA) and ribosomal RNA (rRNA), and small peptides. Mitochondrial biogenesis involves complex transcriptional machinery, requiring mtDNA and nuclear DNA replication [80]. Peroxisome-proliferator-activated receptor γ co-activator-1α (PGC-1α) plays an important role in mitochondrial biogenesis since it activates nuclear respiratory factors 1 and 2 (NRF1;2), which then activate mitochondrial transcription factor A (TFAM), responsible for the transcription and replication of the mtDNA [81]. The mitochondrial content of the cell is determined by the balance between biogenesis and degradation events [77]. Mitophagy is the cellular process by which dysfunctional mitochondria undergo turnover, along with the elimination of long-lived proteins and other damaged organelles, essential for maintaining cardiac function [73]. In addition, mitochondrial quality control mechanisms are dependent on balanced fusion and fission events that result in alterations in mitochondrial shape which are constantly occurring because of the metabolic adaptations needed to supress the energetic needs. Therefore, mitochondria are considered dynamic organelles [82]. The functionality of mitochondria can be restored by fusion events with neighboring and intact mitochondria. When mitochondria are severely damaged and unable to undergo fusion, damaged mitochondria are selectively removed by mitophagy [36]. Fusion is regulated by proteins present in the outer mitochondrial membrane, such as mitofusin 1 and 2 (MFN1;2) and the IMM, such as optic atrophy 1 (OPA1). Fusion is influenced by post-translational modifications including mitofusin ubiquitination. Fission events are regulated by dynamin-related protein 1 (DRP1), a cytosolic GTPase, mitochondrial fission factor (MFF), which is phosphorylated by AMP-activated kinase (AMPK), and mitochondrial fission 1 protein (Fis1) [83] (**Fig. 1.4**).

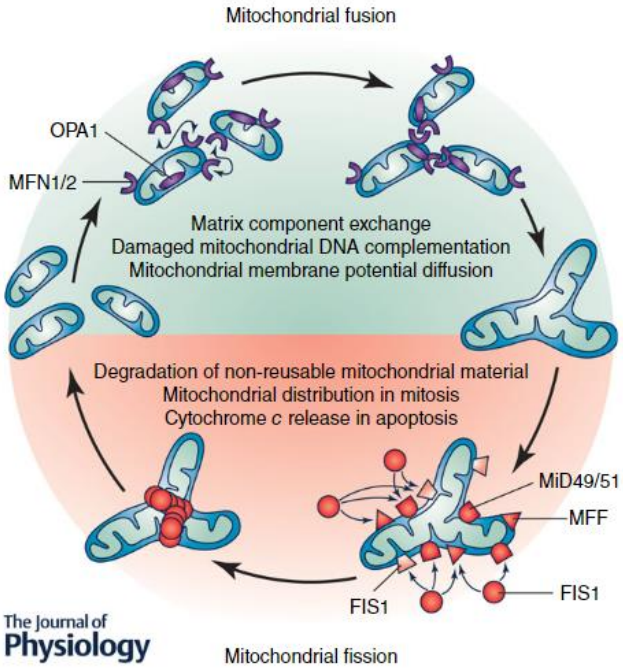


Fig. 1.4 *| Representative scheme of mitochondrial fusion and fission events. Adapted from Vasquez-Trincado et al.* [83].

1.4.6. Oxidative and Nitrosative Stress.

Reactive oxidative and nitrosative species (ROS; RNS) are "two-faced" products since these can, depending on the biological context, act as redox signalling or damaging molecules, potentially affecting macromolecules in the cells, including nucleic acids, proteins and lipids, and induce apoptotic pathways [81,84]. ROS include the superoxide anion ($^{\bullet}O_2^-$), peroxide ($^{\bullet}O_2^{-2}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and hydroxyl ion (OH^-). RNS include peroxynitrite ($ONOO^-$), peroxynitrous acid (HNO_3), nitrate (NO_3^-), nitrite (NO_2^-), among others. RNS react three times faster than $^{\bullet}O_2^-$ and have a longer half-life [84,85].

Oxidative stress is characterized by an imbalance between a local overproduction of ROS or lower antioxidant capacity of the organ, or both [86]. There are several sources of ROS, but these are originated mainly from the ETC [84]. In this context, there is a possibility that electrons could escape from the ETC (mainly from complex I and III) and react with oxygen, producing the radical superoxide anion ($^{\bullet}O_2^-$). This radical is, in part, responsible for lipid peroxidation, which is a process that starts with free radicals' attack of phospholipids or polyunsaturated FAs, resulting in the formation of aldehydes, ketones, alkanes, and carboxylic acids, such as malondialdehyde (MDA) and 4-Hydroxynonemal (4-HNE). These lipid peroxidation products are highly reactive, serving as biomarkers of lipid peroxidation [87]. Cardiolipin, an abundant phospholipid in the mitochondrial membrane, which is critically involved in membrane fluidity and mitochondrial

function, undergoes peroxidation. Alterations in the cardiolipin pool were shown to play a role in heart disease [88]. Lipid peroxides modulate membrane properties and impair the function of targeted proteins, indicating that cardiolipin could play a role in cardiometabolic impairment in MO offspring [89]. A connection between uncoupling proteins (UCP) abundance and a protective effect from ROS deleterious effects has been established, it is thought that UCP2 and 3 can decrease ROS production [90]. Furthermore, upregulation of mitochondrial UCPs can lead to an increase in FA oxidation in rodent models. UCP2 and UCP3 are able to uncouple oxidative phosphorylation and ATP production by dissipating the mitochondrial proton gradient in the OXPHOS [90].

Nitrosative stress occurs when increased levels of NO are verified. Nitric oxide, a vasodilator and angiogenic factor, is produced from L-Arginine in a catalyzed by NO synthase (NOS) reaction. The reaction of superoxide with $NO^{\bullet}$ forms $ONOO^-$, which is the most abundant and cytotoxic RNS [85]. RNS also react reversibly with the polar and hydrophilic thiol groups of cysteine residues. This reaction is called S-nitrosylation, forming S-nitrotyrosol, altering the enzymatic function. The S-nitrosylation of complex I has been pointed out as a cardioprotective mechanism, by inhibiting complex I activity, reducing the escape of electrons [91];[92]. Protein tyrosine residues are susceptible to attacks from RNS, especially from peroxynitrite. Thus, the profile of 3-nitrotyrosine is a biomarker of nitrosative stress [93].

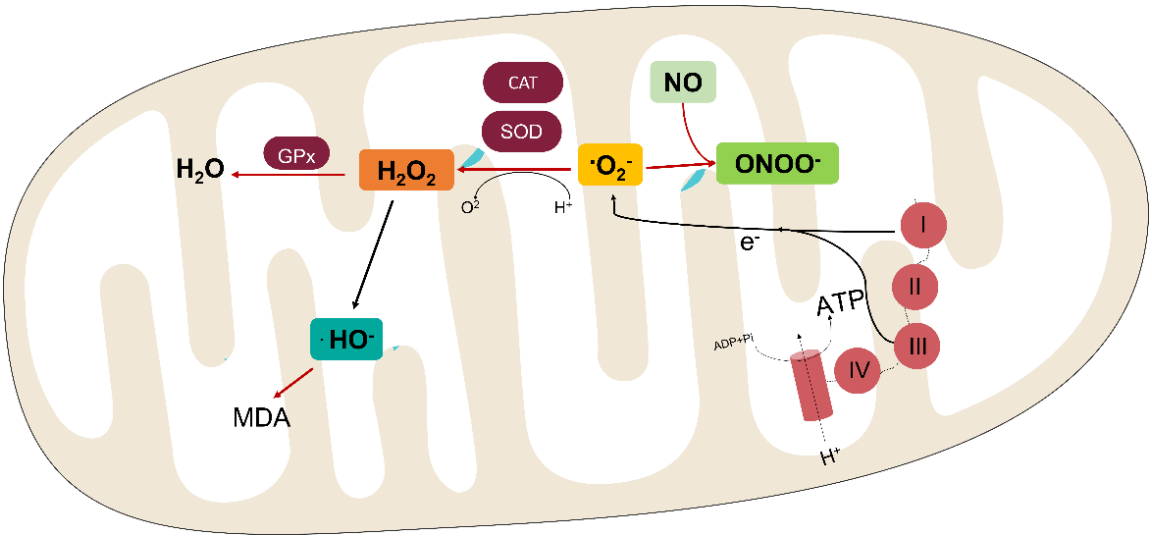


Fig. 1.5 *| Reactive oxygen and nitrogen species formation, and antioxidant defenses.$ONOO^-$: Peroxynitrite; NO: nitric oxide $\cdot O_2$: superoxide anion; CAT: Catalase; SOD: Superoxide dismutase; H2O2: Hydrogen peroxide; Gpx: Glutathione peroxidase family; . $\cdot HO$: Hydroxil radical; MDA: Malondialdheyde.*

Antioxidant capacity results from non-enzymatic and enzymatic antioxidants that together maintain the balance of the redox state of the cell. Superoxide can be converted into H_2O_2 by superoxide dismutase (SOD). SOD are a class of enzymes that convert superoxide in hydrogen peroxide (H_2O_2). The mitochondrial SOD isoform is Mn-SOD [84]. This molecule is constituted by 4 subunits with one manganese atom each, and it oxidizes from Mn (III) to Mn (II). H_2O_2 can either form hydroxyl radicals by Fenton's reaction, reacting with Fe (II) or could be broken down into water by glutathione peroxidase (GPx) and catalase. The GPx (1-4) family is responsible for the reduction of hydroperoxides to water. Catalase is an oxidoreductase enzyme constituted by four subunits, containing a group heme and a molecule of nicotine adenine dinucleotide phosphate hydrogen (NADPH) each. This antioxidant enzyme is present mainly in the peroxisomes. The non-enzymatic antioxidants are obtained through supplementation and diet, and includes vitamins A, C, E, selenium, zinc manganese, and magnesium [94] (**Fig. 1.5**).

1.5. Maternal obesity and offspring cardiac disease programming *in utero*.

Despite better health care systems, CVD increased by approximately 50% in children and young adults in recent decades [95]. Although several epidemiological studies explore the relation between low-birth weight (BW) and increased risk for CVD development, it is recognized that CVD incidence occurs at both ends of the birth weight spectrum, in a U-shaped curve behavior [96]. Under the light of DOHaD, increased number of registered premature deaths caused by CVD could be related to the abrupt rise in MO and overnutrition in the womb. Specifically, an epidemiological follow-up study led by Reynolds in 2013 in the UK showed that MO human offspring between the ages of 36-62 years old were more likely to be admitted in the hospital due to cardiovascular events and have higher risk of premature death than those of lean mothers [97]. Therefore, it has become urgent to understand the mechanisms underlying MO and the offspring development of CVD earlier in life, and how to prevent it. The offspring's cardiac function's long-term adverse effects are partially caused by the occurrence of biochemical, structural, morphological, and metabolic function adaptations of the offspring's cardiovascular system in response to induced stress *in utero*, imprinting the memory for cardiac dysfunction [61]. The study of the influence of maternal lifestyle on the cardiac function of the offspring is an interesting starting point to explore fetal programming of health and disease.

1.5.1. Maternal obesity-induced stress in the placenta and intra-uterine environment modulation.

Placenta is a key driver of fetal growth. Early-life exposure to MO-environmental stimuli programs alterations in the placental structure and function. In 2014, Saben et al. suggested a

lipotoxic placental environment in human obesogenic pregnancies [95]. Through RNA-sequencing, altered levels of genes related to placental lipid metabolism (*DKK1, ANGPTL4, INSIG1, NRIP1*), were verified in comparison with lean mothers' placentas. Neutral lipid staining suggested lipid accumulation for obese mothers' placentas [95]. Maternal saturated fatty acid and carbohydrate consumption could lead to a state of cardiac glucolipotoxicity, whereas increased levels of maternal glucose and an excess of circulating lipids lead to lipid accumulation as TGs in the placenta [19]. Lipotoxicity could lead to oxidative stress, by originating species such as lipid peroxides, oxidized lipoproteins and oxysterols that could exacerbate glucose intolerance, insulin resistance and cardiomyopathy [19].

The determination of oxidant/antioxidant markers in human MO placentas, such as RNS, ROS, catalase, SOD, MDA, by Malti et al., demonstrated an imbalanced redox status associated with MO [98]. Additionally, Wallace et al. demonstrated increased levels of a marker of hypoxia, carbonic anhydrase IX, in the placentas of diet-induced obese mice [99].

MO modulates and creates an adverse intrauterine environment characterized by lipotoxicity, hypoxia and/or oxidative stress, affecting placental development and normal function. Despite these findings, it is essential to mention that in moderate concentrations, ROS and RNS play a vital role in placental formation (trophoblast proliferation, and angiogenesis [100]), thus being vital for embryonic and fetal development. Exacerbated concentrations of these free radicals may be harmful since, in the first trimester of gestation, the placental antioxidant defenses are low, which could easily lead to a pro-oxidative state, quickly affecting fetal development. Placental malfunction can severely impact organogenesis, especially cardiogenesis. Investigation in fetal sheep hearts revealed that placental dysfunction led to decreased cardiomyocyte cell cycle activity and differentiation rate, indicative of a more immature myocardium, with potentially fewer cardiomyocytes, which could lead to a dysfunctional heart [101] .

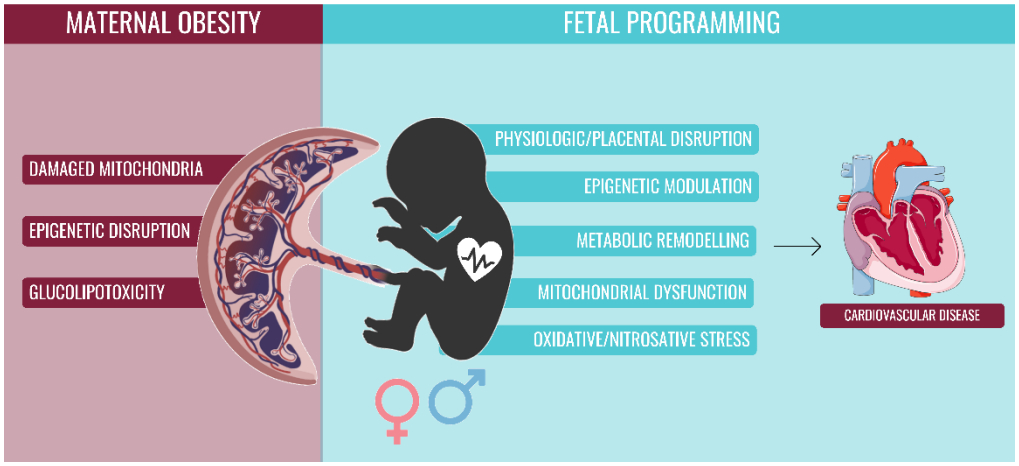


Fig. 1.6 | *Maternal obesity-induced cardiovascular disease programming in the offspring.*

1.5.2. Maternal obesity impacts cardiogenesis and offspring cardiac physiology.

It has been shown that MO compromises fetal heart and structure [38]. Thickening of the left ventricular (LV) free wall, myocyte hypertrophy, thickened intima walls and impaired vascular function, diastolic and systolic dysfunction, and contractile dysfunction were reported in MO offspring mice and maternal high-fat diet (HFD) in non-human primate offspring [102–105]. In another study, female rat offspring exposed to MO displayed elevated systolic and diastolic blood pressure at 26 and 51 weeks of age, whereas altered blood pressure was not observed for male offspring [106]. Cardiac physiology abnormalities induced by MO are also sex-specific. Smaller LV relative wall thickness and higher myocardial performance were registered for female offspring of HFD mice dams. Contrastingly, male offspring of the same generation presented higher LV relative wall thickness and no differences in myocardial performance [107]. A meta-analysis of 13 human studies concluded that exposure to diabetes, a common complication of obesity, *in utero* is associated with increased systolic and diastolic blood pressure in male but not in female offspring [108]. Nonetheless, all these studies support that in response to MO-adverse *in utero* environment, fetal heart remodelling occurs in a sex different manner. Maternal HFD modulates the expression of crucial blood pressure regulatory factors that influence cardiac structure, and this remodelling could represent a compensatory mechanism to maintain cardiac function.

1.5.3. Epigenetic disruption in maternal obesity progeny.

Epigenetic mechanisms have been implied in disease programming since these are responsible for defining the adult phenotype. A solid relationship was previously demonstrated between the disruption of epigenetic factors and fetus adaptations to maternal nutrition/placental environment that could persist later in life [109]. Likewise, Heijmans et al. showed altered DNA methylation patterns in insulin growth factor 2 (IGF2), a maternally inherited gene in individuals exposed to famine prenatally, supporting the theory that early-life environmental conditions can lead to epigenetic changes that persist throughout life, showing the apparent involvement of epigenetic dysregulation influenced by maternal nutrition [109]. Dysregulation of epigenetic modifications such as DNA methylation and post-translational histone modification have been described for MO offspring [36]. A study in Sprague Dawley rat MO offspring performed one and ten days after birth has gathered evidence of downstream signalling of the Class IIa HDAC - myocyte enhancer factor 2D (MEF2) axis via AMPK, which is responsible for the regulation of genes involved in FA oxidation [110]. Class IIa HDAC are phosphorylated by AMPK, which is activated during times of cardiac metabolic stress to increase energy production. Afterward, class IIa HDAC are exported to the nucleus, with the expression of MEF2-dependent genes that respond to hypertrophy marker genes *ANP* and *BNP*. Increased expression of these genes was found and increased protein expression of enzymes involved in FA oxidation, suggesting transcriptional reprogramming [111]. All of these studies suggest altered epigenetics due to *in utero* conditions. These

altered epigenetic mechanisms could lead to memory imprinting for CVD development in offspring and its manifestation in adulthood or even early in life, contributing for the increased CVD incidence in children and young adults in the recent decades.

1.5.4. Tracking Early Cardiac Metabolic Dysfunction in MO Offspring.

1.5.4.1. The quality of maternal diet influences the offspring metabolism.

A maternal balanced diet is required for maternal health and to avoid the development of MO. However, when this is not enforced, several adverse outcomes for the mother and offspring are expected [112,113]. It has been described that maternal diet quality could modulate offspring's metabolic profile [112,113]. Concerning the quality of maternal lipid intake, studies have shown that maternal intake of omega-3 FAs during gestation and lactation decreased serum concentrations of TAG, total cholesterol, and hepatic catalase protein levels in 21-week-old rat pups. In contrast, a maternal diet rich in trans fatty acids impairs offspring's liver glucose tolerance [112]. A low dietary glycemic index (GI) and carbohydrate diet (high fiber, low sugar) have been linked to benefits for the offspring's cardiovascular health [114]. However, excessive maternal intake of protein leads to amino acid imbalanced levels, impacting L-Arg levels [114]. Consistently, impaired L-Arg/NO metabolic pathway was verified for overweight and obese pregnant women [115]. Due to the angiogenic and vasodilator properties, the role of NO in cardiac mitochondrial function, and the connection with cardiac pathological hypertrophic mechanisms (*section 1.4.2.*), the study of a potential impaired NO pathway in an obesogenic pregnancy and the expected adverse consequences for offspring health have gained relevance in recent obstetric research [116].

1.5.4.2. Maternal obesity modulates the offspring's cardiac insulin signalling pathway.

Insulin resistance is a key contributing factor to MO-induced cardiac lipotoxicity. However, the opposite is also true. Cardiac lipotoxicity could lead to insulin resistance since toxic FA metabolites have the ability to impair myocardial insulin signalling [19]. Specifically, the cardiac tissue of 8 weeks old MO offspring mice showed cardiac hypertrophy associated with hyperinsulinemia and activation of insulin signalling pathways through increased phosphorylated Akt-1 (Ser473) and mTOR (Thr 2448) [104]. These changes could potentially lead to LV reconstruction at an accelerated level which could, in turn, lead to heart failure [104]. Moreover, in the cardiomyocytes of MO offspring mice, decreased Akt phosphorylation in Thr172 and AMPK activity and phosphorylation of IRS-1 in Ser307 were shown to be significantly enhanced [117]. Elevated phosphorylation of IRS-1 in Ser307 in hearts from fetuses born to obese sheep were also established along with impaired insulin sensitivity and myocardial dysfunction [19]. Nonetheless, all of this support that impairment in cardiac insulin signalling pathway induced by MO is a key contributing factor to MO-induced offspring cardiac dysfunction.

1.5.4.3. Dysregulation of glucose and fatty acid oxidation in the MO offspring.

The myocardium presents a reduced ability to synthesize FAs so it depends on the uptake of free FAs or lipoproteins to maintain cardiac lipid homeostasis and energy metabolism [86]. Evidence of incomplete β-oxidation of long-chain fatty acids and altered oxidative metabolites in myocytes differentiated from stem cells derived from the umbilical cord from MO offspring at birth was found [118]. Regarding lipotoxicity, enzymes that participate in long-chain fatty acid oxidation were upregulated to keep up with the high demands of the increased fatty acid substrates. Nonetheless, this upregulation is not enough to withstand the increased load of FA through oxidative phosphorylation in obesity, resulting in the accumulation of fatty acyl-CoA in the mitochondria [89]. Moreover, newborn mice offspring cardiomyocytes from HFD and streptozotocin mothers showed lower response to exogenous palmitate, a substrate for mitochondrial fatty acid oxidation, indicating that fatty acid oxidation might be impaired in the cardiac mitochondria of these offspring [105]. Overall, incomplete or impaired FA oxidation may induce lipotoxicity. This dysregulation is observed in the hearts of MO offspring, indicating impairment of key energy pathways in MO offspring cardiac function.

1.5.4.4. Altered mitochondrial function in the origin of cardiovascular disease programming.

Cellular bioenergetics play a crucial role in the pathophysiology of cardiovascular disease. Mitochondrial function and structure are highly susceptible and responsive to environmental alterations [73]. It has been suggested that mitochondria show developmental plasticity, undergoing adaptations depending on the internal cellular surrounding environment, supporting the DOHaD theory. Therefore, mitochondria could play a pivotal role in developmental programming and disease manifestation later in life [92]. If mitochondria become dysfunctional, at least the production of energy in the cardiomyocyte and the production of cell-specific components needed for normal cell function becomes unbalanced. An imbalance between energy production and energy demand, and a disturbance in energy transfer networks play an important role in various pathologies [119].
Adverse *in utero* environment could lead to progressive impairment of cardiac mitochondrial function. The starting point can be an affected OXPHOS function, limited ATP supply and increased generation of ROS and RNS, which then lead to mtDNA and cellular components damage, inciting ROS/RNS leak and aggravated mitochondrial dysfunction [73]. The biological processes resulting from an insult that impacts mitochondrial biology in early life are the same as those modulating mitochondrial biology in adulthood [73]. Being an organ with high energy demand, the heart requires its fuel to keep an optimal contractile activity. Therefore, changes in cardiac metabolism have been linked to oxidative stress, mitochondrial dysfunction, and mitophagy [105]. Proposed mechanisms for mitochondrial dysfunction include impairment in ETC complex activities, defects in supermolecular assembly of ETC complexes (supercomplexes), oxidative

stress, decreased expression of cardiolipin, impaired TCA anaplerosis, and mitochondrial uncoupling [120].

1.5.4.4.1. Maternal obesity affects the cardiac mitochondrial dynamics of offspring.

Cardiac mitochondrial dynamics is a quality control process, allowing mitochondrial health. An unbalance of fission and fusion events has been linked to cardiometabolic complications, contributing to an increased number of dysfunctional mitochondria. Borengasser et al. showed that transcripts for key mitochondrial genes, including OPA1, MFN1, MFN2 were reduced in the livers of 16-week-old MO offspring [120]. Moreover, Saben et al. showed OPA1 and DRP1 decreased levels in skeletal muscles of MO offspring mice [121]. In addition, Mdaki et al. showed fragmented and low-membrane potential mitochondria in cardiomyocytes of maternal HFD mice offspring with little or no fusion or fission events, evidencing severe damage that could signal cells for apoptosis [103]. Fusion protein expression has shown to be sex-specific in maternal HFD rat offspring [82]. Female MO-offspring presented higher mitochondrial fusion protein levels (MFN-1 and OPA1), which may confer a cardioprotective effect. Males presented post-translational modifications which are known to affect dynamism and influence mitophagy-mediated cell death, suggesting that maternal HFD leads to a sex-divergent response [72]. Since mitochondrial dynamics are fundamental to cardiac development, maturation and function, impairment of these events could lead to developmental programming of the cardiac tissue, leaving a cardiometabolic imprinting, impacting cardiac health at birth and throughout the lifetime.

1.5.4.4.2. Altered cardiac mitochondrial bioenergetics in maternal obesity offspring.

Mitochondrial dysfunction is often characterized by an impairment in respiratory chain complexes efficiency and a reduction in their coupling with ATP synthesis. Three-month-old maternal high fat-high sugar (HFHS) diet male mice offspring showed reduced cardiac respiration via complex I, complex II and complex II+III [107]. An abnormal expression of genes involved in mitochondrial dynamics, decreased mitochondrial ETC complex activity and expression, citrate synthase activities, and decreased mitochondrial respiration affect cardiac energy production, impacting the offspring's cardiac function.

1.5.5. The Role of Oxidative and Nitrosative Stress in The Offspring Heart.

Oxidative and nitrosative stress is implicated in several diseases. A pro-oxidative cellular state has been pointed as one of the significant indicators for obstetric and fetal complications. From the intrauterine environment and placenta (*section 1.5.1*), cardiac development (*section 1.5.2*), to the adult stage, findings have implicated oxidative stress in the origin and perpetuation of CVD in MO offspring. Increased ROS production and increased levels of MDA have been reported in diet-induced MO rat and mice offspring. Interestingly, both studies have in common decreased

levels of NO production, which suggests that NO and nitrosative stress may play a crucial role in CVD programming. Reduced SOD2 expression was observed in the aortas of 6-month-old rat offspring of obese dams [90]. In heart dysfunction, increased circulating FAs have been positively correlated with increased UCP2 and UCP3 expression [122]. During MO, lipid peroxidation can lead to calcium overload and increased mitochondrial uncoupling, leading to cardiac dysfunction [123]. In fact, MDA was increased in cardiomyocytes of newborn Sprague Dawley MO-rats [105]. Thus, the maternal effects of MO indeed affect the offspring postnatally, pointing to oxidative stress as a mechanism for the development of CVD in MO offspring.

1.6. Therapeutic Approaches for Disease Programming.

In order to counteract MO adverse effects on the offspring, several studies suggested positive impacts of maternal exercise, nutritional interventions, and supplementation on obese mothers' offspring health, improving cardiometabolic function and gut microbiota [110,111].

1.6.1. The impact of maternal physical exercise practice during gestation.

For non-pregnant obesogenic individuals, regular exercise practice has been linked to improved cardiovascular system function [124]. Improved metabolic adaptations such as increased FA oxidation are verified for the hearts of exercised Sprague-Dawley rats [125]. Moreover, mitochondrial biogenesis is increased in the cardiac tissue of exercised mice [126], possibly due to AMPK enhancement and subsequent activation of mitochondrial PGC1α. In addition, an increased ability of cardiac mitochondria to oxidize FAs, increasing the capacity for ATP synthesis was verified in exercised mice [127]. Insulin sensitivity is also improved in the whole body of human individuals who display regular physical exercise practice, being verified increased Akt activation and translocation of GLUT4 [128]. The benefits of the maternal practice of physical exercise in offspring's health have been suggested through recent years [129]. Maternal physical exercise prevents the rise in TGs and reduces leptin and fat mass in MO offspring albino Wistar rats [130]. Maternal physical exercise also improved the offspring expression of hepatic mitochondrial genes involved in mitochondrial biogenesis, fatty acid metabolism and TCA cycle activity of maternal HFD offspring at 8 months-old Sprague Dawley rats [131]. Nonetheless, maternal physical exercise in obesogenic pregnancy could improve the cardiovascular health of male offspring by preventing pathological hypertrophy and dysfunction with enhanced calcium handling proteins, showing its benefits for offspring cardiac function and structure [132]. Although the benefits of maternal physical exercise practice were shown to improve the metabolic health of the liver, skeletal muscle, and pancreas function for both young and adult mice and rat model animals and enhanced cardiac function [133], its beneficial effects for offspring's cardiac metabolism remain unclear.

1.6.1.1. Gestational exercise during obesity might positively modulate offspring cardiac NO pathway.

Impaired NO metabolic pathway during pregnancy could increase the risk offspring development of NCDs in later life, including CVD [121], since a deficit in NO levels, induced by MO may persist in the postnatal stage through adulthood [133–135] with possible adverse cardiovascular outcomes. Maternal physical exercise practice during an obesogenic pregnancy increased NO derivates (nitrites and nitrates) serum levels of 14-week-old male offspring fed the western diet compared to offspring born to sedentary mothers [136,137]. Nitric oxide-derivates, such as nitrates and nitrites, increased levels induced by maternal exercise practice may be in the origin of an improved cardiovascular function of the offspring. This study highlights the relevance of exploring NO-linked mechanisms in CVD and maternal interventions in the offspring endothelium function and in the context of cardiac pathological hypertrophy and cardioprotective mechanisms.

1.7. Sexual Dimorphism.

The cardiometabolic risk and CVD risk factors are sex-specific (*section 1.2.1*). It is demonstrated that increased CVD risk could start during an early exposure to maternal stress factors, such as MO. The available data implicating maternal diet-induced obesity and offspring outcomes are either male-only-cohorts or mixed-sex cohorts, being the latter less frequent. This is because very few studies are focused on exploring the sex differences in the cardiac metabolism. However, evidence has shown that maternal stress factors, including MO, have different impacts on the offspring's physiologic and metabolic parameters according to sex, in murine and non-humane primate animal models. It has become necessary to start including sex as a variable to unravel the mechanisms behind sex-specific programming, optimize health care, and include a much more specific MO-offspring medical follow-up.

1.7.1. The Role of Epigenetic Mechanisms in Sexual Dimorphism.

Epigenetic mechanisms modulate the sex-specific gene expression in response to an adverse intrauterine environment. It is well established that epigenetic mechanisms control pathological cardiac hypertrophy, gene expression control, and genome stability. A genome-wide study found that one type of DNMT (KDM4A) is increased for knock-out mice with the hypertrophic phenotype with pathological hypertrophy and heart failure [137,138]. These control the promotor for the expression of NFATc4. This could explain the sex-specifc cardiac response since some DNMTs are encoded by the X and Y-chromosomes, which are mainly activated by estrogen. Interestingly, diet-induced MO-associated DNA methylation patterns are more pronounced in the offspring livers for females than for males [20]. Thus, it is possible that offspring gene methylation and/or demethylation profiles induced by MO partly control the sex-specific response.

Nevertheless, it seems important to highlight the levels of sex steroid hormones with its influence on the epigenetic response and the potential role in CVD sex-specific response in MO offspring (*section 1.7.2.*).

1.7.2. Sex Steroid Hormones.

Sex steroid hormones play a vital role in the organism. These are already expressed in an early-embryonic stage. Sex hormones can regulate gene expression and may play a role in predisposition to disease development. Sex-differences in physiology and metabolism are important factors that likely define the differences between rates of cardiometabolic disease risk among men and women [138]. Estrogens are prime sex hormones that play an important role in protein modification, gene regulation and cellular process modulation. It has been shown that mitochondria are important targets of estrogen. Indeed, estrogen receptors α ALPHA and β are present in mitochondria and can affect mitochondrial bioenergetics and the anti-apoptotic signalling [139]. In addition, sex steroid hormones 17β-estradiol (E_2) positively influences cholesterol and lipid metabolism. Female hormone-estrogen plays an essential role in the cardioprotective effect [140].

1.7.2.1. The Role of Mitochondrial Connexin 43.

Connexin 43 (Cx43) is regulated by E_2. Cx43 is a gap junction protein that is highly expressed in human cardiomyocytes, and it is responsible for muscle synchronized contraction 142 and for cardioprotection. Mitochondrial Cx43 is present in SMM the cardiac muscle 143. Besides interacting with other proteins that are essential for mitochondrial function, Cx43 regulates mitochondrial respiration, oxygen consumption, Ca^{2+} homeostasis and K^+ fluxes [141]. Ischemia-reperfusion (I/R) injury occurs when the blood returns to the heart after lower oxygen supply provoked by a myocardial ischemia [142]. Heart mitochondria play an essential role in determining the severity of the I/R injury since these organelles are responsible for providing energy to support cardiac contractility during a few seconds, and display the mitochondrial ATP-sensitive potassium channels that play a crucial role in the protection against I/R injury [142]. Due to its action on cardiac mitochondria, Cx43 is essential in regulating the recovery in a sex-specific way. Given that E2 regulates Cx43, male mice hearts are more likely to be protected by Cx43, because on the one hand, myocardial Cx43 is more expressed in females than in male rats [143] and, on the other, after male and ovariectomized female Cx43 knockout with posterior E_2 administration, showed that E_2 mediated-cardioprotection involves Cx43 and is sex-specific [140]. This data suggests that Cx43 could play a role in mitochondrial function-sex specific response not only in response to an I/R injury, but also to MO-induced cardiac function dysregulation in the offspring, which may justify in part, the mitochondrial sex-specific responses observed for MO-offspring.

Hypothesis and Aims

Considering that CVD is the number one cause of death worldwide along with the abrupt rise in CVD development in young adults, it has become urgent to start exploring and investigating approaches that potentially reduce CVD-related deaths or ideally, when possible, altogether avoid CVD development. Obesity is a significant risk factor for CVD development. However, this relation should take larger proportions. Currently, it seems urgent to acknowledge that, in some cases, the origin of CVD might start *in utero*. Specifically, MO modulates the intrauterine environment, further imprinting alterations in the offspring's heart structure and function. These MO-induced adaptative alterations might endure overtime and lead to CVD development in the offspring's early phase of life. Increasing attention has been directed towards cardiac mitochondrial metabolism and its role in the development of CVD in MO offspring. From the placenta to the adult offspring, it has been suggested that MO-induced mitochondrial mild dysfunction plays an essential role in offspring heart disease development, in a sex-divergent manner. Gestational exercise practiced during an obesogenic pregnancy has the potential to modulate MO-offspring cardiac metabolic function and act as a potential therapeutic effect to prevent future CVD development in MO offspring in a sex-specific fashion. To unravel this, our first aim was to 1) characterize biochemical, structural and morphological adaptations of MOEx offspring cardiovascular system; 2) unravel whether MOEx modulates offspring's cardiac metabolic and mitochondrial function and 3) unravel the contribution of oxidative and nitrosative stress in early CVD programming. Along with this, we aim to identify the sexual dimorphism present in the evaluated parameters and unravel how ex modulates the cardiac response to maternal physical exercise practice during MO.

Chapter 2 – Materials and Methods

2.1. Reagents.

All of the used reagents (Table 2.1) were of the highest available grade of purity and all th e aqueous solutions were prepared in ultrapure water (type I, Milli-Q Biocel A10 with pre-treatment via Elix 5, Millipore). For nonaqueous solutions, ethanol (99.5%, Sigma-Aldrich, Barcelona, Spain) or dimethyl sulfoxide (DMSO, Sigma-Aldrich) were used as solvents.

Table 2.1. | *Used reagents to the experimental procedure performance and respective reference.*

Reagent	CAS number	Reference	Supplier
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
BioRad - DC Protein Assay kit	-	5000116	Bio-Rad
Blue bromophenol	62625-28-	092K3720	Sigma-Aldrich
BSA (Bovine Serum Albumin)	9048-46-8	A7906	Sigma-Aldrich
Clarity Western ECL Substrate	-	170-5061	Bio-Rad
DTT (Dithiothreitol)	03/12/3483	D9779	Sigma-Aldrich
EDTA (Ethylenediamine Tetraacetic Acid)	381-92-6	ED2SS	Sigma-Aldrich
EGTA	67-42-5	E4378	Sigma-Aldrich
Eosin Y	15086-94-9	E4009	Sigma-Aldrich
FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone)	55-60-2	C2759	Sigma-Aldrich
Glutamic acid	56-86-0	G8415	Sigma-Aldrich
Glycerol	56-81-5	G5516	Sigma-Aldrich
Glycine	56-40-6	120070010	Acros Organics
Hematoxylin solution	-	1.05175	Merck Millipore
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	7365-45-9	H4064	Sigma Aldrich
Isopropanol	67-63-0	190764	Sigma-Aldrich
KCl (Potassium Chloride)	7447-40-7	P9541	Sigma-Aldrich
KOH (Potassium hydroxide)	1310-58-3	P5958	Sigma-Aldrich
Malic acid	97-67-6	M7397	Sigma-Aldrich
Methanol	67-56-1	M/4000/17	fisher bioreagents
MgCl2 (Magnesium Chloride)	7786-30-3	223211000	ThermoFischer

Na2HPO4 (Sodium Phosphate dibasic)	7558-79-4	S5136	Sigma-Aldrich
NaCl (Sodium Chloride)	7647-14-5	BP152-1	fisher bioreagents
NADPH	2646-71-1	A1395,0100	Panreac
NaF (Sodium Fluoride)	7681-49-4	27859.293	VWR
NaH2PO4 (Sodium Phosphate monobasic)	7558-80-7	S5011	Sigma-Aldrich
NAM (Nicotinamide)	98-92-0	N0636	Sigma-Aldrich
NaOH (Sodium hydroxide)	1310-73-2	S8045	Sigma-Aldrich
Oligomycin	1404-19-9	O4876	Sigma-Aldrich
PIC (Protease inhibitor cocktail)	-	P8340	Sigma-Aldrich
Pierce™ BCA 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
Rotenone	83-79-4	R8875	Sigma-Aldrich
SDS (Sodium Dodecyl Sulphate)	151-21-3	MB01501	nzytech
Sodium orthovanadate	13721-39-6	S6508	Sigma-Aldrich
Succinic acid	110-15-6	S3674	Sigma-Aldrich
Tetramethyl ethylenediamine (TEMED)	110-18-9	MB-03501	NZYtech
TPP+ (Tetraphenylphosphonium)	2001-45-8	218790	Sigma-Aldrich
Tris Base	77-86-1	BP152-1	fisher bioreagents
TritonX-100	9002-93-1	327371000	Acros Organics
Trizma-HCl	1185-53-1	T3253	Sigma-Aldrich
Tween-20	9005-64-5	P9416	Sigma-Aldrich
β-mercaptoethanol	60-24-2	M3148	Sigma-Aldrich

2.2. Animal model.

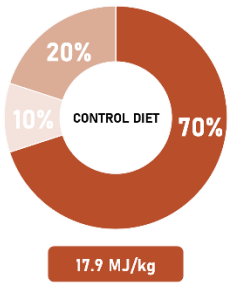
2.2.1. Ethics.

The Ethical Committee of the Institute for Research and Innovation in Health (i3S) the Faulty of Sport of the University of Porto, and the National Government Authority (No. 0421/000/000/2018) approved all animal experiments and interventions. Animal procedures 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), conducted in animal care-approved facilities in the Institute for Research and Innovation in Health (i3S). Outbred 7-week-old Sprague Dawley rats, 44 females (150 – 200

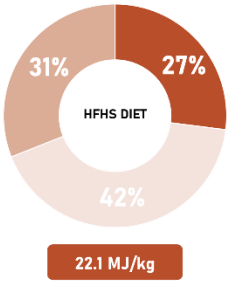
g) (CD-SIFE05SS - R/SPF CD) and 20 males (CD-SIMA05SS - R/SPF CD), were purchased from Charles River Laboratories (L'Arbresle, France). Upon arrival, the rats were submitted to one week acclimatization to the new environment, which also served as quarantine period. After this period, animals were paired in polyethylene type III-H cages with appropriate bedding and environmental enrichment. The animals were kept on 12 h of light/dark cycles. The room temperature was maintained at 21-22ºC, humidity at 50-60%, noise level below 55dB, and *ad libitum* access to food (D12450K, Germany) and water.

2.2.2. Animal intervention – diets and exercise.

A Sprague-Dawley MO rat model was used (**Fig. 2.1.**). Maternal obesity was achieved with a HFHS diet, starting at 6 weeks before impregnation and lasting until lactation. The control group was fed with a standard chow diet composed by 70% of protein, 20% sugar, and 10% fat (10 kJ% fat, no sucrose addition - E157452–047). The high fat-high sugar (HFHS) diet was composed by 27% protein, 31% sugar, and 42% fat (D12451 II) (**Fig. 2.2.**) and was given either to sedentary and exercised dams. At 13 weeks of age, the mating occurred with fertile male. The 3-week period of pregnancy coincided with the exercise protocols for the MOEx group. Before the pregnancy period initiated, all female rats went through the acclimatization to treadmill exercise. Once the impregnation period started, there was a 1-week period of adaptation to exercise, where the intensity of the training was gradually increased from 20 minutes to 60 minutes a day until the speed of 18 m/minute was achieved. For the following 2 weeks, the mothers practiced exercise for 60 minutes, with the gradual increase of the speed until 21m/minute. Exercise was practiced on an adapted motor-driven treadmill (LE8700, Panlab Harvard Apparatus, MA, USA) and with free access to running-wheels (circumference = 103.73 cm, Type 304 Stainless steel, Tecniplast, Casale Litta, Italy) connected to a counter (ECO 701 Hengstler, Lancashire, UK) to mimitize voluntary physical exercise activity. The exercise protocol was practiced 6 days a week for 20-60 minutes during the dark cycle.



Crude Nutrients	(%)
Crude protein (N x 6.25)	18.2
Crude fat	4.1
Crude fiber	5.0
Crude ash	5.3
N free extracts	62.9
Starch	47.0
Sugar	1.0



Crude Nutrients	(%)
Dry matter	93.9
Crude protein (N x 6.25)	22.5
Crude fat	23.1
Crude fiber	5.7
Crude ash	5.9
N free extracts	36.8
Starch	6.6
Sugar	20.4
Dextrin	11.1

Fig. 2.1 | *Maternal control and high fat-high sugar (HFHS) diet composition.*

At 16 weeks of age, female pregnant rodents delivered their offspring who were kept on a normal chow diet and were not submitted to exercise. Dams delivered the offspring naturally. The litter size was reduced to 6 and one male and one female offspring from each litter was randomly selected for the 32 weeks of age time-point and considered as biological unit. After the nursing period (3 weeks) the offspring were weaned (**Fig. 2.2.**). The offspring were euthanized at 32 weeks of age, and cardiac tissue and blood plasma collected. The experimental procedures were performed in the cardiac tissue of 32-week-old offspring. This age corresponds to the young adult period in a human life, with an approximate age of 24 years old (young-adult stage) [144]. The experimental groups comprise rats born to control mothers (F1-Control), rats born to obese mothers (F1-MO), and rats born to obese and exercised during pregnancy mothers (F1-MOEx).

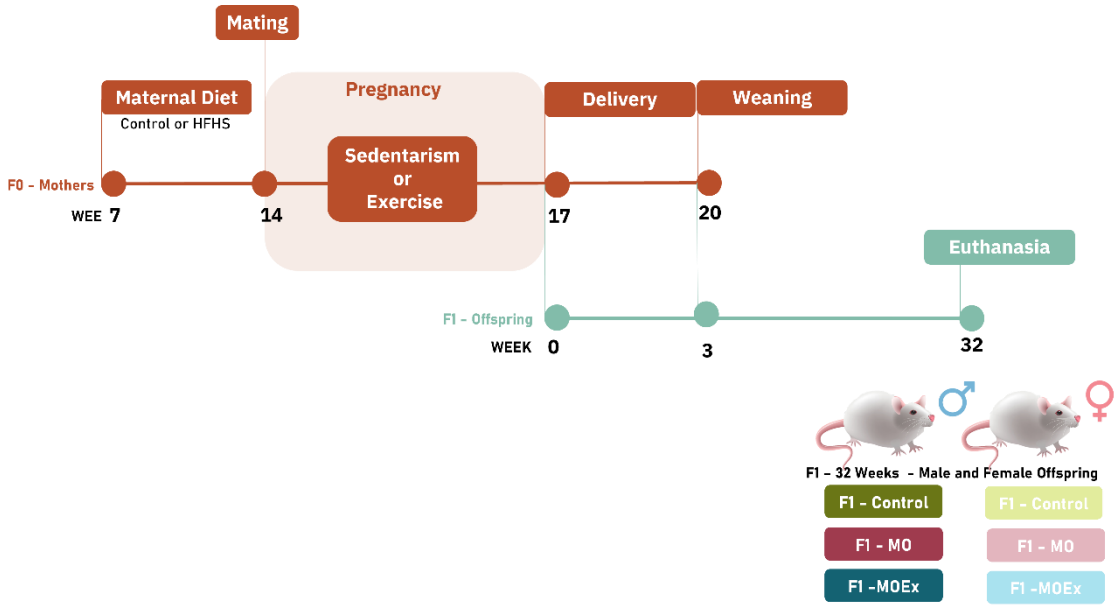


Fig. 2.2 | *Timeline of maternal and offspring treatment.*

2.2.3. Experimental size.

Six mother's offspring (n = 6) constitute each group (F1-Control; F1-MO; F1-MOEx). One female and one male offspring per mother were analysed, making it a total of 12 offspring (n = 12) per group. Taking into consideration that sex-divergent responses will be assessed, an experimental size of 6 (n = 6) per sex and group was considered.

2.2.4. Animal euthanasia and tissue collection.

The offspring nursing period occurred for 3 weeks. The offspring bodyweight was determined previous to euthanasia. Animal euthanasia was performed between 8:00 and 10:00 a.m.

(GMT) under anesthetic conditions. Firstly, after overnight fasting, the animals were placed in an induction camera with 1.5 L/m O_2, 5% isoflurane, and then kept under anesthesia 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. The heart was removed, weighed, rinsed with ice-cold PBS (NaCl, KCl, Na_2HPO_4, and KH_2PO_4) and segmented for different experimental protocols.

2.2.4.1. Blood plasma collection.

Animal blood was collected from the inferior vena cava and centrifuged (3000 x g, 10 minutes, 4ºC) in EDTA-treated tubes, and was stored afterwards at -80ºC until biochemical and metabolomic analysis.

2.2.4.2. Cardiac tissue collection for biochemical analysis.

A segment of the heart was snap-frozen in liquid nitrogen and stored at -80ºC for further use.

2.2.3.2. Cardiac tissue collection for optical microscopy.

A segment of the heart was fixed by immersion in 4% paraformaldehyde pH 7.4, dehydrated in a graded series of alcohol percentages (70-100), and inserted in parafilm blocks for further analysis.

2.2.4.4. Cardiac tissue collection 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% free fatty acid Bovine Serum Albumin (A4503, Sigma; Saint Louis, USA) (HIB^+) and finely minced to facilitate the blood removal and posterior mitochondrial isolation.

2.3. Methodological procedures.

2.3.1. Biochemical characterization of the blood plasma.

Lipid profiles, including glucose, TGs, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and cholesterol were analyzed by tandem mass spectrometry.

2.3.2. Optical microscopy.

Optical microscopy images were obtained with a microscope (Nikon ECLIPSE Ci), and a camera (Nikon Europe BV, Amsterdam, Netherlands).

2.3.3 Heart 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.

2.3.4 Hematoxylin-Eosin (H&E) staining.

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 sained in Gill Hematoxylin for 1 minute. After thoroughly washing for 4 minutes, the samples were stained in Eosin Y 1% for 1 minute and washed with water for 2 minutes. The samples were then dehydrated through alcohols and cleared with xylene. The last step was the mounting of the histological slides performed by Sakura Tissue-Tek GLS.

2.3.5. Masson's Trichrome staining.

Masson's Trichrome staining method allows the detection of collagen fibers of paraffin-embedded tissues by staining the collagen fibers blue and the nuclei black. First, the heart tissue was deparaffinized, hydrated, and washed with distilled H_2O. The samples were then treated with Bouin's solution for 30 minutes 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 minutes, the samples were washed for 10 minutes. Subsequently, the heart sections were stained with Ponceau-fuchsin solution for 2 minutes and washed with distilled H2O. For differentiation, a phosphomolybdic-phosphotungstic acid solution was used for 15 minutes. The samples were stained with Aniline blue solution for 5 minutes and rapidly washed in distilled water afterward. 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.

2.3.6. Immunohistochemistry.

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

2.3.2. Cardiac mitochondria bioenergetics characterization.

2.3.2.1. Isolation of cardiac mitochondria.

Heart mitochondria were isolated according to a previously described protocol by Pereira et al. [145] and adapted from Silva and Oliveira [146]. Minced heart was washed with HIB+ to remove the contaminating blood. Minced blood-free tissue was then resuspended in 20 mL of HIB+ and homogenized with a Potter-Elvehjem homogenizer at 4ºC. HIB+ medium was supplied with 0.2 mL protease solution (subtilisin fraction type VIII, Sigma; Saint Louis, USA) and then removed from the homogenate by centrifugation at 13,000 x g for 10 minutes at 4ºC. The supernatant was discharged, and the pellet was gently resuspended in HIB+ and homogenized. The suspension was centrifuged at 800 x g for 10 minutes at 4ºC. The supernatant (mitochondrial fraction) was collected and centrifuged at 10,000 x g for 10 minutes at 4ºC. The pellet was 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 again at 10,000 x g for 10 min at 4ºC. The supernatant was discharged, and the pellet (isolated mitochondria) was resuspended in 100-300 μL HWB. Isolated mitochondria were kept on ice and used for bioenergetic assays within 3h after isolation.

2.3.2.2. Protein quantification.

For mitochondrial protein quantification, the biuret method was used. This colorimetric method allows the quantification of polypeptide chains, through the detection of the color resultant from the reaction between copper sulphate with biuret, in the presence of compounds with peptide bonds in alkaline conditions. The assays were performed in 96 multi-well plates and after 15 minutes of incubation at 22ºC, the absorbance was read at 595 nm. BSA was used for the standard curve.

2.3.2.3. Mitochondrial membrane potential assessment.

Mitochondrial membrane potential was assessed using a TPP+ selective electrode containing tetraphenylboron as an ion exchanger and as reference, a saturated calomel electrode, as previously described by Moreno et. al, 2015. TPP+ was used for this assay given its ability to move freely across membranes due to its positive charge disguised by 4 aromatic rings. This allows to follow TPP+ accumulation in mitochondria, which develop a negative charge in the matrix due to the ejection of protons. Thus, the value for $\Delta\Psi$ can be measured by determining the distribution of TPP+ across the mitochondrial inner membrane. The addition of ADP causes a depolarization of the mitochondrial membrane, due to the entrance of protons through ATP synthase ($\Delta\Psi$ ADP). The lag phase corresponds to the period of time of repolarization while the ADP is totally phosphorylated by ATP synthase.

This assay was performed with 1 mL of Heart Reaction Buffer (130 mM Sucrose, 10 mM HEPES, 65 mM KCl, 2.5 mM KH_2PO4, 20 μM EGTA, at pH=7.4). Mitochondrial protein was added to the chamber at a final concentration of 0.5 mg/mL. The substrates used for complex I assays were glutamate 0.5 mM and malate 1 mM (G/M). The substrate used for complex II assays was succinate and 1 mM. Rotenone 1.5 μM was used to inhibit complex I. To determine ΔΨ ADP, ADP 30 mM was added.

2.3.2.4. Mitochondrial respiration.

This assay allows to measure the consumption of oxygen by mitochondria, since this molecule is the final electron acceptor of the electron transfer chain through complex IV that produces water and pumps protons, being an interesting approach to evaluate OXPHOS and ETC.

Mitochondrial O_2 consumption capacity was assessed using a Clark-type electrode (OxyGraph). This type of electrodes is constituted by a platinum electrode (anode) and a silver one (cathode), which is from the electrical signal is originated. The oxygen diffuses through an oxygen permeable Teflon-based membrane to the platinum electrode, and the cathode is reduced. The current is proportional to the oxygen concentration in the solution. The signal is then registered to a computer.

This assay was performed with 1 mL of Heart Reaction Buffer (130 mM Sucrose, 10 mM HEPES, 65 mM KCl, 2.5 mM KH_2PO4, 20 μM EGTA, at pH=7.4). Mitochondrial protein was added to the chamber at a concentration of 0.5 mg/mL, corresponding to the basal respiration state, where only endogenous substrates are being used (state 2). The substrates used for complex I (NADH dehydrogenase-ubiquinone oxidoreductase) assays were glutamate 0.5 mM and malate 1 mM (G/M). The substrate used for complex II (succinate dehydrogenase-ubiquinone oxidoreductase) assays was succinate 1 mM Rotenone 1.5 μM was used to inhibit complex I. ADP 30 mM was added to the chamber to induce mitochondrial phosphorylative state (state 3), stimulating increased O2 consumption due to increased ETC activity. To assess the maximum mitochondrial oxygen consumption, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) 0.1 mM was used. This compound is used due its protonophore character, allowing the passage of protons through the mitochondrial inner membrane, which stimulates the electron transport by the ETC, resulting in maximum oxygen consumption rate. This corresponds to the uncoupled phase. State 4 occurs when the ADP is fully phosphorylated, meaning that O2 consumption rates returns to a very similar state to the basal respiration state. ADP/O ratio was calculated. This indicates how many oxygen atoms are consumed to fully phosphorylate the quantity of ADP added to the chamber, being a good indicator of the mitochondrial respiration efficiency. The respiratory control ratio (RCR) was also calculated through the ratio between state 3 and 4. This is an indicator of the mitochondrial coupling efficiency between compound oxidation through the ETC and the phosphorylation of ADP.

2.3.3 Western blot.

Cardiac tissue samples (approximately 20 mg) were homogenized two times during 20 seconds in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 1% TritonX-100) at 4ºC, supplemented with 2.5% of the protease inhibitor cocktail (PIC) (104 mM AEBSF, 80 µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, and 1.5 mM Pepstatin A), 20 mM sodium fluoret (NAF), 10 mM nicotinamide (NAM), 5 mM sodium butyrate, 0.5% DOC, 0.5 mM PMSF, and orthovanadate. Homogeneization was achieved using an Ultra-TurraX homogenizer from IKA (Staufen, Germany). The samples were centrifuged to maximum speed (1320 x g, for 10 min, 4ºC) and the supernatant was collected. Protein was quantified using the BCA assay, using standard curves of BSA. Protein sample final concentration was 1. mg/mL. Laemmli buffer 6x, 375 mM Tris-HCl (pH 6.8), 9% SDS, 50% glycerol, 9% beta-mercaptoethanol, 0.03% bromophenol blue, and 300 mM DTT were added to each sample, reaching to a final concentration of 1.3 mg/m per sample. Afterward, the samples were boiled at 95ºC, for 5 minutes and stored at -20ºC until posterior utilization. To proceed to the protein separation according to the molecular weight, 20 µg of protein were loaded to the 12% acrylamide/bysacrylamide gel. Electrophoresis was ran at room temperature using a running buffer 1x, constituted of 25 mM Tris, 192 mM glycine, 0.1% SDS) in a dilution of 1x at constant intensity of 30 mA for each gel for, approximately, 45-60 minutes in a Mini-PROTEAN tetra Cell (Bio-Rad). Precision Plus Protein Dual Color Standards was ran in parallel with the samples. Gel protein transference occurred for 1 hour and 40 minutes at 90V, to previously methanol activated PVDF membranes for 30 seconds, 1 minute in distilled water, and 5 minutes in transfer buffer 1x. Transfer efficiency was evaluated by Ponceau S staining. Afterwards, the PVDF membranes were blocked with 5% BSA in washing buffer 1x (Tris 20 mM, pH 8.0, NaCl 150 mM, Tween-20 0.1% (w/v) for 1 hour, depending on the antibody manufacturer recommendations. After membrane blocking, membranes were incubated overnight at 4ºC with the primary antibodies (1:1000 or 1:500 in washing buffer 1x) (**Table 2.2**.). The membranes were then washed with washing buffer in 3 cycles of 5 minutes each with washing buffer 1x, and incubated with the respective secondary antibody at room temperature, which were prepared in washing buffer 1x (1:2000) (**Table 2.3.**). After another 3 cycles of 5-minute membrane washing, membrane development was achieved through luminescence after a 5-minute incubation with the clarity western ECL substrate in a VWR UV spectrum. Image analysis was performed using the TotalLab TL120 software (Nonlinear Dynamics, Newcastle, UK), using the background substract rolling ball of either 50 or 500, according to each image. The volume of the bands was used to compare proteins levels between different lanes. Data was normalized with the Ponceau S staining and reported in relation to F1-Control (mean of female and male groups).

Table 2.2 *| Primary antibodies and respective reference code.*

Protein	Residue	UniProt Entry Code	Manufacturer Code	Supplier	Host Specie	MW kDa	Dilution
Akt1	-	P31749	cs-2967	Cell Signaling	Mouse	60	1:1000
AMPKα	-	P54646	cs-2603	Cell Signaling	Rabbit	62	1:1000
Bad	-	Q92934	cs-9292	Cell Signaling	Rabbit	23	1:500
Beta Catenin	-	P35222	cs-9582	Cell Signaling	Rabbit	92	1:1000
CAT-1	-		sc-515782	Santa Cruz	Mouse	70	1:1000
CD36	-	P16671	sc-7309	Santa Cruz	Mouse	88	1:1000
CREB-1	-	P16220		Santa Cruz	Mouse	43	1:1000
DRP1	-	O00429	BDBioSci - 611113	BD Bio Sciences	Mouse	79-84	1:1000
Fis-1	-	Q9Y3D6	sc-B76447	Santa Cruz	Mouse	17	1:1000
GSK3αβ	-	P49840; P49841	sc-7291	Santa Cruz	Mouse	47; 51	1:1000
Mfn-1	-	Q8IWA4	sc-50330	Santa Cruz	Rabbit	86	1:1000
NFATc4	-	Q14934	sc-271597	Santa Cruz	Mouse	140-160	1:1000
Nitrotyrosine	-	N/A	sc-32757	Santa Cruz	Mouse	N/A	1:1000
OPA1	-	O60313	sc-30573	Santa Cruz	Goat	120	1:1000
OXPHOS human cocktail	-	N/A	ab110411	ab cam	Mouse	18; 22; 29; 48; 54	1:1000
p70 S6 kinase α	-	P23443	sc-8418	Santa Cruz	HRP-conjugated antibody	70	1:500
phospho-Akt	Ser473	P31749	cs-4060	Cell Signaling	Rabbit	60	1:1000
phospho-Akt	Thr308	P31749	cs-2965	Cell Signaling	Rabbit	60	1:1000
phospho-AMPKα	Thr172	P54646	cs-2531	Cell Signaling	Rabbit	62	1:1000

phospho-Bad	Ser112	Q92934	cs-9296	Cell Signaling	Mouse	23	1:500
phospho-CREB-1	Ser133	P16220	sc-81486	Santa Cruz	Mouse	43	1:1000
PGC1a	-	Q9UBK2	ST1203	Millipore	Rabbit	113	1:1000
phospho-GSK3αβ	Ser21/9	P49840; P49841	cs-9327	Cell Signaling	Rabbit	46; 51	1:1000
phospho-NFATc4	Ser168/170	Q14934	sc-135771	Santa Cruz	Mouse	140-160	1:1000
phospho-p70 S6 kinase	Ser389	P23443	cs-9205S	Cell Signaling	Rabbit	70	1:500
SDHA	-	P31040	sc-59687	Santa Cruz	Mouse	71	1:1000
TFAM	-	Q00059	sc-23588	Santa Cruz	Goat	25	1:1000
TOM20	-	Q15388	sc-11415	Santa Cruz	Rabbit	20	1:1000

Table 2.3 | *Secondary antibodies and respective reference code.*

Antibody	Description	Company	Manufacturer code	Host specie	Dilution
Anti-Goat	rabbit@goat	Santa Cruz	2771	Rabbit	1:2000
Anti-Mouse	goat@mouse	Santa Cruz	2008	Goat	1:2000
Anti-Rabbit	goat@rabbit	Santa Cruz	2007	Goat	1:2000

2.4. Data analysis and statistics

The software GraphPad Prism 8.0 (GraphPad Software, Irvine, CA, USA) was used for the data analysis. All results are expressed as median, first quartile (Q_1), and third quartile (Q_3) with three significant digits. Each animal was considered an experimental unit. To assess the effect of maternal physical exercise practice during an obesogenic pregnancy, the following comparisons were made: between male F1-Control *vs.* F1-MO, male F1-Control *vs.* F1-MOEx, male F1-MO *vs.* F1-MOEx, female F1-Control *vs.* F1-MO, female F1-Control *vs.* F1-MOEx, female F1-MO *vs.* F1-MOEx, male and female F1-Control *vs.* F1-MO, male and female F1-Control *vs.* F1-MOEx, male and female F1-MO *vs.* F1-MOEx. In addition, to explore the sex-specific response the following comparisons were made: male F1-Control *vs.* female F1-Control, male F1-MO *vs.* female F1-MO, male F1-MOEx *vs.* female F1-MOEx. The normality distribution of the results was evaluated through the Shapiro-Wilk normality test. If $\alpha \geq 0.05$, the results were considered normal, and the parametric unpaired student *t-test* was performed or else, the Mann-Whitney test was used.

For all of the statistical analysis, a p-value of < 0.05 was considered statistically significant (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.001$ or #$p < 0.05$; ## $p < 0.01$; ###$p < 0.001$; #### $p < 0.001$ or § $p < 0.05$; §§ $p < 0.01$; §§§ $p < 0.001$; §§§§ $p < 0.001$). The symbol * represents

a statistically significant result in comparison with F1-Control of the same sex; the # symbol represents a statistically significant result in comparison with F1-MO of the same sex; and the § symbol represents a statistically significant result in comparison with the same group of the opposite sex.

Chapter 3 – Results

3.1. The effect of maternal interventions on the offspring's physiology.

For this study, maternal and offspring's physiologic parameters were evaluated **(Fig. 3.1.)**. The consumption of a HFHS diet led to an obese phenotype with increased maternal weight gain during pregnancy when compared to control fed mothers **(Fig. 3.1.A) (**(F0-MO: median = 156.5; Q_1 = 141.0; Q_3 = 169.3) *vs.* (F0-Control: median = 127.0; Q_1 =78.0; Q_3 = 130.0); p = 0.006).

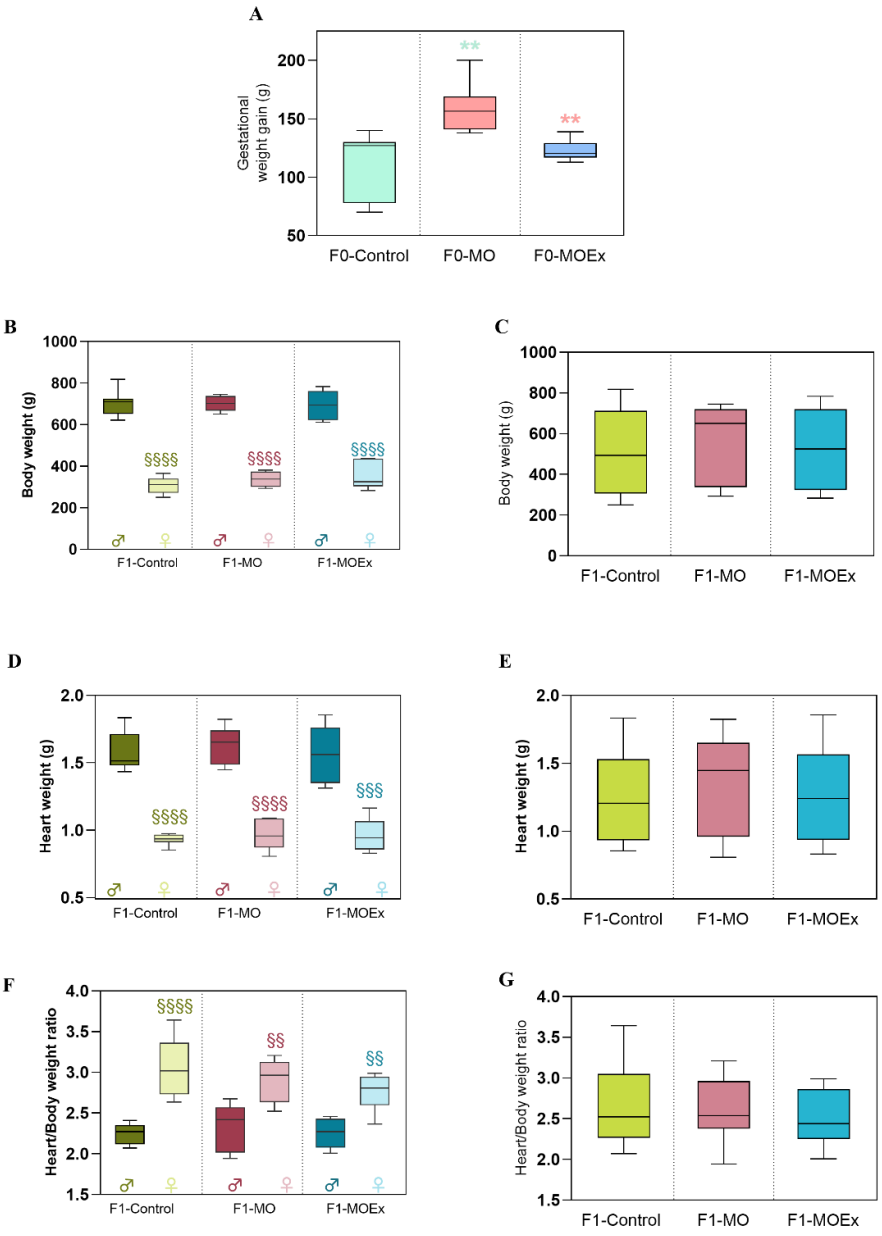


Fig. 3.1 | Effects of maternal obesity and gestational exercise practice on physiological parameters of 24-week-old mothers and 32-week-old offspring. *F0 – Control: mothers fed a standard-diet; F0-MO: sedentary mothers fed a HFHS diet; F0-MOEx: exercised mothers fed a HFHS diet; F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Gestational weight gain. Data previously published in Jelena S. et al*[147]*;* ***(B)*** *Offspring sex-specific body weight at 32-weeks-old;* ***(C)*** *Offspring body weight at 32 weeks-old;* ***(D)*** *Offspring*

sex-specific heart weight at 32 weeks-old; ***(E)*** *Offspring heart weight at 32-weeks-old;* ***(F)*** *Offspring sex-specific heart weight to body weight ratio;* ***(G)*** *Offspring heart weight to body weight ratio. Data are expressed as medians, Q1, Q3, min and max. The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; #* ***vs. F1-MO****; §****vs. opposite sex of the same group.***

In turn, maternal gestational weight gain was decreased for mothers that were intervened with exercise during obesogenic pregnancy (F0-MOEx; median = 120.5; Q1 = 116.8; Q3 = 129.3), in comparison with F0-Control (F0-MOEx *vs.* F0-MO); p = 0.0042.

The offspring's body **(Fig. 3.1.B,C)** and heart weight **(Fig. 3.1.D,E)** were different according to sex. The body weight of female offspring born to lean mothers was lower in comparison with F1-Control male; ((F1-Control female; median = 310.0; Q1 = 271.0; Q3 = 340.0) *vs.* (F1-Control male; median = 711.0; Q1 = 652.0; Q3 = 725.0); p < 0.0001), which is also observed for both F1-MO female (median = 338.0; Q1 = 300.0; Q3 = 374.0) *vs.* F1-MO male (median = 701.5; Q1 = 666.8; Q3 = 738.3); p < 0.0001 and for F1-MOEx female (median = 325.0; Q1 = 304.0; Q3 = 436.3) *vs.* F1-MOEx male (median = 693.5; Q1 = 622.3; Q3 = 762.0); p < 0.0001. This behaviour is also verified for the offspring heart weight. Female offspring showed decreased heart weight in comparison with male offspring, and this is observed for every group (F1-Control female (median = 0.9360; Q1 = 0.9110; Q3 = 0.9670) *vs.* F1-Control male (median = 1.514; Q1 = 1.482; Q3 = 1.712)); p < 0.0001; (F1-MO female (median = 0.9580; Q1 = 0.8705; Q3 = 1.087) *vs.* F1-MO male (median = 1.652; Q1 = 1.488; Q3 = 1.741); p < 0.0001)); (F1-MOEx female (median = 0.9430; Q1 = 0.8585; Q3 = 1.067) *vs.* F1-MOEx male (median = 1.561; Q1 = 1.350; Q3 = 1.760); p = 0.0001)). Consistently, the ratio between heart and body weight **(Fig. 3.1.F,G)** is increased for female offspring of every experimental group, in comparison with male offspring of the same group (F1-Control female (median = 3.019; Q1 = 2.729; Q3 = 3.362) *vs.* F1-Control male (median = 2.273; Q1 = 2.117; Q3 = 2.352)); p = 0.0001; (F1-MO female (median = 2.962; Q1 = 2.633; Q3 = 3.130) *vs.* F1-MO male (median = 2.417; Q1 = 2.015; Q3 = 2.572); p = 0.009)); (F1-MOEx female (median = 2.807; Q1 = 2.596; Q3 = 2.947) *vs.* F1-MOEx male ; median = 2.271; Q1 = 2.076; Q3 = 2.432); p = 0.018)). No differences between intervention groups were identified, indicating the maternal interventions did not alter the offspring's body and heart weight at 32 weeks of age.

Despite no variations in the cardiac muscle mass between the experimental groups was found, a cardiac histological analysis was performed to assess potential anatomical structural alterations **(Fig. 3.2.)**.

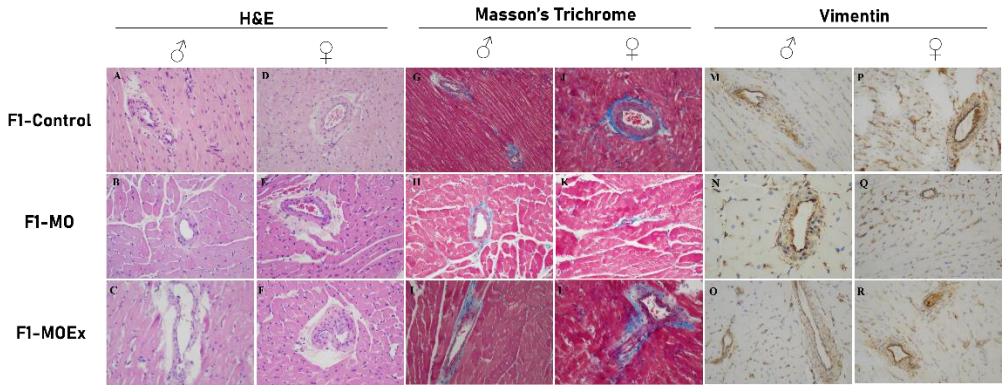


Fig. 3.2 | Optical microscopy and immunohistochemistry preliminary data of a cardiac tissue section of the 32-week-old offspring from control, obese and sedentary, and obese and exercised mothers. (A-F) *Haematoxylin and eosin staining of (A) F1-Control male; (B) F1-MO male; (C) F1-MOEx male; (D) F1-Control female;* ***(E)*** *F1-MO female; (F) F1-MOEx female;* ***(G-L)*** *Masson's trichrome staining of (G) F1-Control male; (H) F1-MO male; (I) F1-MOEx male; (J) F1-Control female; (K) F1-MO female; (L) F1-MOEx female;* ***(M-R)*** *Vimentin staining of (M) F1-Control male; (N) F1-MO male; (O) F1-MOEx male; (P) F1-Control female; (Q) F1-MO female; (R) F1-MOEx female. Amplification (x 200). The presented figures are representative images of the overall acquired data by a certified pathologist without quantification.*

The histological results corroborate the latter observations of cardiac sexual dimorphism (*section 3.1.*). Masson's Trichrome staining in the cardiac tissue of the offspring showed an enlargement of the perivascular matrix in the female offspring groups (F1-Control; F1-MO; F1-MOEx female) **(Fig. 3.2. J, K, L)** in comparison with male offspring groups (F1-Control; F1-MO; F1-MOEx male) **(Fig. 3.2. G, H, I)** without correlation with diet nor diet and exercise.

3.2. Metabolic end-points characterization in the blood plasma of 32-week-old offspring.

The blood plasma biochemical analysis (**Table 3.1.**) revealed that, overall, maternal exercise practice during obesogenic pregnancy ameliorated the MO-induced F1-generation metabolic syndrome phenotype, in a sex-specific way. This is strongly evident for female offspring. Female offspring born to obese mothers had increased TGs levels in comparison with F1-Control female (F1-MO female; median = 131.5; Q1 = 106.5; Q3 = 156.5) *vs.* F1-Control female (median = 60.00; Q1 = 55.00; Q3 = 78.00); p = 0.0001). Both male (F1-MOEx male; (median = 85.30; Q1 = 61.50; Q3 = 101.8) and female (F1-MOEx female; median = 77.70; Q1 = 58.50; Q3 = 94.25) of obese and exercised mothers presented lower TGs levels in comparison with F1-MO male (F1-MO male; median = 113.5; Q1 = 102.8; Q3 = 125.8) and female (F1-MO female; median = 131.5; Q1 =106.5; Q3 = 156.5), respectively (p = 0.0319; p = 0.0039). MOEx female offspring TGs levels were similar to control levels. For this parameter, the maternal practice of physical exercise during pregnancy reversed the MO-induced impact. Female offspring born to lean mothers (F1-Control female) presented lower TGs concentration than male offspring (F1-Control male; median = 127.0; Q1 = 91.00; Q3 = 137.0) (F1-Control female *vs.* F1-Control male; p = 0.0016).

Female offspring born to obese and exercised mothers presented increased HDL levels in comparison with female offspring born to sedentary/obese mothers (F1-MOEx female; median = 63.00; Q1 = 55.00; Q3 = 69.75) *vs.* F1-MO female (median = 40.50; Q1 = 36.25; Q3 = 43.50); p = 0.0001), which, in turn, was decreased in comparison with female offspring born to lean mothers (F1-MO female *vs.* F1-Control female; median = 54.00; Q1 = 48.50; Q3 = 61.00); p = 0.0018). On the contrary to female offspring, and highlighting a sex-specific response, HDL levels were decreased for F1-MOEx male in comparison with F1-MO male (F1-MOEx male (median = 47.60; Q1 = 46.00; Q3 = 50.75) *vs.* F1-MO male (median = 57.00; Q1 = 53.00; Q3 = 70.00); p = 0.0096). Female offspring born to obese and sedentary mothers (F1-MO female) presented lower HDL levels in comparison with male offspring from the same group (F1-MO female *vs.* F1-MO male; p = 0.0003). Reversely, female from obese and exercised mothers (F1-MOEx female) had higher HDL plasma levels comparing with male offspring (F1-MOEx female *vs.* F1-MOEx male; p = 0.0025).

LDL levels were tendentially decreased in F1-MO female (median = 16.00; Q1 = 14.00; Q3 = 20.00) *vs.* F1-Control female (median = 20.00; Q1 = 18.25; Q3 = 27.00); p = 0.0882. LDL levels for F1-MOEx female were increased comparing with F1-MO female (F1-MOEx female (median = 22.00; Q1 = 19.75; Q3 = 24.00) *vs.* F1-MO female; p = 0.0174). The MO-induced LDL levels in the female offspring were reversed with the practice of maternal physical exercise during pregnancy to control levels. Female offspring born to exercised and obese mothers (F1-MOEx female) LDL blood plasma levels were increased in comparison with male offspring (F1-MOEx male; n = 14.50; Q1 = 13.50; Q3 = 15.50) from the same maternal intervention (F1-MOEx female *vs.* F1-MOEx male; p = 0.0002).

Cholesterol blood plasma concentrations were increased for male offspring born to exercised and obese mothers in comparison with male offspring sedentary and obese mothers (F1-MO male; (F1-MOEx male; median = 65.50; Q1 = 57.50; Q3 = 68.25) *vs.* F1-MO male (median = 76.50; Q1 = 67.75; Q3 = 94.75); p = 0.0287).

The atherogenic index (AI) calculated through the ratio between TGs and HDL [148], indicated that overall, female offspring born to lean (F1-Control female; median = 1.091; Q1 = 0.8246; Q3 = 1.143) and exercised/obese mothers (F1-MOEx female; median = 1.297; Q1 = 0.8505; Q3 = 1.594), had decreased AI in comparison with male offspring of the same group (F1-Control male; median = 2.636; Q1 = 2.395; Q3 = 3.323); (F1-MOEx male; median = 2.037; Q1 = 1.777; Q3 = 2.408), (F1-Control female *vs.* F1-Control male, p < 0.0001 ; F1-MOEx female *vs.* F1-MOEx male, p = 0.0069). In addition, female offspring born to sedentary and obese mothers (F1-MO female; median = 2.435; Q1 = 1.884; Q3 = 3.040) presented higher AI in comparison with control (F1-MO female *vs.* F1-Control female, p = 0.0004). A tendential lower CVD risk for both sexes in MOEx offspring in comparison with F1-MO male was observed (median = 2.436; Q1 = 2.173; Q3 = 2.787); female (F1-MOEx male; female *vs.* F1-MO male; female; p = 0.088, p =

0.0050), which is translated in decreased risk for CVD, since this index has a positive association with CVD risk. Specifically, for female offspring, the AI indicates that maternal physical exercise during MO might protect the offspring of CVD development (**Table 3.1.**).

Table 3.1. | ***Impact of maternal obesity and gestational exercise practice on 32-week-old offspring biochemical blood plasma parameters.*** *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet. Data are expressed as median ± standard deviation. The comparison between groups was performed using parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control; # vs. F1-MO; §vs. opposite sex of the same group.***

Blood plasma biochemical parameter	F1-Control male (n = 7)	F1-Control female (n = 7)	F1-MO male (n = 6)	F1-MO female (n = 5)	F1-MOEx male (n = 6)	F1-MOEx female (n = 6)
Glucose ($mg.dL^{-1}$)	110.0 ± 9.6	102.0 ± 11.1	110.0 ± 18.3	110.0 ± 15.6	102.0 ± 9.4	105.5 ± 5.9
Triglycerides ($mg.dL^{-1}$)	127.0 ± 39.8	60.0 ± 11.9§§	115.5 ± 17.0	131.5 ± 26.6****	82.6 ± 20.5*#	77.7 ± 20.0##
LDL ($mg.dL^{-1}$)	14.0 ± 5.1	20.0 ± 4.5	16.0 ± 7.4	16.0 ± 3.3	14.5 ± 1.6	22.0 ± 2.8#§§§
HDL ($mg.dL^{-1}$)	44.0 ± 9.6	54.0 ± 6.9	57.0 ± 8.6*	40.5 ± 4.1**§§§	47.6 ± 3.3##	63.0 ± 8.2§§§ ###
Cholesterol ($mg.dL^{-1}$)	68.6 ± 10.0	87.0 ± 14.0§§	76.5 ± 16.0	91.0 ± 10.0	65.5 ± 6.1 #	93.5 ± 10.3####
Atherogenic Index	2.6 ± 0.5	1.1 ± 0.3§§§§	2.4 ± 0.4	2.4 ± 0.6***	2.0 ± 0.4*	1.3 ± 0.4##§§

3.3. Gestational exercise-induced alterations on offspring cardiac metabolism.

Offspring plasma biochemical analysis revealed precious information concerning the metabolic state of each individual, given that it indicates the levels of metabolites that participate in important metabolic pathways. As reported in *section 1.4.4.1.*, the insulin signalling pathway plays an essential role in glucose and fatty-acids oxidation. Thus, the activation and levels of proteins involved in the insulin signalling pathway and glycogen synthesis were measured. Due to its central role in several cellular responses, Akt activation was measured (**Fig. 3.3.**).

The cardiac activation of Akt in the Ser473 residue (p-AktSer473) does not seem to be altered in the offspring hearts by maternal interventions (**Fig. 3.3.E, F)**. In spite of this, the activation of Akt in the Thr308 (**Fig. 3.3.A, B)** residue was decreased for F1-MOEx male comparing with F1-Control male; (F1-MOEx male (median = 0.7185; Q1 = 0.3844; Q3 = 0.8460)

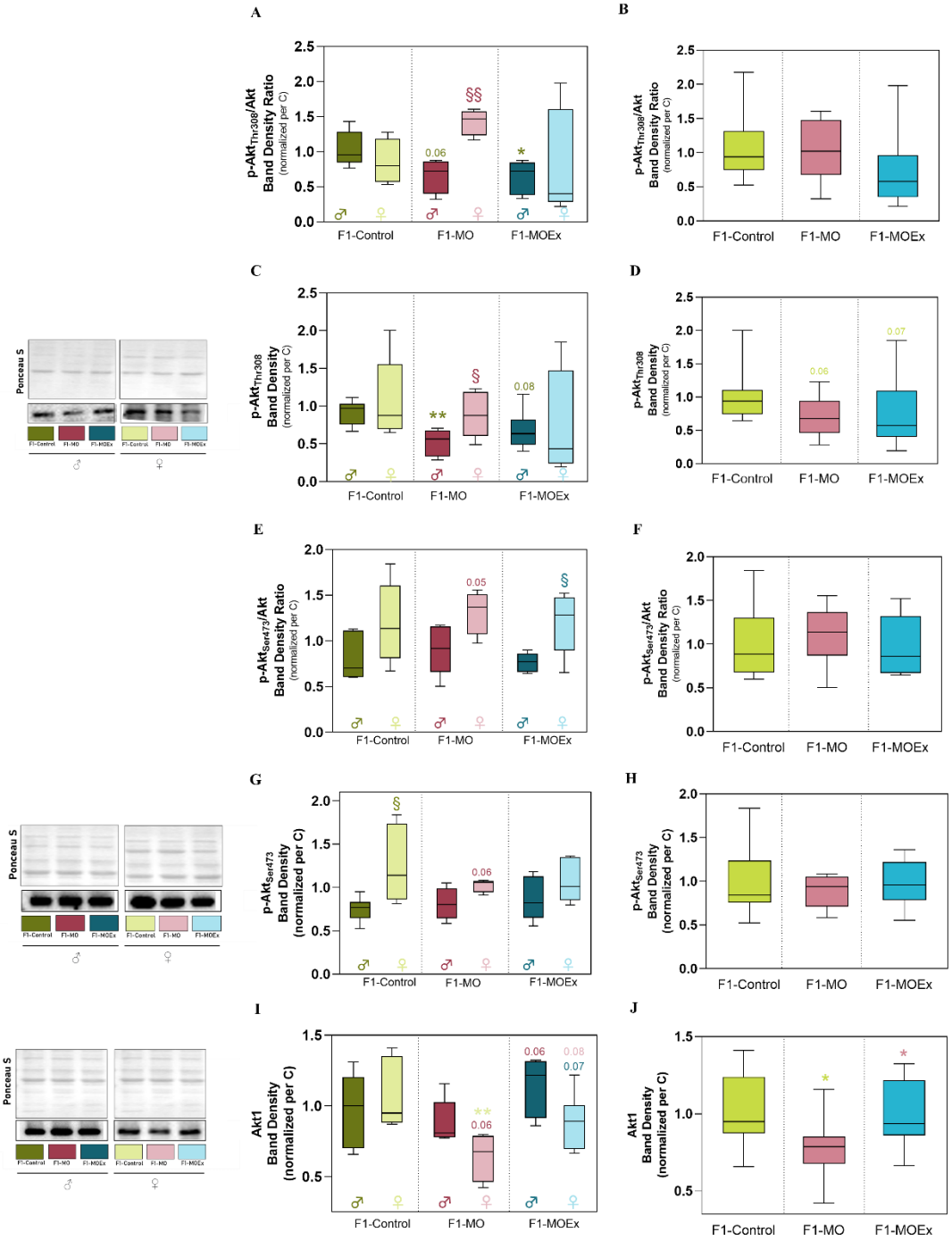
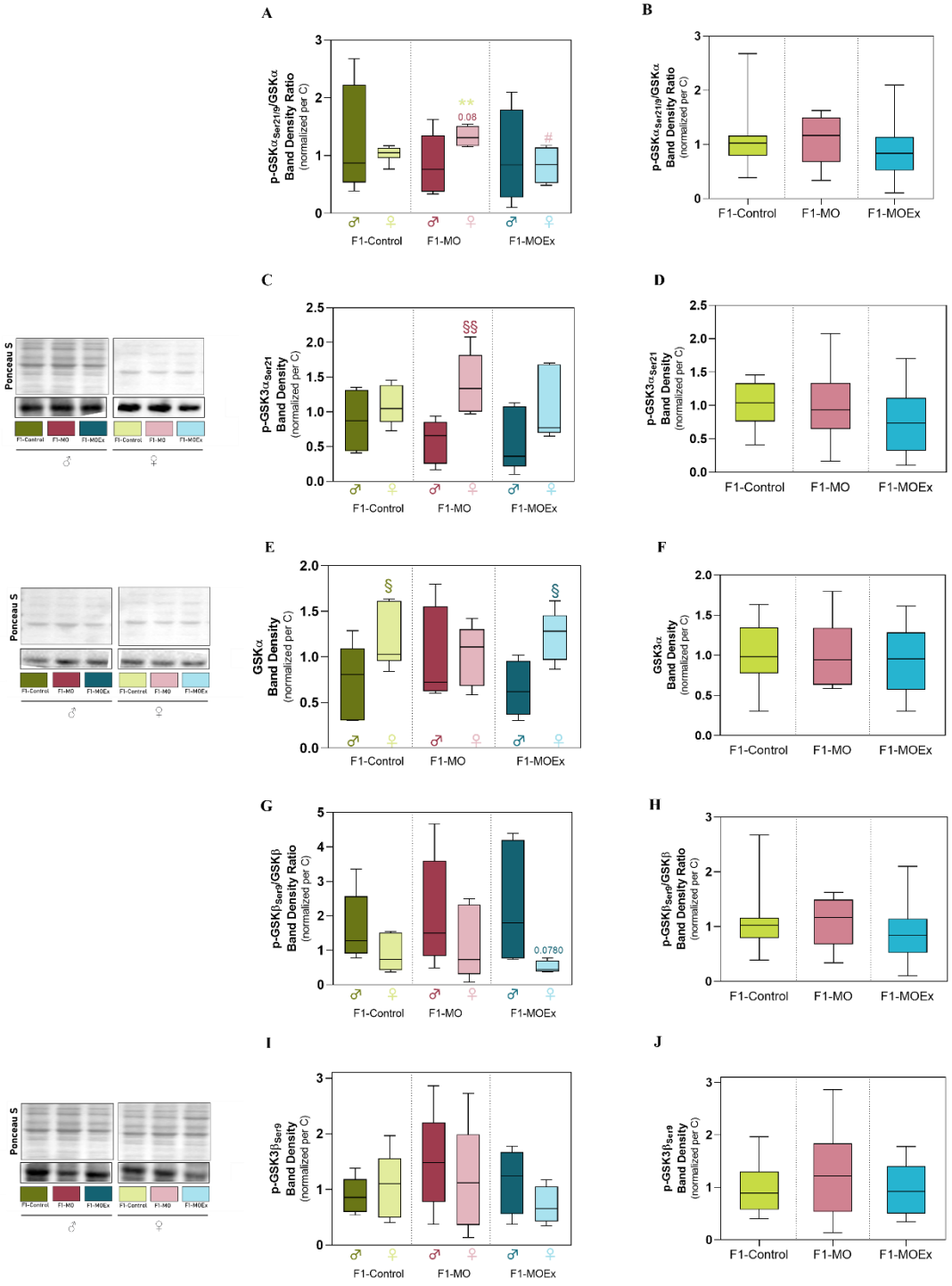


Fig. 3.3 | Modulation of Akt activation in the hearts of 32-week-old offspring by maternal obesity and gestational exercise practice during obesogenic pregnancy. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific activated Akt, calculated through the ratio between p-Akt(Thr308)/Akt1;* ***(B)*** *Activated Akt, calculated through the ratio between p-Akt(Thr308)/Akt1;* ***(C)*** *Sex-specific phosphorylated Akt in the Thr308 residue;* ***(D)*** *Phosphorylated Akt in the Thr308 residue;* ***(E)*** *Sex-specific activated Akt1, calculated through the ratio between p-Akt(Ser473)/Akt1;* ***(F)*** *Activated Akt, calculated through the ratio between p-Akt(Ser473)/Akt1;* ***(G)*** *Sex-specific phosphorylated Akt in the Ser473 residue;* ***(H)*** *Phosphorylated Akt in the Ser473 residue;* ***(I)*** *Sex-specific total Akt1;* ***(J)*** *Total Akt1. Data are expressed as medians, Q1, Q3, min and max. Data were normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 4). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control; # vs. F1-MO; §vs. opposite sex of the same group.***

vs. F1-Control male (median = 0.9529; Q1 = 0.8441, Q3 = 1.284), p = 0.0316), as indicated by the ratio between the phosphorylation of Akt in the Thr308 residue (p-AktThr308) and total levels of Akt (**Fig. 3.3.A)**. A tendential decrease was also observed for p-AktThr308 levels (**Fig. 3.3.C, D)** (F1-MOEx male (median = 0.6344; Q1 = 0.4872; Q3 = 0.8180) *vs.* F1-Control male (median = 0.9692; Q1 = 0.7570; Q3 = 1.029); p = 0.082). In addition, p-AktThr308 levels seemed to be decreased for F1-MO male in comparison with F1-Control male (F1-MO male (median = 0.5614; Q1 = 0.3303; Q3 = 0.6758) *vs.* F1-MOEx male, p = 0.0036). For both the ratio and p-AktThr308 relative levels, respectively, F1-MO female (median = 1.464; Q1 = 1.232; Q3 = 1.576 were increased in comparison with F1-MO male (median = 0.7228; Q1 = 0.3999; Q3 = 0.8609) (F1-MO female *vs.* F1-MO male; p = 0.0027, p = 0.0438). Total Akt1 levels (**Fig. 3.3.I, J)** are significantly decreased for F1-MO female (median = 0.6772; Q1 = 0.4628; Q3 = 0.7890) when compared with F1-Control female (median = 1.000; Q1 = 0.7020; Q3 = 1.204; p = 0.0061). Tendentially increased total Akt1 levels are verified for both sexes for F1-MOEx (F1-MOEx male *vs.* F1-MO male, p = 0.056), (F1-MOEx female *vs.* F1-MO female, p = 0.083). Maternal physical exercise practice affects the offspring's cardiac Akt phosphorylation in the Thr308 residue, but not in the Ser473 residue.

To further disclose the impact of the altered phosphorylation of Akt in the Thr308 residue, the activation of downstream proteins was evaluated (**Fig. 3.4)**. Besides the increased levels of GSK3α (**Fig. 3.4.E, F)** for F1-Control female comparing to F1-Control male (F1-Control female *vs.* F1-Control male: median = 1.032; Q1 = 0.9550; Q3 = 1.613 *vs.* median = 0.8075; Q1 = 0.3035; Q3 = 1.095; p = 0.0476) and F1-MOEx female *vs.* F1-MOEx male (F1-MOEx female *vs.* F1-MOEx male: median = 1.280; Q1 = 0.9673; Q3 = 1.456 *vs.* median = 0.6175; Q1 = 0.3661; Q3 = 0.9559; p = 0.0144) no differences were detected regarding GSK3β (**Fig. 3.4.K, L)**. Despite the apparent increased inactivation of p-GSK3α (**Fig. 3.4.A, B)**, indicated by the ratio between phosphorylated GSK3α in the residue Ser21 and total GSK3α for F1-MO female *vs.* F1-Control female (median = 1.400; Q1 = 1.232; Q3 = 1.556 *vs.* median = 0.8685; Q1 = 0.8293; Q3 = 1.096; p = 0.0010) and decreased inactivated levels of GSK3α for F1-MOEx female *vs.* F1-MO female (median = 0.6720; Q1 = 0.5436; Q3 = 1.020 *vs.* median = 1.400; Q1 = 1.232; Q3 = 1.556; p = 0.0047), this does not seem to be associated with the altered phosphorylation of Akt in the Thr308 residue. β – catenin relative expression (**Fig. 3.4.A, B)** is decreased for F1-MOEx females in comparison with F1-Control females (F1-MOEx *vs* F1-Control females; median = 0.9203; Q1 = 0.7381; Q3 = 1.087 *vs.* median = 1.176; Q1 = 1.011; Q3 = 1.363; p = 0.048). In addition, female offspring of the rest of the groups seemed to have increased β – catenin levels in comparison with male offspring from the respective group (F1-Control female *vs.* F1-Control male (median = 0.6971; Q1 = 0.5339 Q3 = 0.9844); p = 0.0390); (F1-MO female (n = 4; median = 1.123; Q1 = 0.9644; Q3 = 1.328) *vs.* F1-MO male (n = 6; median = 0.9012; Q1 = 0.6336; Q3 = 0.9151; p = 0.0159).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p-GSKα Ser219/GSKα Band Density Ratio (normalized per C)
p-GSK3α Ser21 Band Density (normalized per C)
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p-GSKβ Ser9/GSKβ Band Density Ratio (normalized per C)
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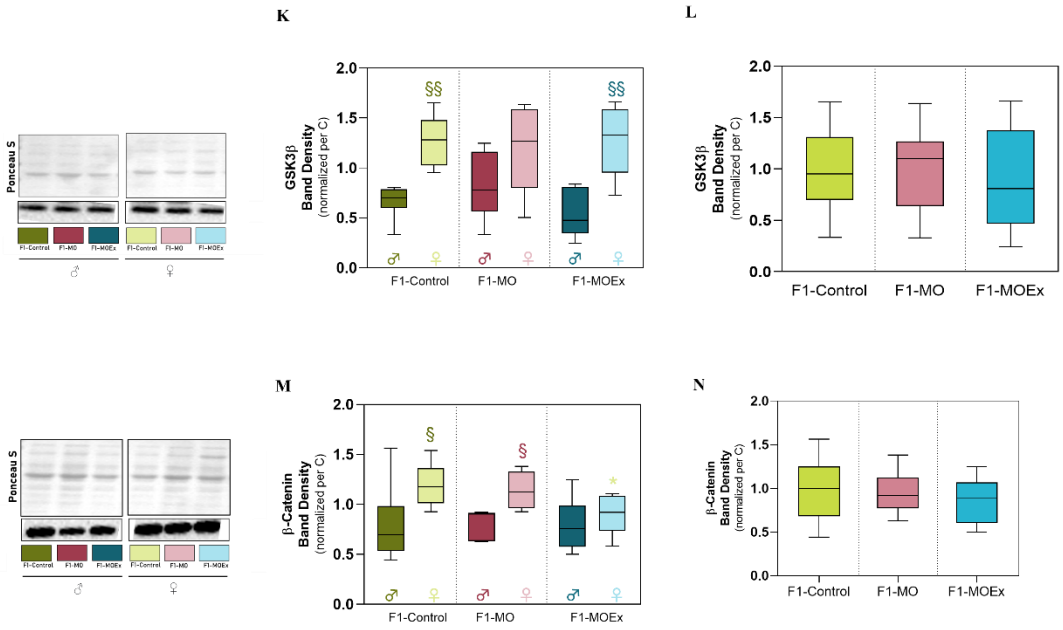
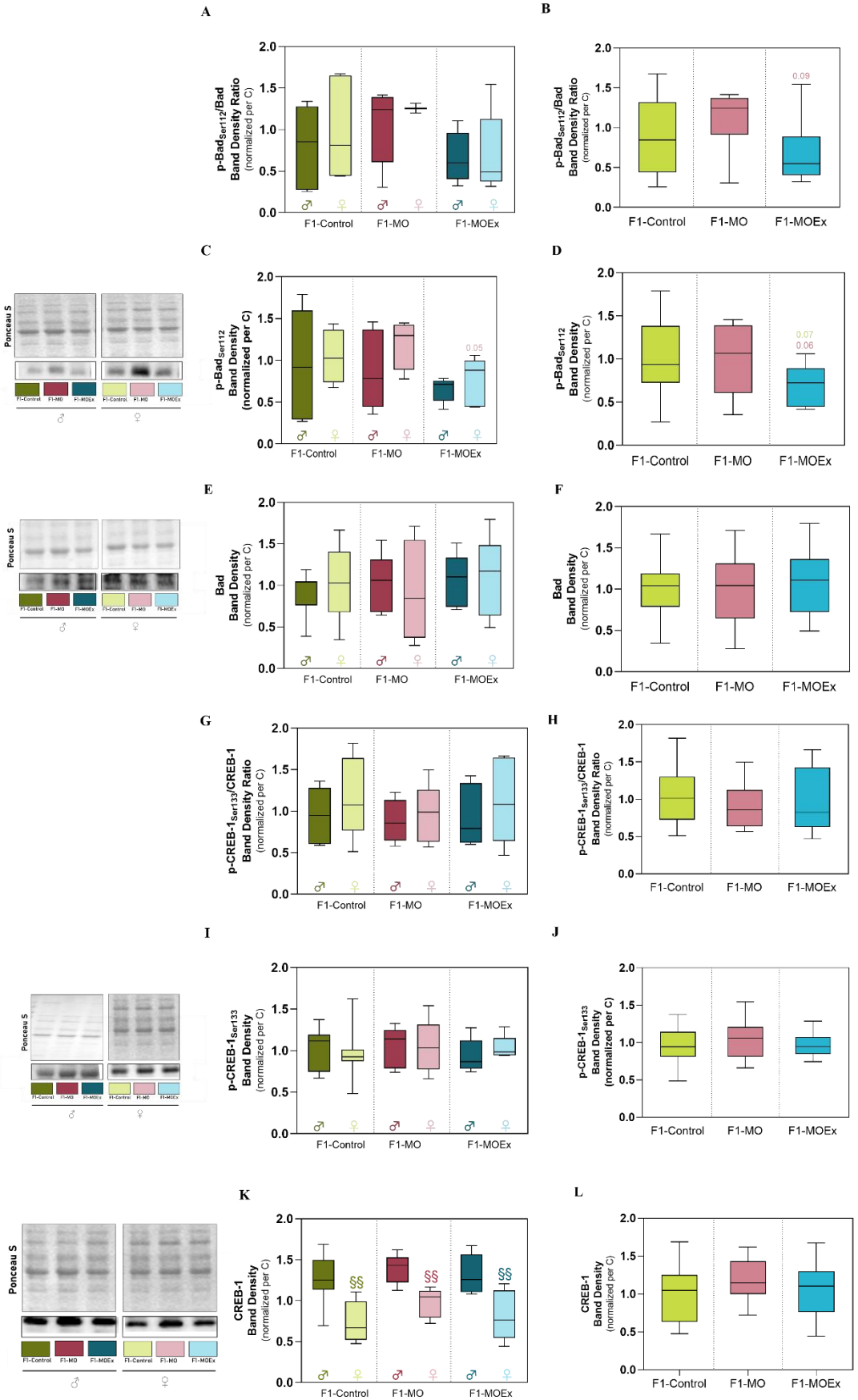


Fig. 3.4 | Cardiac modulation of GSK3α/β and β-catenin in 32-week-old offspring by maternal obesity and gestational exercise practice during obesogenic pregnancy. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific inactivated GSK3α, calculated through the ratio between p- GSK3α (Ser21)/GSK3α;* ***(B)*** *Inactivated GSK3α, calculated through the ratio between p- GSK3α (Ser21)/ GSK3α;* ***(C)*** *Sex-specific phosphorylated GSK3α in the Ser21 residue;* ***(D)*** *Phosphorylated GSK3α in the Ser21 residue;* ***(E)*** *Sex-specific total GSK3α;* ***(F)*** *Total GSK3α;* ***(G)*** *Sex-specific GSK3β, calculated through the ratio between p- GSK3β(Ser9)/GSK3β;* ***(H)*** *Inactivated GSK3β, calculated through the ratio between p-GSK3β (Ser9)/GSK3β;* ***(I)*** *Sex-specific phosphorylated GSK3β in the Ser9 residue;* ***(J)*** *Phosphorylated GSK3β in the Ser9 residue;* ***(K)*** *Sex-specific total GSK3β;* ***(L)*** *Total GSK3β;* ***(M)*** *Sex-specific β-catenin;* ***(N)*** *β-catenin. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 4). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control;*** *#* ***vs. F1-MO;*** *§* ***vs. opposite sex of the same group.***

The inhibition of apoptotic pathways is also regulated by Akt. Considering the verified decreased activation of Akt in the Thr308 residue it was interesting to explore whether an altered Akt activation would impact apoptotic mechanisms **(Fig. 3.5).** The activation of Bad (measured through the ratio between the phosphorylated Bad in the Ser112 residue and total Bad) (**Fig. 3.5.A, B)** and CREB-1 (ratio between the phosphorylated CREB-1 in the Ser133 residue and total CREB-1) (**Fig. 3.5.G, H)**, which is also involved in the apoptotic response, were measured. Neither of the activated, phosphorylated nor total protein levels seemed to be significantly affected between groups. However, it is important to highlight the evident sex-dependent response for CREB-1 total protein levels (**Fig. 3.5.K, L)**. Overall, female offspring presented decreased levels in comparison with the male offspring from the respective group (F1-Control female *vs.* F1-Control male: median = 0.6697; Q1 = 0.5222; Q3 = 0.9913 *vs.* median = 1.249; Q1 = 1.135; Q3 = 1.498; p = 0.0065; F1-MO female *vs.* F1-MO male: median = 1.045; Q1 = 0.7965; Q3 = 1.120 *vs.* median = 1.434; Q1 = 1.226; Q3 = 1.528; p = 0.0066; F1-MOEx female *vs.* F1-MOEx male: median = 0.7662; Q1 = 0.5481; Q3 = 1.125 *vs.* median = 1.258; Q1 = 1.106; Q3 = 1.566; p = 0.0081).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p-Bad$_{Ser112}$/Bad Band Density Ratio (normalized per C)
p-Bad$_{Ser112}$ Band Density (normalized per C)
Bad Band Density (normalized per C)
p-CREB-1$_{Ser133}$/CREB-1 Band Density Ratio (normalized per C)
p-CREB-1$_{Ser133}$ Band Density (normalized per C)
CREB-1 Band Density (normalized per C)
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F1-MOEx
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0.07
0.06
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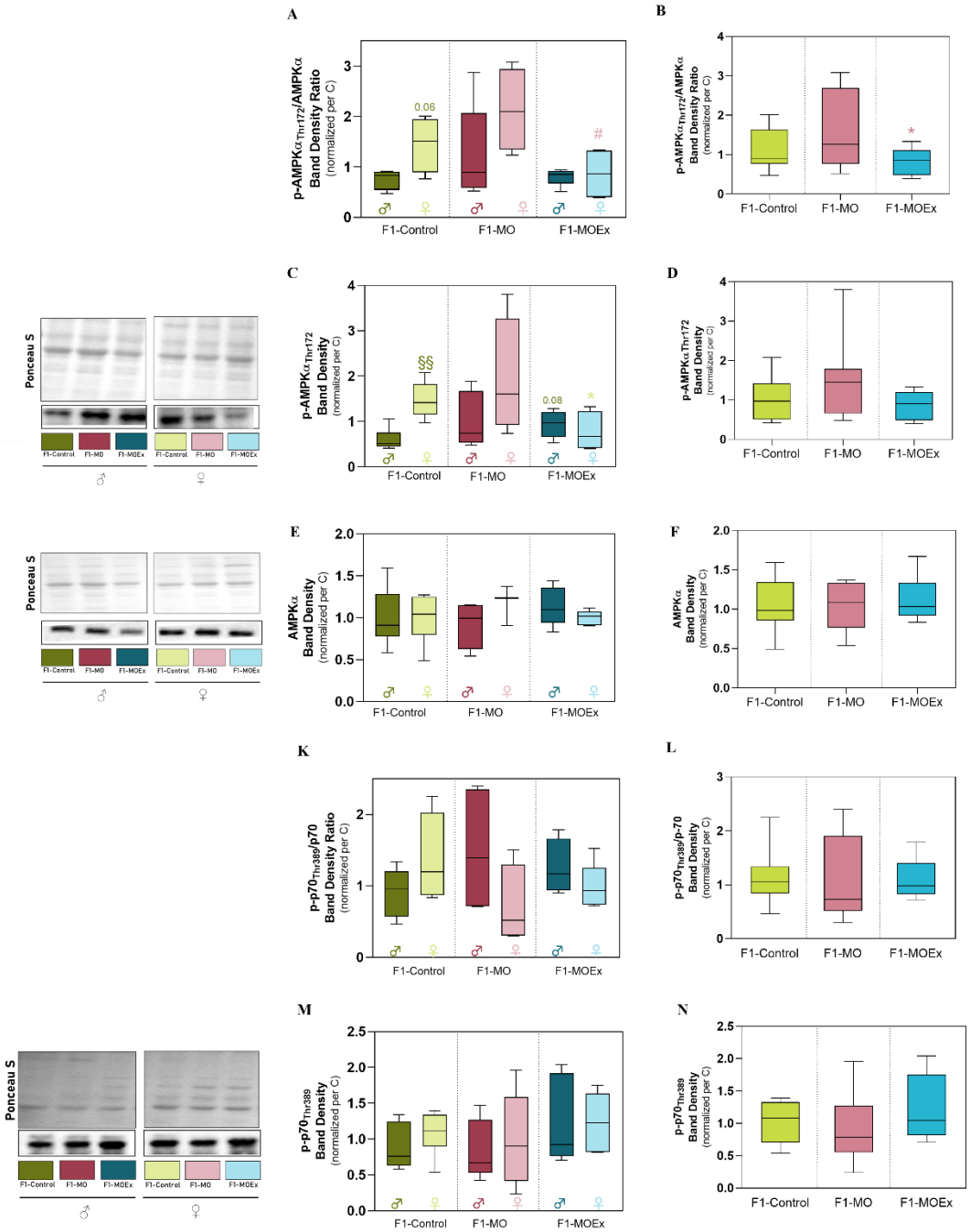
Fig. 3.5 | Cardiac relative band density of of apoptosis-related proteins Bad and CREB-1 in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific activated Bad, calculated through the ratio between p-Bad(Ser112)/Bad;* ***(B)*** *Activated Bad, calculated through the ratio between p-Bad (Ser112)/Bad;* ***(C)*** *Sex-specific phosphorylated Bad in the Ser112 residue;* ***(D)*** *Phosphorylated Bad in the Ser112 residue;* ***(E)*** *Sex-specific total Bad;* ***(F)*** *Total Bad;* ***(G)*** *Sex-specific activated CREB-1, calculated through the ratio between p-CREB1 (Ser133)/CREB1;* ***(H)*** *Activated CREB-1, calculated through the ratio between p-CREB1 (Ser133)/CREB1;* ***(I)*** *Sex-specific phosphorylated CREB-1 in the Ser 133 residue;* ***(J)*** *Phosphorylated CREB-1 in the Ser133 residue;* ***(K)*** *Sex-specific total CREB-1;* ***(L)*** *Total CREB-1. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney ($n \geq 4$). Normality was evaluated with Shappiro-Wilk test. *,#,§ $p \leq 0.05$; **,##,§§$p \leq 0.01$; ***,###,§§§$p \leq 0.001$; ****,####,§§§§ $p \leq 0.0001$.* **** vs. F1-Control; # vs. F1-MO; §vs. opposite sex of the same group.***

Since AMPK regulates energy metabolism and homeostasis, the indirect evaluation of the activation of this protein was measured. The results indicate that the activation of AMPKα determined through the p-AMPK(Thr172)/AMPKα ratio (**Fig. 3.6. A, B)** was decreased for F1-MOEx female in comparison with F1-MO female (F1-MOEx *vs.* F1-MO female: median = 0.8675; Q1 = 0.4047; Q3 = 1.323 *vs.* median = 2.095; Q1 = 1.347; Q3 = 2.940; p = 0.04). Although these verified differences in the ratio, p-AMPK(Thr172) levels (**Fig. 3.6. C, D)** were tendentially increased for male offspring born to exercised/obese mothers (F1-MOEx *vs.* F1-Control male; p = 0.0823). Stressing the p-AMPK(Thr172) sex-specific response, since this parameter is decreased for the females of the same groups (F1-MOEx *vs.* F1-Control female: median = 1.608; Q1 = 0.9326; Q3 = 3.272 *vs.* median = 1.417; Q1 = 1.154; Q3 = 1.824; p = 0.04).

Besides the sex-specific response for the total p70 levels (**Fig. 3.6. O, P)** for F1-MO female *vs.* F1-MO male (F1-MO female *vs.* F1-MO male: median = 1.535; Q1 = 0.8313; Q3 = 1.946 *vs.* median = 0.7943; Q1 = 0.6108; Q3 = 0.9544; p = 0.033) and F1-MOEx female *vs.* F1-MOEx male (F1-MOEx female *vs.* F1-MOEx male: median = 1.150; Q1 = 1.108; Q3 = 1.572 *vs.* median = 0.7804; Q1 = 0.6298; Q3 = 0.9043; p = 0.002), no differences among groups were found for activated p70 levels.

Given that maternal physical exercise practice affects circulating metabolites (*section 3.2.*), including TGs, LDL, and HDL, the relative levels of the lipid transporter CD36 in the cardiac tissue of the 32-week-old offspring was measured (**Fig. 3.6. Q, R)**. The results evidence the sex-specific response, being cardiac F1-Control female CD36 levels higher than for male offspring (F1-Control female *vs.* F1-Control male: median = 1.079; Q1 = 0.8589; Q3 = 1.382 *vs.* median = 0.7605; Q1 = 0.5976; Q3 = 1.000; p = 0.048). In addition, the results suggest decreased CD36 relative levels for male offspring born to obese/ exercised mothers (F1-MOEx male; n = 5; median = 0.3309; Q1 = 0.2689; Q3 = 0.6389) in comparison with male offspring from lean mothers (F1-MOEx male *vs.* F1-Control male; p = 0.04).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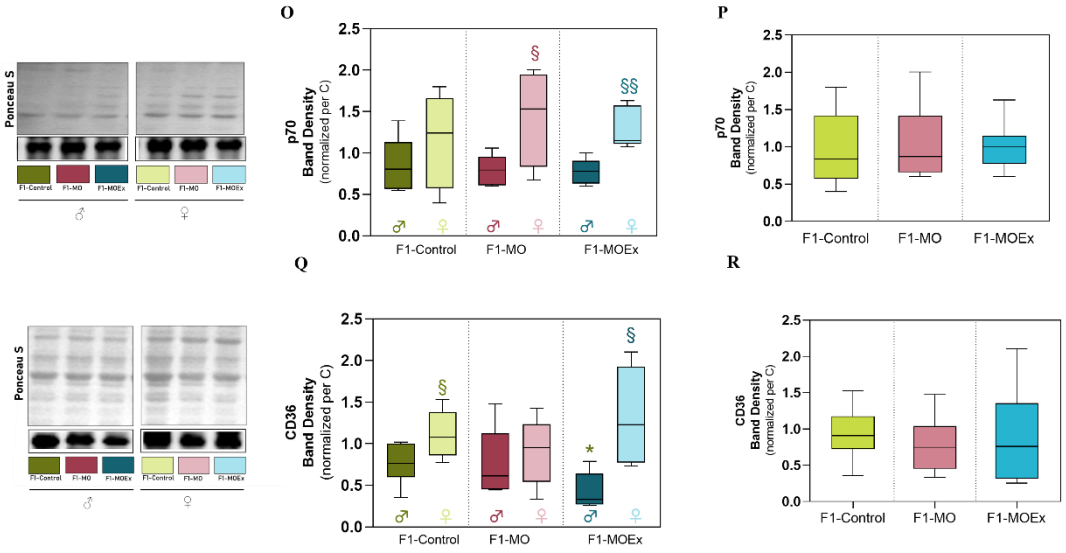


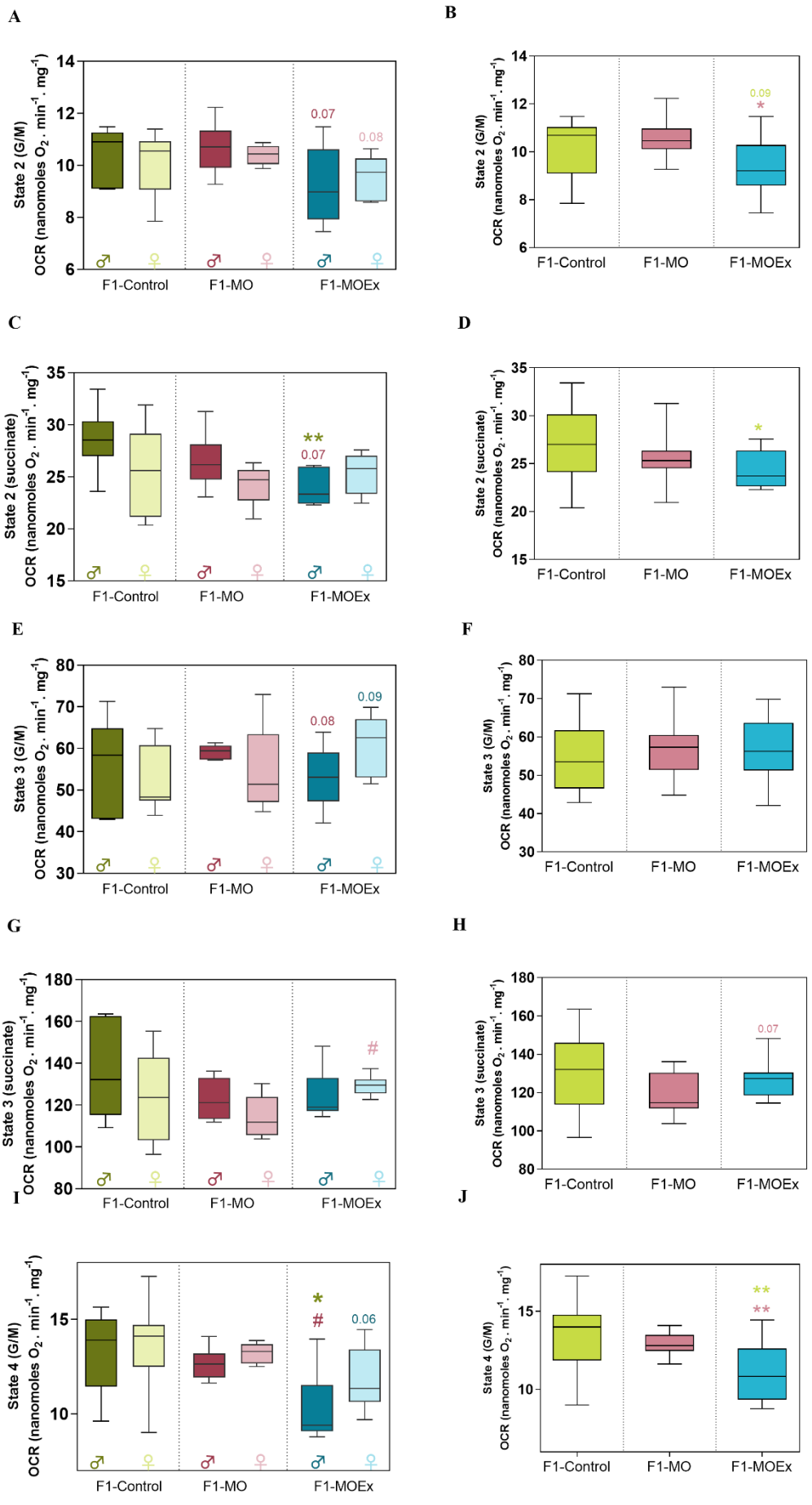
Fig. 3.6 | Cardiac relative band density of activated, phosphorylated, and total AMPK, p70 and CD36 in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific activated AMPK, calculated through the ratio between p-AMPK(Thr172)/AMPKα;* ***(B)*** *Activated AMPK, calculated through the ratio between p-AMPK(Thr172)/AMPK;* ***(C)*** *Sex-specific phosphorylated AMPK in the Thr172 residue;* ***(D)*** *Phosphorylated AMPK in the Thr172 residue;* ***(E)*** *Sex-specific total AMPKα;* ***(F)*** *Total AMPKα;* ***(K)*** *Sex-specific activated p70, calculated through the ratio between p-p70 (Thr389)/p70;* ***(L)*** *Activated p70, calculated through the ratio between p-p70 (Thr389)/p70;* ***(M)*** *Sex-specific phosphorylated p70 in the Thr389 residue;* ***(N)*** *Phosphorylated p70 in the Thr398 residue;* ***(O)*** *Sex-specific total p70;* ***(P)*** *Total p70;* ***(Q)*** *Sex-specific CD36 relative expression;* ***(R)*** *CD36. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 4). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****;* ***# vs. F1-MO****;* ***§vs. opposite sex of the same group.***

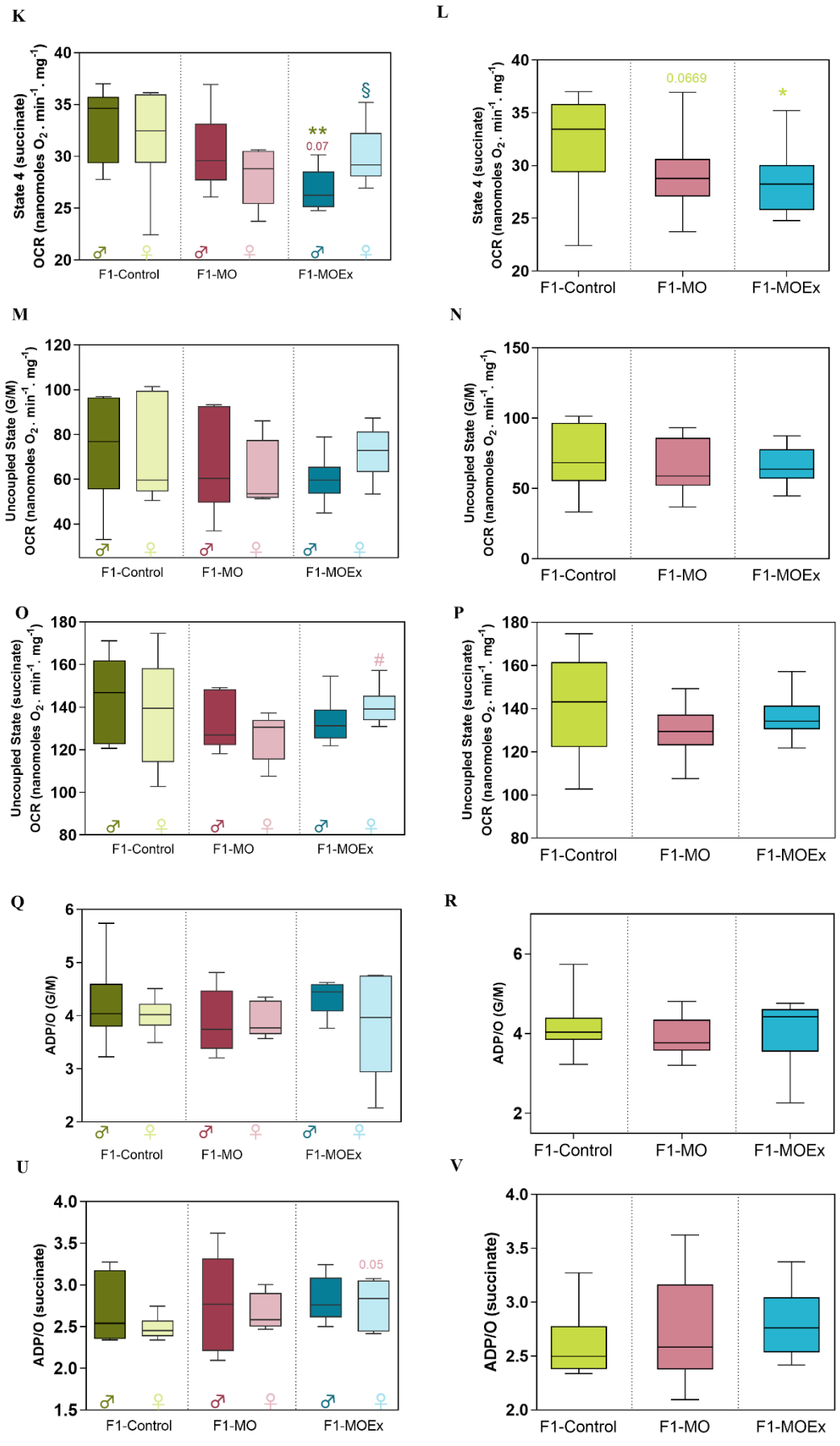
3.3.1. The impact of gestational exercise practice during obese pregnancy in the offspring's cardiac mitochondrial fitness.

The transport of long and medium-chain FAs is mediated by CD36. FAs then enter β-oxidation to produce acetyl-CoA, which then fuels the mitochondrial TCA cycle. From the TCA cycle, the MRC accepts electrons resultant from NADH and succinate oxidation (*section 1.4.5.1.*). Thus, since maternal physical exercise during obese pregnancy affected lipid metabolites (*section 3.2.*) and CD36 levels (*section 3.3.*), the electron flow and proton leaking in the MRC could also be altered. Thus, mitochondrial bioenergetics was assessed (**Fig. 3.7)**. The oxygen consumption rate (OCR) in the state 2 (basal respiration state) was decreased for offspring of exercised/obese mothers compared to offspring of sedentary/obese mothers when supported by G/M (**Fig. 3.7.B)**. The mitochondrial state 2 respiration supported by succinate (**Fig. 3.7. C, D)** is decreased for male offspring from exercised/obese male offspring compared to control (F1-MOEx *vs.* F1-Control male: median = 23.34; Q1 = 22.44; Q3 = 25.98 *vs.* median = 28.54; Q1 = 27.00; Q3 = 30.33; p = 0.008). These male offspring also present tendentially decreased OCR levels in comparison with F1-MO male (F1-MOEx *vs.* F1-MO male: p = 0.07).

Along with this, state 4 OCR was lower for male offspring of exercised/obese mothers in comparison with lean mothers' offspring when using the substrates G/M (**Fig. 3.7. I, J)** (F1-MOEx male; median = 9.405; Q1 = 9.082; Q3 = 11.52; (F1-Control male; median = 13.90; Q1 = 11.45; Q3 = 14.99); (F1-MOEx *vs.* F1-Control; p = 0.02)) and succinate (**Fig. 3.7. K, L)** (F1-MOEx male; median = 26.25; Q1 = 25.12; Q3 = 28.56; F1-Control male; median = 34.63; Q1 = 29.36; Q3 = 35.76) (F1-MOEx *vs.* F1-Control; p = 0.003)). In addition, for male offspring, state 4 OCR was also decreased in comparison with F1-MO male when stimulated with the substrates for complex I (F1-MOEx male *vs.* F1-MO male (median = 12.63; Q1 = 11.92; Q3 = 13.19); p = 0.0411) and tendentially decreased when succinate was used (F1-MOEx *vs.* F1-MO (median = 29.71; Q1 = 27.68; Q3 = 33.16; p = 0.0665). Considering the ADP stimulated OCR, State 3, statistically significant differences were only detected when succinate was utilized (**Fig. 3.7. G, H)**. Female offspring born to exercised/obese motherspresent increased State 3 OCR when compared to female offspring born to sedentary/obese mothers(F1-MOEx female; median = 129.6; Q1 = 125.5; Q3 = 132.3) *vs.* F1-MO female; median = 111.8; Q1 = 105.7; Q3 = 123.9); p = 0.0107). The respiratory control ratio, calculated through the ratio between state 3 and 4 for the same subtract indicates that the mitochondrial coupling efficiency between the ETC oxidation and the ADP phosphorylation was increased for offspring of exercised/obese mothers (F1-MOEx male; median = 5.246; Q1 = 4.760; Q3 = 5.624; F1-MOEx female; median = 5.069; Q1 = 4.687; Q3 = 5.377) compared with F1-Control (male; n = 7; media = 4.255; Q1 = 3.969; Q3 = 4.496; female; median = 3.802; Q1 = 3.460; Q3 = 4.528) for both sexes when stimulated with the substrates for complex I (**Fig. 3.7. X, Z)** (p = 0.0023; p = 0.0059). Using succinate as substrate (**Fig. 3.7. AB, BC)**, this is only verified for male offspring cardiac mitochondria (F1-MOEx male; median = 4.642; Q1 = 4.515; Q3 = 4.797 *vs.* F1-Control male; median = 4.126; Q1 = 3.935; Q3 = 4.429; p = 0.005) and in comparison with F1-MO male (median = 4.021; Q1 = 3.810; Q3 = 4.416; p =0.0039). The OCR during uncoupled state was higher for female offspring from exercised/obese mothersin comparison with sedentary/obese mothers' female offspring when using succinate (**Fig. 3.7. O, P)**; (F1-MOEx female; median = 139.1; Q1 = 133.9; Q3 = 145.5) *vs.* F1-MO female (F1-MO female; median = 130.6; Q1 = 115.4; Q3 = 134.1); p = 0.0429).

The mitochondrial membrane depolarization induced by the addition of ADP was smaller for offspring of exercised/obese mothers (F1-MOEx male; median = 17.37; Q1 = 16.35; Q3 = 19.59; F1-MOEx female; median = 15.89; Q1 = 14.10; Q3 = 17.59) when compared with offspring of lean mothers (F1-Control male; median = 19.83; Q1 = 18.84; Q3 = 26.92; F1-Control female; median = 20.20; Q1 = 18.08; Q3 = 21.15) when substrates for complexes I were used (**Fig. 3.8. E, F)** (F1-MOEx male, female *vs.* F1-Control male, female; p = 0.0350; 0.0328). When succinate was used (**Fig. 3.8. G, H)**, this effects only remains significant for the male offspring; F1-Control male, (F1-MOEx male (F1-MOEx male; median = 11.84; Q1 = 10.90; Q3 = 14.43) *vs.* F1-Control male;


A
State 2 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
14
12
10
8
6
0.07
0.08
♂
♀
F1-Control
F1-MO
F1-MOEx
B
State 2 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
14
12
10
8
6
0.09
*
F1-Control
F1-MO
F1-MOEx
C
State 2 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
35
30
25
20
15
**
0.07
F1-Control
F1-MO
F1-MOEx
D
State 2 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
35
30
25
20
15
*
F1-Control
F1-MO
F1-MOEx
E
State 3 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
80
70
60
50
40
30
0.08
0.09
F1-Control
F1-MO
F1-MOEx
F
State 3 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
80
70
60
50
40
30
F1-Control
F1-MO
F1-MOEx
G
State 3 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
180
160
140
120
100
80
#
F1-Control
F1-MO
F1-MOEx
H
State 3 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
180
160
140
120
100
80
0.07
F1-Control
F1-MO
F1-MOEx
I
State 4 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
15
10
*
#
0.06
F1-Control
F1-MO
F1-MOEx
J
State 4 (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
15
10
**
**
F1-Control
F1-MO
F1-MOEx


K
State 4 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
40
35
30
25
20
**
0.07
§
F1-Control
F1-MO
F1-MOEx
L
State 4 (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
40
35
30
25
20
0.0669
*
F1-Control
F1-MO
F1-MOEx
M
Uncoupled State (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
120
100
80
60
40
F1-Control
F1-MO
F1-MOEx
N
Uncoupled State (G/M)
OCR (nanomoles O2 . min-1 . mg-1)
150
100
50
0
F1-Control
F1-MO
F1-MOEx
O
Uncoupled State (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
180
160
140
120
100
80
#
F1-Control
F1-MO
F1-MOEx
P
Uncoupled State (succinate)
OCR (nanomoles O2 . min-1 . mg-1)
180
160
140
120
100
80
F1-Control
F1-MO
F1-MOEx
Q
ADP/O (G/M)
6
5
4
3
2
F1-Control
F1-MO
F1-MOEx
R
ADP/O (G/M)
6
4
2
F1-Control
F1-MO
F1-MOEx
U
ADP/O (succinate)
4.0
3.5
3.0
2.5
2.0
1.5
0.05
F1-Control
F1-MO
F1-MOEx
V
ADP/O (succinate)
4.0
3.5
3.0
2.5
2.0
F1-Control
F1-MO
F1-MOEx

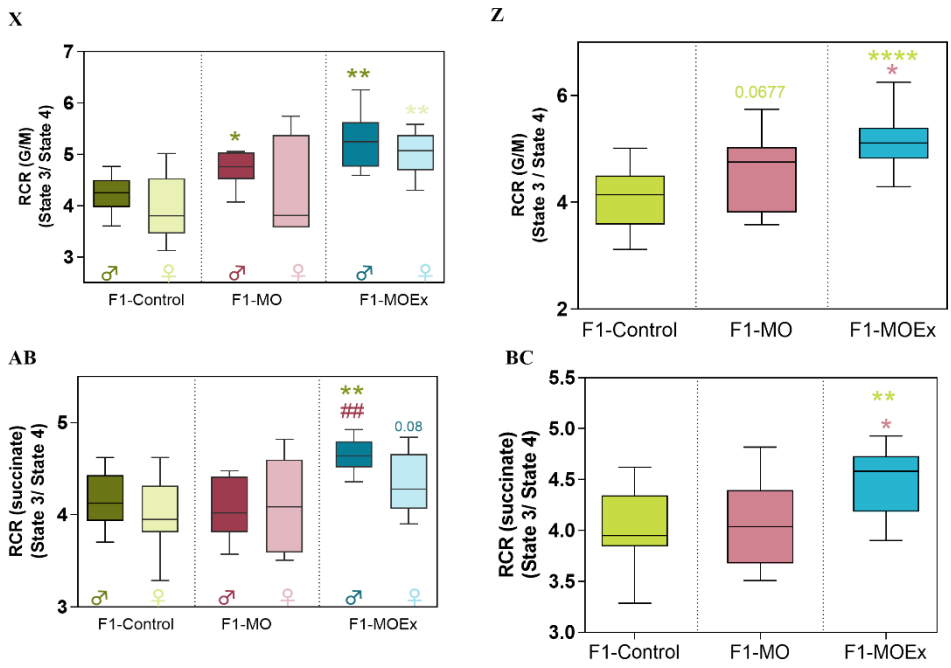
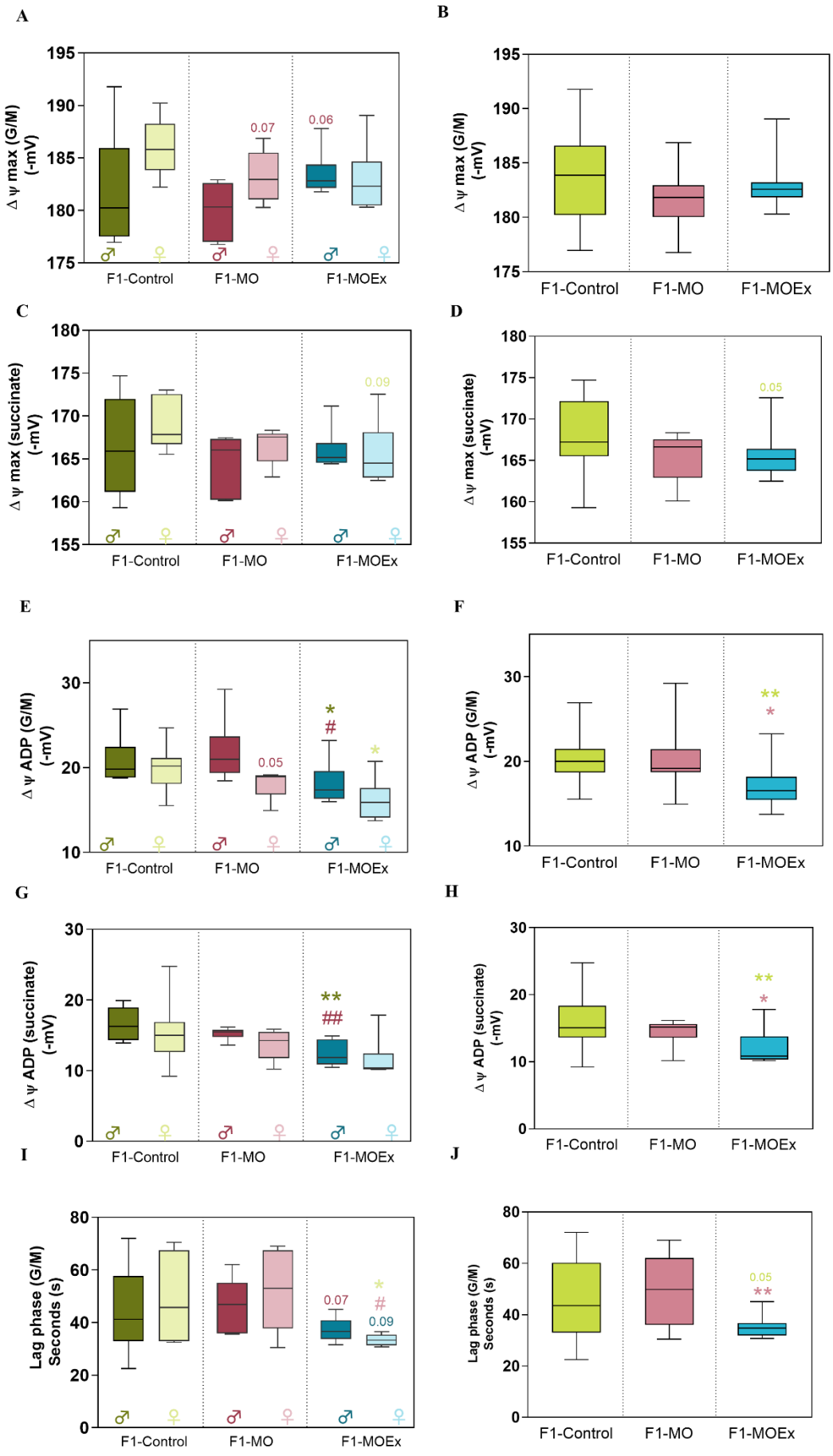


Fig. 3.7 | Cardiac mitochondrial bioenergetics for 32-week-old offspring from maternal obesity or maternal gestational exercise practice during obesogenic pregnancy. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific state 2, G/M were used as substrates;* ***(B)*** *State 2, G/M were used as substrates;* ***(C)*** *Sex-specific state 2, succinate was used as substrate;* ***(D)*** *State 2, succinate was used as substrate;* ***(E)*** *Sex-specific state 3, G/M were used as substrates;* ***(F)*** *State 3, G/M were used as substrates;* ***(G)*** *Sex-specific state 3, succinate was used as substrate;* ***(H)*** *State 3, succinate was used as substrate;* ***(I)*** *Sex-specific state 4, G/M were used as substrates;* ***(J)*** *State 4, G/M were used as substrates;* ***(K)*** *Sex-specific state 4, succinate was used as substrate;* ***(L)*** *State 4, succinate was used as substrate;* ***(M)*** *Sex-specific uncoupled state, G/M were used as substrates;* ***(N)*** *Uncoupled state, G/M were used as substrates;* ***(O)*** *Sex-specific uncoupled state, succinate was used as substrate;* ***(P)*** *Uncoupled state, succinate was used as substrate;* ***(Q)*** *Sex-specific ADP/O ratio, G/M were used as substrates;* ***(R)*** *ADP/O ratio, G/M were used as substrates;* ***(U)*** *Sex-specific ADP/O ratio, succinate was used as substrate;* ***(V)*** *ADP/O ratio, succinate was used as substrate;* ***(X)*** *Sex-specific RCR, G/M were used as substrates;* ***(Z)*** *RCR, G/M were used as substrates;* ***(AB)*** *Sex-specific RCR, succinate was used as substrate;* ***(BC)*** *RCR, succinate was used as substrate. Data are expressed as medians, Q1, Q3, min and max. The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control**; **# vs. F1-MO**; **§vs. opposite sex of the same group.***

median = 16.24; Q1 = 14.30; Q3 = 18.92), p = 0.0044), and also in comparison with F1-MO male, (F1-MOEx male *vs.* F1-MO male (median = 15.42; Q1 = 14.76; Q3 = 15.76), p = 0.0064).

Female offspring born to exercised/obese mothers complete the total phosphorylation of ADP faster, recovering quicker the cardiac mitochondrial ΔΨ than offspring of sedentary/obese mothers and lean mothers when stimulated with complex I (**Fig. 3.8. I, J)** (F1-MOEx female; median = 33.38; Q1 = 31.31; Q3 = 35.38); (F1-MO female; median = 53.00; Q1 = 37.75; Q3 = 67.50); (F1-Control female; median = 45.75; Q1 = 33.00; Q3 = 67.50) (F1-MOEx female *vs.* F1-MO female; p = 0.0151), (F1-MOEx *vs.* F1-Control; p = 0.0489) and complex II substrate (**Fig. 3.8. K, L)** (F1-MOEx female; median = 21.00; Q1 = 20.06; Q3 = 22.31); (F1-MO female; median = 28.13; Q1 = 23.31; Q3 = 30.50); (F1-Control female; median = 25.50; Q1 = 20.25; Q3 = 31.50) (F1-MOEx female *vs.* F1-MO female; p = 0.0095), (F1-MOEx *vs.* F1-Control; p = 0.0389).


A
B
C
D
E
F
G
H
I
J
Δ ψ max (G/M) (-mV)
Δ ψ max (succinate) (-mV)
Δ ψ ADP (G/M) (-mV)
Δ ψ ADP (succinate) (-mV)
Lag phase (G/M) Seconds (s)
F1-Control
F1-MO
F1-MOEx
0.07
0.06
0.09
0.05
0.07
0.09
0.05
*
#
**
##

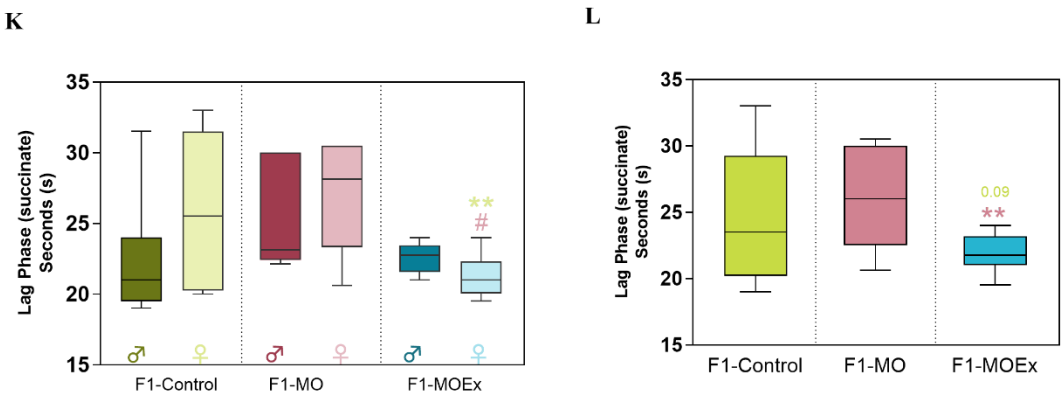


Fig. 3.8 | Cardiac mitochondrial membrane potential modulation in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific maximum membrane potential, G/M were used as substrates;* ***(B)*** *Maximum membrane potential, G/M were used as substrates;* ***(C)*** *Sex-specific maximum membrane potential, succinate was used as substrate;* ***(D)*** *Maximum membrane potential, succinate was used as substrate;* ***(E)*** *Sex-specific membrane potential induced with ADP, G/M were used as substrates;* ***(F)*** *Membrane potential induced with ADP, G/M were used as substrates;* ***(G)*** *Sex-specific membrane potential induced with ADP, succinate was used as substrate;* ***(H)*** *Membrane potential induced with ADP, succinate was used as substrate;* ***(I)*** *Sex-specific lag phase, G/M were used as substrates;* ***(J)*** *Lag phase, G/M was used as substrate;* ***(K)*** *Sex-specific lag phase, succinate was used as substrate;* ***(L)*** *Lag phase, succinate was used as substrate. Data are expressed as medians, Q1, Q3, min and max. The comparison between groups was performed using a parametric t-test or Mann-Whitney ($n \geq 5$). Normality was evaluated with Shappiro-Wilk test. *,#,§ $p \leq 0.05$; **,##,§§$p \leq 0.01$; ***,###,§§§$p \leq 0.001$; ****,####,§§§§ $p \leq 0.0001$.* **** vs. F1-Control**; **# vs. F1-MO**; **§vs. opposite sex of the same group.***

Since offspring cardiac mitochondrial bioenergetics were modulated by maternal habits during gestation, selected OXPHOS subunits expression was also measured to determine if OXPHOS subunits abundance was programmed by the maternal the interventions and contribute to the altered respiratory performance in the offspring hearts **(Fig. 3.9 A, B)**. Offspring's cardiac tissue subunit ATP5A (**Fig. 3.9. A)**, a subunit of ATP synthase, was reduced by maternal obesity when compared with control (F1-MO *vs.* F1-Control: median = 0.9267; Q1 = 0.7148; Q3 = 0.9486 *vs.* median = 0.9825; Q1 = 0.9141; Q3 = 1.101; p = 0.04). On the other hand, SDHA relative expression levels (**Fig. 3.9. B)**, a subunit of complex II, was increased for male offspring born to exercised/obese mothers comparing with male offspring born to lean mothers (F1-MOEx male (F1-MOEx male; median = 1.155; Q1 = 1.071; Q3 = 1.277) *vs.* F1-Control male (F1-Control male; median = 0.9913; Q1 = 0.8385; Q3 = 1.188); p = 0.0459).

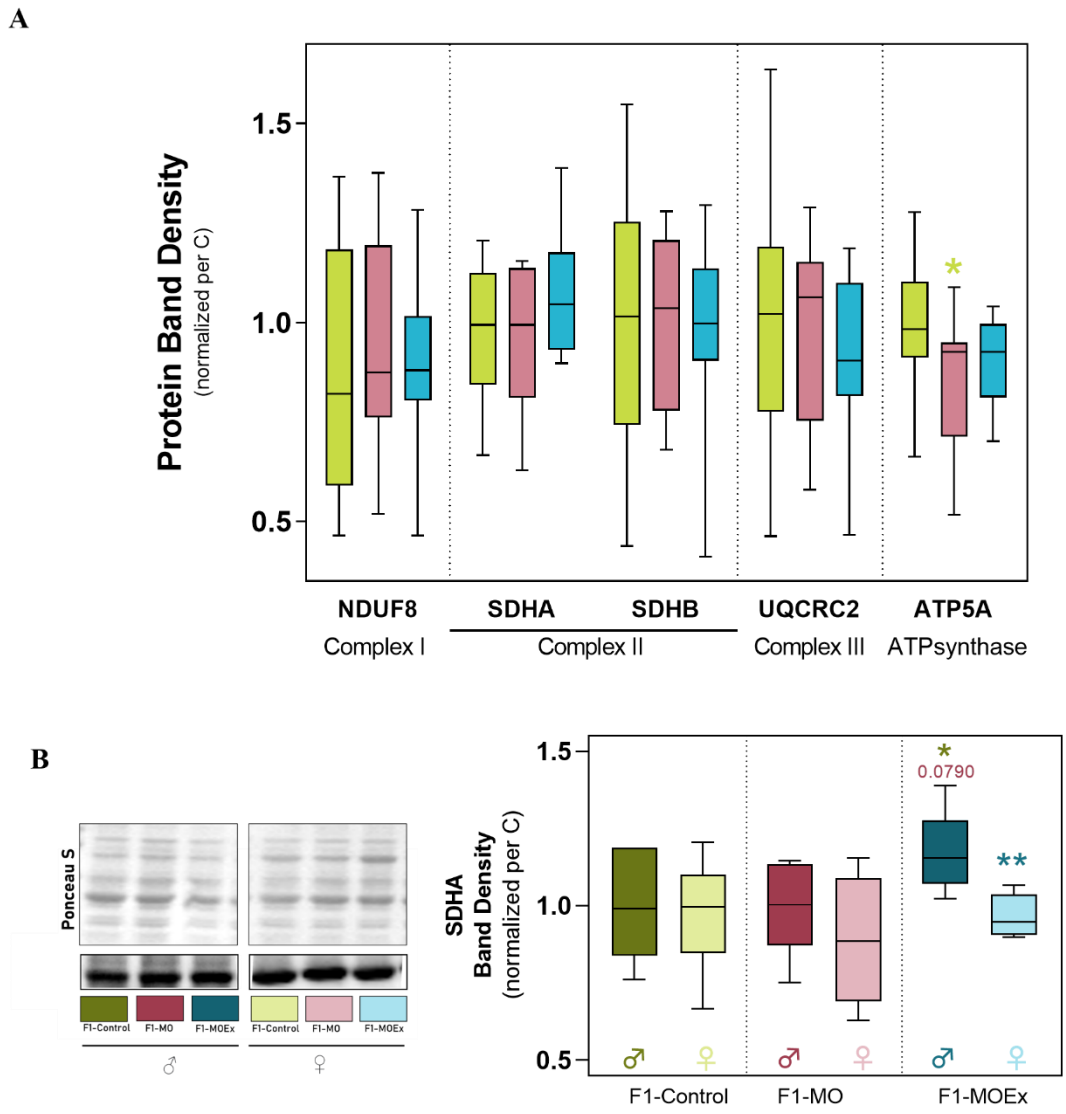


Fig. 3.9 | Cardiac relative band density of mitochondrial OXPHOS complex subunit in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Relative expression levels of complex I subunit NDUF8; complex II subunits SDHA and SDHB; complex III subunit UQCRC2; ATPsynthase subunit ATP5A. The sex-specific data for each measurement is fully disclosed on the supplementary data* ***S.2.0****.****(B)*** *Sex-specific SDHA relative protein expression. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; #* ***vs. F1-MO****; §****vs. opposite sex of the same group.***

To deepen explore how gestational exercise during an obesogenic pregnancy affects cardiac mitochondrial function, the relative levels of mitochondrial dynamics and biogenesis-related proteins were measured. Cardiac MFN-1 relative levels (**Fig. 3.10, A, B**) were increased for female offspring born to exercised/obese mothers compared to female offspring born to lean mothers; (F1-MOEx female (F1-MOEx female; median = 1.073; Q1 = 0.9929; Q3 = 1.233) *vs.* F1-Control female (F1-Control females; median = 0.8313; Q1 = 0.6561; Q3 = 1.031); p = 0.0157).

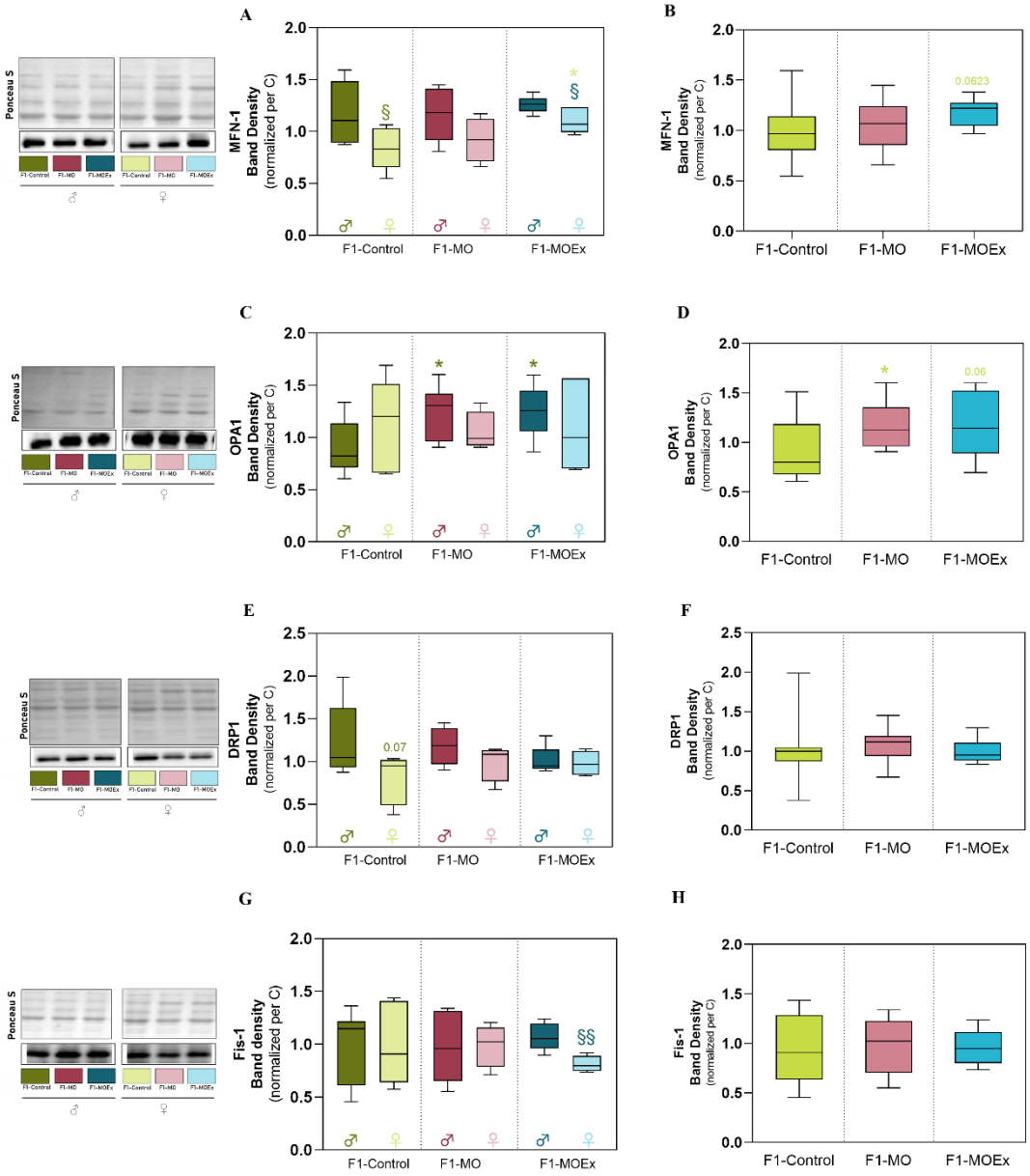


Fig. 3.10 | Cardiac relative band density of mitochondrial dynamics-related proteins in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific MFN-1;* ***(B)*** *MFN-1;* ***(C)*** *Sex-specific OPA1;* ***(D)*** *OPA1;* ***(E)*** *Sex-specific DRP1;* ***(F)*** *DRP1;* ***(G)*** *Sex-specific Fis-1;* ***(H)*** *Fis-1. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; # **vs. F1-MO****; § **vs. opposite sex of the same group.***

Overall, female offspring presented decreased levels of cardiac MFN-1 in comparison with male offspring, especially female offspring born to lean and exercised/obese mothers (F1-Control female *vs.* F1-Control male (median = 1.104; Q1 = 0.8924; Q3 = 1.484); (F1-MOEx female *vs.* F1-MOEx male, p = 0.0185). OPA1 relative levels only exhibit cardiac modulation by maternal habits for the male offspring (**Fig. 3.10, C, D**) being increased for male MOEx offspring compared with

offspring of lean mothers (F1-MOEx male; median = 1.258; Q1 = 1.059; Q3 = 1.451) *vs.* F1-Control male (F1-Control male; median = 0.8247; Q1 = 0.7143; Q3 = 1.1359); p = 0.0340). Interestingly, F1-MO males also present increased OPA1 levels in comparison with F1-Control male (F1-MO male; median = 1.307; Q1 = 0.9626; Q3 = 1.420) *vs.* F1-Control, p = 0.0374). Besides the tendential sex-different levels for cardiac DRP1 (**Fig. 3.10, E, F**) between the offspring born to lean mothers (F1-Control female *vs.* F1-Control male; p = 0.0728) and the decreased cardiac levels of Fis-1 (**Fig. 3.10, G, H**) between F1-MOEx female (median = 0.7967; Q1 = 0.7477; Q3 = 0.8896) *vs.* F1-MOEx male (median = 1.052; Q1 = 0.9600; Q3 = 1.195, p = 0.0077), no major differences were detected for these proteins.

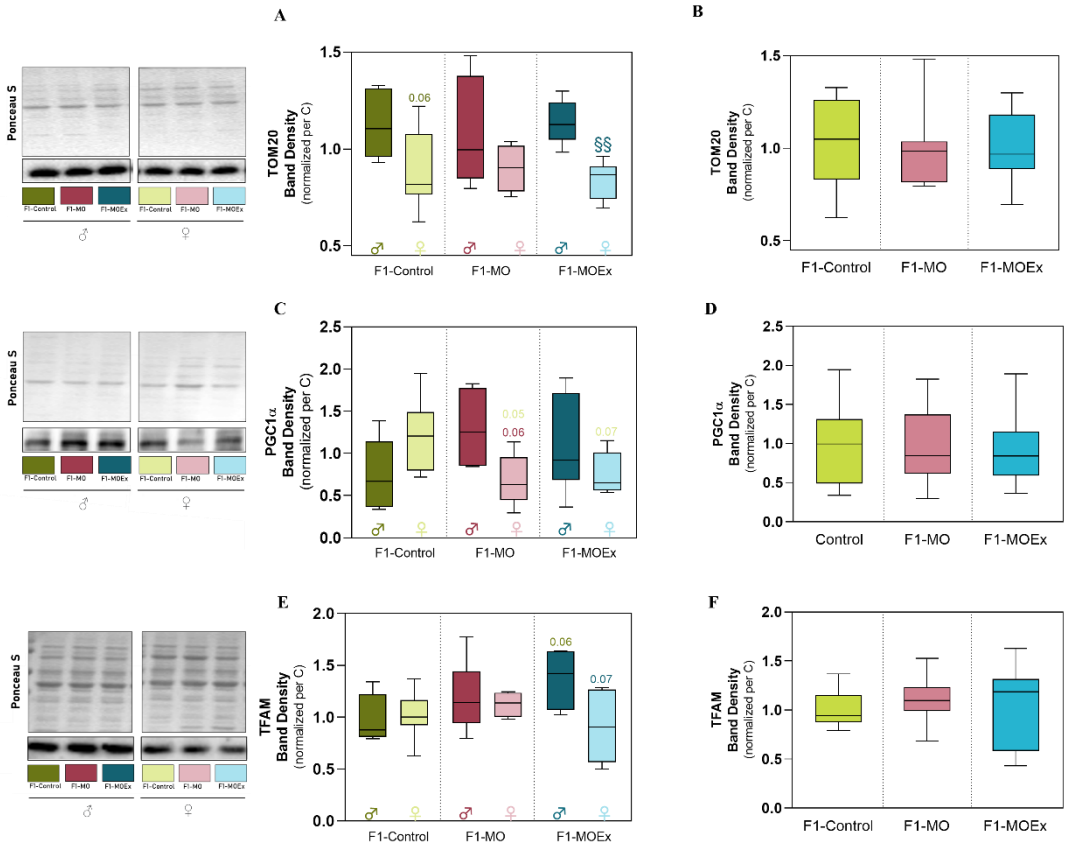


Fig. 3.11 | Cardiac relative band density of cardiac mitochondrial mass indicator and biogenesis-related proteins in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific TOM20;* ***(B)*** *TOM20;* ***(C)*** *Sex-specific PGC1α;* ***(D)*** *PGC1α;* ***(E)*** *Sex-specific TFAM;* ***(F)*** *TFAM. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 5). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; # **vs. F1-MO****; § **vs. opposite sex of the same group.***

The TOM20 relative expression (**Fig. 3.11, A, B**) levels did not revealed alterations in the cardiac tissue of the offspring due to maternal physical exercise practice, even though the sex-specific response was evident (F1-Control female (median = 0.8181; Q1 = 0.7646; Q3 = 1.078) *vs.* F1-Control male (median = 1.104; Q1 = 0.9590; Q3 = 1.312), p = 0.0580; (F1-MOEx female (median = 0.8657; Q1 = 0.7427; Q3 = 0.9101) *vs.* F1-MOEx male (median = 1.127; Q1 = 1.048; Q3 = 1.240, p = 0.0011). Cardiac PGC1α levels (**Fig. 3.11, C, D**) were tendentially decreased for both female offspring born to sedentary/obese mothers and exercised/obese mothers (F1-MOEx female; median = 0.6519; Q1 = 0.5624; Q3 = 1.013) compared to female offspring born to lean mothers (F1-Control female; median = 1.208; Q1 = 0.7960; Q3 = 1.490), (F1-MO female *vs.* F1-Control female, p = 0.0522; F1-MOEx female *vs.* F1-Control female; p = 0.0757).

TFAM relative cardiac expression (**Fig. 3.11, E, F**) was tendentially increased for male offspring of exercised/obese mothers *vs.* male offspring born to lean mothers (F1-MOEx male *vs.* F1-Control male; p = 0.0653). There is also a tendential decrease in TFAM relative levels between F1-MOEx female (median = 0.9048; Q1 = 0.5669; Q3 = 1.267) and F1-MOEx male offspring (F1-MOEx female *vs.* F1-MOEx male, p = 0.0661) highlighting the sex-divergent response.

3.4. Effect of nitrosative stress in response to gestational exercise practice during MO.

Oxidative and nitrosative stress can contribute for mitochondrial dysfunction (*section 1.4.6*). The cardiac 3-nitrotyrosine relative levels (**Fig. 3.12, A, B**) were lower for male offspring born to exercised/obese mothers than male offspring born to lean mothers (F1-MOEx *vs.* F1-Control male: median = 0.585; Q1 = 0.4254; Q3 = 0.9014 *vs.* median = 1.099; Q1 = 1.038; Q3 = 1.346; p = 0.0069). Given this result, proteins involved in the NO pathway were measured. Although the levels of cardiac CAT-1 (**Fig. 3.12, C, D**) were not altered for F1-MOEx, CAT-1 was tendentially increased for F1-MO male in comparison with F1-Control male (F1-MO male *vs.* F1-Control male: median = 1.346; Q1 = 1.278; Q3 = 1.405 *vs.* median = 1.145; Q1 = 0.8778; Q3 = 1.363; p = 0.0959).

Given that NO also controls the hypertrophic response (*section 1.4.2.*), the activation of NFATc4 was evaluated (**Fig. 3.12, E, F**). The results showed that the cardiac ratio of phosphorylated NFATc4 (Ser168,170)/total NFATc4 was tendentially lower for male offspring born to exercised/obese mothers (compared to male offspring born to lean mothers (F1-MOEx *vs.* F1-Control male: median = 0.7854; Q1 = 0.4188; Q3 = 1.004 *vs.* median = 1.221; Q1 = 0.8808; Q3 = 1.542; p = 0.0666).

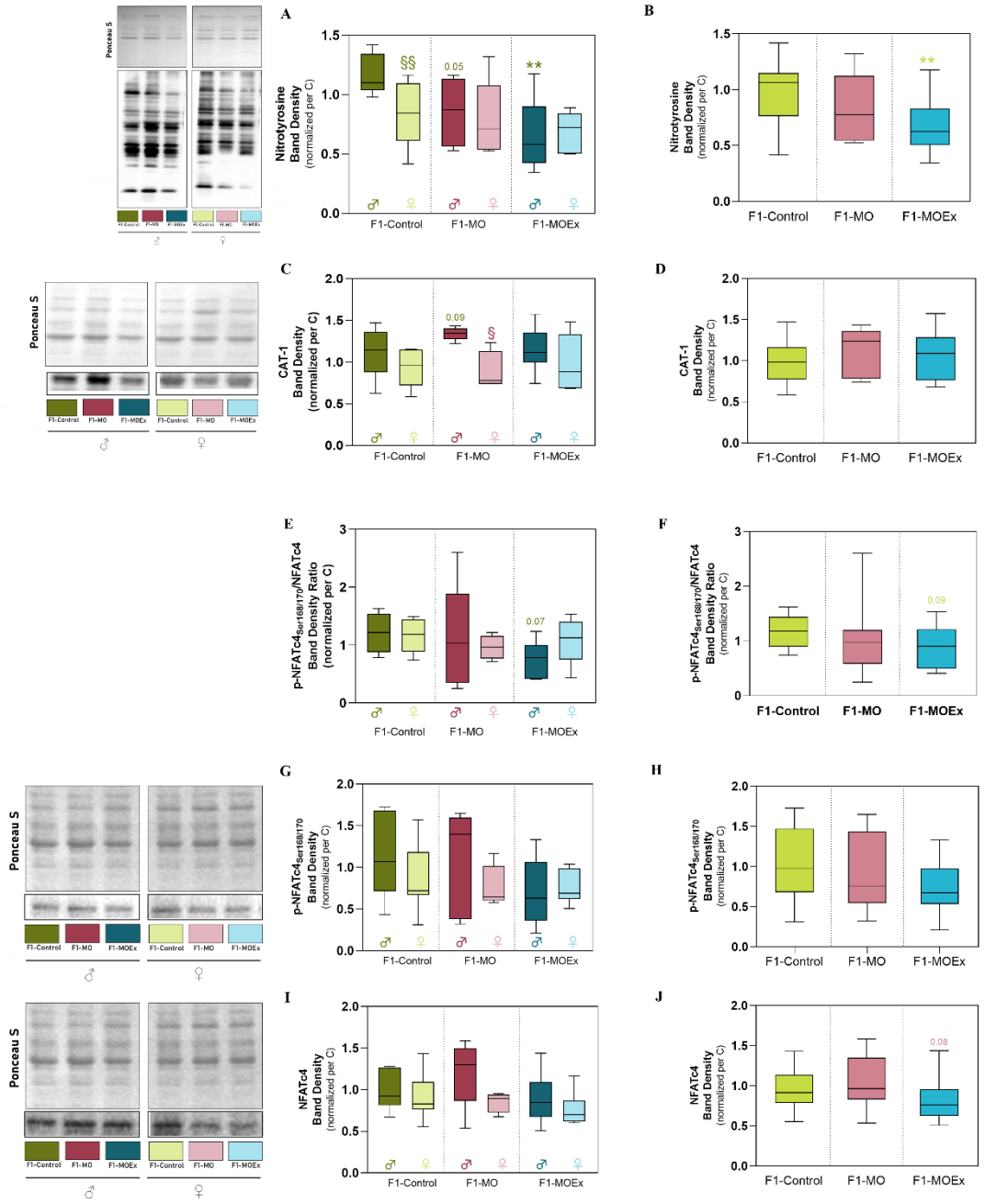


Fig. 3.12 | Cardiac relative band density of cardiac nitrosative stress, and NO pathway-related proteins in 32-week-old offspring from obese mothers and obese and exercised mothers. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific 3-nitrotyrosine;* ***(B)*** *3-nitrotyrosine;* ***(C)*** *Sex-specific CAT-;* ***(D)*** *CAT-1;* ***(A)*** *Sex-specific inactivated NFATc4, calculated through the ratio between p-NFATc4(Ser168/170)/NFATc4;* ***(B)*** *Inactivated NFATc4, calculated through the ratio between p-NFATc4(Ser168/170)/NFATc4;* ***(C)*** *Sex-specific phosphorylated NFATc4 in the Ser168/170 residues;* ***(D)*** *Phosphorylated NFATc4 in the Ser168/170 residues. Data are expressed as medians, Q1, Q3, min and max. Data was normalized with Ponceau S staining and the protein expression represented relative to the mean of F1-Control group (male and female). The comparison between groups was performed using a parametric t-test or Mann-Whitney* ***(n ≥ 5)****. Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****;* ***# vs. F1-MO****;* ***§vs. opposite sex of the same group***

Chapter 4 – Discussion

The innovation of this work was the use of an animal model that mimetics obesity and, simultaneously, is exposed to maternal physical exercise practice during pregnancy to prevent MO-induced pathology. This becomes of great advantage for unravelling the mechanisms by which MO programs the offspring's cardiovascular function, that can perpetuate cardiometabolic impairments over the life-course of the offspring, and ultimately, result in an increased risk of CVD development at some point in the offspring's lifetime. The used animal model also allows the collection of offspring cardiac tissue to study specific molecular pathways to evaluate the potential role of maternal physical exercise practice as a non-pharmacological therapeutic approach by attenuating or reversing the MO-induced cardiac deleterious effects. The present section is dedicated to discussing whether maternal physical exercise practice modulates the MO-induced responses, and whether it could be used as a preventive strategy. This work adds a new perspective in the fetal programming research field that, to the present knowledge, remains to be explored.

4.1. Lower gestational weight gain induced by exercise during maternal obesity and the offspring's physiologic sex-specific response.

In this rat animal model of MO, a high maternal weight gain during gestation for sedentary/obese mothers was verified due to the type of the diet (*section 2.1*). In humans, an atypical gestational weight gain in obese and normoglycemic mothers is associated with the maternal body mass and size [149]. Obese pregnant women tend to gain more weight during gestation than lean pregnant women[150], which meets the results obtained in this study. Nonetheless, the most interesting feature about this parameter was that for pregnant rats exposed to HFHS diet and exercise practice during pregnancy, gestational weight gain was very similar to those of lean pregnant rats, indicating that exercise practice during obesogenic pregnancy reversed the MO-induced excessive gestational weight gain. In humans, physical exercise practice seemed to avoid an abnormal gestational weight gain in a lean pregnancy, which was associated with a decreased fat mass and led to the offspring's decreased body weight [151].

However, in this present study, both maternal interventions did not affect the absolute body weight of the 32-week-old offspring between groups. This is consistent with a 2018 cohort study, where maternal physical exercise practice during obesity did not affect the infants' body weight at 180 days of age [152]. The sex-specific response in the 32-week-old offspring body weight was evident in this study. Male offspring from each group were heavier than female offspring. Indeed, these observations were also made for Sprague-Dawley rats born to mothers given a Control and HF diet [152]. These sex differences seem to be innate, possibly due to a different composition in adiposity between males and females and fat distribution (*section 1.1.1.*). As for the

heart weight, no differences between groups were detected at 32 weeks of age, but it was verified that the heart weight exhibit sexual dimorphism. It is reasonable to associate a higher heart weight with a higher heart size. Physiologically, women tend to have a smaller heart than men[153], which agrees with the presented data. However, this parameter should be complemented with the information for the cavities size and mass of the LV for better conclusions. Indeed, the preliminary data obtained by optical microscopy was suggestive that neither of the maternal interventions interferes with the offspring's cardiovascular morphology at 32-week-old. Most importantly, these data corroborate that heart physiology is different according to sex. These physiological differences might reflect the sexual dimorphism and indicate that sex is an important influencer of heart size. The heart weight to body weight ratio is an important measure and is currently used to characterize myocardial hypertrophy [154,155]. Given the data obtained for body and heart weight, the heart to body weight ratio results are consistent, with increased ratio values for female offspring regardless of the groups.

4.2. Gestational exercise during an obesogenic pregnancy prevents the development of metabolic syndrome-related blood indicators in the offspring.

The metabolic analysis in blood plasma samples of the 32-week-old offspring revealed changes among treatment groups in a sex-specific way. Female F1-MO displays a biochemical profile that suggest increased predisposition to metabolic syndrome development, due to increased levels of triglycerides, and decreased levels of HDL, which behaviour is considered as a biomarker of metabolic disease risk. Female F1-MO seemed to present higher predisposition to CVD, as indicated by the AI. For all of these parameters, maternal physical exercise practice during gestation recovered the levels to control, indicating a potential positive impact. This suggests that female offspring born to sedentary and obese mothers might begin to develop metabolic syndrome at a young-adult age. This phenotype is prevented by maternal physical exercise practice during an obesogenic gestation, possibly avoiding the early development of CVD, indicated by decreased F1-MOEx AI.

The development of metabolic syndrome in male F1-MO is not as evident. However, the protective action of maternal physical exercise also ameliorated some biochemical parameters. F1-MOEx triglycerides and HDL levels are decreased compared to F1-MO, indicating an adaptative and possible beneficial response from maternal physical exercise practice. The decreased AI for F1-MOEx also indicates a lower predisposition for CVD, reinforcing the positive impact of gestational exercise practice during an obesogenic pregnancy.

4.3. Behind the protective effect of gestational exercise during MO: the case of the offspring's cardiac insulin signalling pathway.

When exposed to an obesogenic environment during fetal development, offspring tend to develop early metabolic disease, namely obesity and diabetes, characterized by metabolic dysfunction. One of the most affected pathways in cardiac tissue due to metabolic disease is the intracellular insulin signalling. Whether MO-offspring present impaired cardiac insulin signalling is unknown. This modulation may be critical for the heart metabolic shift between lipid and glucose metabolism, characteristic of diabetes.

The presented data indicates decreased levels of activated Akt in Thr308 residue, a distal insulin signalling pathway component, for F1-MOEx males compared with F1-Control. It should be noted that to observe physiological increases in Akt activity, only a small proportion (~ 10%) needs to be phosphorylated [156]. It has been suggested that MO programs offspring's cardiac hypertrophy through Akt-ERK-mTOR pathway activation in mice[104]. In this present study, a lower Akt activation in the Thr308 was not coincident with the phosphorylation in the Ser473 residue. Thus, less Akt activation induced by gestational exercise during MO-pregnancy may have a possible positive cardiac outcome in the offspring, although further data is needed to confirm this hypothesis.

Indeed, the impact of a less activated Akt was assessed by evaluating downstream proteins. GSK3, which is involved in glycogen synthesis regulation, is inhibited through phosphorylation by Akt. The phosphorylated/total protein of GSK3α was increased for F1-MO female, and this parameter is even reversed to control levels with the maternal practice of gestational exercise during MO. This indicates that female offspring born to sedentary and obese mothers might have decreased rate of glucose storage as glycogen and that gestational exercise promotes a higher rate of glucose storage capability and, potentially, metabolic plasticity in the heart. This is not directly related to the inhibition of Akt, since for female offspring the activated Akt levels did not differ between groups, however, GSK can be phosphorylated at the same residue by other kinases (e.g. PKA, PKC) [157].

β – catenin, which is targeted for degradation by GSK3, was decreased for F1-MOEx females. This result is consistent with the decreased GSK3α phosphorylation for MOEx offspring, indicating that the interaction of GSK3α and β – catenin [158] might be occurring in the cardiac tissue of female F1-MOEx offspring. This could indicate that maternal physical exercise practice during MO induces an increased β – catenin degradation in the cardiac tissue of F1-MOEx offspring, which might be associated with increased GSK3α inhibition.

In addition, Akt also plays an essential role in cellular survival by inhibiting pro-apoptotic proteins. The activated Bad levels did not differ between groups, nor the activated CREB-1 levels. The decreased Akt activation in the Thr308 did not affect the relative expression levels of

these pro-apoptotic proteins in any experimental groups, thus it seems unlikely that apoptotic mechanisms were occurring in the young-adult offspring born to MO independently of maternal exercise. Interestingly, the literature suggests that a maternal high-fat diet in Sprague-Dawley rats induces 1-day-old offspring's cardiomyocyte apoptosis [159]. However, in the cardiac tissue of 1-month old offspring, these differences are not observed [159]. This study together with the present data indicate that pro-apoptotic mechanisms might not occur in young-adult offspring cardiac tissue. In this study, gestational exercise practice during obesity did not influence the offspring's cardiac apoptotic mechanisms. To evaluate the AKT-mTORC1 signalling pathway, the mTORC1 activation was evaluated by the downstream phosphorylation of p70, however, no alterations between groups were found suggesting that AKT is not stimulating the mTORC1 downstream signalling.

The AMPK signalling pathway is cardioprotective due to its rapid response during stress and to cellular metabolic status. AMPK activation induces cardiac fatty acid oxidation and glucose uptake[104]. The cardiac tissue of sheep offspring born to obese mothers present decreased activated AMPK levels[160]. There are no studies involving gestational exercise practice and its effects on the cardiac function of offspring where AMPK levels are evaluated. In the livers of mice[161] and sheep[162] offspring born to exercised mothers, activation of AMPK (p-AMPKThr172) was increased, which was associated with a positive effect of gestational exercise. Despite this result, the activation of cardiac AMPK by Thr172 phosphorylation in this animal model was not altered for F1-MO and was even decreased for female F1-MOEx. It must be taken into consideration that the exercised mothers were also obese. In this case, maternal physical exercise practice did not stimulate the cardiac AMPK activation, possibly even induced a slightly deleterious effect by reducing the activated AMPK levels.

It has been previously described that besides translocating lipids and being responsible for FA uptake, CD36 also regulates the activation of AMPK[104]. Given the altered blood plasma levels of lipid metabolites and the decreased AMPK activation for F1-MOEx, relative CD36 expression levels were evaluated. The cardiac CD36 levels for male offspring born to exercised/obese mothers were decreased. Data regarding MO/Exercised-CD36 expression levels modulation in the offspring's heart are still lacking. However, this is suggestive that gestational exercise during MO has a beneficial effect, by reducing CD36 expression levels, thus potentially reducing FA uptake and accumulation. The activated AMPK decreased levels appeared to be independent of CD36 decreased expression, since the differences were observed for female or male offspring, respectively.

Overall, it seems that maternal physical exercise positively affects the cardiac metabolism of the MO-offspring with the effects prevailing, at least, until 32 weeks of age. Even though AMPK was less activated, maternal physical exercise practice during MO pregnancy might have modulated the GSK3/ β – catenin pathway, by decreasing GSK3α

phosphorylation, and promoting degradation of β – catenin. GSK3α altered levels did not seem to be associated with a decreased activation of Akt, which, to a greater extent, did not interfere with any of the evaluated downstream targets nor the activation of anti-apoptotic or AKT-mTORC1 mechanisms. Moreover, maternal physical exercise practice seemed to induce decreased cardiac CD36 levels expression, suggesting that FA uptake may be reduced, indicating a potentially beneficial effect.

4.3. The remodelling of offspring's cardiac mitochondrial function induced by maternal physical exercise practice during MO.

Gestational exercise practice during MO had a positive impact on the young-adult offspring's cardiac mitochondrial bioenergetics. The tendentially decreased basal respiration state (state 2) for male and female F1-MOEx with G/M and succinate as substrates, respectively, indicate that this effect was induced by maternal physical exercise practice during pregnancy. However, this might not just be attributed to exercise since the state 2 measure only takes into account endogenous substrates. Indeed, for ΔΨ max no differences were found between groups. Despite this, the depolarization induced by the addition of ADP is decreased for both male and female offspring of exercised/obese mothers, which is consistent with the fact that cardiac mitochondria of offspring of exercised mothers recover their ΔΨ faster than offspring from sedentary/obese mothers after the total phosphorylation of ADP with complex I and II substrates, as indicated by the lag phase.

Also, state 4 OCR is lower for male offspring of exercised mothers when stimulated with substrates for complex I and II. These alterations for the F1-MOEx group indicate positive bioenergetic adaptations of MO-offspring cardiac mitochondria to maternal physical exercise practice. This is highlighted by increased RCR for male and female offspring of exercised and obese mothers with the substrates for complex I, which indicates a better coupling efficiency between electron transport and phosphorylation of ADP in offspring born to exercised MO-mothers. With succinate as substrate, only male offspring showed increased RCR. Although these parameters suggest positive implications of maternal physical exercise practice for MO-offspring mitochondrial bioenergetics, these observations do not necessarily indicate better efficiency of the mitochondrial phosphorylative system since the ADP/O ratio seemed to be unaltered between groups.

Previous studies have shown that cardiac mitochondrial bioenergetics are crucial in mediating the development of cardiac pathologies [163]. Indeed, a study with mice indicates that the cardiomyocytes of offspring born to HF-fed mothers had impaired mitochondrial bioenergetics, with decreased OCR in the basal state [105]. The same effect was measured for F1-MOEx in this study; however, this might not just be attributed to maternal physical exercise in a MO context. Although cardiac complex II subunit SDHA relative expression levels were increased for

male F1-MOEx compared to F1-Control, these mitochondrial bioenergetic alterations induced by maternal physical exercise were not accompanied by alterations in the cardiac expression of the other subunits of the OXPHOS in F1-MOEx offspring. Importantly, the lack of MOEx-induced alterations in the OXPHOS complex subunits does not signify that the offspring's cardiac OXPHOS activity is not affected by maternal physical exercise during MO. Indeed, OXPHOS complex subunit evaluation was performed in cardiomyocytes of mice F1 offspring born to HFHS fed mothers, and no differences in the expression levels were detected for HFHS-offspring, although cardiac mitochondrial abnormalities were detected through mitochondrial high-resolution respirometry[107]. In the same way, MO negatively affects the offspring's cardiac mitochondrial bioenergetics without any alterations in the OXPHOS complex subunit levels, gestational exercise practice could also positively induce alterations in the offspring's cardiac mitochondrial bioenergetics without interfering with the expression levels of the OXPHOS complex subunits.

Cardiac mitochondrial membrane potential alterations induced by MOEx might indicate a role of mitochondrial dynamics to recover the membrane potential. Although the TOM20 relative expression levels, an indicator of the mitochondrial mass, were decreased for F1-Control and F1-MOEx females in comparison with males from the respective group and no differences were detected between the other groups, an increase in protein markers for cardiac mitochondrial fusion was observed, marked by increased levels of MFN-1 for female F1-MOEx and OPA1 for male offspring. Increased mitochondrial fusion events might be a consequence of the altered mitochondrial membrane potential parameters. It has been described increased OPA1 levels in newborn rat male offspring born to HFD fed mothers [82]. Of note that for OPA1 levels, F1-MO offspring presented increased levels as well. This may represent an already adaptative response to MO of offspring cardiac mitochondrial network to increase fusion events. Along with OPA1, MOEx also promoted increased MFN-1 levels, which could indicate increased fusion events. However, the activity of these proteins is highly dependent on post-translational modifications, thus more data must be obtained to better conclusions. The tendentially decreased levels of PGC1α in female F1-MOEx suggest lower mitochondrial biogenesis in this group. In addition, the tendentially increased levels of TFAM for male F1-MOEx contradicts a general effect of maternal physical exercise in MO-offspring. This highlights the sex-specific response, since maternal physical exercise altered both TFAM and PGC1α expression levels, but the response was very different for male and female offspring. The verified increased fusion events may result from a compensatory mechanism induced by gestational exercise practice to counteract the MO-induced mitochondrial dysfunction [83]

4.4. The role of RNS and nitrosative stress in offspring's cardiac response to gestational exercise during obesity.

Mitochondrial dysfunction could be a result or a cause of oxidative and nitrosative stress. For male F1-MOEx, 3-nitrotyrosine levels were decreased, highlighting that maternal physical exercise practice, during MO, prevents nitrosative stress, reducing the potential biomolecule damage. Although nitrosative stress is characterized by increased levels of NO, this metabolite is required to regulate the antihypertrophic response. Maternal physical exercise practice induced a tendential decreased phosphorylation of NFATc4. This indicates that the rate of NFATc4 nuclear translocation is tendentially increased. To further evaluate whether hypertrophy development is, in fact, occurring, functional evaluation is needed in the future. Of note that exercise practice induces a hypertrophic response (*section 1.4.2.*), which might justify this result along with increased levels of NO-related damage. Indeed, maternal physical exercise practice increased the serum levels of NO in the F1-generation in mice at 14-weeks-old[139]. However, in this study, MOEx during an obesogenic pregnancy produced no differences for CAT-1 levels, which suggests unaltered transport of the NO precursor, L-Arginine, by maternal physical exercise practice in the cardiac tissue of the MO-offspring. Further studies are needed to determine the exact role of NO and nitrosative stress in the cardiac response of the offspring to MO and to MO combined with maternal physical exercise practice.

Overall, the data provided by this study suggests that the innate physiology and morphology of each individual is highly associated with the individual's sex, which possibly influences the response to a pathological insult. Moreover, maternal physical exercise practice, during an obese pregnancy, was demonstrated to exert a potential positive modulation of the offspring's cardiac function, by preventing markers of metabolic syndrome development, by inducing a cardiac metabolic remodelling with the improvement of cardiac mitochondrial fitness. These parameters altogether might result in a phenotype that benefits the offspring and reduces or even, prevents the MO-induced increased risk of CVD development.

Chapter 5 – Conclusions and Future Perspectives

It is well-established that MO induces deleterious alterations in fetal development, which predispose the offspring to CVD development in early life. It is even possible to link the rise in MO with increased CVD-related events in young-adults through recent years. However, it remains undefined the future steps of action among the medical community to prevent the evolution of this trend.

This work contributed for the knowledge that physical exercise practice for obese pregnant women might be a possible solution in preventing CVD-related episodes in the offspring. The evidence collected suggest the potential benefits of maternal physical exercise practice during MO in the offspring's cardiac function. Altogether, this project, at this phase, indicates that maternal physical exercise practice during an obesogenic pregnancy leads to alterations in offspring's blood plasma parameters which are associated with a lower risk for CVD development. Maternal physical exercise induced mitochondrial bioenergetic adaptations, with possible increased fusion events and altered biogenesis, which indicates possible positive implications for the cardiovascular health of the young-adult offspring exposed to MO. However, further complementary investigation is required to sustain these results, especially regarding the prevention of oxidative and nitrosative stress.

The prevention of nitrosative stress could be a result of complex I inhibition through S-nitrosylation by NO, which has been described to have a cardioprotective effect by reducing electron scaping through the MRC complex I [92]. Thus, the evaluation of the S-nitrosylation in complex I could be of major interest as a complementary result for this work. Moreover, the role of NO has been extensively described in the hypertrophic response and in vascular origin of CVD. Thus, the potential role of MOEx in reversing the offspring's NO levels and inducing a positive cardiovascular response in the offspring should be taken into consideration. To this extent, NO-derivates levels and NOS activity should be measured, as well as the activity of soluble guanylyl cyclase (*section 1.4.2.*) (**Fig. 5.1**).

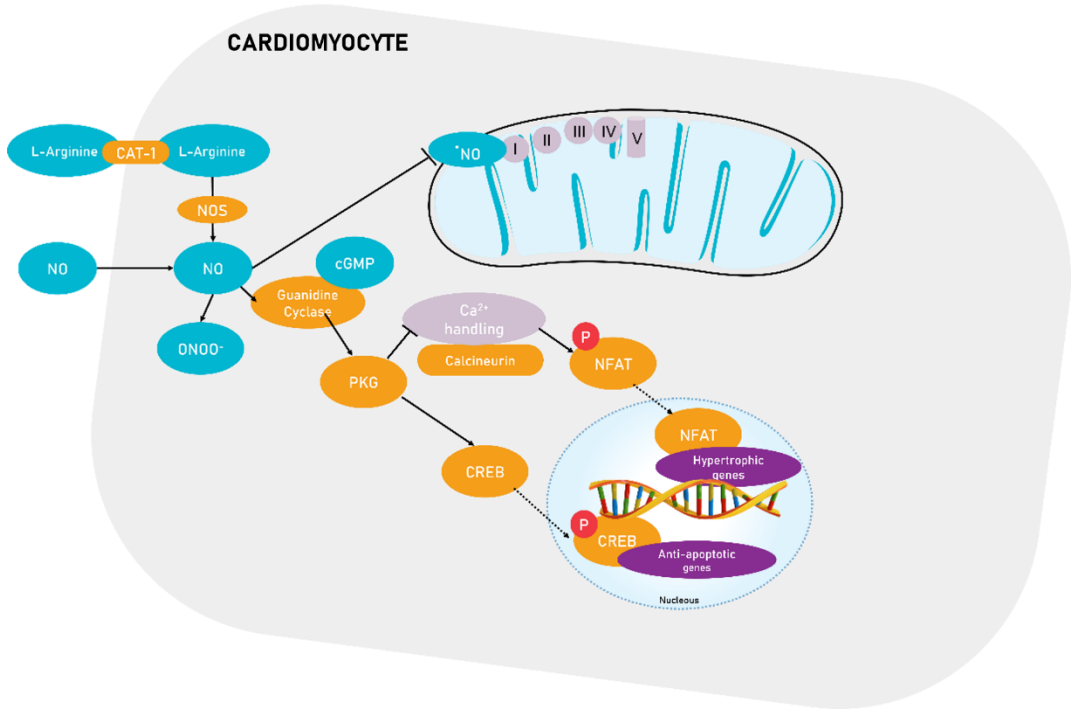


Fig. 5.1 | *Nitric oxide signalling* ***pathway in the cardiomyocyte, including NO-mediated anti-hypertrophic response and NO-induced cardioprotection.*** *CAT-1: cationic amino acid transporter 1; NOS: nitric oxide synthase; NO: nitric oxide; $ONOO^-$: peroxynitrite; cGMP: 3',5' - cyclic guanosine monophosphate ;PKG: protein kinase G; NFAT: nuclear factor of activated T-cells ; CREB: Cyclic AMP-responsive-element-binding protein.*

Concluding, this work corroborates a clear origin of CVD in the womb, and made possible to provide new data regarding the beneficial effects of maternal physical exercise during MO (**Fig. 5.2**), which, to the current knowledge, remain highly undefined. The prosecution of investigation in this topic can result in a new preventative non-pharmacological solution for MO-offspring premature CVD development.

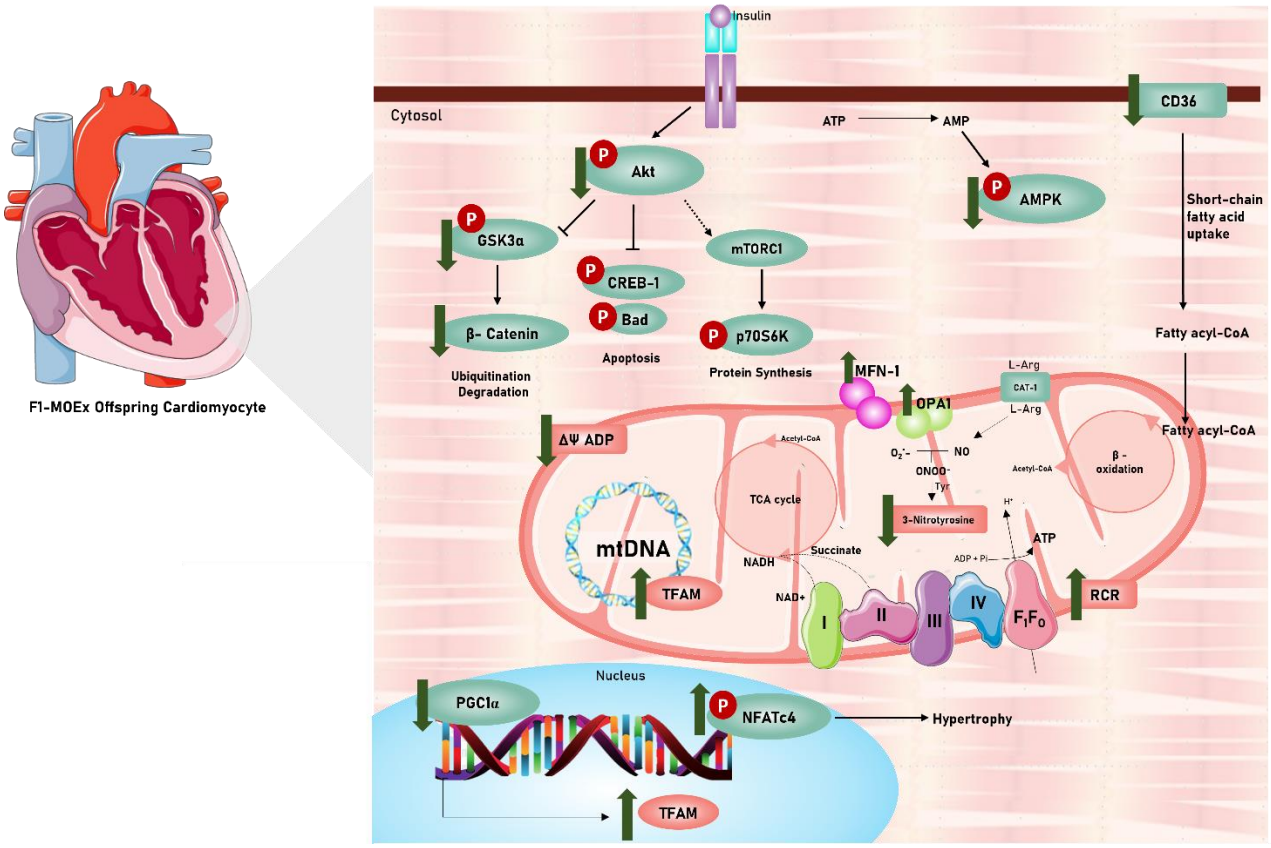


Fig. 5.2 | The impact of maternal physical exercise practice during an obesogenic pregnancy on the cardiac metabolism of 32-week-old offspring. *CD36: Cluster of differentiation 36; AMPK: AMP-kinase; GSK3α: Glycogen synthase kinase 3 α; CREB-1: Cyclic AMP-responsive-element-binding protein; mTORC1: Mammalian target of rapamycin; TFAM: Mitochondrial transcription factor A; PGC1α: Peroxisome-proliferator-activated receptor γ co-activator-1α*; NFATc4: Nuclear translocator of activated T cells 4*; ΔΨ ADP: Mitochondrial membrane potential induced by ADP; mtDNA: Mitochondrial DNA; MFN-1: Mitofusion-1; OPA1: Optic atrophy 1; $ONOO^-$: Peroxynitrite.*

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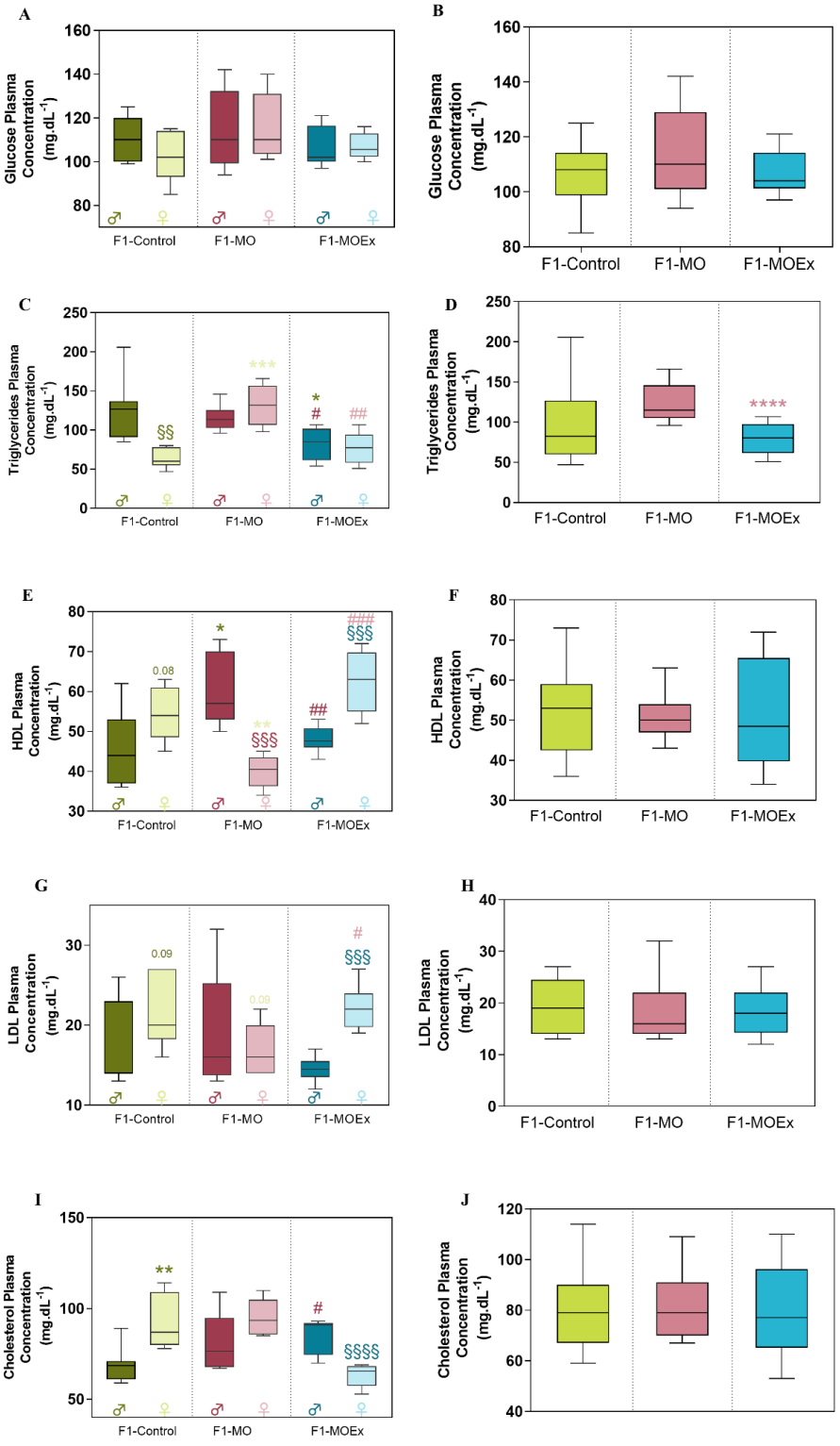
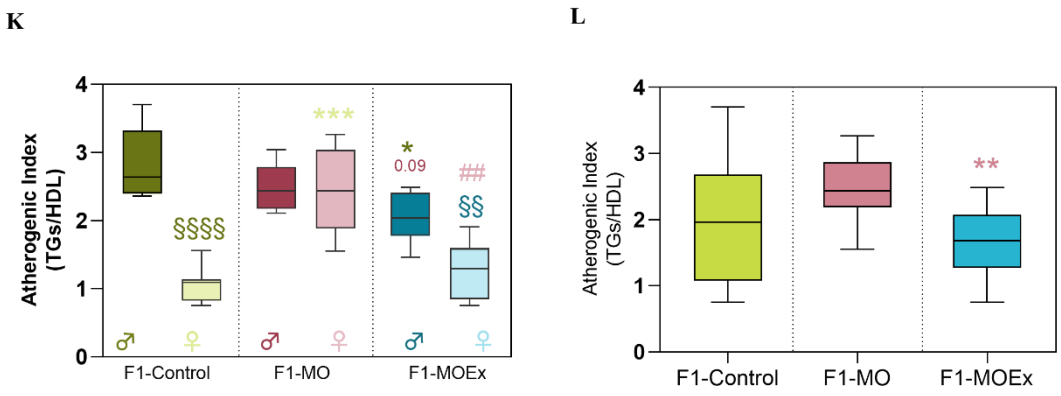
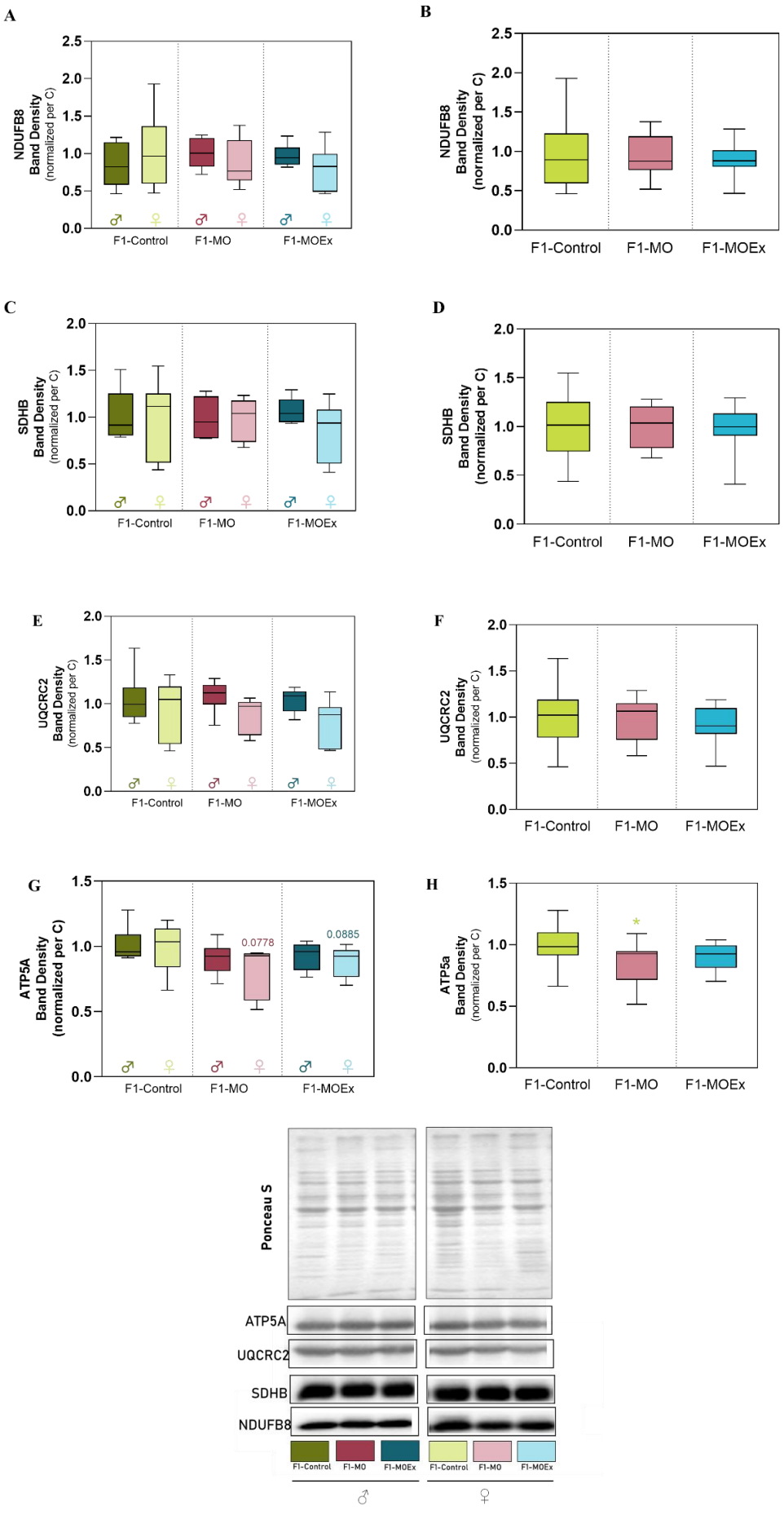
Supplementary Data





S.1.0 | ***Biochemical blood plasma parameters characterization of 32-week-old offspring.*** *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific glucose blood plasma concentration (mg/dL);* ***(B)*** *Glucose blood plasma concentration (mg/dL);* ***(C)*** *Sex-specific triglycerides (TG) blood plasma concentration (mg/dL);* ***(D)*** *Triglycerides (TG) blood plasma concentration (mg/dL);* ***(E)*** *Sex-specific high-density lipoprotein (HDL) blood plasma concentration (mg/dL);* ***(F)*** *High-density lipoprotein (HDL) blood plasma concentration (mg/dL);* ***(G)*** *Sex-specific low-density lipoprotein (LDL) blood plasma concentration (mg/dL);* ***(H)*** *Low-density lipoprotein (LDL) blood plasma concentration (mg/dL);* ***(I)*** *Sex-specific cholesterol blood plasma concentration (mg/dL);* ***(J)*** *Cholesterol blood plasma concentration (mg/dL);* ***(K)*** *Sex-specific atherogenic index (AI), calculated through the ratio TG/HDL;* ***(L)*** ***A****therogenic index (AI), calculated through the ratio TG/HDL. Data are expressed as median ± standard deviation. The * symbol represents a statistically significant result in comparison with F1-Control of the same sex; the # symbol represents a statistically significant result in comparison with F1-MO of the same sex; and the § symbol represents a statistically significant result in comparison with the same group of the opposite sex. The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 4). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; *# vs. F1-MO*; *§vs. opposite sex of the same group.*


A
NDUFB8 Band Density (normalized per C)
F1-Control
F1-MO
F1-MOEx
B
C
SDHB Band Density (normalized per C)
D
E
UQCRC2 Band Density (normalized per C)
F
G
ATP5A Band Density (normalized per C)
0.0778
0.0885
H
ATP5a Band Density (normalized per C)
*
Ponceau S
ATP5A
UQCRC2
SDHB
NDUFB8
♂
♀

S.2.0 | 32-week-old offspring cardiac relative band density of OXPHOS complex subunits. *F1-Control: offspring born to mothers fed a standard-diet; F1-MO: offspring born to sedentary mothers fed a HFHS diet; F1-MOEx: offspring born to exercised mothers fed a HFHS diet.* ***(A)*** *Sex-specific NDUFB8 relative band density;* ***(B)*** *NDUFB8 relative band density;* ***(C)*** *Sex-specific SDHB relative band density;* ***(D)*** *SDHB relative band density;* ***(E)*** *Sex-specific UQCRC2 relative band density;* ***(F)*** *UQCRC2 relative band density;* ***(G)*** *Sex-specific ATP5A relative band density;* ***(H)*** *ATP5A relative band density. Data are expressed as medians, Q1, Q3, min and max. The comparison between groups was performed using a parametric t-test or Mann-Whitney (n ≥ 4). Normality was evaluated with Shappiro-Wilk test. *,#,§ p≤0.05; **,##,§§p≤0.01; ***,###,§§§p≤0.001; ****,####,§§§§ p≤0.0001.* **** vs. F1-Control****; #* ***vs. F1-MO****;* ***§vs. opposite sex of the same group.***