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Gestational exercise and intergenerational inheritance. Metabolic and functional outcomes in rat liver and skeletal muscle

Jorge Beleza

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DOCTORAL THESIS

GESTATIONAL EXERCISE AND INTERGENERATIONAL
INHERITANCE. METABOLIC AND FUNCTIONAL
OUTCOMES IN RAT LIVER AND SKELETAL MUSCLE.

Jorge Beleza

JORGE MANUEL DE SOUSA BELEZA

Barcelona, 2022



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DOCTORAL THESIS

Jorge Beleza

Barcelona, 2022

Universitat de Barcelona Facultat de Biologia
Departament de Biologia Cel·lular, Fisiologia i
Immunologia

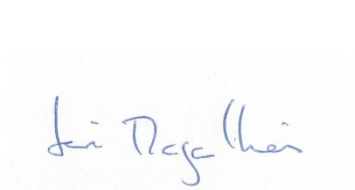
Gestational exercise and intergenerational inheritance. Metabolic and functional outcomes in rat liver and skeletal muscle.

Memoria presentada por Jorge Belezza para optar al grado de Doctor por la
Universidad de Barcelona.

Dr. José Magalhães
Director

Dr. Joan Ramon Torrella Guio
Director and tutor

Jorge Belezza
Doctoral student



Barcelona, 2022

O passado e o futuro deixam de ter significado quando atravesso uma perigosa fenda no gelo ou quando, durante um segundo, alguma maravilha do mundo natural me cativa. A fração de segundo e a eternidade não têm de ser contraditórias. O tempo fica abolido e ambas podem ser vividas num só e único momento.

ERLING KAGGE

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Abstract

Maternal environmental exposure during gestational period may affect offspring risk for chronic diseases. Accordingly, maternal metabolic disturbances induced by high-fat high-sucrose (HFHS) diets can have a negative impact on offspring liver and skeletal muscle (SM) tissue phenotypes. In contrast, gestational exercise (GE) seems to favorably modulate deleterious stimuli induced by HFHS-diet consumption, not only in mothers but also in their offspring. However, the mechanisms associated to intergenerational inheritance of favorable metabolic outcomes in the offspring are far to be understood, as well as the role of epigenetic regulation in the offspring program. In order to contribute to increase the scientific knowledge in this field, the present PhD thesis comprises two literature review publications and three animal-based experimental papers that were developed using a gestational diabetes mellitus (GDM) model. Therefore, the goal was to analyze the potential role of an exercised model that combines voluntary free-running wheel (FRW) with forced endurance exercise, against liver and SM offspring metabolic and functional impairments. Our data revealed that GE counteracted the negative impact of HFHS maternal diet and sedentarism in offspring liver and SM morphology. Moreover, offspring from HFHS exercised mothers exhibited an improvement in liver and SM parameters of mitochondrial biogenesis, oxidative phosphorylation subunits protein content and respiratory capacity, associated to improvements in maximal exercise performance. Additionally, improvements in biomarkers of mitochondrial performance seems to be regulated, at least in part, by the role of miRNAs through epigenetic modifications inherited by the offspring generation. Ultimately, these findings shed light on the importance of maternal lifestyle, specifically the relevance of GE, on the phenotypic programming of the offspring against GDM-associated metabolic impairments and ultimately to an enhanced functional performance. Hopefully, evidence from the present PhD thesis can contribute to encourage mothers to engage in an active lifestyle before and during pregnancy aiming positive outcomes in their offspring.

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Abbreviations

AMPk	5' adenosine monophosphate-activated protein kinase
AUC	Area under the curve
BLC	Blood lactate concentration
BW	Body weight
C	Control
CCA	Number of capillaries per 1,000 μm^2 of FCSA
CS	Citrate synthase
DOHaD	Developmental origins of health and disease
FCSA	Fiber cross-sectional area
FG	Fast glycolytic
FIG	Fast intermediate glycolytic
FRW	Free-running wheel
FOG	Fast oxidative glycolytic
GDM	Gestational diabetes mellitus
G/M	Glutamate/malate
HFD	High fat diet
HFHS	High-fat high-sucrose
HIIT	High-intensity interval training
ME	Maternal exercise
miRNA	MicroRNA
MWT	Maximal workload test
NAFLD	Non-alcoholic fat liver diseases
NCF	Number of capillaries per fiber
NRF2	Nuclear factor-erythroid factor 2-related factor 2
OXPHOS	Oxidative phosphorylation
PE	Physical exercise
PGC-1 α	Peroxisome proliferator-activated receptor- γ coactivator-1 α
RCR	Respiratory control ratio
SED	Sedentary
SM	Skeletal muscle

SOL	Soleus
S/R	Succinate/rotenone
TA	Tibialis anterior
T2DM	Type 2 diabetes <i>mellitus</i>
TFAM	Mitochondrial transcription factor A

1. General Introduction

1.1. Background

Physical exercise

Physical exercise (PE) is currently recognized as an undoubted effective strategy to prevent and counteract the epidemics of chronic metabolic disorders, associated to sedentarism, that western countries are dealing with in the XXI century (Pedersen and Saltin 2015). In fact, recent publications from the American College of Sports Medicine (Liguori 2020) and the World Health Organization (WHO 2020) summarized the strong body of evidence showing the positive impact of PE in conditions such as cardiovascular disease, type-2 diabetes, hypertension, stroke, osteoporosis, metabolic syndrome, and different types of cancer. Additionally, PE also has benefits on mental health (Schuch et al. 2016; Marques-Aleixo et al. 2021), delays the onset of dementia (Livingston et al. 2017; Bernardo et al. 2016), and can contribute to general well-being (Das and Horton 2012). Moreover, the evidence bases for PE in subpopulations, namely in pregnant women, has shown a reduction in common complications during pregnancy associated with minimal risks (Birsner and Gyamfi-Bannerman 2020). In fact, maternal exercise (ME) is significantly associated with the reduction of some metabolic complications in pregnancy, like excessive gestational weight gain and development of gestational diabetes mellitus (GDM) (Du et al. 2019).

From a physiological perspective, performing PE induces an increased metabolic stress to the human organism, challenging the homeostasis during the time that exercise is performed, which can be further extended for minutes, hours or days after the end of exercise session (Hargreaves and Spriet 2020). To face the physiological challenges imposed by PE, tissues have the capacity to structurally, functionally and metabolic adapt (Mukund and Subramaniam 2020). The most pronounced and direct physiological adaptations can be observed in the effector systems directly related to PE, of which locomotor, cardiovascular and nervous systems are examples (Booth et al. 2015; Marques-Aleixo et al. 2021; Ascensão et al. 2011). Adaptations to PE occur at both molecular and macroscopic levels, ranging from phenotypic alterations in the skeletal muscle (SM) fiber type

composition to the modulation of key molecular pathways, all with the goal of preparing the organism for similar future events and aiming at improving performance (Jacobs et al. 2013; Lundby and Jacobs 2016). Besides the direct impact of PE in the effector systems, currently it is well established that SM communicates with other tissues, operating as an endocrine-type organ (Murphy, Watt, and Febbraio 2020; Pedersen et al. 2003). The contractile activity of SM resulting from PE practice, induces the release of several muscle-derived molecules (myokines) into the blood stream (Pedersen et al. 2003), which reach the different non-contractile tissues through tissue-specific receptors and activate multiple signaling cascades that modulate tissue phenotype (Beleza et al. 2018; Lee and Jun 2019).

Some of the cellular adaptations associated to PE are mediated by alterations in gene expression that are orchestrated by epigenetic modifications (Jiménez-Chillarón et al. 2015). Although cells from an organism share identical DNA, its expression may be driven by distinct epigenetic-related influences, such as DNA methylation, histone modifications and microRNA (miRNA) regulation (Barrès and Zierath 2016; McGee and Hargreaves 2019). DNA methylation and histone modifications involve a direct influence at chromatin level, while miRNAs regulate the cell's protein expression by modulating mRNA stability and the efficiency of translation (Widmann, Nieß, and Munz 2019). In this context, PE has the ability to modulate the epigenetic landscape of different cells, thus regulating the delicate balance between gene expression or silencing, under the control of transient or stable transcriptional or post-transcriptional mechanisms (McGee and Walder 2017). In fact, data highlight the epigenetic-related role of miRNAs in the post-translational regulation of gene expression in SM response to exercise (Davidsen et al. 2011). Both resistance and endurance training seem to be correlated with the expression of different miRNAs that have important roles in distinct biological pathways (Davidsen et al. 2011; Xu et al. 2015; Russell et al. 2013; Aoi et al. 2010).

The modulation induced by PE described above is recognized as having an important role in the prevention and treatment of several pathological conditions like cardiac dysfunction, hyperglycemia, obesity-induced adiposopathy and liver

diseases such as non-alcoholic fat liver diseases (NAFLD) (Gonçalves et al. 2014; Rocha-Rodrigues, Rodríguez, et al. 2017; Ascensão et al. 2005). Furthermore, regarding many pathophysiological conditions there is strong evidence of a curvilinear inverse dose-response relationship between PE and major positive health-related outcomes (Liguori 2020; WHO 2020). However, despite the clear positive effect of PE, a strong research effort has been made to better ascertain the most accurate PE guidelines in terms of frequency, intensity, duration, type, volume and progression of interventions (Pescatello, Riebe, and Thompson 2014). Nonetheless, due to the complexity of factors involved in the exercise prescription and in the interindividual response to PE, doubts remain about the guidelines to follow in some of the specific health-related complications mentioned above.

Because of human-related ethical constraints regarding several experimental conditions, excision and biochemical study of particular tissues, exercise physiologists have been using different exercise-based models in rodents to mimic the physiological impact of PE both on systemic and at different levels of cellular organization. In fact, different forced exercise models have been used in rodents enabling to increase the scientific knowledge in the biomedical field. Additionally, voluntary self-paced motor behavior models, such as 24-h access to free-running wheels (FRW), have been used to minimize (mal)adaptations associated to sedentarism or pathological conditions (Fonseca et al. 2011; Gonçalves et al. 2014). However, despite the widespread use of FRW, there is a lack of knowledge regarding the truly physiological and biochemical impact of this model on voluntarily exercised animals, mainly due to the lack of control in exercise intensity and duration.

Physical exercise and mitochondria

Mitochondria play a crucial role in the maintenance of cellular homeostasis and ultimately contributes for the Human general health status (Beleza et al. 2018; Magalhães et al. 2014). Besides the classical role as the “powerhouses of the cell”, mitochondria have a pivotal role in the regulation of several cellular physiological processes like modulation of redox status, pH control, calcium

homeostasis, cell signaling, osmotic regulation and control of programmed cell death (Gnaiger 2020; Skulachev 1999). Moreover, among the distinct effects of PE on the organism, the modulation of mitochondrial network seems to play a pivotal role in exercise adaptations. In fact, a PE-induced increase in energy demand can activate mitochondrial remodeling processes and further improve mitochondrial network performance. For instance, mitochondria appear to adapt specifically to the type of metabolic stress applied throughout exercise. Endurance training increases the oxidative capacity to oxidize fatty acids and thereby save limited carbohydrate stores from being depleted (Brooks and Mercier 1994), while high-intensity exercise training induces global improvements in respiratory capacity (Jacobs et al. 2013). Additionally, PE modulates mitochondrial ability to operate in the control of cell signaling, death and quality control. In fact, one of the classic cells' adaptations to exercise is the increase in mitochondrial content and stimulation of mitochondrial biogenesis, being the peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) the master regulator of this process (Pilegaard, Saltin, and Neufer 2003). Also, preserving healthy mitochondria and consistent removal of damaged and non-functional ones is a critical point in the maintenance of mitochondrial turnover, metabolic efficiency and homeostasis. Similarly to many other stimuli causing mitochondrial network remodeling, this dynamic process in response to PE is regulated by fusion, fission and auto(mito)phagic events (Konopka et al. 2014). Altogether, these quality control mechanisms allow the cells to be more metabolically capable and functionally efficient, being able to respond to physiological challenges imposed by PE. Likewise, among the several strategies used to induce a positive mitochondria modulation, several studies from our laboratory have proved that PE is one of the most efficient non-pharmacologic strategies, namely, with a positive impact in several tissues with a high metabolic activity like heart, liver, adipose tissue and SM (Ascensão et al. 2006; Gonçalves et al. 2016; Rocha-Rodrigues et al. 2018; Lumini-Oliveira et al. 2009).

Gestational exercise

The concept of developmental origins of health and disease (DOHaD) highlights the importance of maternal environmental exposure during gestational period, which may affect the offspring risk for chronic diseases (Armitage, Poston, and Taylor 2008; Falcão-Tebas et al. 2012; Falcão-Tebas et al. 2019). In fact, some environmental exposures, such as maternal malnutrition, may increase the offspring predisposition to the development of metabolic disorders in adulthood (Langley-Evans and McMullen 2010; Armitage, Poston, and Taylor 2008; Falcão-Tebas et al. 2019). Accordingly, maternal metabolic disturbances induced by high fat diet (HFD) consumption during gestation can have a negative impact on the offspring generation and may increase the propensity to impaired glucose tolerance and insulin sensitivity, as well as the predisposition to obesity development later in the offspring life (Bayol, Simbi, and Stickland 2005; Kruse et al. 2013; Masuyama and Hiramatsu 2012). These (patho)physiological consequences in the offspring result from progressive development of less resistant phenotypes in several tissues and organs, including liver and SM, and are usually perpetrated later in life (WHO 2001; Warner and Ozanne 2010; Li et al. 2016). In fact, maternal metabolic impairment can program liver and SM mitochondrial dysfunction in the offspring generation (Saben et al. 2016; Vacca et al. 2015; Pessayre et al. 2012; Szendroedi, Phielix, and Roden 2011). Considering the pivotal role of mitochondria in the cellular physiology, their dysfunction may be one of the most relevant markers of metabolic diseases progression; however, data reporting the mechanism that elucidate about the impact of maternal lifestyle, particularly during the gestational period are still scarce.

Recently, research regarding gestational exercise (GE) provided strong evidence about its health-related positive benefits, not only for mothers, but also for their offspring (Beleza et al. 2021; Gilbert et al. 2012; Mottola and Artal 2016; Stevanović-Silva et al. 2021). GE can prevent adverse effects associated to maternal environmental exposure on offspring metabolic health (Stanford et al. 2015; Laker et al. 2014) and even reduce the risk for NAFLD and obesity when offspring are exposed to HFD consumption (Sheldon et al. 2016; Wasinski et al.

2015). Rodent's studies demonstrated that ME improves offspring long-term glucose homeostasis (Carter et al. 2012; Mottola 2013; Harris, Baer, and Stanford 2018; Stanford et al. 2018) and increases the expression of SM mitochondrial content and biogenesis signaling markers (Raipuria, Bahari, and Morris 2015). Furthermore, GE increases offspring volition for self-paced FRW exercise (Eclarinal et al. 2016), although the mechanistic explanation of how GE improves offspring metabolic health through intergenerational inheritance, as well as mitochondrial-associated metabolic modulation, is so far unknown. Nevertheless, some studies suggest that epigenetic-related programming associated with ME contributes, at least in part, to mitigate disturbances associated with several pathophysiological conditions at distinct levels of cellular organization in the offspring (Stevanović et al. 2020).

Considering the beneficial effects of GE mentioned above, behavior's changes regarding to the practice of exercise during pregnancy should be adopted as part of the huge parental responsibility with their offspring and with the society. Nevertheless, additional research is needed to investigate the effects of exercise on pregnancy-specific outcomes and to clarify the most effective GE counseling methods, like optimal intensity and frequency able to counteract HFD-associated metabolic disorders and the deleterious intergenerational inheritance. This PhD thesis is aimed to collaborate to the enlargement of the current knowledge in this field.

1.2. List of Scientific Publications

This thesis is based on the following scientific publications which are referred to in the text by their Roman numerals:

Theoretical Background

- I. **Beleza J**, Rizo-Roca D, Ascensão A, Magalhães J. (2018). **Targeting Mitochondria with Sweat: Improving Mitochondrial Function with Physical Activity**. In Oliveira, P (Ed) *Mitochondria Biology and Experimental Therapeutics*. Springer, USA, pp. 379-406.
- II. **Beleza J**, Stevanovic J, Coxito P, Costa RC, Ascensão A, Torrella JR, Magalhães J. (2021). **Building-up fit muscles for the future**. *European Journal of Clinical Investigation*, 116, 1-20.

Experimental Work

- III. **Beleza J**, Albuquerque J, Santos Alves E, Fonseca P, Santocildes G, Stevanovic J, Rocha-Rodrigues S, Rizo-Roca D, Ascensão A, Torrella JR, Magalhães J. (2019). **Self-paced free-running wheel behavior impact on rats' functional, physiological, biochemical and morphological features**. *Frontiers in Physiology*, 10, 593.
- IV. Stevanovic J, **Beleza J**, Coxito P, Pereira SP, Rocha H, Gaspar T, Gartner F, Correia S, Martins MJ, Guimarães T, Martins S, Oliveira OJ, Ascensão A, Magalhães J. (2021). **Maternal high-fat high-sucrose diet and gestational exercise modulate hepatic fat accumulation and liver mitochondrial respiratory capacity in mothers and male offspring**. *Metabolism Clinical and Experimental*, 116, 1-20.
- V. **Beleza J**, Stevanovic J, Coxito P, Costa RC, Ascensão A, Torrella JR, Magalhães J. (2022). **Gestational exercise increases male offspring maximal workload capacity early in life**. SUBMITTED TO: *International Journal of Molecular Science*.

1.3. PhD thesis organization

The present PhD thesis is based on the scientific publications indicated above. The document starts with a book chapter (I) where the current knowledge about the effects of PE in the modulation of mitochondrial physiology is reviewed, and where the distinct mechanisms involving mitochondria in contractile tissues are explored, as well as the pleiotropic repercussions affecting also non-contractile tissues. Additionally, the role of PE in improving several mitochondrial features and its implication against disease-related deleterious consequences is also reviewed. Finally, special attention is addressed to the emerging role of epigenetic-based regulation induced by PE that, at least in part, contributes to target mitochondrial function.

In work II a special attention was dedicated to review the existing knowledge about the impact of parental exercise and the intergenerational offspring metabolic programming. Moreover, in this review our focus was on the SM modulation induced by PE and specifically the particular role of the epigenetic mechanisms that orchestrate the SM offspring inheritance of positive outcomes induced by the exercise habits of parental generation.

Regarding the experimental part of this PhD thesis, we start by presenting a paper (III) where the characterization of a FRM exercise model is provided. This work contributed to improve the knowledge of the physiological impact of FRW in rodents. Here we compared the FRW with a high-intensity interval training (HIIT) exercise model and we demonstrated that both models induced similar SM and cardiac adaptations. Additionally, FRW induced an improvement in animal's ability to perform maximal aerobic exercise, which is in line with others exercise-forced models, such as HIIT. Moreover, results from work III provided us with scientific robustness to design an innovative exercise intervention model aimed to significantly increase dams physical activity level by using a combination of FRW with an endurance training on a treadmill. This intervention was later implemented in the experimental works IV and V.

In works IV and V we carried out a research in which female rats were fed with a HFHS diet prior to mating, in order to induce a specific metabolic environment before pregnancy that would, in the particular conditions of pregnancy, prompt

the development of metabolic remodeling, characteristic of GDM. Thereafter, we analyzed the impact of GE against GDM-induced metabolic impairments, being the beneficial effect induced by GE clear in both mothers and offspring. Markers of mitochondrial function, content and biogenesis were evaluated in liver and SM tissues. An improvement in the ability to perform maximal aerobic exercise was found in the offspring from exercised mothers. Additionally, these functional alterations were associated to improvements in mitochondrial features. Ultimately, some of the offspring metabolic and functional improvements induced by GE seems to be orchestrated by epigenetic regulation, namely associated to miRNAs expression.

2. Aims

2.1. General Aims

To analyze the impact of GE against GDM-induced metabolic and functional impairments in maternal and offspring. Additionally, to identify some of the mechanisms that can regulate intergenerational transmission of metabolic and functional alterations induced by GE.

2.2. Specific Aims

1. To clarify the physiological impact of a FRW model from a central and peripheral point of view, namely the cardiac and skeletal muscle adaptations.
2. To investigate the potential metabolic benefits of GE against GDM in liver and SM tissues.
3. To better understand the implication of GE in offspring capacity to perform maximal exercise efforts.
4. To ascertain the mitochondrial modulation induced by GE in mothers' and offspring's liver and SM tissues.
5. To elucidate the underlying mechanisms involved in the intergenerational transmission of cross-tolerance through GE to the offspring against GDM-associated metabolic disorders.
6. To gather strong evidence and translational power to encourage mothers to engage in an active lifestyle before and during pregnancy.

3. Publications

I

Beleza J, Rizo-Roca D, Ascensão A, Magalhães J. (2018). Targeting Mitochondria with Sweat: Improving Mitochondrial Function with Physical Activity. In Oliveira, P (Ed) *Mitochondria Biology and Experimental Therapeutics*. Springer, USA, pp. 379-406.

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

Targeting Mitochondria with Sweat: Improving Mitochondrial Function with Physical Activity



Jorge Beleza, David Rizo-Roca, António Ascensão, and José Magalhães

Abstract It is well established that physical exercise imposes increased levels of mechanical and metabolic stress to the human organism, altering the homeostasis and stimulating the inherent ability of tissues to structurally and functionally adapt to cope with the inflicted challenges. These adaptations usually result in an increased resistance against the harmful effects characterizing senescence as well as those associated with disease conditions. These include muscle myopathies, cardiac dysfunction induced by ischaemia-reperfusion, obesity, diabetes or toxicants exposure, liver and neurodegenerative diseases. In fact, exercise may directly alter cellular energy status or increase mechanical load in contractile tissues, such as skeletal and cardiac muscles, and may also indirectly induce an endocrine-like effect through the release of distinct molecules by striated muscles, which may exert consequent stimulation in non-contractile tissues, such as brain, liver or adipocytes. Mitochondrial remodelling is among the most important mechanisms targeted by exercise that contribute to the mentioned protective phenotype. The powerful influence of exercise in mitochondrial physiology include favourable changes in bioenergetics and substrate utilization, alterations in redox homeostasis, changes in network dynamics through biogenesis, fusion and fission mechanisms, an important involvement in the control of cellular death mechanisms as well as influence in cell signalling, autophagy-related renewal and quality control processes. The present review analyses the effects of exercise in the modulation of mitochondrial physiology, examining distinct proposed mechanisms targeting mitochondria and potentially responsible tissue boosting and consequent defect rescuing. The emerging role of epigenetic-based contribution to these cross-tolerance effects is also addressed.

Keywords Exercise · Mitochondrial remodelling · Tissue protection · Aging · Disease · Epigenetic

Jorge Beleza and David Rizo-Roca contributed equally to this work.

J. Beleza · D. Rizo-Roca · A. Ascensão · J. Magalhães (✉)

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

e-mail: jmaga@fade.up.pt

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379

1 Introduction

It is well established that physical exercise imposes an increased level of metabolic stress to the human organism, altering the homeostasis during the time exercise is performed, which can be further perpetrated during minutes, hours or days after the completion of exercise. To cope with the physiological challenges imposed by physical exercise, tissues have an inherent ability to structurally and functionally adapt. The most pronounced and direct physiological adaptations can be observed in the effector systems directly related to physical exercise, of which locomotor, cardiovascular and nervous systems are examples. In particular, the physiological adaptations occurring in the skeletal muscle system are well documented (Booth et al. 2015; Lundby and Jacobs 2016). Skeletal muscle is a very specialized tissue with a remarkable plasticity and adaptability to the physiological constraints imposed by contractile activity leading to several structural, functional and metabolic alterations, and ultimately promoting alterations in key molecular pathways that impact tissue phenotype. Besides the direct effect of contractile activity on skeletal muscle itself and in the cardiac tissue, the metabolic and mechanical alterations induced by physical exercise can have pleiotropic repercussions affecting also non-contractile tissues. This endocrine-like effect results, among other reasons, from the release of mediator proteins secreted by muscle cells to the blood stream (the so-called myokines) (Pedersen et al. 2003; Hoffmann and Weigert 2017), including interleukin 6 (IL-6), brain derived neurotrophic factor (BDNF) and irisin, which have specific receptors on the different organs and further activate multiple signalling cascades (Fig. 1). The disruption of homeostasis caused by physical exercise boosts tissue phenotype, i.e., augmenting the metabolic efficiency and/or increasing resistance against harmful stimuli, thus allowing to consider exercise as an outstanding strategy aiming at preserving cellular, tissue and organ viability and able to mitigate distinct stressful conditions (Fiuza-Luces et al. 2013; Pedersen and Saltin 2015). Accordingly, work from our laboratory and from others provide morphologic, functional and molecular evidence of the protective and therapeutic effect of exercise in different contractile and non-contractile tissues, including skeletal and cardiac muscles, brain, liver and adipose tissue, against harmful conditions associated with senescence, drug exposure and pathological states (Ascensão et al. 2005a; Lumini et al. 2008; Santos-Alves et al. 2014; Gonçalves et al. 2014a; Marques-Aleixo et al. 2016; Rocha-Rodrigues et al. 2017). Due to their important roles and interactions with several essential features of cell physiology, special attention to mitochondrial-mediated mechanisms targeted by physical exercise has been devoted (Ji et al. 2016). Among the distinct effects of physical exercise on the organism, the modulation of mitochondrial network seems to play a pivotal role in exercise adaptations. For instance, due to the crucial mitochondrial role in cellular energy production, an increase in energy demand promoted by physical exercise activates a considerable amount of mitochondrial remodelling processes leading to improvements in the mitochondrial network performance, seen as the ability to produce energy more efficiently, to more actively operate in the

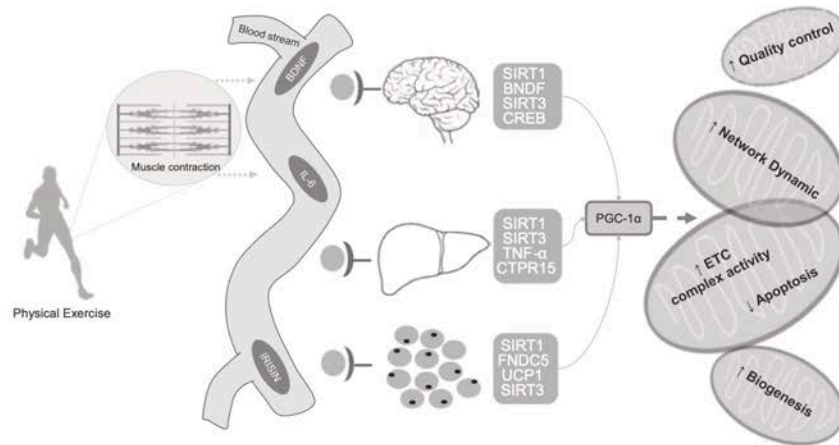


Fig. 1 Examples of how physical exercise-induced skeletal muscle endocrine-like effect may result in influenced mitochondrial phenotype in non-contractile tissues (Brain; Liver and Adipose Tissue). Muscle contractile activity promoted by physical exercise leads to the release of several muscle-derived molecules (myokines) into the blood stream, including brain-derived neurotrophic factor (BDNF), interleukin 6 (IL-6) and FNDC5/irisin. These myokines reach the different non-contractile tissues through tissue-specific receptors activating multiple signalling cascades that modulate tissue phenotype. Some of the most suggested molecules that are modulated in the different tissues as a consequence of myokine interaction include: sirtuin 1 (SIRT1), brain-derived neurotrophic factor (BDNF), sirtuin 3 (SIRT3) and cAMP response-element-binding protein (CREB) in the brain; SIRT1, SIRT3, tumor necrosis factor alpha, (TNF- α) and myonectin (CTPR15) in the liver; and SIRT1, fibronectin type III domain-containing protein 5 (FNDC5), uncoupling protein 1 (UCP1) and SIRT3 in adipose tissue (for references see: Fiuza-Luces et al. 2013; Iizuka et al. 2014; Schnyder and Handschin 2015; Kapilevich et al. 2015; Hoffmann and Weigert 2017). Ultimately, these molecules activate the master regulator of mitochondrial physiology, peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), therefore, activating a considerable amount of mitochondrial remodelling processes leading to improvements in the mitochondrial network performance through positive modulation (up-arrow) of quality control, network dynamic, electron transport chain (ETC) complex activity and biogenesis and downregulation (down-arrow) of apoptotic signalling markers. The mitochondrial remodelling in liver, brain and adipose tissues, further contributes for increasing the resistance of these non-contractile tissues against harmful stimuli, such as those resulting from aging process, toxins exposure and disease conditions

control of cell signalling, death and quality control (Fig. 2). One of the classic adaptations to exercise is the increase in mitochondrial content, being peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) the master regulator of this process of mitochondrial biogenesis (Booth et al. 2015). Also crucial to the adaptive process of mitochondrial turnover and metabolic efficiency is the ability to preserve healthy mitochondria and remove the damaged and non-functional reticulum. Similarly to many other stimuli causing mitochondrial network remodelling, this dynamic process in response to exercise is regulated by fusion, fission and auto(mito) phagic events (Konopka et al. 2014). Altogether, these quality control mechanisms

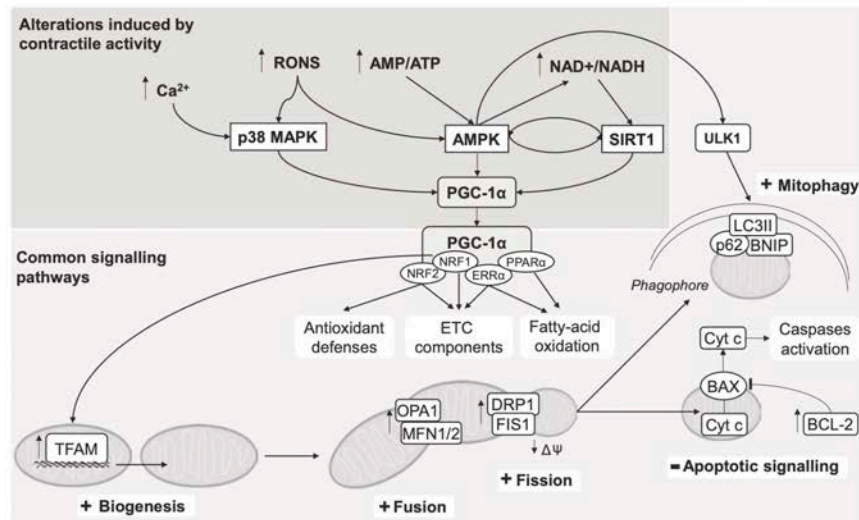


Fig. 2 Main alterations induced by contractile activity in cellular homeostasis, which activate different signalling pathways that converge in the mitochondrial master regulator PGC-1 α . After its activation, PGC-1 α interacts with multiple transcription factors (such as NRF1 and 2, ERR α and PPAR α) which result in a myriad of cellular outcomes, including (1) the formation of new mitochondria, (2) increased mitochondrial dynamics, (3) boosted antioxidant defences, (4) increased content of ETC subunits, (5) enhanced fatty-acid oxidation, and (6) anti-apoptotic adaptations. Furthermore, exercise-induced activation of AMPK leads to the phosphorylation of ULK1, stimulating the mitophagy of damaged mitochondria. Overall, these adaptations boost the tissue oxidative capacity. RONS reactive oxygen and nitrogen species, p38 p38 mitogen-activated protein kinase, AMPK AMP-activated protein kinase, SIRT1 Sirtuin 1, PGC-1 α peroxisome proliferator-activated receptor- γ coactivator-1 α , NRF1 nuclear respiratory factor 1, NRF2 nuclear factor erythroid 2-related factor 2, ERR α estrogen-related receptor α , PPAR α peroxisome proliferator-activated receptor α , TFAM mitochondrial transcription factor A, OPA1 optic atrophy 1, MFN 1 and 2 mitofusin 1 and 2, respectively, DRP1 dynamin-related protein 1, FIS1 mitochondrial fission protein 1, cyt c cytochrome c, $\Delta\psi$ mitochondrial membrane potential

allow the cells to be more metabolically capable and functionally efficient, which are essential requisites to decrease fatigability and to improve endurance capacity in striated muscles. Furthermore, as will be addressed in the next subsections of this chapter, the above-mentioned alterations in non-contractile tissues caused by exercise, which are mainly mediated by circulating cytokines, also translate in mitochondrial remodelling in liver, brain and adipose tissue, further contributing to increase the resistance of these non-contractile tissues against disease-related deleterious consequences (Marques-Aleixo et al. 2016; Gonçalves et al. 2016; Rocha-Rodrigues et al. 2016).

In summary, among the different strategies to modulate mitochondrial physiology, physical exercise is now considered one of the most effective non-pharmacological interventions showing multiple beneficial features to improve several cellular

processes (Fiuza-Luces et al. 2013). These include the control of cellular redox status, cellular signalling, calcium homeostasis, cell differentiation and death, and autophagy-related renewal processes.

In the next subsections, we will address how exercise can interfere with physiological and metabolic processes by “shaping and fitting” the mitochondrial network.

2 An Overview of How Physical Exercise Targets Different Features of Mitochondrial Structure, Metabolism and Function

2.1 Mitochondrial Bioenergetics

During exercise, substrate utilization depends on exercise modality, intensity and duration. Thus, the power output determines the ATP demand, while intensity and duration determine the percentage use of each metabolized fuel (lipids or carbohydrates) and its predominant source (hepatic, intramuscular and circulating). Generically, circulating free fatty acids (FFA) (supplied by adipocyte lipolysis) and glucose (either from oral ingestion or hepatic glycogenolysis) are the main substrates during exercise performed at low or moderate intensity (van Loon et al. 2001). On the other hand, during high intensity exercise, the fuel source shifts to a higher glucose contribution both through anaerobic glycolysis and pyruvate oxidation, and a larger contribution of muscle glycogen (van Loon et al. 2001). Hence, exercise can greatly challenge the muscle energy homeostasis. For example, in strenuous aerobic exercise, muscle blood flow and oxygen consumption can increase up to >30-fold, leading to an estimated tricarboxylic acid cycle (TCA) cycle flux increase of 100-fold (Gibala et al. 1998).

It has been extensively described that endurance training induces metabolic adaptations leading to increased content and/or activity of proteins and enzymes involved in glycolysis, glucose transport, TCA cycle, mitochondrial ATP production, and transport and oxidation of fatty acids (Schantz et al. 1983; Holloszy and Coyle 1984; Egan and Zierath 2013), which overall leads to a more efficient oxidative phosphorylation. Furthermore, endurance-trained subjects exhibit, at a given intensity, a relative decrease in circulating glucose and muscle glycogen utilization and greater reliance in fatty acid oxidation (Henriksson 1977; Jansson and Kaijser 1987), which is accompanied by increased content of fatty acid transporters, such as CD36 (Talanian et al. 2010), and increased levels of β -oxidation regulators, including medium-chain and very long chain acyl-CoA dehydrogenases (Horowitz et al. 2000). This decrease in carbohydrate use during submaximal exercise is a consequence of training-induced increased mitochondrial content and function, and allows the organism to spare glycogen; therefore reducing its depletion within the muscle and improving endurance performance and fatigue resistance.

Regardless the acetyl-CoA source, endurance training increases the TCA cycle flux (Befroy et al. 2008), leading to increased production of NADH and succinate, which are oxidized in the respiratory chain. Furthermore, it also induces an increase of the malate-aspartate shuttle (Schantz et al. 1986), which carries reducing equivalents across the mitochondrial membranes. These chronic exercise-based adaptations have the ultimate purpose of improving the abundance of electrons for “fuelling” the electron transport chain (ETC) and prompt cells with a more powerful and efficient aerobic machinery.

2.2 Mitochondrial Biogenesis

As stated before, endurance training is associated with an increase of the mitochondrial content (Hood et al. 2016). Tissues respond to perturbations in the energy status, adjusting the mechanisms of ATP production to meet the energy demand. Among these mechanisms, the increase in mitochondrial content and oxidative phosphorylation rate are central to the metabolic plasticity. The expansion of the mitochondrial reticulum occurs through the induction of mitochondrial biogenesis. The first evidence of exercise-induced mitochondrial biogenesis was suggested by John Holloszy in 1967, when he observed a significant increase of cytochrome c content in skeletal muscle of trained rats (Holloszy 1967). Since then, many studies have demonstrated that different modalities of physical exercise can induce mitochondrial biogenesis not only in skeletal muscle and heart, but also in non-contractile tissues, such as brain, liver and adipose tissue (Little et al. 2011). To assess this, several techniques and biomarkers have been developed over the last five decades, either by direct analysis (estimating the organelle volume in relation to total cellular volume) or by indirect evaluation (quantifying the content of mitochondrial proteins, such cytochrome c, or measuring the activity of enzyme markers, of which cytochrome c oxidase and citrate synthase are examples) (Schwerzmann et al. 1989; Larsen et al. 2012).

Accordingly, in the classic papers of Hickson (1981) and Dudley et al. (1982) using a rat model, it was reported that the magnitude of exercise-induced mitochondrial biogenesis was dependent of the intensity, duration and frequency of the exercise sessions, as well as the recruited fibre type. For many years, mitochondrial biogenesis has been associated with endurance training. For instance, Ingjer (1979) reported significantly higher mitochondrial content regardless the fibre type in quadriceps muscle cross-sections of six elite cross-country skiers compared to untrained subjects. Similarly, 6 weeks of endurance training on bicycle ergometers induced a 40% increase in mitochondrial volume/density of *vastus lateralis* (Hoppeler et al. 1985). Increases in mitochondrial enzymes have also been described in response to short-term endurance training programs. For example, 7–10 days of cycle ergometer exercise (2 h/day at 60–70% of $\text{VO}_{2\text{max}}$) resulted in a 30% increase of citrate synthase activity, β -hydroxyacyl-CoA dehydrogenase and carnitine acetyltransferase in both men and women (Rodas et al. 2000). However,

there is growing evidence that other types of training protocols, namely high-intensity interval training (HIIT) can also result in similar responses regarding mitochondrial biogenesis (Burgomaster et al. 2008; Jacobs and Lundby 2013; MacInnis et al. 2016). In addition, a significant amount of data also suggest that physical exercise increases the mitochondrial content in cardiac tissue (Marques-Aleixo et al. 2015b). As suggested above, physical exercise, namely endurance training can also exert systemic effects in an endocrine-like fashion (Fig. 1), therefore inducing mitochondrial biogenesis even in non-contractile tissues (Little et al. 2011). The first evidence of exercise-induced mitochondrial biogenesis in the adipose tissue of rats after 10 weeks of daily swimming came from Stallknecht in 1991 (Stallknecht et al. 1991), which was further confirmed in more recent successive studies (Sutherland et al. 2009; Rocha-Rodrigues et al. 2017). Intriguingly, the evidence for training-induced mitochondrial network adaptations in liver were more controversial. In fact, while some previous data suggested no alteration or even a decrease in the liver mitochondrial content after voluntary physical activity and endurance training (Terblanche et al. 2001; Matiello et al. 2010), more recent studies have clearly shown the opposite (Haase et al. 2011; Santos-Alves et al. 2015). Similarly and despite some controversy (Herbst et al. 2015), physical exercise also seems to stimulate mitochondrial biogenesis in brain (Boveris and Navarro 2008; Dietrich et al. 2008; Safdar et al. 2011; Marques-Aleixo et al. 2015a), although studies are still scarce.

The signalling pathways that mediate the induction of mitochondrial biogenesis by chronic exercise rely in an orchestrated series of events, involving both nuclear and mitochondrial genomes, which begins immediately after the first bout of exercise (Perry et al. 2010). It involves the upregulation of several transcription factors, such as the nuclear respiratory factor 1 (NRF1), the estrogen-related receptor (ERR) and their co-activator PGC-1 α and p53, as well as the incorporation of both mitochondrial and nuclear gene products into the expanding organelle reticulum (Hood et al. 2016). Among the 37 genes encoded by mitochondrial DNA (mtDNA), only 13 encode for proteins of the electron transport chain (Taanman 1999), being the transcription of these genes mediated by the exercise-induced PGC-1 α upregulation (Perry et al. 2010; Scarpulla 2011). On the other hand, the upstream regulation of PGC-1 α by exercise in the skeletal muscle is modulated by signalling kinases that are activated by events generated during the contractile activity, such as cytosolic Ca²⁺, mechanical stress, accumulation of AMP and RONS (reactive oxygen and nitrogen species) (Carter et al. 2015). Among several kinases, p38 MAPK (p38 mitogen-activated protein kinase) and AMPK (AMP-activated protein kinase) are particularly relevant. Elevated cytosolic Ca²⁺ results in CaMKII (Ca²⁺/calmodulin-dependent protein kinase II)-mediated activation of p38 MAPK (Wright et al. 2007), which stimulates upstream transcription factors of PGC-1 α , such as activating transcription factor 2 and myocyte enhancer factor-2 (Puigserver et al. 2001; Cao et al. 2004). On the other hand, the rise of the AMP/ATP ratio mediated by exercise activates AMPK, which can directly phosphorylate and activate PGC-1 α (Jäger et al. 2007). Furthermore, AMPK increases NAD⁺ levels leading to the activation of SIRT1 (Sirtuin 1) (Cantó et al. 2009), a NAD-dependent

deacetylase that also contributes to the activation of both AMPK and PGC-1 α (Ruderman et al. 2010). The sensitivity of cells to such early events explains why mitochondrial biogenesis signalling begins early during the onset of exercise.

The role of PGC-1 α in mitochondrial biogenesis is so prominent that it is usually referred as the master regulator of mitochondrial biogenesis and oxidative capacity (Fig. 2). In fact, the overexpression of PGC-1 α in transgenic mice leads to fibre type transition from fast glycolytic to slow oxidative fibres (Lin et al. 2002), evidencing the crucial role of this transcriptional co-activator on deep mitochondrial metabolic and morphological changes.

Through the described mechanisms, physical exercise leads to the accumulation of mitochondrial and nuclear encoded proteins required for the assembling of the respiratory chain subunits, as well as other proteins necessary for mitochondrial function (Hood et al. 2016). In addition to an increase in oxidative capacity, concomitant with an increase in ETC complexes content, the formation of new mitochondria facilitates the elongation of the mitochondrial network, which has a positive impact in tissue metabolic capacity and overall bioenergetics. Furthermore, new mitochondria are required to replace the damaged fragments, contributing to the maintenance of a healthy and functional mitochondrial reticulum.

Taken together, growing evidence suggest that physical exercise can exerts a positive influence on mitochondrial network of contractile and some non-contractile tissues, although the underlying mechanisms remain to be completely understood.

2.3 Mitochondrial Network Dynamics and Quality Control

As mentioned in previous sections, when targeted by stimuli, mitochondria are highly dynamic organelles that can change their morphology to cope different energy demands or to activate specific signalling pathways. In fact, it has been suggested that fragmented mitochondria are preferentially found under low energy requirements and low respiratory activity, whereas mitochondrial fusion increases under higher energy demanding conditions leading to mitochondrial reticulum elongation, which allows a better transmission of electrogenic events and distribution of oxygen and metabolites (Westermann 2012).

Accordingly, the mitochondrial network seems to be remodelled under the cellular energy stress imposed by physical exercise. It has been extensively described that endurance training induces an expansion of the mitochondrial reticulum (Carter et al. 2015); therefore optimizing mitochondrial capability to deal with cellular bioenergetics needs. However, despite the considerable amount of work reporting increases in mitochondrial content and function induced by exercise (Hood et al. 2016), few studies devoted specific attention to its dynamics, particularly in human subjects. Cartoni et al. (2005) found increased mRNA levels of *MFN1* and *MFN2* (mitofusin 1 and 2, respectively) in muscle biopsies obtained from trained cyclists 24 h after endurance exercise. On the other hand, Perry et al. (2010) reported progressively increased content of specific proteins associated to dynamic-related

signalling processes, namely MFN1, FIS1 (mitochondrial fission 1 protein) and DRP1 (dynamin-related protein 1) after a HIIT program. Similarly, Garnier et al. (2005) described a positive correlation between the training status and the skeletal muscle mRNA levels of *MFN2* and *DRP1* of healthy and diseased humans. These results strongly suggest that different types of exercise can effectively modulate mitochondrial dynamics, increasing both fusion and fission activities. Interestingly, also in the context of physical exercise, both fusion and fission can lead to adequate mitochondrial turnover and result in cellular beneficial adaptations, despite their antagonistic direct implications on mitochondrial network.

In close association with the mechanisms of mitochondrial dynamics, the mitochondrial quality control machinery is also considered a key player in mitochondrial turnover. In fact, among the processes associated with tissue adaptation and survival, mitophagy, which can be activated by increased oxidative stress and damage, encompasses a series of events that ultimately result in the elimination of dysfunctional mitochondria from the network to preserve mitochondrial bioenergetics. In the last few years, some studies, especially performed in rodents, have focused on the effects of exercise in mitochondrial autophagy. Data suggest that both acute and chronic exercise modulate the expression of proteins associated with auto(mito)phagy signalling, such as LC3II, p62 and BNIP3 (Pagano et al. 2014; Vainshtein et al. 2015). Interestingly, Lira et al. (2013) suggested that increased basal mitophagy is necessary for exercise-induced skeletal muscle adaptations, such as mitochondrial biogenesis, and for the improvement of physical performance.

The molecular mechanisms by which exercise modulates the expression of these mitochondrial dynamics and quality control-related proteins have not been completely elucidated. It is clear, however, that PGC-1 α , AMPK, RONS and Ca²⁺ signalling play an important role in the exercise-induced mitochondrial dynamics (Fig. 2). For instance, in addition to its well-known role in mitochondrial biogenesis, PGC-1 α activation induces the expression of MFN2 (Soriano et al. 2006). Furthermore, AMPK also activates ULK1, a crucial protein for the autophagy induction (Egan et al. 2011). On the other hand, Ca²⁺-dependent calcineurin dephosphorylates DRP1 at its serine 637, promoting its activation and translocation to the mitochondrial surface (Cribbs and Strack 2007). Thus, it seems that these processes are regulated by the same signalling pathways that are responsible for most of the known exercise-induced skeletal muscle adaptations, suggesting that mitochondrial morphology, dynamics and quality control are an important part of the orchestrated muscle cell response to contractile activity.

In conclusion, there is growing evidence that exercise has a great impact in the mitochondrial dynamics and quality control, not only as epiphenomena, but due to the intrinsic properties of the mitochondrial network, which plays an important role in cellular bioenergetics, signalling and homeostasis. However, reported data and suggestions are limited to mRNA and protein expression. Therefore, extrapolations from these results should be taken with caution, and the application of more advanced techniques is needed to contrast the current hypothesis.

2.4 Involvement of Mitochondria in Cell Fate

In addition to its central role in energy metabolism, mitochondria are key organelles that also participate in distinct but overlapping pathways involved in cellular death, including apoptosis (for references and details see Wang and Youle 2009) and necrosis (Whelan et al. 2012). Interestingly, endurance training has been shown, particularly in rodent models, to modulate the expression of some proteins usually involved in apoptotic signalling pathways towards a more anti-apoptotic phenotype. For example, 8 weeks of endurance training downregulated *Bax* mRNA and upregulated *Bcl-2* mRNA as well as increased the protein content of ARC (apoptosis repressor with caspases recruitment domain protein) in rat *soleus* muscle (Siu et al. 2004). Similarly, increased BCL-2 and decreased AIF (apoptosis inducing factor) content were found in the *soleus* muscle and left ventricle of voluntary wheel-exercised rats (Vainshtein et al. 2011). Furthermore, H₂O₂-induced increase of BAX, cytochrome c and AIF was ablated in the left ventricle of these exercised animals. In accordance, chronic electrostimulation of *tibialis* anterior rat muscle (3 h/day/7 days) did not induce alterations in the BAX/BCL-2 ratio, although increased ARC content and less cytochrome c release were observed under both basal and stressful (exogenous ROS treatment) conditions (Adhihetty et al. 2006).

In addition to the direct modulation of anti- and pro-apoptotic proteins, exercise-induced adaptations of mitochondrial morphology and dynamics also seem to confer a protective phenotype against apoptosis. It is well-known that fragmented mitochondria are more prone to the release of the cytochrome c (Suen et al. 2008) and that mitochondrial fission machinery proteins, such as DRP1 and FIS1 are involved in programmed cell death (Frank et al. 2001). Thus, exercise-induced increased rate of mitochondrial fusion and concomitant network elongation not only contributes to energy-production efficiency, but also confers a more apoptotic-resistant phenotype. In fact, it has been described that stress-induced degradation of MFN2 leads to mitochondrial fragmentation and enhanced apoptotic cell death (Leboucher et al. 2012). In contrast, exercise-induced upregulation of PGC-1 α seems to indirectly contribute to mitigate this deleterious phenotype. Adhihetty et al. 2007 reported increased apoptotic susceptibility in denervated skeletal muscle, which was associated to reduced PGC-1 α expression and mitochondrial content. These results were further confirmed in a *Pgc-1 α* KO rat model (Adhihetty et al. 2009). Additionally, lack or decrease of PGC-1 α content was associated with increased RONS production, which also contributes to the activation of the apoptotic signalling cascade (Simon et al. 2000). As will be discussed in the next subsection, physical exercise also boosts cellular antioxidant defences and protects against oxidative stress and damage (Radak et al. 2017), therefore contributing to the aforementioned apoptotic-resistant phenotype.

In summary, physical exercise, particularly endurance training, increases resistance against apoptosis through the direct/indirect regulation of anti- and pro-apoptotic proteins, namely by remodelling the mitochondrial reticulum and by decreasing oxidative stress.

2.5 Mitochondria as Active Players in the Regulation of Cell Signalling: Dual Role of RONS and Antioxidants

During the contractile activity, mitochondria not only increase ATP synthesis, but also the production of RONS. For many years, mitochondrial RONS were considered mere harmful by-products of respiration. Surprisingly, Reid et al. (1993) reported that increased doses of antioxidant treatment (superoxide dismutase and catalase) progressively decreased the ability of isolated fibre bundles from rat diaphragm to generate force, which suggested that moderate amounts RONS have a determinant impact on excitation-contraction coupling and are necessary to achieve optimal contractile function. Thereafter, data from several studies reinforced the central involvement of RONS in signalling pathways, including gene transcription, mitochondrial dynamics and activation of apoptosis.

While acute strenuous exercise has been associated with excessive oxidative stress and tissue deleterious consequences, chronic exercise plays an important role in the organism adaptive response to exercise via RONS signalling, including the increase of antioxidant defences. For example, NRF2 (nuclear factor erythroid 2-related factor 2), a master regulator of antioxidant responses, is activated by RONS during physical exercise (Dembele et al. 2009). In fact, NRF2 is consistently shown to be upregulated after exercise in rodents (Gounder et al. 2012) as well as in active humans (Ballmann et al. 2014; Scott et al. 2015). Upon activation, NRF2 regulates the transcription of more than 200 genes, including genes encoding glutathione reductase, glutathione peroxidase and NADPH oxidase. Similarly, RONS-mediated activation of forkhead-box O (FOXO) stimulates the transcription of genes coding for antioxidant enzymes, such as superoxide dismutase-2 (SOD2) and catalase under exercise conditions (Kavazis et al. 2014; Klotz et al. 2015). In addition, factor nuclear kappa B (NFκB) has also been shown to be stimulated by exercise-derived RONS, therefore activating the transcription of antioxidant-related genes and increasing the expression of manganese-dependent superoxide dismutase (MnSOD), inducible nitric oxide synthase and glutathione (Morgan and Liu 2011). Thus, exercise-derived mitochondrial RONS production exhibit a dose-dependent hormetic behaviour, being high doses associated to cellular deleterious effects and adequate amounts related to beneficial and adaptive responses that can protect from following oxidative insults.

In addition to this autoregulatory behaviour, RONS also mediate signalling pathways involved in mitochondrial network remodelling and quality control, such as mitochondrial biogenesis, fission and mitophagy. Accordingly, RONS stimulate the activation and transcription of PGC-1α through p38 MAPK and AMPK (Kang et al. 2009), whereas high-doses of antioxidant supplementation seems to ablate the exercise-induced upregulation of PGC-1α, NRF1 and TFAM (mitochondrial transcription factor A) as well as the concomitant increase in mitochondrial content (Gomez-Cabrera et al. 2008). Similarly, vitamin C and vitamin E supplementation blocked the exercise-dependent upregulation of PGC-1α, SOD1 and SOD2, glutathione peroxidase preventing health-promoting effects of physical exercise in

humans (Ristow et al. 2009). As multiple studies demonstrated that exacerbated mitochondrial RONS production positively correlates with mitochondrial fission and fragmentation (Yu et al. 2006; Jendrach et al. 2008), therefore stimulating apoptotic signalling, the exercise-induced protective effects against oxidative stress could also contribute to the anti-apoptotic phenotype in trained subjects.

Altogether, these data show that, within a physiological range, chronic exercise-derived mitochondrial RONS are necessary for the cellular adaptive response against oxidative stress as well as for the regulation of several mitochondrial pathways.

3 Physical Exercise Antagonizes Mitochondriopathies Characterizing Aging and Diseased Conditions

Considering physical exercise as an important stimulus that targets and boosts several mitochondrial features, including those related to morphology, biochemistry and function, it is clearly expected that it may represent a very interesting tool for abrogating the harmful consequences associated with the physiopathology of aging and disease. In fact, assuming that most of the previously referred mechanisms associated with cellular and tissue dysfunction in disease, such as exacerbated inflammatory response, increased oxidative stress and damage, apoptosis, altered auto(mitophagy), mitochondrial dynamics and quality control are also regulated by physical exercise, in this subsection we propose to analyse the cross-tolerance effects of different types of exercise against the mitochondriopathies characterizing aging and some diseased conditions. These include cardiac dysfunction induced by ischaemia-reperfusion (IR), doxorubicin (DOX) treatment and hyperglycaemia, liver diseases such as NAFLD (non-alcoholic fat liver diseases) or induced by anti-inflammatory drugs, neurodegenerative diseases and obesity-induced adiposopathy. We here intended to discuss the potential of physical exercise (preventive and/or therapeutic) against these deleterious effects in several features of mitochondrial physiology, and also to address the potential implications of the epigenetic regulation on exercise-induced mitochondrial adaptations.

3.1 Aging

It is sufficiently clear that senescence causes deterioration of cellular function in all tissues, increasing their susceptibility to dysfunction and disease. Also, among the numerous theories associated with the aetiology and pathophysiology of this process is the increased oxidative stress (Dröge 2002), in which mitochondria have a recognized role as a source and target of the generated RONS. With increasing age, mitochondrial function suffers alterations that predispose them to enhance production of RONS, mainly at the level of the respiratory chain. As known, the interaction of these species with other molecules composing the phospholipidic

structure of membranes, proteins and nucleic acids of DNA that lack protective histones, causes damage and consequent dysfunction, phenomena that are further exacerbated by the diminished antioxidant capacity of aged cells. As physical exercise, particularly the chronic forms, is known to decrease the levels of oxidative stress by both decreasing RONS production and boosting antioxidant capacity (through the increase of inducible scavenging molecules) at several levels of tissue and cell organization, including in mitochondria, we will further discuss in brief some examples of how chronic physical exercise mitigates the increased aging-induced organ and tissue dysfunction/damage by targeting mitochondria.

For instance, in addition to the exercise-induced benefits against the incompetent autonomic activity in aged hearts through re-sensitization of β -adrenergic receptors, improvements in the diminished calcium handling and hypertrophy, as well as in the increased fibrosis, exercise also restores/attenuates the compromised mitochondrial function characterizing senescence (Roh et al. 2016). These ameliorations were seen through increased mitochondrial respiration and decreased RONS production (Bo et al. 2014), increased PGC-1 α expression and associated signalling pathways (Leick et al. 2010; Bayod et al. 2012; Kang et al. 2013), mitochondrial dynamics and quality control factors (Koltai et al. 2012) and mitochondrial DNA (Safdar et al. 2011) in aged animals submitted to chronic exercise programs. It is proposed that exercise enhances antioxidant defences and restores redox homeostasis in aging tissues, at least in part, via NRF2, activating antioxidant response genes (Gounder et al. 2012). NRF2 is also co-activated by PGC-1 α under oxidative stress conditions as PGC-1 α induces GPx1 (Glutathione peroxidase 1) and SOD2 in models of neurodegeneration (St-Pierre et al. 2006). It was recently suggested that exercise training in old mice improves brain mitochondrial capacity by targeting electron transport chain function and mitochondrial dynamics without affecting mitochondrial biogenesis (Gusdon et al. 2017).

Age-related sarcopenia and muscle dysfunction in humans were antagonized by 9 weeks of either neuromuscular electrical stimulation or leg press, being the improved muscle function and structure induced by training stimulations associated to increased protein levels of mitochondrial calcium uniporter and of fusion protein OPA1 (optic atrophy 1) (Zampieri et al. 2016). Also, other results indicate that the exercise training-related functional improvements found in aged muscles were associated with changes in mitochondrial proteins involved in redox and calcium homeostasis, bioenergetics, remodelling and dynamics (Figueiredo et al. 2009; Alves et al. 2010; White et al. 2016).

To further unravel evidence for a mitochondrial polymerase γ -independent mtDNA repair pathway mediated by exercise, Safdar et al. (2015) reported that the tumour suppressor protein p53 translocates to mitochondria and facilitates mtDNA mutation repair and mitochondrial biogenesis in response to endurance training of moderate intensity. Corroborating these data, genetic-based approaches showed that exercise failed to prevent mtDNA mutations, to induce mitochondrial biogenesis, to preserve mitochondrial morphology, to reverse sarcopenia, or to mitigate premature mortality in mutator mice with muscle-specific deletion of p53. In addition to previous research from David Hood's laboratory suggesting mitochondrial p53 as

an important mediator, although not necessary, for mitochondrial adaptations induced by voluntary activity, including biogenesis (Saleem et al. 2009), and demonstrating that acute endurance exercise induce p53 translocation to mitochondria where it may serve to positively modulate the activity of TFAM (Saleem and Hood 2013), the results from Safdar et al. (2015) established p53 as a novel target for exercise-mediated maintenance of the mtDNA genome and suggest mitochondrially targeted p53 as a therapeutic mechanism counteracting aging and diseases of mitochondrial aetiology. Detailed and comprehensive reviews on the known mechanisms of how exercise mitigates mitochondrial senescence, particularly in skeletal muscle are available (Carter et al. 2015; Joseph et al. 2016).

3.2 Cardiac Dysfunction

The direct important role of mitochondrial physiology abnormalities in heart failure is attested by many studies, justifying a recent consensus report. In fact, experts in the field recently pointed out that, despite the pathophysiology of heart failure-associated diseases is complex, mitochondrial dysfunction seems to be a crucial therapeutic target to improve cardiac function (Brown et al. 2016). The emerging treatments with the potential to improve the function of the failing heart by targeting mitochondria include many pharmacological approaches, being physical exercise probably the most effective non-pharmacological countermeasure. In fact, several studies demonstrated that distinct forms of physical exercise, including acute single pre-conditioning bout, short and long-term endurance training, intermittent exercise and HIIT as well as voluntary physical activity mitigate (by preventing and/or treating) the deleterious effects characterizing cardiac diseases and toxicity, targeting mitochondria in the adaptive process (for references see Ascensão et al. 2007, 2011a, b; Powers et al. 2014a, b). Considering IR injury as a model of cardiac damage, against which exercise has shown to be protective, several mechanisms and related candidate proteins have been described to contribute to the more resistant phenotype induced by exercise rescuing several harmful effects of IR. These include the improvement in mitochondrial antioxidant and anti-apoptotic signalling profile (Ascensão et al. 2005b; Kavazis et al. 2008, 2009; Magalhães et al. 2013), adaptations in proteins known to be involved in the composition and/or sensitizing the mtPTP (mitochondrial permeability transition pore) (Lumini-Oliveira et al. 2011; Magalhães et al. 2014), probably leading to increased capacity of cardiac mitochondria to tolerate calcium (Marcil et al. 2005; Ciminelli et al. 2006) and, despite controversy (Brown et al. 2005), the inhibition of mitochondrial K_{ATP} channels (Quindry et al. 2010). The mentioned positive adaptations observed in trained animals, which correlate with improved haemodynamic endpoints, also translate into boosted mitochondrial respiratory performance (Leichtweis et al. 1997; Ascensão et al. 2006a, b; Lee et al. 2012; Alleman et al. 2016), meaning that, among other performance features, cardiac tissue is more capable to oxidize substrates and to produce ATP. It was also recently reported that regular treadmill exercise inhibits mitochondrial

accumulation of cholesterol and oxysterols during myocardial IR in wild-type and *ob/ob* mice, concomitantly improving oxidative phosphorylation (Musman et al. 2016).

The protective mitochondrial phenotype induced by physical exercise against the damage caused by IR events is also evident against DOX exposure (Ascensão et al. 2012b), a very effective anticancer agent against several malignancies, but limited by the dose dependent dilated cardiomyopathy. Data from our group and others revealed the benefits of acute and chronic exercise, as well as of voluntary physical activity, against the deterioration of functional capacity and structure of hearts from animals exposed to acute single bolus and sub-chronic dosages of DOX (Ascensão et al. 2005a, 2006a, 2011b, Chicco et al. 2005, 2006; Marques-Aleixo et al. 2015b). Similarly to IR, these ameliorations in hearts/mitochondria from DOX treated animals were accompanied by favourable adaptations in several molecular markers related to antioxidant capacity, calcium-induced mtPTP opening, mitochondrial biogenesis and dynamics as well as quality control and apoptotic signalling (Kavazis et al. 2010; Dolinsky et al. 2013; Marques-Aleixo et al. 2015b). Actually, exercise-induced cardioprotection could also be associated with an improvement of mitochondrial quality control (Campos et al. 2012; Sun et al. 2013). Exercise increases cardiac mitochondria renewal and remodelling by promoting mitochondrial biogenesis, fusion, and auto(mito)phagy in order to eliminate damaged mitochondria (Sun et al. 2013). Together with redox-related and anti-apoptotic adaptations, the mitochondrial network adjustments provided by chronic physical exercise can be critical in the attenuation or reversion of DOX-induced cardiac and mitochondrial toxicity (Dolinsky et al. 2013; Smuder et al. 2013).

3.3 Liver Diseases-Induced Toxicity

It is likewise known that liver mitochondria are structurally and functionally affected by several deleterious conditions that include obesity and high-fat diet-induced NAFLD as well as by exposure to toxicants, such as anti-inflammatory drugs (Fromenty et al. 2004; Wei et al. 2008; Pessayre et al. 2010) and that increased aerobic fitness and physical exercise represent valuable counteracting tools against the resulting deleterious effects (Rector and Thyfault 2011; Gonçalves et al. 2013; Ascensão et al. 2013). Thyfault et al. (2009) clearly showed that rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and increased susceptibility to hepatic steatosis and injury compared to high aerobic capacity animals. Moreover, accumulating evidence agrees that increased voluntary physical activity, but particularly endurance training, is able to positively modify liver tissue and mitochondrial phenotypes from diseased or injured conditions towards more resistant ones. Accordingly, several works from our lab demonstrated that mitochondrial features and endpoints from wild-type animals (Santos-Alves et al. 2015) and rats exposed to high-fat diet induced non-alcoholic steatohepatitis (Gonçalves et al. 2014a, b, 2016) or *in vitro* anti-inflammatory drugs (Ascensão et al. 2012a;

Santos-Alves et al. 2014) were positively modulated by exercise/increased physical activity. These features include histopathological hallmarks, mitochondrial membrane phospholipidic composition, *in vitro* respiratory capacity, transmembrane potential fluctuations, calcium resistance-dependent mtPTP and apoptotic signalling, oxidative stress and damage markers, mitochondrial dynamics comprising biogenesis, fusion and fission, as well as alterations in (auto)mitophagy signalling molecules, closely related to quality control mechanisms.

3.4 Obesity-Induced Adiposopathy

While in conditions of obesity and insulin resistance mitochondrial reticulum in adipose tissue is clearly compromised; physical exercise is proposed to increase mitochondrial content (Townsend et al. 2017), thus augmenting the metabolic activity of this tissue and contributing to important energy expenditure and functional health benefits. These physical exercise-related adaptations seem to be based, at least in part, on the lipolysis-dependent increased AMPK-PGC-1 α and consequent mitochondrial biogenesis. In fact, exercise stimulates AMPK, PGC-1 α and the overexpression of UCP1 (uncoupling protein 1), important hallmark of adipose tissue browning (Boström et al. 2012; Mottillo et al. 2016). Additionally, despite some controversy (Norheim et al. 2014) it has been suggested that exercise prompts the secretion of catecholamines, and several myokines, such as FNDC5, IL-6 or meteorin-like, which seem to be important contributors to mitochondrial-associated “beiging/browning” of white adipose tissue (Stanford et al. 2015; Ost et al. 2016). Accordingly, recent data from our group reported that physical exercise modulated muscle and adipose tissue myokines expression and brown adipose-like phenotype in rats fed a high-fat diet, and improved epididymal white adipose tissue mitochondrial complex subunits as well as markers of mitochondrial biogenesis and dynamics (Rocha-Rodrigues et al. 2016, 2017). Chronic exercise-induced adipose tissue mitochondrial biogenesis is known to occur in a eNOS-dependent manner as exercise increased mitochondrial DNA, mitochondrial enzymes, and expression of NRF1, TFAM and PGC-1 α in subcutaneous adipose tissue in wild type, which was not evident in eNOS null animals (Trevellin et al. 2014). These changes occurring in adipose tissue phenotype in response to exercise training may be part of the mechanism by which exercise improves whole-body metabolic health, despite human studies failed to observe increased adipose tissue mitochondrial content and functional capacity to oxidize fat after high-intensity training (Larsen et al. 2015).

3.5 Neurodegenerative Diseases

Despite some controversy (Herbst et al. 2015), regular physical activity and exercise may also play an important role in the modulation of brain mitochondrial machinery (Marques-Aleixo et al. 2015a), thus mitigating mitochondrial malfunction

characterizing aging and neurodegenerative diseases (Marques-Aleixo et al. 2012; Bernardo et al. 2016). In accordance, cross-tolerance studies have been designed to test physical exercise rescues mitochondrial defects characterizing several pathophysiological conditions as follows. Recently, Gusdon et al. (2017) reported that endurance training increased brain cortex mitochondrial complex I activity and the expression of DRP1 in aged mice, confirming early results pointing out training-induced increased mitochondrial biogenesis signalling markers in most of the brain regions (Steiner et al. 2011). Reinforcing the role of mitochondrial mediating mechanisms and target therapies in neurodegenerative diseases, Cheng et al. (2016) found that neurons lacking the mitochondrial deacetylase SIRT3 were more vulnerable to dysfunction and degeneration in mouse models of epilepsy and Huntington's disease. In contrast, the running wheel-induced hippocampal SIRT3 overexpression modulated mitochondrial protein acetylation and bolster neuronal resistance to oxidative stress and apoptosis. Moreover, long-term endurance training significantly improved the cognitive function and reduced the expression of amyloid β -42 peptide in APP/PS1 transgenic mouse model of Alzheimer Disease (Bo et al. 2014). Concomitantly, improved mitochondrial respiratory function by increasing the complexes I, and IV and ATP synthase activities, whereas attenuated ROS generation and mtDNA oxidative damage were observed in APP/PS1 mice. Furthermore, the impaired mitochondrial antioxidant enzymes and mitochondrial 8-oxoguanine DNA glycosylase-1 (OGG1) activities seen in the transgenic mice were reversed with training. Also, the acetylation levels of mitochondrial OGG1 and MnSOD were markedly suppressed in transgenic mice after exercise training, in parallel with increased content of SIRT3.

In addition to senescence and neurodegenerative diseases-associated mitochondrial defects, exercise has also been effective in counteracting toxicants-induced brain mitochondrial dysfunction and related mechanisms. For instance, brain cortex and cerebellum mitochondrial modifications associated with cumulative subchronic DOX administration, including perturbations in mitochondrial oxidative phosphorylation capacity, increased oxidative stress and damage, augmented mPTP opening susceptibility and apoptotic signalling and deregulation of mitochondrial quality control were positively modulated by long-term physical exercise performed before and during treatments (Marques-Aleixo et al. 2016). Moreover, 3,4-methylenedioxymethamphetamine (MDMA)-induced impairments in cognitive and brain mitochondrial function, namely in acquisition and retention of spatial memory, RONS production, mitochondrial membrane potential, mitochondrial swelling, mitochondrial outer membrane damage, cytochrome c release, and ADP/ATP ratio, were mitigated by physical exercise (Taghizadeh et al. 2016).

4 Is There a Role for Epigenetic Modifications in Exercise-Targeting Mitochondrial Function?

Some of the favourable effects associated with physical exercise are mediated by alterations in gene expression, which are in part orchestrated by epigenetic modifications to DNA (histone modifications and DNA methylation) and by the

expression/repression of non-coding miRNAs (McGee et al. 2009; Denham et al. 2014; Jiménez-Chillarón et al. 2015). Through these processes, physical exercise regulates the expression of genes associated to a myriad of cellular events, such as metabolic and inflammatory processes, aging, cancer, and central nervous system degeneration. Reported regulated genes include *PGC-1 α* , pyruvate dehydrogenase lipoamide kinase isozyme 4, apoptosis-associated speck-like protein containing a CARD, and BDNF (Pareja-Galeano et al. 2014). It has been proposed that physical exercise regulates the epigenetic landscape indirectly through a metabolic remodeling, involving alterations in the production of TCA cycle intermediates that are involved in epigenetic engineering (Donovan and Miller 2011). Moreover, Barrès et al. (2012) showed that high-intensity acute exercise decreased human skeletal muscle DNA methylation and increased the expression of the respective genes associated to mitochondrial biogenesis, such as *PGC-1 α* and *TFAM*.

Whether exercise-induced epigenetic modifications can be transmitted to subsequent generations and cause a persistent modulation of gene expression with consequences at mitochondrial level remains questionable. It is known that physical exercise performed during pregnancy improves long-term health of the offspring in later life through increased mitochondrial fitness in brains and hearts. Actually, Chung et al. (2017) recently reported that maternal exercise up-regulated genes encoding proteins associated with mitochondrial dynamics, namely NRF1, NRF2 and MFN1 expression, and increased ATP production and cytochrome c oxidase activity while decreased H₂O₂ levels on foetal mouse hearts. Suggestions exist that maternal exercise alters mitochondrial enzymatic activity, improves mitochondrial antioxidant defence capacity and markers of mitochondrial biogenesis in brains of the offspring (Park et al. 2013; Marcelino et al. 2013). Moreover, physical exercise performed before pregnancy improves indices of embryos development, which were accompanied by increases in ATP levels, mitochondrial membrane potential and mtDNA copy number, and a decrease in H₂O₂ concentration (Xu et al. 2016). In this study, methylation levels of CpG sites on imprinting control regions of imprinted genes (*Igf2*, *Igf2r*, *Meg 3* and *H19*) in the embryos of exercised groups were normalized when compared to those of the non-exercised groups. Additionally, global DNA and histone methylation (H3K4m2, H3K9m3 and H3K36m) confirmed that physical activity in pre-pregnancy facilitates the maintenance of epigenetic modifications (Xu et al. 2016). Six weeks of voluntary wheel running prior to and during pregnancy prevented maternal high-fat diet-induced skeletal muscle *Pgc-1 α* promoter hypermethylation and increased its expression as well as its target genes, an effect that correlated with an improvement of age-associated metabolic dysfunction observed at 9 months of age in the offspring (Laker et al. 2014).

In addition to transcription factors activity, DNA methylation and histone modification, the influence of physical exercise on transcription and translation processes is also related to the expression of miRNAs (Kirby and McCarthy 2013; Russell and Lamon 2015). Despite the recognition that the findings to date, supporting a role for miRNAs in muscle plasticity, remain for the most part correlative, several studies reported that endurance training-modulated animal and human miRNAs (miR-494, miR-696 and miR-761) predicted to target genes that control mitochondrial biogenesis, including *PGC-1 α* , *TFAM* and *FOXJ3* (Aoi et al. 2010; Yamamoto et al. 2012; Xu et al. 2015).

5 Conclusions

In summary, from the literature consulted, it is clear that increased physical activity and endurance training boost skeletal and cardiac muscles, liver, brain and adipose tissues, increasing its resistance to aging and disease-induced injury by targeting mitochondrial network physiology and related mechanisms. Among the different mechanisms by which physical exercise improves molecular, functional and morphologic mitochondrial features, it is tempted to state that epigenetics has also an important and emergent role.

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¹Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain.

²Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal.

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



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REVIEW ARTICLE

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Building-up fit muscles for the future

Transgenerational programming of skeletal muscle through physical exercise

Jorge Beleza¹ | Jelena Stevanović-Silva² | Pedro Coxito² | Rui Carlos Costa³ |
António Ascensão² | Joan Ramon Torrella¹ | José Magalhães²

¹Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

²Laboratory of Metabolism and Exercise (LaMetEx), Faculty of Sport, Research Centre in Physical Activity, Health and Leisure (CIAFEL), University of Porto, Porto, Portugal

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

Correspondence

Jorge Beleza, Secció de Fisiologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain. Email: jmsbeleza@gmail.com

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Abstract

'Special issue - In Utero and Early Life Programming of Aging and Disease'. Skeletal muscle (SM) adaptations to physical exercise (PE) have been extensively studied due, not only to the relevance of its in situ plasticity, but also to the SM endocrine-like effects in noncontractile tissues, such as brain, liver or adipocytes. Regular PE has been considered a pleiotropic nonpharmacological strategy to prevent and counteract the deleterious consequences of several metabolic, cardiovascular, oncological and neurodegenerative disorders. Additionally, PE performed by parents seems to have a direct impact in the offspring through the transgenerational programming of different tissues, such as SM. In fact, SM offspring programming mechanisms seems to be orchestrated, at least in part, by epigenetic machinery conditioning transcriptional or post-transcriptional processes. Ultimately, PE performed in the early in life is also a critical window of opportunity to positively modulate the juvenile and adult phenotype. Parental PE has a positive impact in several health-related offspring outcomes, such as SM metabolism, differentiation, morphology and ultimately in offspring exercise volition and endurance. Also, early-life PE counteracts conceptional-related adverse effects and induces long-lasting healthy benefits throughout adulthood. Additionally, epigenetics mechanisms seem to play a key role in the PE-induced SM adaptations. Despite the undoubtedly positive role of parental and early-life PE on SM phenotype, a strong research effort is still needed to better understand the mechanisms that positively regulate PE-induced SM programming.

KEYWORDS

early-life exercise, epigenetics, maternal exercise, offspring, paternal exercise

1 | INTRODUCTION

The skeletal muscle (SM) is a tissue primarily responsible for the force production and movement in the human body representing approximately 40% of total body weight.¹ Nowadays, SM is referred as a very specialized tissue with remarkable

plasticity,² therefore, one of the most studied tissues regarding physical exercise (PE) adaptations.³

Physical exercise imposes muscle stress that can disrupt homeostasis during the exercise and recovery periods.⁴ To cope with this physiological challenge, acute and chronic muscle structural, functional and metabolic adaptations occur.² Ultimately, alterations in key molecular pathways

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impact tissue phenotype preparing muscle towards similar future events and improving performance.¹ The impact of PE on muscle plasticity may be expressed as changes in muscle endurance and strength.² However, muscle adaptations are related to PE dose-response control such as frequency, intensity, time, type, volume and progression (the so-called FITT-VP principle).⁵

Considering the crucial mitochondrial role in several cellular metabolic processes, a central point in the improvement of SM 'fitness' is the remodelling of mitochondrial network.³ These adaptations allow the muscle cells to be more functionally efficient with positive repercussions on SM phenotype and performance.⁶ In addition, PE-induced SM contractile activity has pleiotropic repercussions on noncontractile tissues,⁶ being this SM endocrine-like effect mediated by several proteins secreted to the bloodstream, the so-called myokines.^{6,7} Therefore, PE is consensually recognized as an important preventive and/or therapeutic nonpharmacological intervention able to mitigate the development or the deleterious effects of a wide range of metabolic, cardiovascular, oncological and neurodegenerative diseases.⁸

Recently, parental PE has also been proposed as having a positive role in the modulation of the offspring phenotype,^{9,10} such as in SM, with consequences in their fitness levels.¹¹ In fact, besides maternal exercise (ME) performed during pregnancy,¹²⁻¹⁶ paternal exercise before conception has also been recently associated with healthy-related outcomes¹⁷⁻²⁰ highlighting the role of PE as a relevant strategy in transgenerational programming of the future generations. Furthermore, PE performed early in life also seems to mitigate adverse constraints inherited from gestational environment and prompt the juvenile with long-lasting healthy benefits throughout adulthood.²¹ Apart from other mechanistic insights, these PE-related adaptations seem to be orchestrated by epigenetic signalling that fine-tune gene expression or silencing under the control of transient or stable transcriptional or post-transcriptional mechanisms.^{22,23}

The aim of this review is to highlight the updated knowledge regarding the role of transgenerational programming and early-life healthy behaviours mediated by PE in the modulation of SM phenotype.

2 | MATERNAL EXERCISE IMPACT IN TRANSGENERATIONAL PROGRAMMING OF SKELETAL MUSCLE OFFSPRING

Discoveries in the field of the next generation's health and early-life environmental exposure shed light on the concept of developmental origins of health and disease. This concept helps to understand the sensitivity of an organism to certain environmental insults during critical periods of development,

such as foetal and perinatal period, that may affect the offspring risk for chronic diseases in a short- or long-term manner.^{21,24,25} An adverse in utero environment (ie, maternal malnutrition, infections, or metabolic and hormonal disorders) might trigger foetal and/or perinatal adaptive responses, leading to persistent physiological and metabolic alterations.²⁶

Different studies using either maternal nutrient restriction^{25,27,28} or increased high-fat diet (HFD) consumption in rodents²⁹⁻³¹ have shown impaired glucose regulation and altered insulin sensitivity in the adult offspring. Moreover, evidence suggests that adverse maternal nutrition during the sensitive periods of pregnancy and lactation can also influence the offspring predisposition to develop distinct pathological conditions, including diabetes,³² nonalcoholic fatty liver disease^{16,33} and obesity later in life.²⁴

During pregnancy, ME may represent a cue by which foetus will cope with a hostile in utero environment (Figure 1). In fact, the benefits of active lifestyle during pregnancy, particularly ME, have been reported not only in mothers, but also in their children.^{34,35} Data from human and animal model studies, demonstrated that ME induced maternal benefits including reduced body weight, improved cardiovascular function and reduced risk for gestational hypertension and hyperglycemia, preeclampsia and cesarean section,^{10,15} whereas the offspring of exercised mothers have reduced risk of excessive body weight and fat mass, as well as improved long-term glucose homeostasis.^{10,14,15,36} Moreover, ME can also improve brain metabolism in the offspring and prevent neurodegenerative-related neurotoxicity and cognitive impairment.³⁷ Interestingly, as will be discussed below, ME can also prevent female offspring insulin resistance in the SM imposed by paternal obesity.¹³ These aforementioned studies highlight the beneficial role of exercise before and/or during pregnancy in offspring short- or long-term health.

The SM has a significant relative 'weight' in whole-body mass, an incontestable structural and physiological relevance. It also plays a central role as an endocrine-type stimulator of distinct signalling pathways in several other tissues preventing chronic noncommunicable diseases, namely during the exercise and post-exercise recovery periods.^{1,4,7} Therefore, conditions that impose constraints to SM normal development or induce degeneration may have a significant negative impact on the organism throughout life, and even in the healthy status of the next generations.

Accordingly, maternal HFD consumption seems to have a deleterious consequence on offspring SM development,^{11,38,39} being associated with reduced gene expression of the specific transcription factor myogenic differentiation 1 (MYOD1) in *gastrocnemius*.⁴⁰ Downregulation of MYOD1, the master regulator of SM differentiation, leads to a reduced number of myogenic cells and may be related to impaired SM development.⁴⁰ In line, Bayol et al²⁹ showed

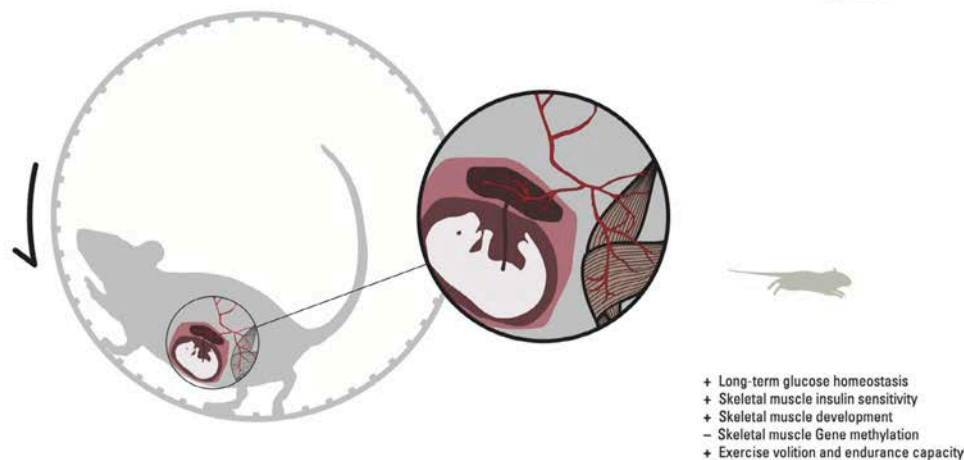


FIGURE 1 Having an active lifestyle and perform exercise during pregnancy induces numerous beneficial health-related outcomes in the offspring generation. Under these 'activated' conditions and/or during the post-exercise recovery periods, a skeletal muscle endocrine-like effect mediated by the muscle contractile activity stimulates the release of several muscle-derived molecules (myokines). Once in the bloodstream, some of these myokines target placenta modulating placental-related physiology, such as placental vascularization¹⁰¹ or are 'transferred' to the foetus circulation promoting the activation of several positive downstream signalling pathways. Among other putative mechanisms described in the text, this may represent one of the molecular pathways that positively modulates the offspring towards a healthier phenotype. Ultimately, the triggering of these signalling cascades induced by maternal exercise, via 'skeletal muscle-derived activation', promotes several valuable molecular, physiological and functional outcomes in the offspring, such as increased skeletal muscle insulin sensitivity with long-term positive consequences on glucose homeostasis, favourable skeletal muscle development and diminished level of several promoters' gene methylation that increases offspring's exercise volition and endurance capacity

that offspring sired by HFD-fed mothers exhibited a 25% reduction in *semitendinosus* muscle cross-sectional area and approximately 20% fewer muscle fibres. Moreover, decreases in capillary density, fibre cross-sectional area and type-IIa muscle fibres mRNA expression has been reported upon maternal HFD consumption, in *gastrocnemius* muscle,¹¹ with negative consequences on offspring muscle force production.^{11,41} Furthermore, maternal HFD consumption induces alterations in SM mitochondrial physiology through a decrease in mRNA expression of several biomarkers of mitochondrial biogenesis and dynamics (peroxisome proliferator-activated receptor- γ coactivator-1 α , PGC-1 α ; nuclear respiratory factor 1 and mitochondrial transcription factor A, TFAM),^{11,42} associated with a downregulation of nuclear-encoded proteins linked to oxidative phosphorylation.⁴³

As SM also play a key role in systemic insulin-sensitivity and glucose uptake,⁴ SM offspring-related metabolic impairments have been associated with deregulated parental nutritional regimens. In fact, maternal HFD-feeding lead to impairments in SM insulin-signalling,^{29,41} glucose and lipid metabolism,³⁸ associated with reduced mRNA expression and content of glucose transporter type 1 and 4, fatty acid synthase and PGC-1 α .^{38,40,44,45} Furthermore, maternal HFD

consumption seems to impair exercise performance in offspring as well as training efficiency early in life.⁴⁵

Bearing in mind the consensual health-related role of PE,⁷ ME has been proposed as a nonpharmacological strategy to counteract or attenuate morphology, function or metabolism downregulation in offspring induced by parental malnutrition.^{10,46} However, as suggested before, even though considerable amount of data from several tissues, particularly liver,¹⁶ adipose tissue,⁴⁷ brain³⁷ and pancreas⁴⁸ has been published, recent research has also been devoted to the impact of ME on offspring's SM.^{11,40} Regarding the development of SM in the offspring of HFD-fed mothers, Raipuria et al⁴⁰ reported that ME reverted HFD-related *gastrocnemius* MYOD1 downregulation in male offspring. Moreover, *gastrocnemius* weight, fibre cross-sectional area, type-IIa muscle fibres mRNA expression and concomitant maximal strength increased in offspring sired from exercised mothers.¹¹

The metabolic mechanisms underlying ME effects on offspring glucose tolerance and insulin-sensitivity in SM have also been studied in different rodent models. Albeit there are discrepancies among these studies due to the use of different strains of mice^{31,36} and rats,^{12,49,50} and different durations and modalities^{13,31,36} of ME, in general, data suggest that ME before and during pregnancy improves glucose tolerance and

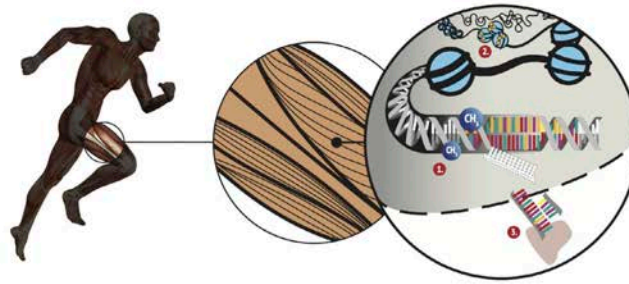


FIGURE 2 In the context of physical exercise, several biochemical and mechanical factors have been associated with the positive responsiveness of skeletal muscle towards a more 'fit' phenotype. Recently, epigenetic-related mechanisms have also been added to this myriad of orchestrated factors that mediate skeletal muscle gene expression in response to physical exercise. In fact, the levels of promoter's DNA methylation (1), post-translational modification of histone proteins (2) and altered microRNA post-transcriptional regulation (3) are considered putative molecular mechanisms that may impact the ability of skeletal muscle to adapt in response to physical exercise or, on the other hand, to mediate downstream regulatory adaptations on non-contractile tissues

insulin sensitivity in the offspring SM.^{9,13,36,49,50} Regarding the different exercise models, although free-running wheel (FRW) has been associated with clear SM-related benefits in health animals,⁵¹ the absence of intensity control may be one the reasons associated with the variability and distinct outcomes described in studies involving metabolic constraints.

Alternatively, treadmill running is a well-established model used during pregnancy to control PE load.^{13,52} In fact, the intensity of exercise performed before and during gestation seems to play a key role in the offspring phenotypic adaptations. Low-intensity treadmill running exercise (55% of maximal aerobic speed) for 4 weeks before and during gestation improved SM glucose tolerance and insulin sensitivity in young male offspring.^{10,46,52} Additionally, Falcão-Tebas et al¹³ showed that maternal treadmill exercise before (60%–75% of VO_{2max}) and during pregnancy (65%–50% of VO_{2max}) increased glucose transporter type 4 protein expression in the offspring, which is in line with some previous studies even when FRW was used,⁴⁰ and may explain, at least in part, the attenuated lower insulin-stimulated glucose uptake in offspring sired by obese fathers. Moreover, citrate synthase activity, a marker of SM mitochondrial content, increased and reactive oxygen species production was normalized in *gastrocnemius* from female offspring, despite the lack of changes in the expression of mitochondrial biogenesis markers, such as PGC1 α and TFAM, which contrasts with data reporting increased mRNA expression of PGC1 α using a FRW model.⁴⁰

Taking into account the positive impact of ME in the offspring SM metabolic phenotype, improvement in exercise volition and performance would also be expected. In contrast with data reporting that maternal HFD reduced fatty acid synthase and glucose transporter type 1 gene expression, together with offspring impairment of exercise performance,⁴⁵ to our knowledge, only one study reported

that ME performed before and during pregnancy positively modulated the propensity of the offspring for PE.⁵³ In fact, female offspring volition for exercise in the FRW was higher during different time points throughout life when compared with offspring from sedentary mothers. Considering the positive health-related outcomes of the fitness levels throughout life,⁵⁴ additional studies exploring offspring exercise ability, particularly maximal aerobic performance, could be of high interest to support the effectiveness of SM-related transgenerational programming of fitness in offspring sired from exercised mothers. In fact, only a very recent study by Son et al¹¹ reported that gestational exercise improved endurance capacity in the offspring.

Taken together, data suggest that ME may have a vital role in offspring's SM programming. However, more studies are necessary to identify the optimal ME training regimens and to fully elucidate the mechanisms responsible for the improvement in offspring SM responses to ME.

3 | EPIGENETIC MECHANISMS ASSOCIATED WITH SKELETAL MUSCLE ADAPTATION TO PHYSICAL EXERCISE

The regulation of gene expression is a fundamental process that establishes and impacts the phenotype induced by different environmental stimuli, including PE, in the different tissues.^{55,56} Nevertheless, although cells from an organism share identical DNA, its expression may be driven by distinct epigenetic-related influences, such as DNA methylation, histone modifications and microRNA (miRNA) regulation (Figure 2). While DNA methylation and histone modifications involve a direct influence at chromatin level, but without

changes in the genome sequence, miRNAs regulate the amount of proteins expressed in cells by modulating mRNA stability and the efficiency of translation.⁵⁷ In this context, PE has the ability to modulate the epigenetic signature mapping of different cells, thus mediating the subsequent modifications at transcription and post-transcription levels that characterizes distinct acute and chronic adaptations.^{56,58,59}

3.1 | Variability of skeletal muscle epigenetic landscape mediated by physical exercise

As illustrated in Figure 2, SM epigenetic landscape modulated by PE fine-tune the delicate balance between gene expression or silencing under the control of transient or stable transcriptional or post-transcriptional mechanisms.^{22,23}

So far, an almost consensual idea supports that different types and regimens of PE, with distinct cohort of healthy and nonhealthy active human subjects⁶⁰⁻⁶³ revert or attenuate the methylation promoter profile of genes involved in crucial metabolic or structural remodelling of Skeletal muscle pathways, such as *Pgc-1α* and *Tfam*, in a dose-dependent manner⁶⁰; thus diminishing protein expression silencing associated with these genes. Moreover, the SM genome-wide DNA hypomethylation pattern (measured as the number of hypomethylated CpG-sites) obtained after a 7-week resistance training program was retained and even exacerbated in the detraining and the retraining period, respectively.⁶³ Ultimately, a 9-day period of bed-rest in healthy active subjects induced an increased methylation of the *Pgc-1α* gene that was attenuated following 4 weeks of training.⁶⁴ This could be particularly interesting since SM is a programmable tissue and can 'remember' early-life mechanical and metabolic stimuli through epigenetic mechanisms that underly SM memory, affecting its function throughout life.⁶⁵

Accordingly, histone modifications associated with phosphorylation, acetylation, and mono-, di- and tri-methylation mechanisms seem to determine distinct spatial relationships between DNA and the histone core, conditioning SM transcriptional activation in response to exercise.⁶⁶ McGee et al⁶⁶ showed that although no difference in total histone deacetylases (HDAC) activity was found following exercise in human SM, alterations in class IIa HDAC function occurred through changes in subcellular localization, as HDAC 4 and 5 nuclear abundance immediately after PE were significantly decreased. This HDAC reallocation out of the nucleus⁶⁷ seems to be orchestrated, at least in part, by phosphorylation kinase cascades, namely AMP-activated protein kinase and the calcium-calmodulin-dependent protein kinase II.⁶⁶ In addition, endurance-trained subjects respond to intense cycling exercise through mitogen-activated protein kinase signalling. Extracellular regulated kinase 1/2 and p38 pathways are associated with activation of mitogen and stress-activated kinase

1 and histone H3 phosphorylation in human *vastus lateralis* from highly trained cyclists, which provides a potential cellular mechanism that facilitates exercise-related gene transcription.⁶⁸ Accordingly, it has been recently showed that increased acetylation levels in H3 were closely related to up-regulation of gene expression involved in resistance exercise-related SM adaptations.⁶⁹

Ultimately, mounting data highlight the epigenetic-related role of miRNAs in the post-translational regulation of gene expression in SM response to exercise.⁷⁰⁻⁷² Although an impressive array of miRNAs is presently under accurate scientific surveillance regarding its impact in exercise-related adaptations,^{73,74} the inter-individual variability of miRNAs expression in SM is still a matter of debate. Studies using resistance training reported muscle mass gain responses that are positively correlated with the expression levels of miR-378 in a high or low responsive manner.⁷⁵ Moreover, data from endurance exercise participants emphasize the SM mRNA regulation of components of the miRNA biogenesis pathway (Drosha, Dicer and Exportin-5),⁷⁴ as well as the role of miRNAs in the stimulation of microvascular remodelling⁶² and metabolic signalling pathways, such as mitochondrial biogenesis.^{73,74,76} While miRNAs molecules are essential cellular mediators of processes related to PE adaptation, secreted miRNAs can also act as signalling molecules mediating intercellular communication via paracrine or endocrine signalling. Cells can release miRNAs to the blood stream associated with lipid and protein carriers, namely extracellular microvesicles, exosomes and lipoproteins⁷⁷⁻⁷⁹ and deliver them in recipient cells thus regulating target gene expression and cell function.⁸⁰ Nevertheless, data are still scarce regarding the intrinsic and accurate mechanisms associated with this circulating miRNA-mediated intercellular communication. In fact, further studies are needed to establish the biological cause-and-effect relationships between circulating and different tissue adaptations during exercise or post-exercise recovery.⁷⁰

3.2 | Epigenome-mediated programming of offspring skeletal muscle through parental physical exercise

Besides their fundamental role as modulators of the gene expression in several organs, epigenetic influence during prenatal and neonatal development has also been proposed to affect offspring metabolic health.⁸¹ Mounting evidence suggest that environmental factors, such as xenobiotics, diets and PE contribute to transgenerational epigenetic inheritance that alters cell/tissue function. Consequently, they may predispose to an increased disease risk or, in contrast, to putative beneficial health-related outcomes later in life.⁵⁷ In this regard, some studies suggest that epigenetic-related programming associated

with ME contributes, at least in part, to mitigate disturbances associated with several pathophysiological conditions at distinct levels of cellular organization in the offspring.³³

Nowadays, it is clear that parental environmental exposure has an influence on programming SM metabolism and phenotype. In fact, epigenetic alterations have been proposed as having a key role in the transgenerational transmission of metabolic impairments.³² Therefore, understanding whether and how epigenetic modifications can manifest downstream implications for offspring in SM may be of extreme importance. Accordingly, Prats-Puig et al.⁸² reported gestational obesity-related alterations in methylation levels of *MYL6*, *MYH11*, *TNNT3*, *TPM2*, *CXCL2* and *NCAM1* genes in the umbilical cord, with implications in infant's fasting glucose levels at 6 years of age, due to hypothetical in utero inappropriate epigenetic programming of SM. Moreover, increased SM miR-15a and miR-15b expression in offspring of mothers with gestational diabetes seems to be associated with foetal programming and consequent predisposition to develop type-2 diabetes and associated cardiometabolic disease later in life.⁸³

In contrast, ME is a promising nonpharmacological strategy capable to reduce the susceptibility of offspring to developing metabolic disease in adulthood, and therefore contributes to break the vicious cycle of transgenerational transmission of maternal-associated malnutrition diseases. Although epigenetic-related mechanisms are the main players proposed so far,^{59,65,84} studies exploring the role of ME in the programming of offspring's landscape are still scarce. Laker et al.⁴⁹ reported that ME attenuated *Pgc-1 α* promoter hypermethylation in the SM of newborns from HFD-mothers, which is of crucial physiological and functional relevance considering the role of PGC-1 α as a master regulator of mitochondrial biogenesis and oxidative metabolism. Moreover, other PGC-1 α -related downstream targets such as glucose transporter type 4, cytochrome c oxidase subunit-4 and cytochrome c were also hypermethylated by the HFD and epigenetically prompted into a more positive phenotype by ME until 12 months of age.⁴⁶ This highlights the life-long protection provided by PE regarding epigenetic-transgenerational programming. In addition, ME induced DNA demethylation of the *Pgc-1 α* with consequent overexpression in *gastrocnemius* offspring muscle PGC-1 α 1/4 isoforms content, mitochondrial biogenesis and improved muscle function.¹¹

Until present, evidences are based on the relevance of ME on offspring health outcomes; however, recent research has shown that fathers' environmental exposure may also play an important role in offspring programming.¹⁷⁻²⁰ Sperm-mediated programming through epigenetic transmission of heritable alterations in chromatin structure may be one potential mechanism by which paternal physiology can impact offspring development. Father's environmental exposure leading to an obese phenotype may impair sperm number

and motility^{85,86} comprising fertility-related outcomes. In addition, the effects of paternal diet are likely to be transmitted to offspring through the sperm.^{85,87} Data from rodent studies showed that paternal nutritional behaviour can also influence both the sperm and offspring DNA methylation profile. In fact, the HFD-fed fathers and their male offspring-related spermatozoa epigenome showed overlapping DNA methylation that compromises distinct signalling metabolic pathways.⁸⁸ Moreover, paternal HFD decreased dimethyl H3-K9 protein content in sperm with further consequences on placental and foetus weight.⁸⁹

In contrast, paternal exercise is positively associated with progressive and total sperm motility^{19,20} (Figure 3). In fact, endurance, resistance and combined training might improve sperm parameters, including viability and motility of sperms through increasing the serum testosterone and luteinizing hormone levels.^{19,20} Recent data showed that reduced DNA methylation induced by exercise is present in germ cells suggesting that these positive outcomes can be transmitted to future generations.⁹⁰ In an elegant study, Guth et al.¹⁷ demonstrated that parental exercise resulted in an increased SM mass and improved glucose tolerance in female offspring. This parental exercise program altered the expression of several genes involved in lipid metabolism and specifically intramuscular lipogenesis in *gastrocnemius* (*Adipoq*, *Cidec* and *Scd1*) throughout two generations of sedentary male offspring. Genes involved in insulin and leptin signalling, as well as in the control of glucose metabolism (*H19*, *Ptpn1*, *Ogt* and *Oga*), were differently methylated in the sperm of paternal trained mice, which was associated with altered expression of these genes in the offspring SM and with an improvement in the metabolic efficiency.^{18,89} Although controversial,^{18,89} the expression of several paternal sperm miRNAs levels may be positively modulated by exercise training.⁹¹ The MiR-503 seems to be positively regulated by PE and have a role in target genes of cyclin family crucial for G₁ to S phase progression with beneficial consequences in oocyte first-stage cleavage events, embryo development and phenotype in the subsequent offspring.⁹¹

Taken together, data published so far highlight the relevant role that parental exercise may also have in the epigenetic-related transgenerational programming of SM offspring. However, a strong research effort should be done to explore the truly effects of epigenetic-related mechanisms that mediate the impact of paternal exercise on offspring phenotype.

4 | EARLY-LIFE EXERCISE COUNTERACTS NEGATIVE IMPACT OF PARENTS' LIFESTYLE

Besides the relevant role of PE before conception and during the gestational period, there is increasing evidence that

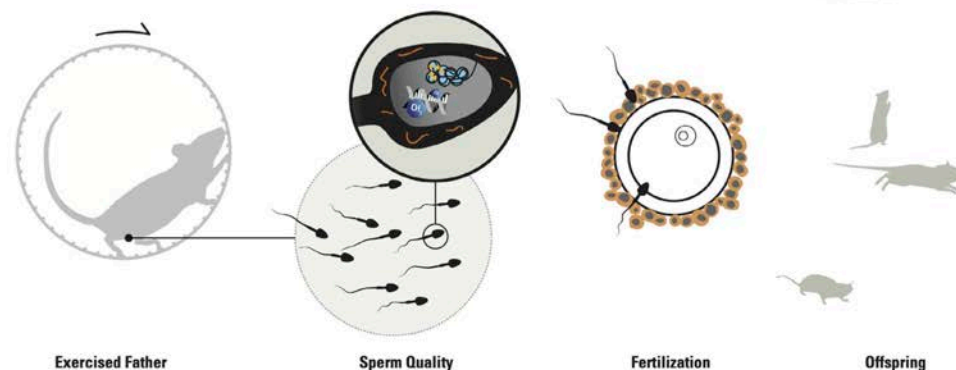


FIGURE 3 Apart from the consensual benefits mediated by maternal exercise performed during pregnancy on several offspring' health-related parameters, the role of paternal exercise has also been recently considered. The adoption of an active lifestyle together with the engagement in physical exercise programs before conception seems to have a beneficial effect on sperm viability and motility. Additionally, paternal exercise also has a positive impact in sperm chromatin structure through alterations in DNA methylation levels, histone proteins modification as well as in the modulation and expression of distinct miRNAs. Some of the paternal-induced epigenetic alterations are likely to be transmitted to the offspring through the germ cells and positively alter the skeletal muscle expression of several genes involved in the offspring glucose and lipid metabolism, that is, contributing to build-up fit muscles for the future

early-life PE may also play a significant impact in the modulation of the juvenile offspring^{21,25,92-94} and adult phenotype,⁹⁵ as developmental programming is not restricted to the gestational period. In fact, the postnatal period is also a critical window of opportunity,^{81,96} being PE performed during childhood negatively correlated to constraints inherited from an adverse gestational environment and induces lower prevalence of chronic diseases in adulthood.^{95,97} In fact, early-life exercise seems to mitigate the deleterious consequences of undernutrition during pregnancy and subsequent intrauterine growth restriction.^{25,92} Huber et al⁹² reported that in offspring exposed to maternal undernutrition, long-term daily moderate exercise reverted the decrease in fatty acid synthase protein expression and consequently improved overall energy expenditure and enhanced SM oxidative capacity. Therefore, the exercise-induced improvement in muscle metabolism contributed to the prevention of the paradoxical obesity development reported in undergrown offspring at birth.⁴¹ Furthermore, exercise engagement from early-life throughout adulthood in this model of undernourished mothers had the same effect in the prevention of obesity, when a moderate level of daily exercise was controlled by a locked FRW model only allowing 56m/day of running distance, which suggests that even moderate daily exercise prevents prenatally induced obesity.⁹⁷

In a model of impaired uterine blood flow-induced low birth weight in offspring, Laker et al⁹³ showed that early-life PE until 9-weeks of age elicited gastrocnemius mitochondrial biogenesis and oxidative capacity, through increased protein expression of PGC-1 α , elevated β -Hydroxyacyl-CoA

dehydrogenase and citrate synthase enzyme activities.⁹³ However, no sustained effects of early exercise on the expression and activity of these proteins were still evident after the cessation of exercise (at 24-weeks of age), compromising the long-term SM beneficial programming induced by early-life exercise.⁴⁸

As previously discussed, similarly to the impact of maternal undernutrition during pregnancy, an excessive caloric intake during gestation is also associated with health-related complications in offspring.^{11,32} Recent works have demonstrated beneficial effects of early-life voluntary exercise on the metabolic profile of offspring from obese mothers with exercise reversing maternal obesity-induced adiposity.⁹⁴ On the other hand, Falcão-Tebas et al²¹ focused on the offspring early-life PE reprogramming effect against paternal HFD consumption. Data revealed that only 4 weeks of exercise early in life normalized whole-body insulin sensitivity and reduced body weight in offspring later in adulthood (at 25-weeks of age), which seems to be influenced by SM adaptations, namely the increased levels of glucose transporter type 1 protein content in *epitrochlearis* muscle in adulthood.²¹ Moreover, improvements in *plantaris* mitochondrial respiration were reported,²¹ suggesting that mitochondrial function 4 months *post-partum* in offspring from HFD parents may have contributed, at least in part, to the normalization of insulin sensitivity in SM. In addition, a work from Patterson et al⁹⁸ showed that a 3-week FRW programme in rats, performed immediately after weaning, prevented the onset of diet-induced obesity for at least 10 weeks after the cessation of exercise training, despite the continued HFD consumption.

Besides the relevance of prenatal exercise on offspring health, recent striking findings show that maternal perinatal exercise (ie during lactation) can also impose metabolic reprogramming in the offspring, through changes in the composition breastmilk. Ribeiro et al⁹⁹ reported that elevated insulin levels in the milk of exercising mothers could, at least partly, contribute to lessening the effects of early-life overfeeding on obesity development of their offspring. The better insight into exercise effects on breastmilk composition was given by a cross-fostering study by Harris et al¹⁰⁰. Findings showed that offspring from sedentary mothers when cross-fostered by exercising dams improved glucose and insulin tolerance, in addition to a decrease in adiposity and body mass, compared to the offspring of exercising mothers nurtured by sedentary dams. It seems that ME expresses some of its beneficial metabolic effects through altering breast milk composition, particularly an oligosaccharide 3'-sialyllactose, which levels correlate positively with exercise.¹⁰⁰ These remarkable cross-fostering data actually demonstrate the role that ME may have on altering breast milk composition and thus, it is possible involvement in the offspring metabolic status.¹⁰⁰ Even though studies on this new aspect of ME mechanisms are in its infancy, it undoubtedly offers a new perspective on the benefits that ME during lactation may also have on the offspring metabolic programming.

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

Although the beneficial role of parental and early-life PE seems to be sufficiently robust in rodent model studies, a strong effort is still needed to highlight the potential translation power of the benefits and related mechanisms into humans.

In addition, further studies considering a variety of experimental set-ups are needed in order to ascertain whether: (1) other organs and systems rather than skeletal muscle benefit from this transgenerational effectiveness of PE stimuli; (2) cellular and subcellular structures of different tissues and organs adapt into a more resistant phenotype, providing a cross tolerance-like effect when deleterious stimuli are applied in vitro, in situ or in vivo; (3) the protective and therapeutic effects of PE are still beneficial when parental or offspring conditions are altered/manipulated, through distinct nutritional, toxicological or environmental interventions.

Nevertheless, the current knowledge seems to be appropriated to establish clinical guidelines and scientifically supported evidence for the promotion of parental PE before and during gestation, as well as offspring PE early in life. In this sense, behaviour changes regarding the practice of PE

before and during pregnancy should be adopted as part of the huge parental responsibility with their offspring and with the society.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

JB, JSS, JRT, and JM: Conceptualization. JB, JSS, and PC: Literature survey. JB, JSS, and JM: Original Draft. JB, JSS, PC, AA, RCC, JRT, and JM: Final editing and approval. RCC: Information visualization. JM and JB: Funding acquisition.

ORCID

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

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

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

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

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

Joan Ramon Torrella  <https://orcid.org/0000-0001-6314-6523>

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

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

²Porto Biomechanics Laboratory (LABIOME), University of Porto, Porto, Portugal,.

³Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain.



Self-Paced Free-Running Wheel Mimics High-Intensity Interval Training Impact on Rats' Functional, Physiological, Biochemical, and Morphological Features

Jorge Beleza^{1*}, João Albuquerque¹, Estela Santos-Alves¹, Pedro Fonseca², Garoa Santocildes³, Jelena Stevanovic¹, Sílvia Rocha-Rodrigues¹, David Rizo-Roca¹, António Ascensão¹, Joan Ramon Torrella³ and José Magalhães¹

¹ Laboratory of Metabolism and Exercise (LaMetEx), Department of Sport Biology, Faculty of Sport, Research Centre in Physical Activity, Health and Leisure (CIAFEL), University of Porto, Porto, Portugal, ² Porto Biomechanics Laboratory (LABIOME), University of Porto, Porto, Portugal, ³ Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

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*Correspondence:

Jorge Beleza
jmsbelza@gmail.com

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Free-running wheel (FRW) is an animal exercise model that relies on high-intensity interval moments interspersed with low-intensity or pauses apparently similar to those performed in high-intensity interval training (HIIT). Therefore, this study, conducted over a 12-weeks period, aimed to compare functional, thermographic, biochemical and morphological skeletal and cardiac muscle adaptations induced by FRW and HIIT. Twenty-four male Wistar rats were assigned into three groups: sedentary rats (SED), rats that voluntarily exercise in free wheels (FRW) and rats submitted to a daily HIIT. Functional tests revealed that compared to SED both FRW and HIIT increased the ability to perform maximal workload tests (MWT-cm/s) (45 ± 1 vs. 55 ± 2 and vs. 65 ± 2). Regarding thermographic assays, FRW and HIIT increased the ability to lose heat through the tail during MWT. Histochemical analyzes performed in *tibialis anterior* (TA) and *soleus* (SOL) muscles showed a general adaptation toward a more oxidative phenotype in both FRW and HIIT. Exercise increased the percentage of fast oxidative glycolytic (FOG) in medial fields of TA (29.7 ± 2.3 vs. 44.9 ± 4.4 and vs. 45.2 ± 5.3) and slow oxidative (SO) in SOL (73.4 ± 5.7 vs. 99.5 ± 0.5 and vs. 96.4 ± 1.2). HIIT decreased fiber cross-sectional area (FCSA- μm^2) of SO (4350 ± 286.9 vs. 4893 ± 325 and vs. 3621 ± 237.3) in SOL. Fast glycolytic fibers were bigger across all the TA muscle in FRW and HIIT groups. The FCSA decrease in FOG fibers was accompanied by a circularity decrease of SO from SOL fibers (0.840 ± 0.005 vs. 0.783 ± 0.016 and vs. 0.788 ± 0.010), and a fiber and global field capillarization increase in both FRW and HIIT protocols. Moreover, FRW and HIIT animals exhibited increased cardiac mitochondrial respiratory control ratio with complex I-driven substrates (3.89 ± 0.14 vs. 5.20 ± 0.25 and vs. 5.42 ± 0.37). Data suggest that FRW induces significant functional, physiological, and biochemical adaptations similar to those obtained under an intermittent forced exercise regimen, such as HIIT.

Keywords: voluntary physical activity, animals, skeletal muscle, mitochondria, heart

INTRODUCTION

The use of animal models is a common practice in exercise-based studies. In fact, exercise physiologists use different animal models, in particular rodents, to study the physiological and biochemical impact of exercise induced by forced running endurance training in treadmill, swimming, or climbing stairs [for a complete guide of training methods and animal models for the study of exercise see Kregel et al. (2006)]. Moreover, voluntary self-paced motor behavior models, such as 24-h access to free-running wheels (FRW), are used to minimize (mal)adaptations associated to sedentarism or pathological conditions (Fonseca et al., 2011a; Gonçalves et al., 2014). Having animals usually housed in small cages, in this FRW model, wheels are introduced in the housing cages and animals are allowed to freely run at chosen intensities and durations (Gonçalves et al., 2014, 2016; Marques-Aleixo et al., 2016). However, despite the widespread use of FRW, there is a lack of knowledge regarding the truly physiological and biochemical impact of this model on voluntarily exercised animals. Moreover, when considering the motor pattern, this model is characterized by short or middle-duration bouts of exercise performed at high speed interspersed by long-lasting pauses and periods of low intensity that, in certain sense, seem to mimic the exercise bouts performed during sessions of high-intensity interval training (HIIT) (Novak et al., 2012).

Recently introduced as an exercise model, HIIT has proved to be a powerful stimulus to induce rapid skeletal muscle remodeling and to enhance skeletal muscle oxidative capacity in humans (Ramos-Filho et al., 2015). Furthermore, HIIT seems to reduce the rate of lactate production during exercise, to increase the capacity of skeletal muscle lipid oxidation (Gibala and Jones, 2013), and to induce an increased post-exercise metabolic rate (Schaun et al., 2017). However, despite the increasing use of HIIT in animal studies (Ramos-Filho et al., 2015; Delwing-de Lima et al., 2017; Freitas et al., 2018; Shirvani and Arabzadeh, 2018), data regarding its overall exercise intensity and metabolic impact is still scarce. Therefore, considering distinct levels of tissue and cellular organization, we here aim to analyze the different skeletal and cardiac muscles response induced by FRW and HIIT. For skeletal muscle, histomorphological features using two different phenotypic specimens, mainly oxidative *soleus* (SOL) and the *tibialis anterior* (TA), will be analyzed. For cardiac muscle, mitochondrial function will be used as an important hallmark of cardiac tissue metabolic fitness and adaptation (Ascensão et al., 2006; Magalhaes et al., 2013, 2014), and bioenergetics endpoints associated with *in vitro* oxygen consumption will be obtained. Moreover, taking in to account that energy expenditure in rats seems to be related with alterations in body temperature (Wanner et al., 2015b), namely in skin temperature (Wanner et al., 2015a), measurements on the animals' tail temperature (T_{tail}) during maximal workload tests will also be performed.

To the best of our knowledge, this is the first study that compares, from a physiological, biochemical, and morphological point of view, the impact of both FRW and HIIT exercise

regimens in a rodent model. Taken together, data will contribute to better understand the impact of these often-used exercise models on skeletal and cardiac muscle and to truly clarify the relevance of the FRW when compared to other more formal and forced exercise animal models, such as HIIT.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were performed in accordance with guidelines for Care and Use of Laboratory Animals in Research advised by Federation of European Laboratory Animal Science Associations (FELASA). The Ethics Committee of the Faculty of Sport, University of Porto and the National Government Authority (*Direção Geral de Alimentação e Veterinária* – N° 0421/000/000/2018) approved the experimental protocol.

Wistar male rats ($n = 24$) aged 7–8 weeks old were obtained from Charles River Laboratories (L'Arbresle, France) and were housed in individual cages under controlled environmental conditions (21–22°C; 50–60% humidity), receiving standard laboratory pellet chow (4RF21, Mucedola, Italy) and water *ad libitum* in 12-h light/dark cycles. After 1 week of quarantine, animals were randomly assigned into three groups ($n = 8/\text{group}$): sedentary (SED), FRW, and HIIT.

Maximal Workload Test (MWT) and $\text{VO}_{2\text{max}}$ Assessment

In the context of the present study, MWT were performed both prior to the training/FRW and at the end of the protocol, being $\text{VO}_{2\text{max}}$ only evaluated in the referred first moment. For these purposes, animals were adapted to a motorized treadmill for 5 days (LE8700, Panlab, Harvard, United States). In the first day, rats were placed on the immobile treadmill to adapt to the new device and in the second day, the treadmill was turned on and the speed and duration were gradually increased during the subsequent 4 days. Moreover, electric-based shock grids were used to stimulate rats to run.

In the MWT, the animals were individually evaluated on the treadmill at an inclination of 15°. The test started at a speed of 15 cm/s, after 5 min the speed was increased to 30 cm/s and then stepped up 3 cm/s every 2 min until maximal workload was achieved. This MWT allowed to achieve $\text{VO}_{2\text{max}}$ -related speed in order to set the different workloads for the HIIT protocol described below, being $\text{VO}_{2\text{max}}$ evaluated on a close chamber treadmill coupled to a gas analyzer (LE405 Gas Analyser, Panlab, Barcelona, Spain). Two criteria were established to confirm maximal workload, namely the exhaustion of the animal and blood lactate concentration higher than 7 mmol/L. Immediately after the end of MWT and in different time points (1, 3 and 5 min after), blood samples were collected from the animals' tail until a maximal value of lactate concentration (Lactate Plus Meter, Nova Biomedical, United States) was achieved. However, to confirm the achievement of $\text{VO}_{2\text{max}}$, two additional criteria were considered, namely the plateau in oxygen consumption and respiratory coefficient higher than 1.

TABLE 1 | Summary of speed achieved during the experimental period.

	Week												Speed (cm · s ⁻¹)
	1	2	3	4	5	6	7	8	9	10	11	12	
Warm up	33	33	33	33	33	33	33	35	35	35	35	35	
Bout 1	45	45	45	45	45	45	47	47	47	47	47	47	
Bout 2	46	46	46	46	47	47	47	49	49	49	49	49	
Bout 3	47	47	47	47	49	49	49	51	51	51	51	51	
Bout 4	48	48	48	48	51	51	51	53	53	53	53	53	

Exercise Protocols

Animals from the FRW group were housed in a polyethylene cage equipped with a running wheel (circumference = 1.05, Type 304 Stainless steel, Tecniplast, Casale Litta, Italy). The rats had free access to the running wheel 24 h/day and the running distance was recorded using a digital counter (ECO 701 Hengstler, Lancashire, United Kingdom).

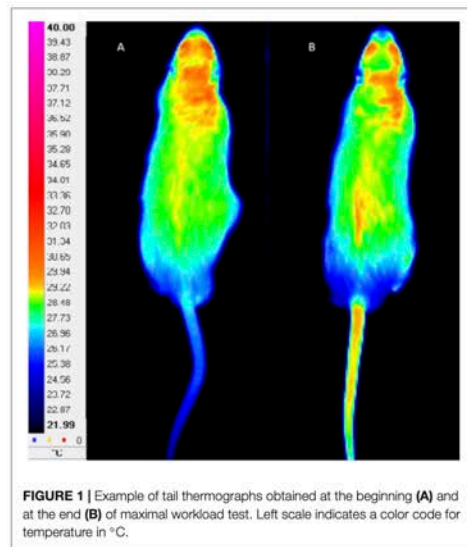
Animals from the HIIT group were exercised during 12 weeks (5 days/week), Monday to Friday, between 10:00 and 12:00 AM, on the motor treadmill at 15° inclination. The HIIT protocol started with 5 min warm up at low speed correspondent to 60% of VO_{2max} , then the rats performed four bouts of 4 min at speeds correspondent to 85–90% of VO_{2max} interspersed by 2 min of active recovery at speed correspondent to 60% of VO_{2max} [adapted from Songstad et al. (2015)]. Nevertheless, during training sessions the speed was gradually increased to meet animals' adaptations throughout the protocol (Table 1) and electric-based shock grids were used to stimulate rats to run. Both SED and FRW animals were placed on a non-moving treadmill to be exposed to the same handling and environmental conditions.

Thermographic Measurements

A thermographic camera (FLIR A325, FLIR Systems, Wilsonville, United States) properly calibrated, presenting the resolution of 320 × 240 dpi, sensitivity of 70 mK and accuracy of ±2%, was used for the acquirement of animal tail thermographs. The distance between the camera and the photographed animal was set at 2 m. In each measurement, the assessment of the T_{tail} was obtained, by the average of the temperature in the whole tail during 5 s (Figure 1). The analysis was performed using the software FLIR ThermaCAM Researcher Pro 2.10®. Thermographs were obtained in the start and at the end of the 12 weeks of exercise regimens (FRW and HIIT), at the beginning (Figure 1A) and in each step until the end of the MWT (Figure 1B).

Muscle Sampling and Histochemical Procedures

All animals were fasted overnight for 12 h with access to drinking water before sacrifice. Animals were weighed at the day of sacrifice and anesthetized with ketamine (90 mg · kg⁻¹) and xylazine (10 mg · kg⁻¹). Right SOL and TA muscles were excised, immediately frozen in pre-cooled isopentane and stored at -80°C until further analysis.

**FIGURE 1** | Example of tail thermographs obtained at the beginning (A) and at the end (B) of maximal workload test. Left scale indicates a color code for temperature in °C.

Muscle samples were mounted in embedding medium (Tissue-Tek®, Sakura Finetek Europe, Zoeterwoude, Netherlands), and cut in serial transverse sections (12–16 μm) using a cryostat (Leica CM3050S, Wetzlar, Germany) at -22°C. Sections were mounted on gelatinized (0.02%) slides and stained for: (1) myofibrillar adenosine triphosphatase (mATPase) following pre-incubation in alkaline (pH 10.7) and acid (pH 4.2) mediums to differentiate between slow and fast-twitch fibers (Brooke and Kaiser, 1970); (2) endothelial adenosine triphosphatase (eATPase) to reveal muscle capillaries (Fouces et al., 1993); and (3) succinate dehydrogenase (SDH) to distinguish between aerobic and anaerobic fibers (Nachlas et al., 1957).

Morphofunctional Measurements

Microphotographs obtained with a light microscope (BX61, Olympus, Tokyo, Japan) connected to a digital camera (DP70, Olympus, Tokyo, Japan) at a magnification of 100x were used to obtain muscle fiber morphofunctional measurements. The analyzed parameters were obtained from transverse cross sections of approximately 5.5 × 10⁵ μm² from three different microphotographs and were treated using an image analyzing software (ImageJ; Rasband 1997–2014). The TA was analyzed following the protocol described in Torrella et al. (2000), represented schematically in Figure 2. First, we chose the major axis of the muscle transverse section and divided its total length into four equal intervals. Oriented from medial to lateral, the end of the first interval was chosen as medial zone whilst the beginning of the fourth interval was chosen as lateral zone.

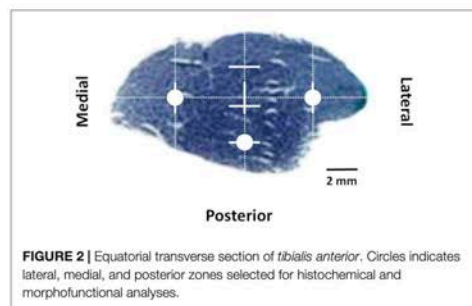


FIGURE 2 | Equatorial transverse section of *tibialis anterior*. Circles indicates lateral, medial, and posterior zones selected for histochemical and morphofunctional analyses.

Subsequently, a secondary orthogonal axis was drawn to cross the central division of the major longitudinal axis. This orthogonal axis was also divided in four equal intervals. Oriented from anterior to posterior, the beginning of the fourth interval was chosen as posterior zone. An image of the equatorial zone of the muscle section was obtained using a light-stereoscope (Olympus, SZ40, Japan) and the sections were divided in a grid-like structure throughout two-dimensional axis to provide the sample areas (or fields) to be studied for each anatomical region (medial, lateral, and posterior). Due to their different fiber type composition and morphometry, data are presented separately for each of these different regions.

Fibers were typified (Figure 3) as slow oxidative (SO), fast oxidative glycolytic (FOG), fast glycolytic (FG) or fast intermediate glycolytic (FIG), with higher SDH staining than FG, but lower staining than FOG fibers.

The fiber cross-sectional area (FCSA) and the shape factor circularity ($SF = 4 \cdot \pi \cdot FCSA / \text{perimeter}^2$) were measured. The following parameters related with fiber capillarization were also obtained: the absolute number of capillaries per fiber (NCF), the relative number of capillaries per 1,000 μm^2 of FCSA (CCA), fiber density (FD, mm^{-1}), capillary density (CD, mm^{-1}), and capillary-to-fiber ratio ($C/F = CD/FD$).

Isolation of Heart Mitochondria

Mitochondria from left heart ventricles were isolated using conventional methods of differential centrifugation as described elsewhere (Bhattacharya et al., 1991). Mitochondrial and homogenate protein contents were determined by the Biuret method calibrated with BSA (Van Norman, 1909). All mitochondrial isolation procedures were performed at 0–4°C. Considering the relatively greater abundance of intermyofibrillar (IMF) (~80%) compared with subsarcolemmal (SS) (~20%) mitochondria within the cells, a potentially dominant role for the IMF subfraction vs. the SS subfraction when studying mitochondrial alterations is expected.

Mitochondrial Oxygen Consumption Assays

Mitochondrial respiratory function was polarographically measured at 30°C, using a Biological Oxygen Monitor system

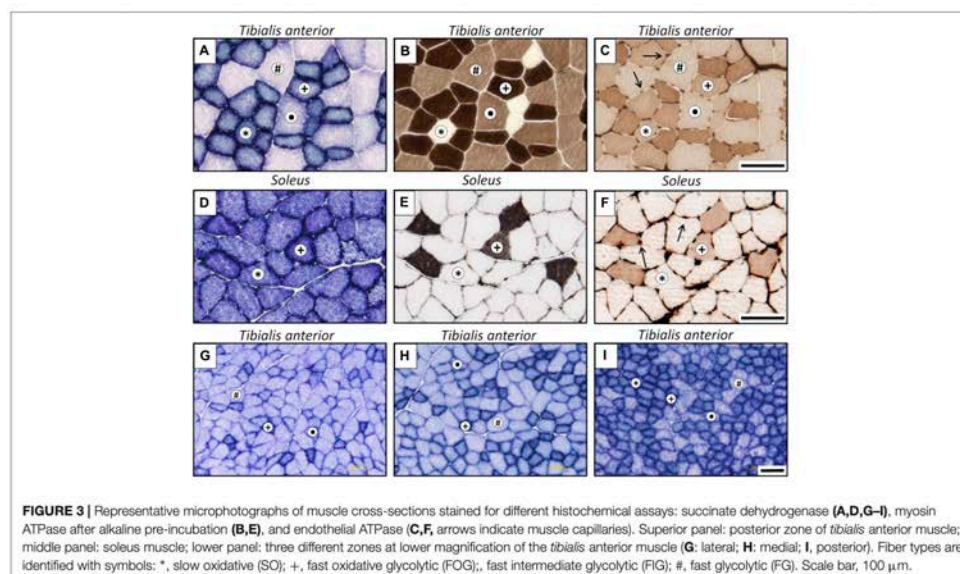


FIGURE 3 | Representative microphotographs of muscle cross-sections stained for different histochemical assays: succinate dehydrogenase (A,D,G–I), myosin ATPase after alkaline pre-incubation (B,E), and endothelial ATPase (C,F, arrows indicate muscle capillaries). Superior panel: posterior zone of *tibialis anterior* muscle; middle panel: soleus muscle; lower panel: three different zones at lower magnification of the *tibialis anterior* muscle (G: lateral; H: medial; I: posterior). Fiber types are identified with symbols: *, slow oxidative (SO); +, fast oxidative glycolytic (FOG); #, fast intermediate glycolytic (FIG); ., fast glycolytic (FG). Scale bar, 100 μm .

(Hansatech Instruments, Norfolk, United Kingdom) and a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, United Kingdom). Reactions were conducted in a 0.75 mL closed glass chamber, thermostated and magnetically stirred containing 0.5 mg of mitochondrial protein in a reaction buffer with 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 mM EGTA, 2.5 mM KH₂PO₄, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate (10 mM) plus malate (5 mM) as substrates for complex I or succinate (10 mM) plus rotenone (4 mM) as complex II-related substrates.

State 3 respiration (oxygen consumption after adding 125 nmol ADP) and state 4 (considered as the rate of oxygen after full ADP phosphorylation) were measured. The respiratory control ratio (RCR, state 3/state 4) and the ADP/O ratio (nmol ADP phosphorylated by natom O consumed), were calculated according to Estabrook (1967), using 474 natom O mL⁻¹ as the value of solubility of oxygen at 25°C in double-distilled water.

Mitochondrial Membrane Potential Measurements

Mitochondrial transmembrane potential ($\Delta\psi$) was indirectly measured based on the activity of the lipophilic cation tetraphenylphosphonium (TPP⁺), through a TPP⁺ selective electrode previously prepared as described by Lumini-Oliveira et al. (2011). Reactions were carried out in 1 mL of reaction buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 mM EGTA, 2.5 mM KH₂PO₄, pH 7.4, supplemented with 3 μ M TPP⁺, and 0.5 mg/mL of protein at the temperature kept at 25°C. For measurements of $\Delta\psi$ with complex I substrates, energization was carried out with 10 mM of glutamate and 5 mM of malate and ADP-induced phosphorylation was accomplished by adding 125 nmol ADP. For measurements of $\Delta\psi$ with complex II substrates, 10 mM succinate supplemented with 4 μ M rotenone were added to the medium containing 3 mM TPP⁺ and mitochondria. The lag phase, which reflects the time required to phosphorylate the added ADP, was also measured for both substrates.

Citrate Synthase Activity in Skeletal Muscle

Citrate synthase (CS) activity was measured in SOL and TA homogenates, as a marker of mitochondrial content and oxidative capacity, using the method described by Srere (1969). Briefly, 10 mg of muscle were homogenized in 100 mL of ice-cold medium containing 75 mM Tris·HCl, 2 mM MgCl₂, and 1 mM EDTA (pH 7.6). The CoA-SH released from the reaction of acetyl-CoA with oxaloacetate was measured by its reaction with a colorimetric agent [5, 5-dithiobis (2-nitrobenzoate)]. The enzymatic activity was measured spectrophotometrically at 412 nm.

Statistical Analysis

Data are reported as the mean $\pm$ SEM (standard error of the mean). Statistical analyses were performed using GraphPad Prism (version 7.0). One-way analysis of variance (ANOVA)

was followed by Bonferroni *post hoc* test to compare differences between groups. In all cases, a value of $p < 0.05$ was considered statistically significant.

RESULTS

Physical Exercise and Body Weight

After the 12 weeks of protocol, FRW animals ran a significantly higher overall day distance than HIIT (Figure 4). Their activity increased from the beginning of the protocol and reached a peak at the 6th week, when a non-significant decrease was observed until the end of the protocol. However, 12 weeks of HIIT decreased body weight, while FRW did not elicit significant alterations in this parameter (Table 2).

Maximal Workload Test-Related Speed, Tail Temperature, and Blood Lactate Concentration

At the end of the 12 weeks, SED animals achieved a lower maximal speed in the MWT (from 57 to 48 cm/s) whereas both FRW (from 54 to 60 cm/s) and HIIT (from 57 to 69 cm/s) increased their maximal speed when compared to baseline (Figure 5A).

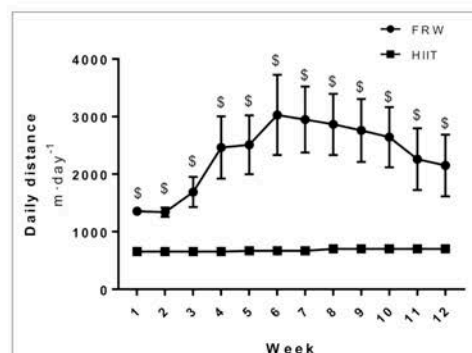
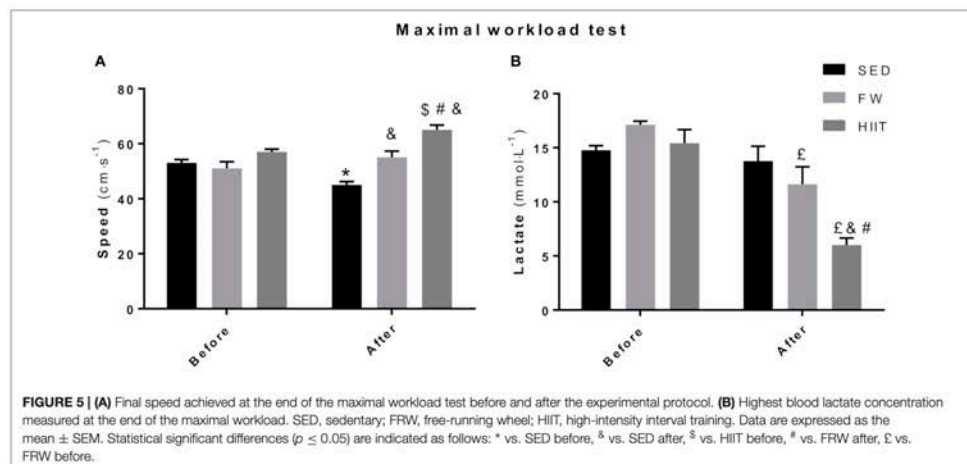


FIGURE 4 | Mean distance covered per day. SED, sedentary; FRW, free-running wheel; HIIT, high-intensity interval training. Data are expressed as the mean $\pm$ SEM. Statistical significant differences ($p \leq 0.05$) are indicated as follows: \$ vs. HIIT.

TABLE 2 | Final body weight and muscle to body weight ratio.

Group	Body Weight (g)	Soleus/Body Weight (mg/g)	Tibialis Anterior/Body Weight (mg/g)
SED	390 $\pm$ 28.11	2.24 $\pm$ 0.16	0.40 $\pm$ 0.04
FRW	411 $\pm$ 4.86	2.24 $\pm$ 0.16	0.53 $\pm$ 0.02
HIIT	354 $\pm$ 5.42*	1.95 $\pm$ 0.08	0.51 $\pm$ 0.05

SED, sedentary; FRW, free-running wheel; HIIT, high-intensity interval training. Data are expressed as the mean $\pm$ SEM. Statistical significant differences ($p \leq 0.05$) are indicated as follows: * vs. SED, # vs. FRW.



No differences between groups in the maximal velocities at the beginning of the protocol were registered. However, significant differences were evident between groups at the end of the 12 weeks. In fact, HIIT and FRW animals reached significantly higher velocities compared to SED. Moreover, a significant difference was also found between HIIT and FRW (69 vs. 60 cm/s, respectively).

To find out how glycolytic energy pathway was modulated by this exercise programs and also in which comparative extent oxidative-related demands may support different exercise intensities and workloads after both FRW and HIIT, maximal blood lactate concentrations (BLC) were measured at the end of the MWT, before and after the 12 weeks of the protocol (Figure 5B). No significant differences were found at the beginning of the protocol in maximal BLC. However, FRW animals showed a lower BLC at the end of MWT after 12 weeks of free access to the running wheel (FRW: 11.63 ± 3.95 mmol/L vs. SED: 13.77 ± 3.35 mmol/L). Moreover, the animals submitted to the HIIT regimen reached a lower BLC at the end of the MWT (HIIT: 6.00 ± 1.85 mmol/L vs. SED: 13.77 ± 3.35 mmol/L and FRW: 11.63 ± 3.95 mmol/L), despite attained a higher maximal speed when compared to those of the SED and FRW groups.

Figure 6 shows the tail temperature (T_{tail}) at the different steps of the MWT before and after the 12 weeks of the intervention protocol. SED animals did not show statistical differences in T_{tail} in any step, with exception of the baseline (Figure 6A). After the training period, FRW animals showed an increased T_{tail} from speeds 36 to 54 cm/s when compared to baseline (Figure 6B). The HIIT regimen induced a significantly decreased T_{tail} at 36 cm/s. However, at speeds ranging from 45 to 51 cm/s, significant increments were obtained when compared to baseline (Figure 6C). A comparison between groups at the end of the 12 weeks in resting conditions (Figure 6D) showed that HIIT animals presented a lower T_{tail} compared to FRW; compared to

FRW and SED at 36 cm/s, but a higher T_{tail} at 45 and 48 cm/s when compared to SED. The FRW animals revealed a higher T_{tail} at 48 cm/s compared to SED.

Citrate Synthase Activity

Figure 7 shows CS activity in TA and SOL muscles as a marker of mitochondrial oxidative capacity. No significant differences were observed on TA after both chronic exercise regimens (Figure 7A). The activity in the SOL muscle was significantly higher in both exercised groups compared to SED control (Figure 7B).

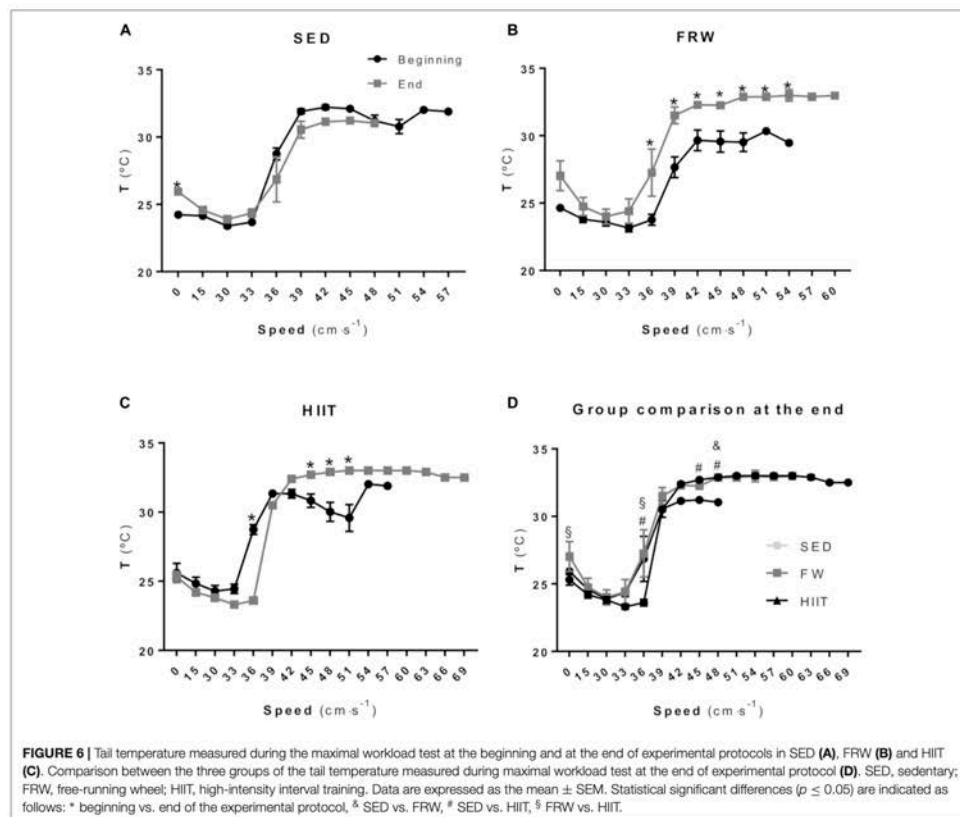
Cardiac Mitochondrial Function

Despite no alterations were found in heart mitochondrial state 3 and 4 when complex I- and II-driven substrates were used after 12 weeks of both training modalities (Figures 8A–D), an increase in RCR with complex I-driven substrates (3.89 ± 0.14 vs. 5.20 ± 0.25 and 5.42 ± 0.37) in FRW and HIIT groups was evident (Figure 8E). When complex II-related substrates were used, HIIT induced an increase in RCR (3.86 ± 0.17 vs. 2.98 ± 0.19 and 3.24 ± 0.05) compared with SED and FRW conditions (Figure 8G). Phosphorylating efficiency, measured as ADP/O ratio, was higher in HIIT group (4.22 ± 0.31 vs. 3.20 ± 0.20 and 3.24 ± 0.16) for complex I-driven substrates (Figure 8F), while no alterations between groups were found when substrates for complex II were used (Figure 8H).

No alterations between groups were found in the transmembrane electrical potential endpoints (maximal potential, repolarization, and lag phase) for both complexes I- and II-related substrates (data not shown).

Skeletal Muscle Fiber Type Distribution

Both chronic exercise models caused alterations in skeletal muscle fiber type distribution in TA and SOL (Figure 9). In the lateral field of TA, the FRW group exhibited an increased



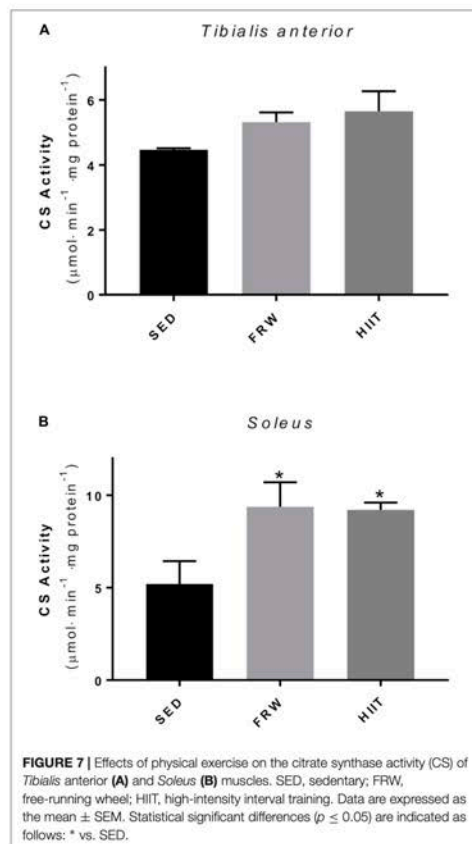
percentage of FOG fibers (36.0 ± 5.0 vs. 14.0 ± 2.2 and vs. 21.9 ± 6.5), while a significant increase in the percentage of FIG fibers was observed in the HIIT group (Figure 9A). Moreover, both FRW and HIIT increased the percentage of FOG fibers in the TA medial field (44.9 ± 4.4 and 45.2 ± 5.3 vs. 29.7 ± 2.3), which is concomitant with the decreased percentage of FG fibers (Figure 9B). Regarding SO fibers, only present in the posterior field, both FRW and HIIT exercise models induced an increased percentage (Figure 9C). Furthermore, in the SOL muscle, FRW, and HIIT elicited a decrease in the percentage of FOG fibers and a corresponding increment of SO fibers (Figure 9D).

Skeletal Muscle Fiber Cross-Sectional Area and Fiber Shape

Despite no evidence of changes in muscle to body weight ratio (Table 2), alterations in FCSA and fiber shape were registered in the present protocol. Figure 10 shows the FCSA from muscles

TA (Figures 10A–C) and SOL (Figure 10D). In TA muscle, both FRW and HIIT induced a significant increase in FCSA of FG fibers in all the muscle regions. This increase was also evident in FIG fibers from the medial (3781.00 ± 146.07 vs. 2966 ± 222.86 and $3569.00 \pm 193.41 \mu\text{m}^2$) and posterior fields (4301.00 ± 137.49 vs. 2192 ± 157.62 and $2796.00 \pm 189.95 \mu\text{m}^2$) in FRW animals, but not evidenced after the HIIT protocol. Both oxidative fiber types (SO and FOG) showed no significant changes in FCSAs after any exercise protocol, except for FOG fibers from the lateral field of HIIT animals, in which a significant reduction in fiber size was observed (1634.00 ± 76.79 vs. 2001.00 ± 61.19 and $1881.00 \pm 42.146 \mu\text{m}^2$). In SOL muscle, a significant reduction in FCSA was measured in SO fibers of HIIT animals (3621.00 ± 237.30 vs. 4350.00 ± 286.90 and $4893.00 \pm 325.00 \mu\text{m}^2$).

Regarding the shape of the fibers (circularity), no significant alterations in the TA lateral and posterior fields were observed (Figures 10E–G). In contrast, a reduction in the circularity of the



FOG and SO fibers HIIT animals was found in the TA medial field (Figure 10F). Furthermore, both exercise models decreased the circularity of SO fibers in the SOL (Figure 10H).

Fiber Capillarization

The FRW and HIIT exercise protocols elicited alterations in individual capillarization of the different fiber types (Figure 11). FRW increased the NCF of FOG fibers in the TA lateral (6.90 ± 0.30 vs. 5.30 ± 0.29 and 6.50 ± 0.38) and posterior fields (8.10 ± 0.43 vs. 5.80 ± 0.50 and 7.10 ± 0.37). Regarding the FIG and FG fibers, the FRW model induced an increase of NCF in the TA medial and posterior fields (Figures 11B,C), while in the lateral field, it only increased the NCF of FG fibers (6.90 ± 0.30 vs. 5.30 ± 0.29 and 6.50 ± 0.38). Furthermore, HIIT increased the NCF of FIG and FG fibers of TA lateral and medial fields. However, in the TA posterior field, HIIT only

increased the NCF of FG fibers (Figures 11A–C). Regarding the SOL muscle, both exercise models increased the NCF of SO fibers (10.90 ± 0.35 and 8.40 ± 0.29 vs. 6.70 ± 0.58). Furthermore, the FRW model also induced an increase in NCF of SO fibers comparing to HIIT (10.90 ± 0.35 vs. 8.40 ± 0.29). However, in the FOG fibers, HIIT induced an increase in NCF comparing to SED and FRW (10.10 ± 0.17 vs. 6.20 ± 0.63 and 5.00 ± 0.22). Additionally, the NCF of FOG fibers decreased in the FRW (Figure 11D).

Regarding CCA, FRW, and HIIT increased the CCA of FOG fibers in the TA medial and lateral fields, while only the HIIT regimen induced an increase of the FOG-related CCA in the TA posterior field (Figures 11E–G). Furthermore, both exercise models increased muscle capillarization of the SO fibers from SOL (Figure 11H).

Capillary Density and Capillary-to-Fiber Ratio

Both exercise models increased CD in all the TA fields. Although this increase was not significant in the posterior region after FRW, the trend is clear (Figure 12A). The same trend for increasing was observed in the SOL muscle in the FRW group and a significantly greater CD after HIIT protocol was noted (Figure 12B).

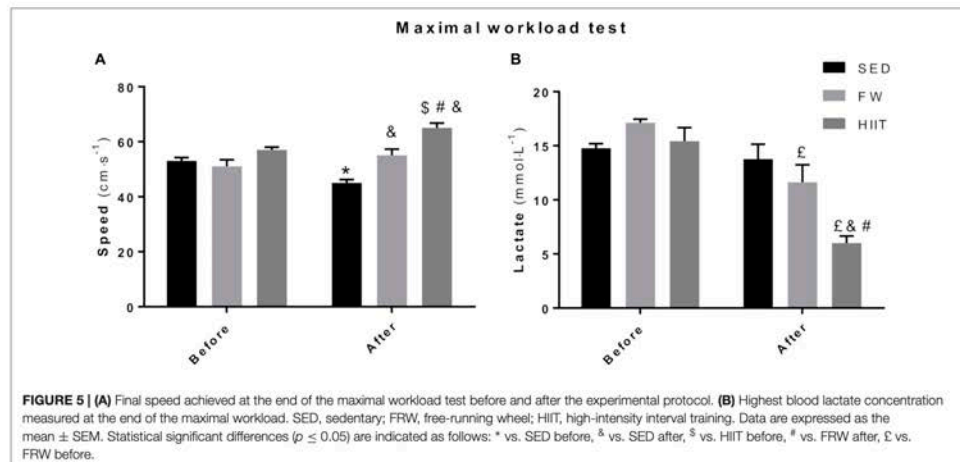
Regarding C/F, the FRW, and HIIT models significantly increased this morphological feature in the three fields of the TA and in SOL (Figures 12C,D).

DISCUSSION

The overall findings of the present study suggest that 12 weeks of FRW, performed in a running wheel-equipped cage, as well as 12 weeks of forced training, using a HIIT protocol in a treadmill, induced similar physiological and biochemical adaptations at different levels of cellular organization in skeletal and cardiac muscles. From both central and peripheral point of view, FRW and HIIT regimens positively modulated animals' functional capacity to perform maximal exercise. Specifically, both protocols induced alterations in the skeletal muscle fiber morphology and metabolism toward a more oxidative phenotype, as well as in cardiac mitochondria, which exhibited a more efficient energetic phenotype.

Maximal Workload Tests

The FRW model has been used in rodents to attenuate the lack of physical activity associated with sedentary way of life (Fonseca et al., 2011a,b; Hyatt et al., 2015). In addition, it is also frequently used to avoid further impairments caused by lack of metabolic and mechanical stimuli or as a specific exercise regimen established to induce organic adaptations and thus, to positively modulate tissue phenotype (Kariya et al., 2004; Marques-Aleixo et al., 2016; Rocha-Rodrigues et al., 2016, 2017). In accordance with data from previous studies (Deloux et al., 2017; Stolle et al., 2017; Zhou et al., 2017), our results suggest that FRW induced significant adaptations regarding animals' physical fitness. In fact, as



No differences between groups in the maximal velocities at the beginning of the protocol were registered. However, significant differences were evident between groups at the end of the 12 weeks. In fact, HIIT and FRW animals reached significantly higher velocities compared to SED. Moreover, a significant difference was also found between HIIT and FRW (69 vs. 60 cm/s, respectively).

To find out how glycolytic energy pathway was modulated by this exercise programs and also in which comparative extent oxidative-related demands may support different exercise intensities and workloads after both FRW and HIIT, maximal blood lactate concentrations (BLC) were measured at the end of the MWT, before and after the 12 weeks of the protocol (Figure 5B). No significant differences were found at the beginning of the protocol in maximal BLC. However, FRW animals showed a lower BLC at the end of MWT after 12 weeks of free access to the running wheel (FRW: 11.63 ± 3.95 mmol/L vs. SED: 13.77 ± 3.35 mmol/L). Moreover, the animals submitted to the HIIT regimen reached a lower BLC at the end of the MWT (HIIT: 6.00 ± 1.85 mmol/L vs. SED: 13.77 ± 3.35 mmol/L and FRW: 11.63 ± 3.95 mmol/L), despite attained a higher maximal speed when compared to those of the SED and FRW groups.

Figure 6 shows the tail temperature (T_{tail}) at the different steps of the MWT before and after the 12 weeks of the intervention protocol. SED animals did not show statistical differences in T_{tail} in any step, with exception of the baseline (Figure 6A). After the training period, FRW animals showed an increased T_{tail} from speeds 36 to 54 cm/s when compared to baseline (Figure 6B). The HIIT regimen induced a significantly decreased T_{tail} at 36 cm/s. However, at speeds ranging from 45 to 51 cm/s, significant increments were obtained when compared to baseline (Figure 6C). A comparison between groups at the end of the 12 weeks in resting conditions (Figure 6D) showed that HIIT animals presented a lower T_{tail} compared to FRW; compared to

FRW and SED at 36 cm/s, but a higher T_{tail} at 45 and 48 cm/s when compared to SED. The FRW animals revealed a higher T_{tail} at 48 cm/s compared to SED.

Citrate Synthase Activity

Figure 7 shows CS activity in TA and SOL muscles as a marker of mitochondrial oxidative capacity. No significant differences were observed on TA after both chronic exercise regimens (Figure 7A). The activity in the SOL muscle was significantly higher in both exercised groups compared to SED control (Figure 7B).

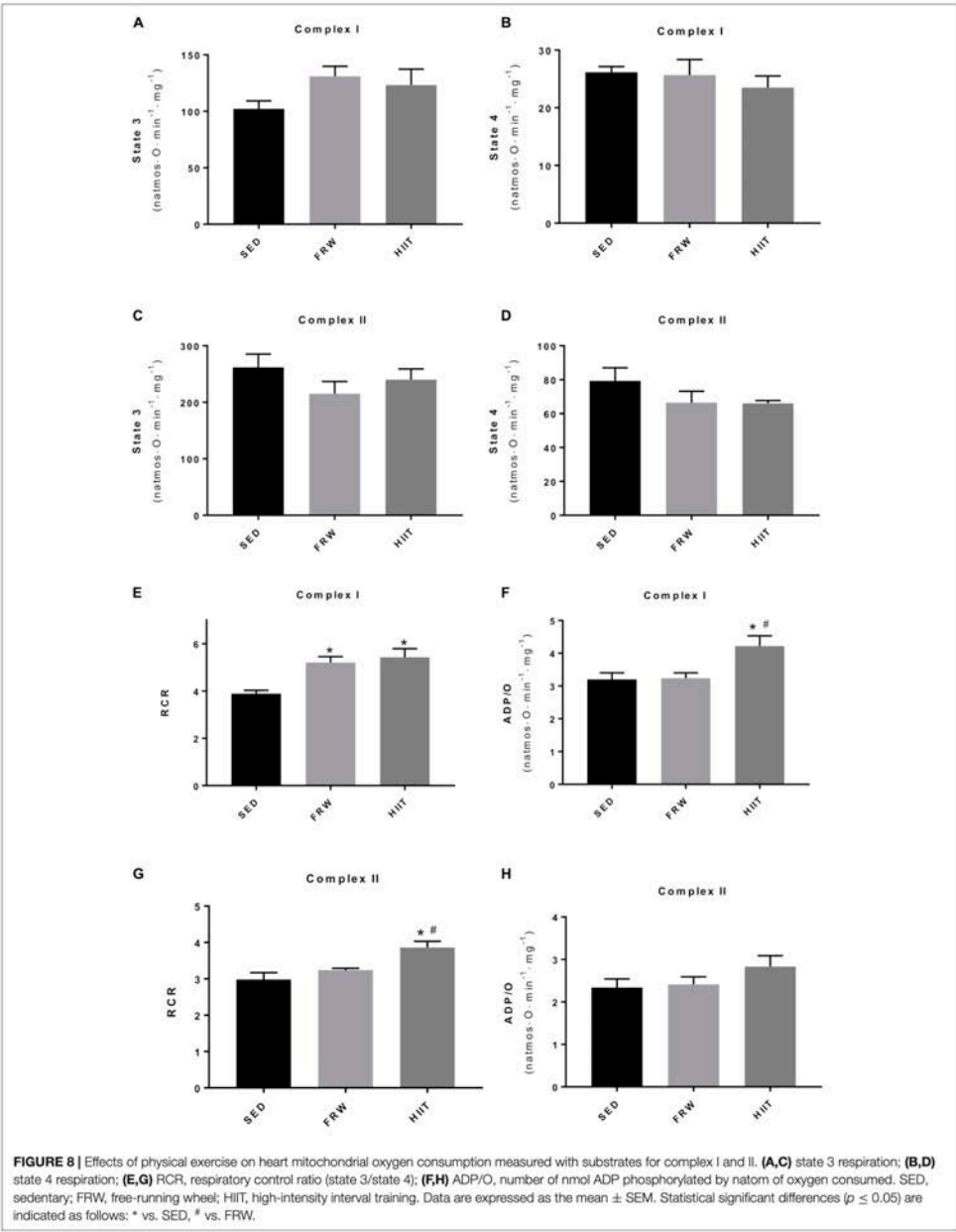
Cardiac Mitochondrial Function

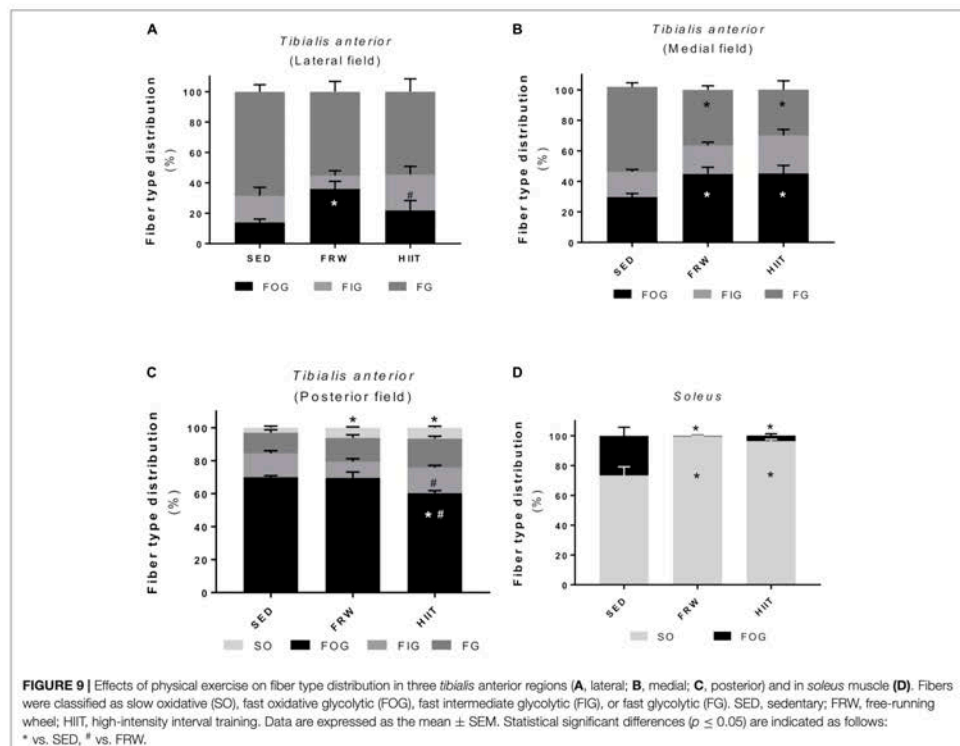
Despite no alterations were found in heart mitochondrial state 3 and 4 when complex I- and II-driven substrates were used after 12 weeks of both training modalities (Figures 8A–D), an increase in RCR with complex I-driven substrates (3.89 ± 0.14 vs. 5.20 ± 0.25 and 5.42 ± 0.37) in FRW and HIIT groups was evident (Figure 8E). When complex II-related substrates were used, HIIT induced an increase in RCR (3.86 ± 0.17 vs. 2.98 ± 0.19 and 3.24 ± 0.05) compared with SED and FRW conditions (Figure 8G). Phosphorylating efficiency, measured as ADP/O ratio, was higher in HIIT group (4.22 ± 0.31 vs. 3.20 ± 0.20 and 3.24 ± 0.16) for complex I-driven substrates (Figure 8F), while no alterations between groups were found when substrates for complex II were used (Figure 8H).

No alterations between groups were found in the transmembrane electrical potential endpoints (maximal potential, repolarization, and lag phase) for both complexes I- and II-related substrates (data not shown).

Skeletal Muscle Fiber Type Distribution

Both chronic exercise models caused alterations in skeletal muscle fiber type distribution in TA and SOL (Figure 9). In the lateral field of TA, the FRW group exhibited an increased

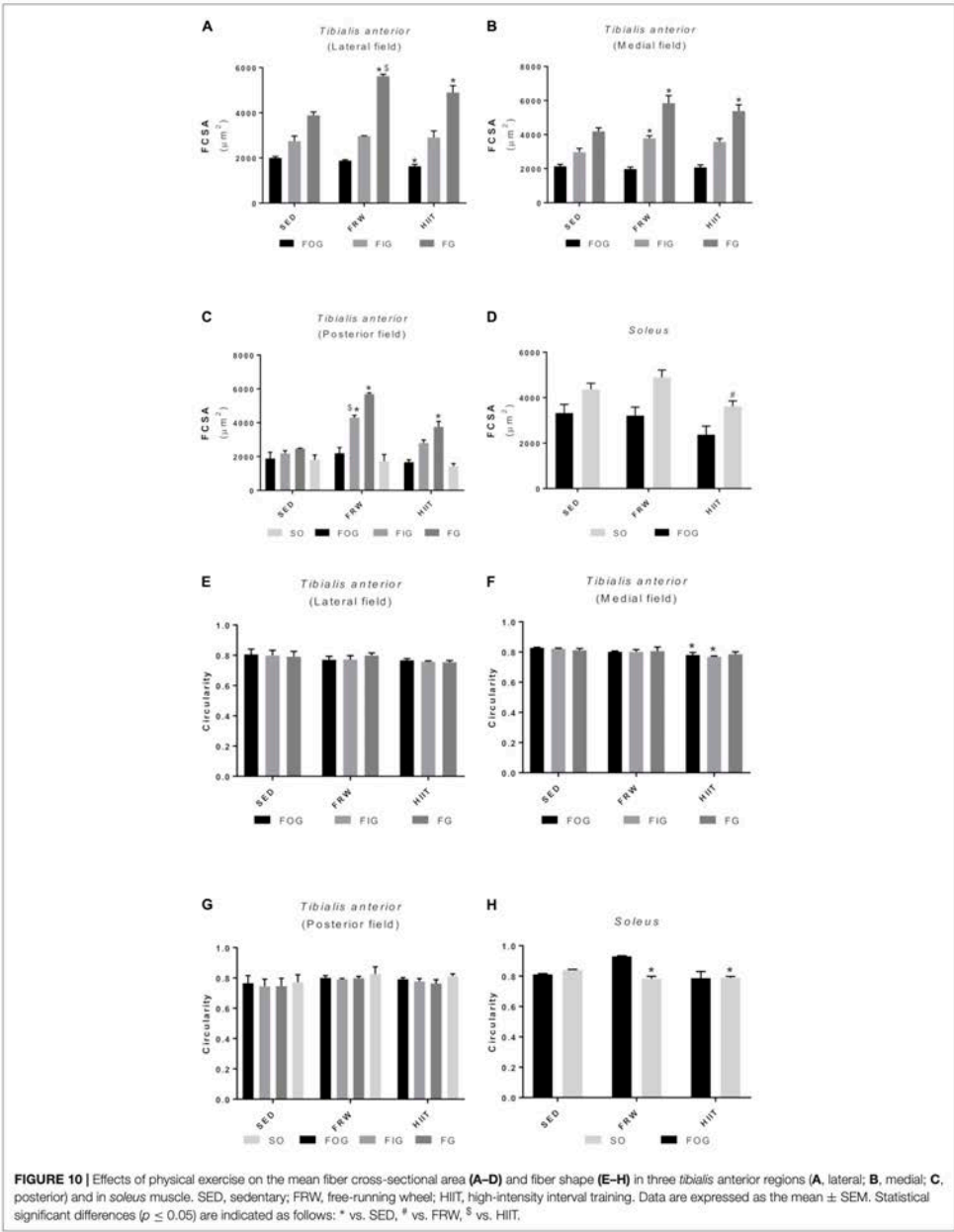


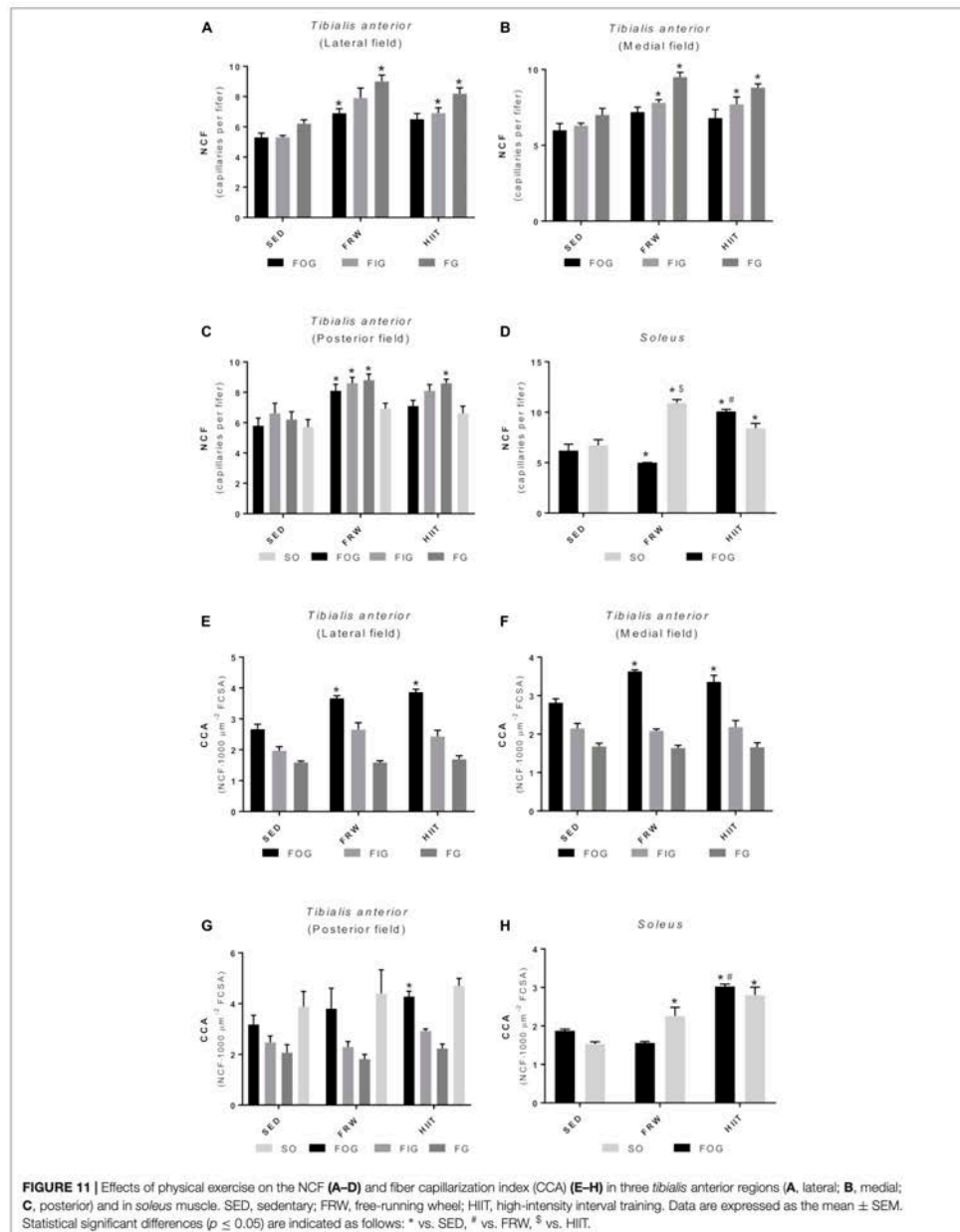


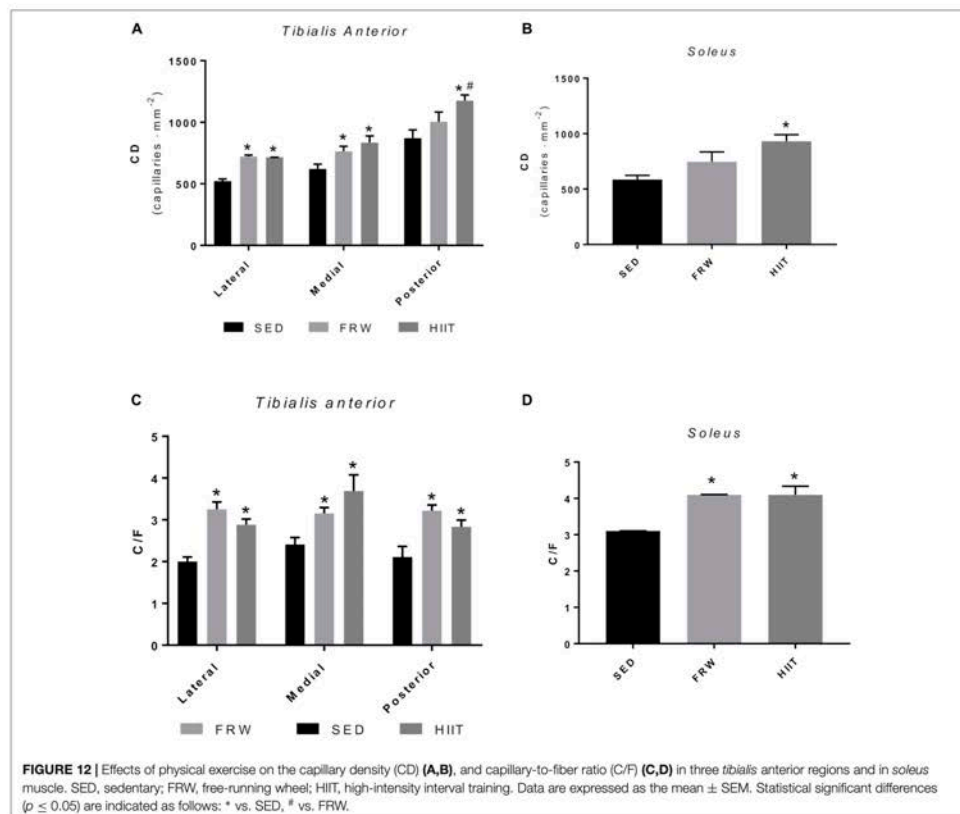
previously mentioned, the adaptations produced by both exercise models were significantly different compared to the SED group along the different analyzed parameters. Our results showed that 12 weeks of HIIT, but also of FRW, induced an increase in the maximal speed evaluated in the MWT, a very important outcome related to animals' functional capacity, even though the maximal speed achieved by HIIT-trained animals was higher than that of FRW. Maximal BLC decreased in both groups, with HIIT presenting lower levels than FRW. These two functional-related parameters are clear indicators of the increased animals' ability to perform maximal exercise and of their metabolic response to high-levels of exercise through an improvement of the aerobic machinery at both central and peripheral levels. From an integrative metabolic perspective, it is possible that the altered skeletal muscle phenotype induced by both chronically intermittent exercise modalities, from more glycolytic into more oxidative-based fibers, might favor an increased efficiency of exercised groups in the lactate metabolism and transformation. This could have occurred through the known autocrine, paracrine, and endocrine links between glycolytic and oxidative metabolisms

sustaining the complex mechanistic concept of lactate shuttle (Ferguson et al., 2018).

Alterations in rats' T_{tail} have been related to exercise intensity (Wanner et al., 2015b). Actually, considering the lack of sweat response in rats, it is known that rats use the blood flow to the tail as a thermoregulatory mechanism (Wanner et al., 2015a). Accordingly, Tanaka et al. (1988) demonstrated that tail vasodilation increases in proportion to the exercise intensity at a range from 45 to 75% of the VO_{2max} ; however, at intensities higher than 75% of the VO_{2max} , this positive linear association does not occur. Therefore, T_{tail} variations in response to similar metabolic activities imposed by treadmill exercise workloads during MWT were used as endpoints of the thermoregulatory response. The T_{tail} started to decrease in the early MWT low-intensity exercise stages and then rapidly increased probably due to skin vasodilatation mechanisms in order to contribute to heat release. This increase in the T_{tail} reached a plateau, which was sustained until the exhaustion (Figure 6). It is of note that, for both exercise protocols, the first significant differences between beginning MWT







and end MWT in T_{tail} were observed at the same speed ($36 \text{ cm} \cdot \text{s}^{-1}$). Moreover, both FRW and HIIT programs resulted in similar kinetics in T_{tail} curves during MWT. Thus, for both exercise protocols, the observed general T_{tail} increase during the 12th week MWT suggest similar thermoregulatory adaptations translated into an increased ability to lose heat by the tail during MWT.

Alterations in Body Weight

The FRW group had higher body weight (in line with sedentary animals) than HIIT animals at the end of the 12 weeks, which can possibly be associated with different weight-related metabolic remodeling features induced by both exercise models. HIIT training has been suggested as a model of exercise that induce body weight reduction in humans, as it comprises high intensity bouts, with consequent increases in energy expenditure (Ramos-Filho et al., 2015; Kong et al., 2016) not only during the course of the exercise itself, but also

in the subsequent period (Schaun et al., 2017). Considering that FRW animals run a significantly higher distance/day than their HIIT counterparts, the global exercise volume performed in the context of the present experimental design did not seem to have *per se* an effective role in the body weight decreases, which is in accordance with other works (Goncalves et al., 2015; Rocha-Rodrigues et al., 2016). Even though we did not measure it in the present study, an empirical assumption suggests that the higher exercise volume accomplished by the FRW rats, in contrast to the historically confirmed influence of continuous long lasting aerobic exercise on body weight (Ascensao et al., 2005), was performed through very short intermittent bouts interspersed by long recovery periods when compared to the 4 min bouts performed by the rats engaged in the HIIT program established in this study, which may compromise its effectivity on body weight alterations. Moreover, despite the methodological care regarding animals' handling and environmental stress imposed in this

study, we cannot exclude the possibility that, at least in part, an additional stress evoked by the forced HIIT program, when compared to FRW, may have influenced animals' metabolism and eating and sleep habits with consequences on body weight alterations.

Morphological and Metabolic Adaptations on Skeletal Muscle

The results were extremely consistent among the different morphometrical and capillarization evaluated parameters (fiber type distribution, fiber size and shape, muscle and fiber capillarization), as well as in the CS activity, suggesting a positive adaptation toward a more oxidative phenotype in both FRW and HIIT compared to the SED hypokinetic animals. In fact, TA and SOL muscles of exercised animals showed an increased percentage of more markedly oxidative fibers (Figure 9), such as FOG in the lateral and medial fields of TA and SO in the posterior field of TA and in SOL. These alterations in the aerobic fiber type percentage were consistent with a decrease in FCSA (Figure 10) of the aerobic fibers (FOG and SO), which has been described as hallmark of morphological muscle modifications under situations where a challenge for oxygen supply was overcome by decreasing cellular diffusion distance to mitochondria (Panisello et al., 2007). On the other hand, FG fibers from TA muscle adapted to the stimulus increasing cross-sectional area in FRW and HIIT groups, suggesting that both models simultaneously induce fiber type-specific adaptations aimed to reduce muscle fatigability while maintaining force production needed during short bouts of high intensity activity.

Moreover, as indicates the reduction in circularity, both exercise models induced elongated transversal profiles in SO fibers from SOL muscle, thus resulting in higher fiber perimeters. As it is well-known that there is a substantial proportion of mitochondria distributed in a subsarcolemmal position (Weibel, 1984), longer fiber perimeters will allow more mitochondria situated at shorter diffusion distances from the capillaries. This, as has been proposed in other animal models, could be seen as a fiber morphometrical modification to facilitate the oxygen supply to mitochondria in situations of high aerobic demand (Johnston, 1982; Torrella et al., 1998). All the previously described modifications were reinforced by an increased fiber capillarization (CCA) and global field capillarization (CD and C/F). Indeed, these results suggest that both FRW and HIIT protocols induced angiogenesis as reflected by higher C/F and NCF values. Considered a crucial biomarker of both mitochondrial content and an important indicator of tissue oxidative fitness, CS activity was determined in skeletal muscles, being its activity augmented in both exercise regimen groups, although only in SOL muscle showed statistically significant differences.

Cardiac Mitochondrial Function

In order to explore the potential impact of both FRW and HIIT models on central-related training adaptations,

cardiac muscle mitochondrial respiratory capacity, an important hallmark reflecting cardiac metabolism and function (Abel and Doenst, 2011), was evaluated. In accordance with our previous studies dealing with models of forced endurance exercise (Ascensao et al., 2005; Magalhaes et al., 2013), our results revealed that FRW and HIIT induced beneficial cardiac mitochondrial adaptations compatible with an increase in the metabolic coupling. Mitochondrial RCR is influenced by several metabolic oxidative features and is recognized as the best general measure of mitochondrial function in isolated mitochondria (Brand and Nicholls, 2011). In the present study, cardiac mitochondria from both FRW and HIIT animals energized with complex I-linked substrates showed higher RCR compared to SED, which suggests that the metabolic coupling between oxygen consumption and ATP synthesis was improved. Nevertheless, when substrates for complex II were used, these improvements were only observed in the HIIT group. Likewise, mitochondrial-related data also showed that HIIT, but not FRW, induced an increase in mitochondrial phosphorylation efficiency in ATP production as measured by ADP/O ratio. This data suggest that physiological central mechanisms were also positively modulated by both HIIT and FRW and certainly contributed, at least in part, for the improved ability to perform maximal exercise through a more toward oxidative phenotype.

CONCLUSION

Data from our study clearly suggest that FRW is an exercise model that induces adaptations comparable, in most cases, with a forced HIIT regimen. In fact, functional and thermoregulatory adaptations, as well as significant alterations in biochemical and morphological phenotype in both skeletal and cardiac muscles were found in FRW-exercise animals when compared to those engaged in a HIIT protocol.

ETHICS STATEMENT

This study was carried out in accordance with guidelines for Care and Use of Laboratory Animals in Research advised by Federation of European Laboratory Animal Science Associations (FELASA). The Ethics Committee of the Faculty of Sport, University of Porto, and the National Government Authority (Direção Geral de Alimentação e Veterinária – N° 0421/000/000/2018) approved the experimental protocol.

AUTHOR CONTRIBUTIONS

JB, AA, JT, JS, and JM conceived and designed the research. JB, JA, ES-A, PF, JS, SR-R, and JM conducted sample collection, processing, and data collection. JB, GS, DR-R, and JT performed image analysis. JB, AA, JS, and JM composed the initial manuscript. JT, AA, and JM revised and approved the final draft.

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IV

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

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

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

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

⁵Department of Biomedicine, Biochemistry Unit, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal.

⁶CNC—Center for Neuroscience and Cell Biology, UC-Biotech, University of Coimbra, 3060-197 Cantanhede, Portugal

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ABSTRACT

Background: Maternal high-caloric nutrition and related gestational diabetes mellitus (GDM) are associated with a high-risk for developing metabolic complications later in life and in their offspring. In contrast, exercise is recognized as a non-pharmacological strategy against metabolic dysfunctions associated to lifestyle disorders. Therefore, we investigated whether gestational exercise delays the development of metabolic alterations in GDM mothers later in life, but also protects 6-week-old male offspring from adverse effects of maternal diet.

Methods: Female Sprague-Dawley rats were fed with either control (C) or high-fat high-sucrose (HFHS) diet to induce GDM and submitted to gestational exercise during the 3 weeks of pregnancy. Male offspring were sedentary and fed with C-diet.

Results: Sedentary HFHS-fed dams exhibited increased gestational body weight gain ($p < 0.01$) and glucose intolerance ($p < 0.01$), characteristic of GDM. Their offspring had normal glucose metabolism, but increased early-age body weight, which was reverted by gestational exercise. Gestational exercise also reduced offspring hepatic triglycerides accumulation ($p < 0.05$) and improved liver mitochondrial respiration capacity ($p < 0.05$), contributing to the recovery of liver bioenergetics compromised by maternal HFHS diet. Interestingly, liver mitochondrial respiration remained increased by gestational exercise in HFHS-fed dams despite prolonged HFHS consumption and exercise cessation.

Conclusions: Gestational exercise can result in liver mitochondrial adaptations in GDM animals, which can be preserved even after the exercise program cessation. Exposure to maternal GDM programs liver metabolic setting of male offspring, whereas gestational exercise appears as an important preventive tool against maternal diet-induced metabolic alterations.

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

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

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

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1. Introduction

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

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

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

2. Material and methods

2.1. Animals

Seven-week-old female Sprague-Dawley rats (150–200 g) (Charles River, L'Arbresle, France), were randomly divided into 2 diet groups: control (C) and high-fat high-sucrose (HFHS) diet. After 7-weeks on respective diets, dams were mated with sedentary C-fed male rats (Charles River, L'Arbresle, France). Pregnant HFHS-fed dams were divided into two subgroups: sedentary animals and animals submitted

to gestational exercise protocol during pregnancy. This resulted in 3 experimental groups ($n = 6$ per group): P-C-S - sedentary C-fed dams; P-HFHS-S - sedentary HFHS-fed dams; and P-HFHS-E - exercised HFHS-fed dams. Dams delivered naturally and litter size was reduced to 3 male and 3 female pups to avoid food competition and effects on pups' body mass. The offspring were weaned after 3-weeks of nursing. Male offspring were assigned to 3 experimental groups ($n = 6$ per group): C—S offspring of sedentary C-fed dams; HFHS-S offspring of sedentary HFHS-fed dams; and HFHS-E offspring of exercised HFHS-fed dams. The offspring from all experimental groups were sedentary and fed with C-diet. Dams were euthanised 8-weeks post-partum and the offspring at 6-weeks of age (Fig. 1A).

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

2.2. Diets and exercise protocol

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

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

2.3. Glucose homeostasis

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

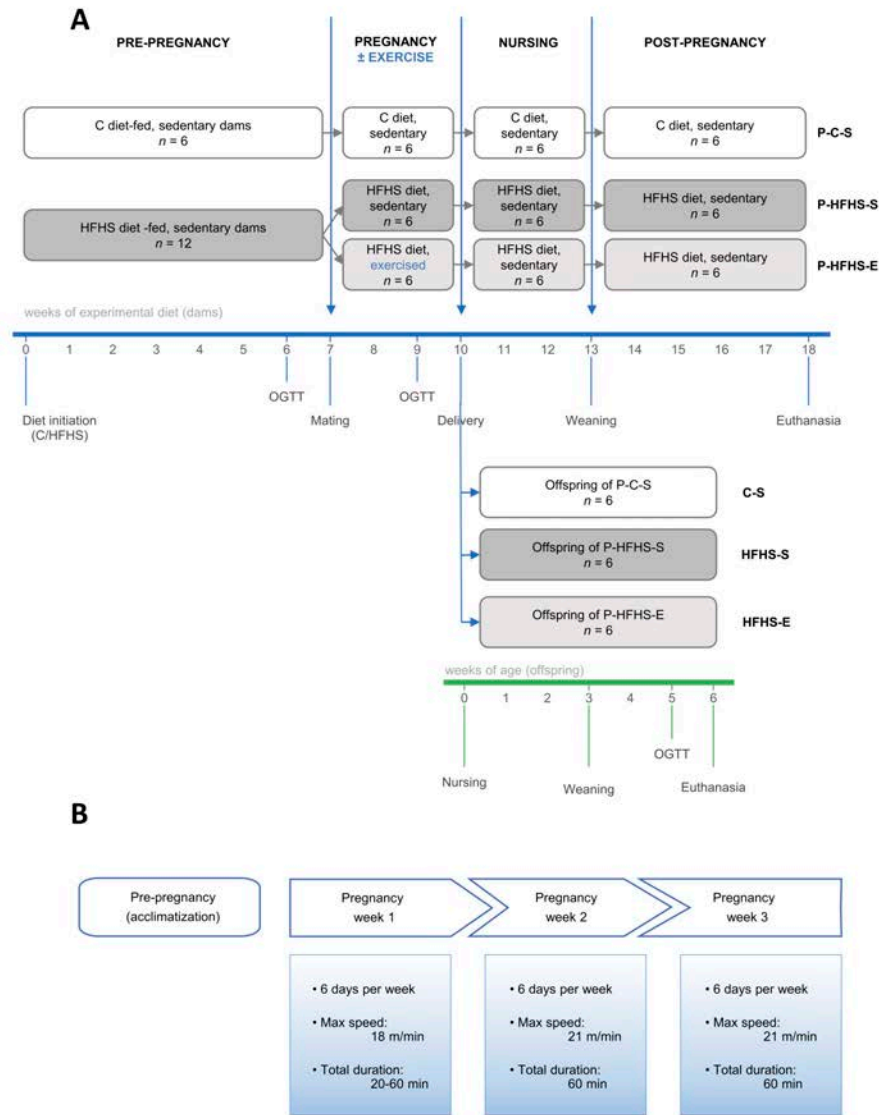


Fig. 1. Experimental (A) and exercise (B) protocol. C – maternal control diet; HFHS – maternal high-fat high-sucrose diet; P – pregnant animals in maternal generation; S – sedentary animals in maternal generation; E – exercising animals in maternal generation. OGTT – oral glucose tolerance test.

related glucose intolerance during pregnancy. In the offspring, OGTT was performed 1-week before euthanasia. Glucose was administered to the rats at a dose of 2 g/kg body weight. To avoid potential harm induced by intraperitoneal injection or oral gavage to (pregnant) mothers during OGTT, glucose was orally administered in the form of non-flavoured jelly (Globo Gelatina Neutra, A Colmeia do Minho,

Portugal) [22]. To avoid novel objects avoidance, rats were trained to eat jelly without glucose. Moreover, since prolonged hypoglycaemia in pregnancy could influence the fetus development, animals were submitted to a 6-h fasting period prior to OGTT. The same fasting period was followed in pre-mating OGTT and in offspring OGTT.

2.4. Animal euthanasia and tissue sampling

Animals were anaesthetized (induction: 5% isoflurane, 1 L/m O₂; maintenance: 2.5% isoflurane, 0.4 L/m O₂) after overnight fasting. The liver was rapidly excised, rinsed with ice-cold PBS, weighed, and separated for light microscopy, mitochondria isolation, and other analysis. The blood was collected from inferior vena cava and centrifuged (3000 ×g, 10 min, 4 °C). Collected plasma was stored at −80 °C until later use for biochemical and metabolomics analysis. In order to reduce performance and detection bias in the following techniques and to avoid researchers from knowing the animals' treatment groups, the animals were labelled with a blinded coding system during data acquisition until the statistical analysis.

2.5. Light microscopy and liver triglyceride content

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

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

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

2.6. Analysis of acylcarnitine levels in plasma and liver homogenates

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

2.7. Isolation of liver mitochondria and mitochondrial respiratory assays

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

Table 1
Animal data and plasma biochemical analyses.

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

Values are means ± SEM (n = 6).

P – pregnant animals (maternal generation); NP – non-pregnant counterparts of maternal generation; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers;

ALT – alanine aminotransferase; AST – aspartate aminotransferase; HDL – high-density lipoproteins; LDL – low-density lipoproteins.

* vs. P-C-S (p < 0.05) or vs. C-S (p < 0.05) in maternal or offspring generation, respectively.

* vs. P-HFHS-S (p < 0.05) or vs. HFHS-S (p < 0.05) in maternal or offspring generation, respectively.

mitochondrial protein in respiration buffer (130 mM sucrose, 10 mM HEPES, 2.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM KH_2PO_4 , 0.1 mM EGTA, pH 7.4). Mitochondrial respiration through electron transport chain (ETC) complex I or complex II was initiated by adding glutamate/malate (G/M, 10 mM and 5 mM, respectively) or succinate (10 mM) plus rotenone (1.5 μM), respectively. State 3 respiration and phosphorylation cycle were obtained by adding ADP (150 nmol) and the rate of oxygen consumption after ADP phosphorylation represented state 4. The state 3/state 4 ratio used to calculate the respiratory control ratio (RCR). ADP/O ratio represented the ratio between the number of nmol ADP phosphorylated by nmol O_2 consumed during state 3. Additionally, the state 4 was measured in the presence of oligomycin (2 μg) in order to inhibit proton influx through the ATP synthase.

2.8. Western blotting

Equivalent amounts (20 μg) of liver mitochondria were denatured in sample loading buffer and separated by SDS/PAGE, followed by transfer to PVDF membranes. Membranes were blocked with non-fat dry milk and afterwards incubated with primary antibodies: anti-OXPHOS cocktail (1:1000; Abcam, ab110413) and anti-UCP2 (1:500; Sigma Aldrich, SAB2501087), and subsequently, with secondary antibodies: horseradish peroxidase-conjugated anti-goat (1:10000; Santa Cruz, sc-2354) or anti-mouse (1:10000; Santa Cruz, sc-2005). Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, #1705061) and

acquired by ChemiDoc-XRS Image System (Bio-Rad). Density was determined by using the Image Lab software 6.0.1 (Bio-Rad). Ponceau S staining was used for normalization of protein loading or transfer differences [26]. The final data were expressed as percentage variation of control values (%P-C-S or %C-S, in maternal and offspring generation, respectively).

2.9. Statistical analysis

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

3. Results

3.1. Maternal and offspring characteristics and glucose homeostasis

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

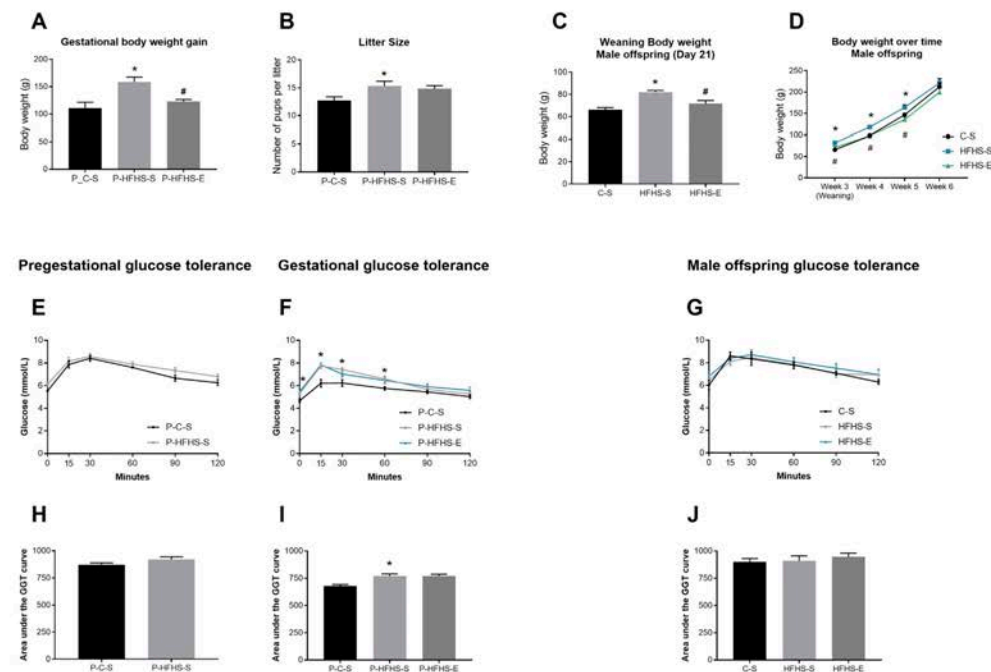


Fig. 2. Effect of maternal high-fat-high-sucrose diet and gestational exercise on maternal body weight gain during gestation period (A), litter size (B) and offspring body weight changes from weaning (C) to the euthanasia point at 6 weeks of age (D), maternal glucose tolerance before pregnancy (E, H)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

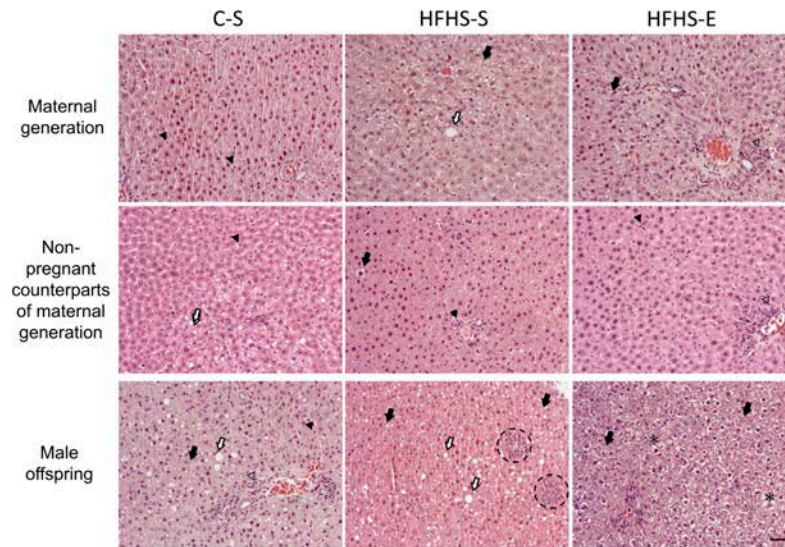


Fig. 3. Representative light micrographs ($\times 200$) of liver tissue from all groups and generations: C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers. The presence of microvesicular steatosis (black arrow), macrovesicular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

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

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

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

3.2. Histological analysis and liver triglyceride content

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

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

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

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

Values are means $\pm$ SEM ($n = 6$).

P – maternal generation; NP – non-pregnant counterparts of maternal generation; C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised mothers.

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

3.3. Plasma and liver acylcarnitine levels

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

3.4. Liver mitochondrial bioenergetics

After 18-weeks of HFHS-diet, sedentary dams did not show any changes in the RCR with G/M- or S/R-related substrates; however gestational exercise increased the RCR ($p < 0.05$, vs. P_HFHS_S), when G/M were used as substrates, which was maintained even after exercise cessation (Fig. 6A-C). The same effect of gestational exercise (P_HFHS_S vs. P_HFHS_E) was noticed in respiratory rates with oligomycin (G/M and S/R) (Fig. 7E). In contrast, maternal HFHS-diet and sedentarism reduced offspring RCR, an effect which was not observed in the offspring of exercised HFHS-fed mothers ($p < 0.05$, vs. P_HFHS_S) (Fig. 6E-G). Respiration with oligomycin (G/M) was greatly increased in offspring of sedentary HFHS mothers when compared to sedentary C mothers, whereas maternal gestational exercise diminished the impact of maternal HFHS (Fig. 8F). The ADP/O ratio was not affected by maternal diet and exercise in both generations (Fig. 7D, H).

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

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

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

4. Discussion

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

4.1. Animal characteristics and model of GDM

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

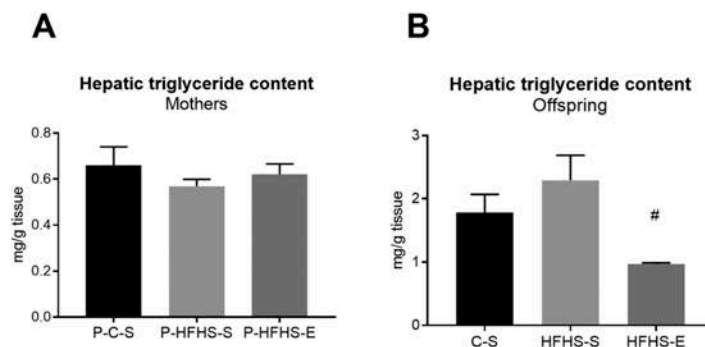


Fig. 4. Effect of maternal high-fat high-sucrose diet and gestational exercise on maternal (A) and offspring (B) liver triglyceride content; P – pregnant animals (maternal generation); C – mothers fed with control diet; HFHS – mothers fed with high-fat high-sucrose diet; S – sedentary mothers; E – exercised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

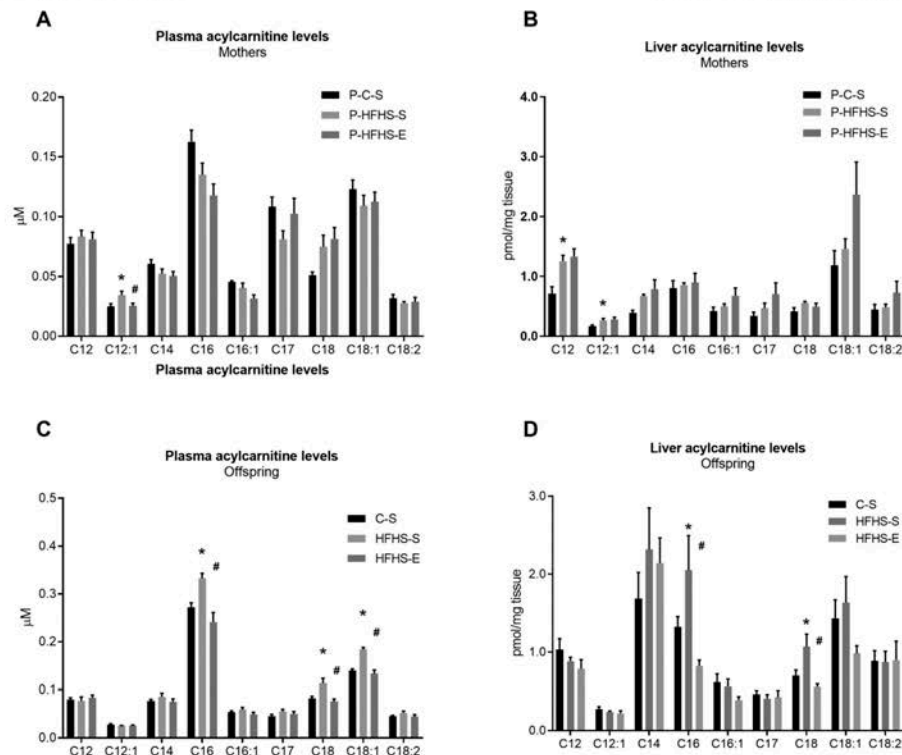


Fig. 5. Effect of maternal high-fat high-sucrose diet and gestational exercise on acylcarnitine levels in (A) plasma and (B) liver in the maternal generation on acylcarnitine levels in plasma (C) and liver (D) in the offspring generation. C – mothers fed with control diet; HFHS – mothers fed with high-fat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

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

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

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

4.2. Hepatic lipid accumulation

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

Mothers

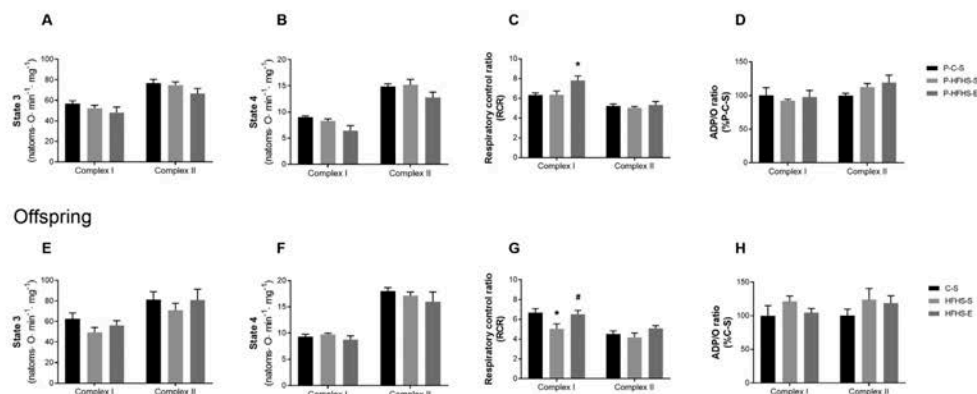


Fig. 6. Effect of maternal high-fat high-sucrose diet and gestational exercise on liver mitochondria (A) state 3 respiration, (B) state 4 respiration, (C), respiratory control ratio (RCR), and (D) ADP/O ratio in the maternal generation; and (E) state 3 respiration, (F) state 4 respiration, (G), respiratory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

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

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

4.3. Liver mitochondrial function

Acylcarnitine profiling is used to describe alterations in mitochondrial β -oxidation in animal models of obesity and IR [40]. In the dams of the present study, HFHS feeding did not alter long-chain acylcarnitine levels. Eventually, the 8 or 12-weeks of HFD did not influence hepatic acylcarnitines due to liver capacity to export the excess of FA to circulation [40,41]. Conversely, offspring have increased exactly those plasma and liver acylcarnitines (C16, C18, and C18:1), that correspond to different FA composition between C and HFHS-diets consumed by mothers.

Considering that fetus receives nutrient supplies, especially glucose and FA, from maternal diet through the placenta [42], these data suggest that increased accumulation of those acylcarnitine in the offspring plasma and liver are a consequence of maternal diet. Furthermore, reduced placental β -oxidation in GDM encourages the accumulation of triglycerides in placenta [37], which may result in an increased transfer of placental FA to the fetus liver FA pool. Fortunately, it seems that maternal gestational exercise emerged as a very effective tool against excessive energy originated from mothers' HFHS-diet. Accordingly, less FA seem to be transferred through placenta to the fetus, resulting in the control levels of acylcarnitines in plasma and liver of the offspring from exercised HFHS mothers. Even though liver and muscle contribute differently to the plasma acylcarnitine pool during exercise, i.e. muscle mainly contribute to plasma medium and long-chain acylcarnitines and liver only for short-chain acylcarnitines [43], our data showed that maternal exercise promoted a decrease in hepatic and plasma long-chain acylcarnitines in the offspring when compared to their sedentary counterparts. Nevertheless, Xu and collaborators provided evidence that upon exercise, skeletal muscle can release long-chain acylcarnitines to plasma, that are further taken by liver [43]. Indeed, plasma levels of acylcarnitine above a certain threshold seem to represent a stimulus for their uptake by the liver [44]. In the liver, however, due to the increased fitness mitochondrial phenotype stimulated by exercise (Fig. 6), long-chain acylcarnitines are efficiently shortened and exported to circulation as short-chain acylcarnitines resulting in the decrease of the hepatic long-chain acylcarnitines levels [43]. Another possible interesting mechanism that could explain this role of exercise includes muscle-secreted myokines [45]. The secretion of myokines such as interleukins (IL)-6, fibroblast growth factor 21 (FGF21), and fibronectin type III-domain containing 5 (FNDC5), later cleaved to irisin, are found to be increased upon skeletal muscle contraction [46]. Irisin may further modulate PPAR α signalling pathways, which consequently regulates lipid metabolism through thermogenesis mechanisms or upregulation of FGF21, which can eventually improve liver insulin sensitivity [47]. Furthermore, considering that PPAR α is also involved in hepatic (acyl)carnitine metabolism [48], this irisin-

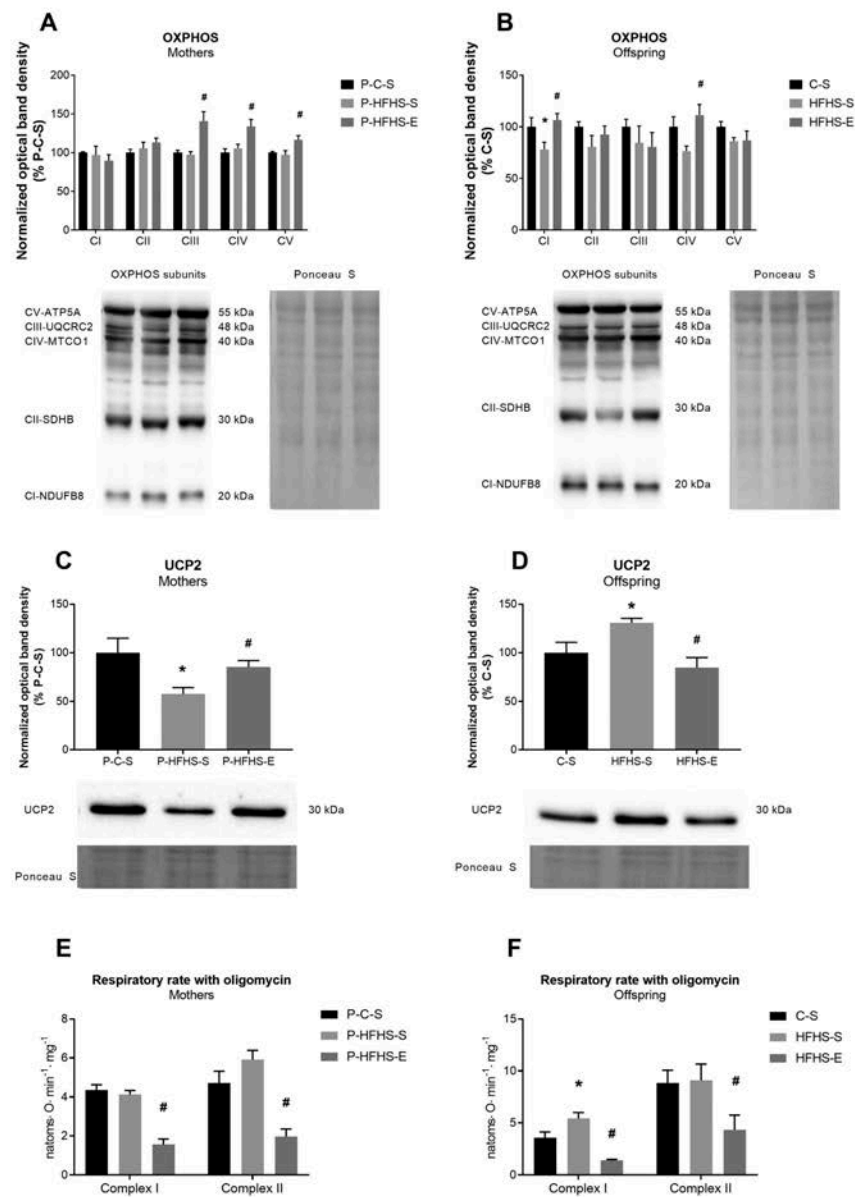


Fig. 7. Effect of maternal high-fat high-sucrose diet and gestational exercise on OXPHOS subunits in (A) maternal generation and (B) offspring generation and UCP2 content in (C) maternal generation and (D) offspring generation; and respiratory rate with oligomycin in (E) maternal generation and (F) offspring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

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

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

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

To our knowledge, there are no data regarding the effect of exercise cessation on liver mitochondrial function of GDM animals. In the present study, HFHS-diet per se did not affect liver mitochondrial function or OXPHOS expression in mothers. Indeed, in other studies, HFD did not alter liver mitochondrial respiratory rates in female rodents [58,59]. Interestingly, we observed that even 8-weeks after exercise cessation, mitochondrial adaptations were maintained, as reflected in

increased RCR values when using complex I-related substrates and increased protein expression of complex III, complex IV, and ATP synthase. Even though increased oxygen consumption was not followed by upregulation of complex I, the increase of complexes III and IV expression may suggest that gestational exercise promoted the formation of ETC supercomplex. In fact, enhanced supercomplex formation was observed in human skeletal muscle upon exercise [60]. This dynamic supercomplex, consisting of complexes I, III, and IV, can represent a mechanism through which mitochondrial ETC might adapt to cell requirements [61]. Within supercomplexes, the stability of complex I seems to be maintained by complex III [62] and complex IV [63], which may explain the improvement of complex I-related RCR due to exercise without any alterations in complex I protein expression.

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

5. Conclusions

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

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

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CRedit authorship contribution statement

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

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

Declaration of competing interest

No conflict of interest.

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¹Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain.

²Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal.

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



Article

Gestational exercise increases male offspring maximal workload capacity early in life.

Jorge Belezai^{1*}, Jelena Stevanović-Silva², Pedro Coxito², Hugo Rocha³, António Ascensão², Joan Ramon Torrella¹, José Magalhães²

¹ Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain;

² Laboratory of Metabolism and Exercise (LaMetEx), Research Centre in Physical Activity, Health and Leisure (CIAFEL), Laboratory for Integrative and Translational Research in Population Health (ITR), Faculty of Sport, University of Porto, Porto, Portugal;

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

* Correspondence:

E-mail: jmsbeleza@gmail.com

Jorge Beleza

Secció de Fisiologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

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Abstract: Mothers' antenatal strategies to improve the intrauterine environment can positively decrease pregnancy-derived interferences. By challenging the mother-fetus unit, gestational exercise (GE) favorably modulates deleterious stimuli, such as high-fat high-sucrose (HFHS) diet-induced adverse consequences for offspring. We aimed to analyze whether GE alters maternal HFHS-consumption effects on male offspring maximal workload performance (MWP) and in some skeletal muscle (soleus-SOL and tibialis anterior-TA) biomarkers associated with mitochondrial biogenesis and oxidative fitness. Male offspring Wistar rats were divided into experimental groups according to mothers' dietary and/or exercise conditions: offspring of sedentary control diet-fed or HFHS-fed mothers (C-S or HFHS-S, respectively) and of exercised HFHS-fed mothers (HFHS-E). Despite maternal HFHS did not significantly alter MWP, offspring from GE dams increased MWP. Lower SOL AMPK levels in HFHS-S were reverted by GE. SOL PGC-1 α , OXPHOS C-I and C-IV subunits remained unaltered by maternal diet, although increased in HFHS-E offspring. Additionally, GE prevented maternal diet-related SOL miR-378a overexpression, while upregulated miR-34a expression. Decreased TA C-IV subunit expression in HFHS-S was reverted in HFHS-E, concomitantly with the downregulation of miR-338. In conclusion, GE in HFHS-fed dams increases offspring MWP, which seems to be associated with the intrauterine modulation of SM mitochondrial density and functional markers.

Keywords: Maternal Exercise; Epigenetics; Mitochondria.

1. Introduction

The concept of developmental origins of health and disease (DOHaD) uncovers the importance of maternal environmental exposure during critical periods of perinatal development, which may affect the offspring risk for chronic diseases in a short- or long-

term manner [1-3]. In fact, adverse in utero environment, such as maternal malnutrition, might prompt offspring maladaptive responses, leading to persistent physiological and metabolic alterations throughout life [4]. Accordingly, maternal high-fat diet (HFD) consumption during gestation and lactation impairs glucose regulation and alter insulin sensitivity in the adult offspring [5-7]. These (patho)physiological consequences in offspring result from progressive development of less resistant phenotypes in several tissues and organs, including skeletal muscle (SM), a key cornerstone in different important adaptive-related axis [8-10] that are usually perpetrated later in life. These direct and indirect key roles of SM can be illustrated by its autocrine, paracrine, and/or endocrine-like effects in local and systemic physiology, but also by its importance in locomotor activities, thereby determining physical fitness and performance features.

Among many subcellular structures influencing SM adaptive routes, particular emphasis is given to mitochondrial network due to its known roles in a plethora of processes, including energy production, inter-compartmental redox regulation, ion buffering, as well as apoptotic and auto(mito)phagy signaling intermediates. Moreover, SM mitochondrial turnover and renewal through dynamic processes of mitochondrial biogenesis, fusion, and fission [10,11] ultimately lead to different (un)fitness resistant phenotypes with consequences in exercise performance, as well as training efficiency early in life [12,13].

Besides being a master tool to improve athlete's performance, physical exercise (PE) has been considered as an important preventive and therapeutic non-pharmacological strategy against several pathological conditions [14,15], including HFD-induced health-related complications [16], and being mitochondrial physiology in the center of the observed beneficial adaptations [17]. In addition, data as been showing that maternal exercise (ME) performed during pregnancy provides health-related benefits not only for mothers, but also for their offspring [18-21]. In fact, rodents studies have demonstrated that ME improved offspring long-term glucose homeostasis [22-25] and increased the expression of SM mitochondrial content and biogenesis signaling markers [26]. Furthermore, ME performed before and during pregnancy may increase offspring volition for self-paced free-running wheel (FRW) exercise [13]. However, the impact of ME exercise on offspring MWP, as well as on mitochondrial-associated metabolic biomarkers is so far unknown. Yet, this concern could potentially be of high interest considering the link between the effectiveness of ME in the offspring intergenerational programming and physical fitness contribution to health outcomes throughout life [27].

Therefore, we here intended to ascertain whether gestational exercise (GE) mitigates the effects of maternal HFHS-consumption on male offspring MWP, and in some SM biomarkers associated with mitochondrial biogenesis and oxidative fitness. These include, among others, usual biomarkers of mitochondrial density (citrate synthase (CS) activity and mitochondrial DNA (mtDNA) content), key regulatory proteins associated with mitochondrial biogenesis signaling (AMPk, PGC-1 α , NRF2 and TFAM), inner mitochondrial membrane proteins known to actively influence electron transport chain activity and oxidative phosphorylation (OXPHOS subunits, ANT), and transmembrane proton gradient and redox environment (UCP3). Also, microRNAs (miRNAs) will be analyzed as important players in the post-transcriptional regulation of gene expression coding for some of these proteins (miR-378a, miR-494, miR-338, mirR-34a) in SM with distinct metabolic phenotypes – soleus (SOL) and tibialis anterior (TA). We hypothesized that GE attenuates the negative effects of maternal HFHS-consumption on offspring MWP, at least in part, through SM mitochondrial remodeling.

2. Results

The present study focused on the effects of GE on MWP and associated SM markers of oxidative metabolic features in 6-wks-old male offspring from dams fed with HFHS diet. Two different SM were considered to study the impact of maternal environmental exposure on distinct SM phenotypes - SOL as a muscle with a marked oxidative phenotype and TA as a muscle with a heterogeneous constitution and function.

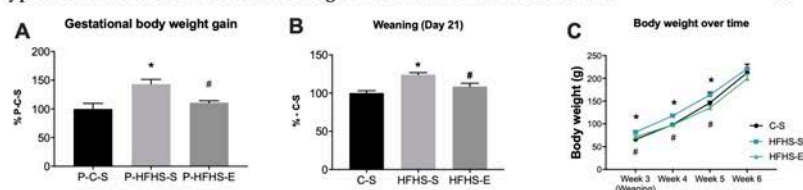


Figure 2: Gestational body weight gain of dams during pregnancy (A); offspring body weight at 21 days of age (B); and offspring body weight from the weaning until 6 weeks of age (C). Control sedentary dams (P-C-S); high-fat high-sucrose sedentary dams (P-HFHS-S); high-fat high-sucrose exercised dams (P-HFHS-E); offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

Table 1. Body weight, SM weight, and plasma biochemical analysis

	C-S	HFHS-S	HFHS-E
Body weight (g)	212.40 $\pm$ 8.74	221.20 $\pm$ 10.02	199.30 $\pm$ 10.23
Soleus/Body Weight (mg/g)	0.47 $\pm$ 0.03	0.38 $\pm$ 0.02*	0.45 $\pm$ 0.01 [†]
Tibialis Anterior/Body Weight (mg/g)	1.60 $\pm$ 0.03	1.52 $\pm$ 0.04	1.74 $\pm$ 0.03 [†]
Glucose (mg/dL)	193.43 $\pm$ 11.60	180.20 $\pm$ 3.44	211.83 $\pm$ 5.78 [†]
Triglycerides (mg/dL)	64.00 $\pm$ 4.78	79.00 $\pm$ 0.63*	51.50 $\pm$ 2.14 [†]

Table 1: Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

Male offspring of sedentary HFHS-fed mothers had significantly higher body weight (BW) at weaning compared to offspring of sedentary C-fed mothers, suggesting that GE prevented the adverse effects of maternal HFHS on offspring BW (Figure 2B). Although, the BW differences between groups were maintained throughout the 2 remaining experimental weeks, no significant statistical differences were found in offspring BW in the 6th week (Figure 2C).

As presented in Table 1, SOL-to-BW ratio was significant lower in HFHS-S group when compared to C-S, but was restored back to baseline values in offspring from exercised mothers. Still, in TA, no significant alterations associated to maternal nutrition were found (C-S vs. HFHS-S), while an increase in TA-to-BW ratio was found in HFHS-E animals compared to sedentary counterparts.

Offspring of HFHS-S mothers exhibited higher blood triglycerides compared to their C-S counterparts (HFHS-S vs. C-S); however, GE despite maternal HFHS-feeding reverted triglycerides levels back to baseline (HFHS-S vs. HFHS-E).

Table 2. Maximal workload test

Table 2: Maximal workload test. Final speed achieved at the end of the maximal workload (Max Speed); time until exhaustion expressed ($T_{\text{Exhaustion}}$); basal blood lactate concentration ($\text{Lactate}_{\text{Basal}}$); highest blood lactate concentration measured at the end of the maximal workload ($\text{Lactate}_{\text{PostEx}}$); basal blood glucose concentration ($\text{Glucose}_{\text{Basal}}$); blood glucose concentration measured at the end

	Max Speed ($\text{cm}\cdot\text{s}^{-1}$)	$T_{\text{Exhaustion}}$ (min)	Lactate _{Basal} (mmol/L)	Lactate _{PostEx} (mmol/L)	Glucose _{Basal} ($\text{mg}\cdot\text{min}/\text{dL}$)	Glucose _{PostEx} ($\text{mg}\cdot\text{min}/\text{dL}$)
C-S	48.57 $\pm$ 1.52	15.31 $\pm$ 2.86	1.81 $\pm$ 0.12	7.66 $\pm$ 0.24	132.21 $\pm$ 3.93	134.00 $\pm$ 6.47
HFHS-S	47.50 $\pm$ 2.36	15.53 $\pm$ 1.33	1.96 $\pm$ 0.90	7.50 $\pm$ 0.34	135.33 $\pm$ 3.00	136.00 $\pm$ 6.59
HFHS-E	56.25 $\pm$ 2.19 [#]	20.67 $\pm$ 1.50 [#]	1.94 $\pm$ 0.90	7.33 $\pm$ 0.80	128.30 $\pm$ 3.09	135.40 $\pm$ 5.05

of the maximal workload ($\text{Glucose}_{\text{PostEx}}$). Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: # vs. HFHS-S.

Table 2 shows performance data, namely maximal speed and time to exhaustion. As can be observed, offspring from HFHS-E group were able to run at higher maximal speed during a longer period of time when compared to their HFHS-S counterparts. No differences between groups were found in basal and post-exercise blood lactate and glucose contents.

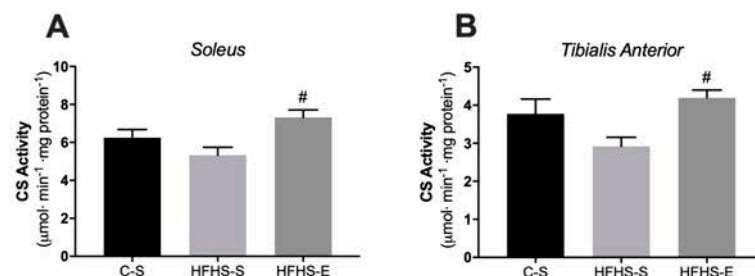


Figure 3: Citrate synthase activity normalized to muscle protein content in Soleus (A) and Tibialis Anterior (B). Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: # vs. HFHS-S.

Figure 3 shows CS activity in SOL and TA muscles. No significant alterations induced by maternal HFHS diet were noted in CS activity of both SM from sedentary dams (HFHS-S vs. C-S). However, offspring from exercised HFHS-fed mothers had a higher CS activity in SOL and TA compared to the offspring of their non-exercised counterparts.

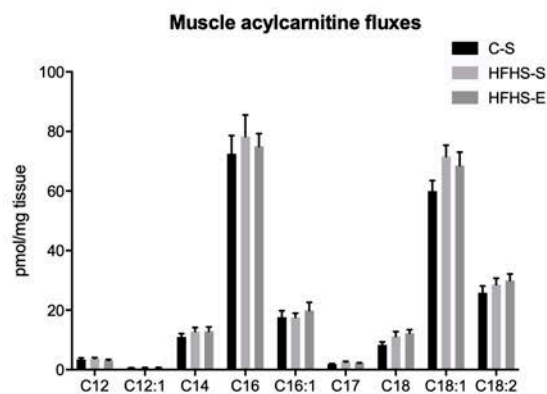


Figure 4: Acylcarnitine fluxes in plasma. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$).

Offspring acylcarnitine fluxes were evaluated in TA to ascertain the role of maternal diet and exercise on fatty acid catabolism (Figure 4); however, no differences were observed in acylcarnitine levels as a response to maternal diet or exercise.

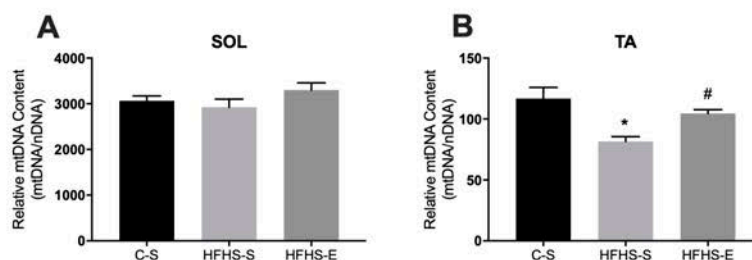


Figure 5: Effects of diet and exercise in mitochondrial DNA content of (A) Soleus (SOL) and (B) Tibialis Anterior (TA) muscles. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

As can be depicted from figure 5, relative mtDNA content was not altered by either maternal exposure to HFHS diet and/or exercise in SOL (Figure 5A), while in TA, HFHS-S offspring group had a lower mtDNA content than both C-S and HFHS-E groups (Figure 5B).

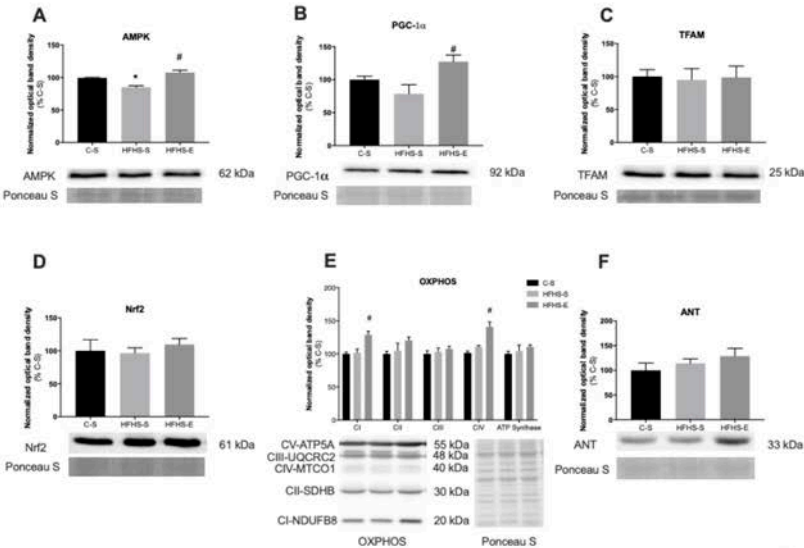


Figure 6: Relative protein content of 5' adenosine monophosphate-activated protein kinase (AMPK) (A); Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (B); Mitochondrial transcription factor A (TFAM) (C); Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) (D); Oxidative phosphorylation subunits (OXPHOS) (E) and Adenine nucleotide translocator (ANT) (F) in soleus muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean ± SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

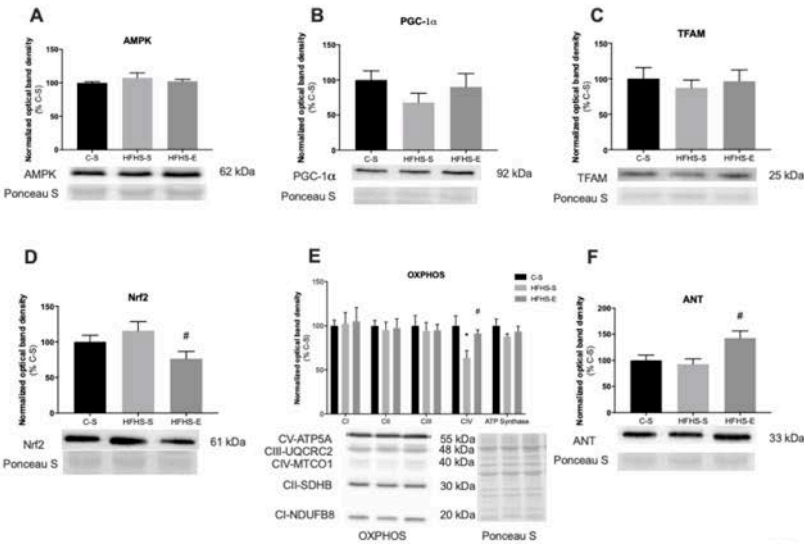


Figure 7: Relative protein content of 5' adenosine monophosphate-activated protein kinase (AMPK) (A); Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (B); Mitochondrial transcription factor A (TFAM) (C); Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) (D); Oxidative phosphorylation subunits (OXPHOS) (E) and Adenine nucleotide translocator (ANT) (F) in tibialis anterior muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean ± SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

Figures 6 and 7 show the relative protein content of some analyzed biomarkers associated with mitochondrial biogenesis and oxidative fitness in SOL and TA, respectively.

As seen in Figure 6A, SOL AMPk protein content was significantly lower in HFHS-S offspring group than C-S, while an increase in HFHS-E vs. HFHS-S group was observed. Despite AMPk decrease in HFHS-S group, no alterations were found in PGC-1α content for the same group. However, offspring of exercised mothers had a higher SOL PGC-1α protein content (Figure 6B). Interestingly, these alterations in SOL AMPk and PGC-1α contents were not observed when TA was analyzed (Figure 7A-B), TFAM protein content was similar in all groups for both muscles (Figures 6C and 7C).

NRF2 was analyzed as an important regulator of mitochondrial uncoupling and biogenesis in conditions of stress. No alterations were found in SOL (Figure 6D), while HFHS-E group showed a decrease in the NRF2 protein content of TA (Figure 7D). Additionally, the content of OXPHOS complexes I-IV subunits and ATP synthase were also determined. Relative protein content in C-I and C-IV subunits were higher in HFHS-E offspring in SOL (Figure 6E). In TA, C-IV subunit content decreased in HFHS-S and was restored back to control values in HFHS-E group (Figure 7E). No alterations were found in other analyzed subunits within groups from both muscles.

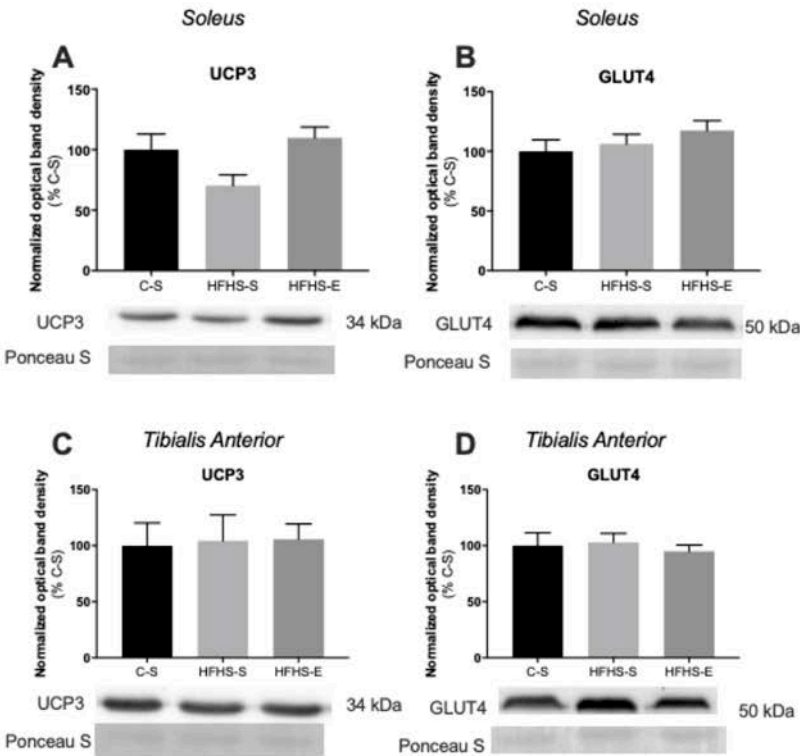


Figure 8: Relative protein content of Uncoupling protein 3 (UCP3) (A) and Glucose transporter type 4 (GLUT4) (B) in Soleus muscle; Uncoupling protein 3 (UCP3) (C) and Glucose transporter type 4 (GLUT4) (D) in Tibialis Anterior muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$).

Regarding GLUT4 and UCP3 protein contents, neither maternal diet nor GE affected them in any of the analyzed muscles (Figure 8).

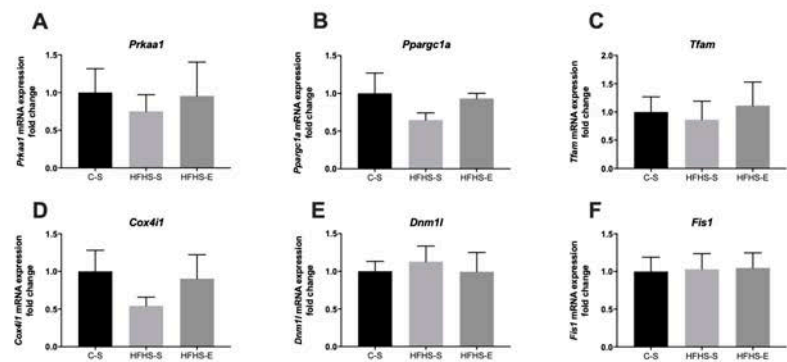


Figure 9: Expression levels of the genes: Prkaa (A) Ppargc1a (B) Tfam (C) Cox4i1 (D) Dnm1l (E) and Fis1 (F) in Soleus muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

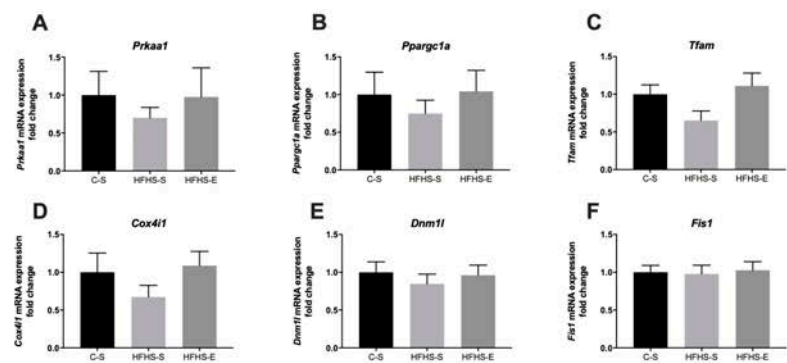


Figure 10: Expression levels of the genes: Prkaa (A) Ppargc1a (B) Tfam (C) Cox4i1 (D) Dnm1l (E) and Fis1 (F) in Tibialis anterior muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

Regarding gene expression, although some tendencies are clearly noted, no statistically significant alterations were found in the expression of the selected analyzed genes, both in SOL and TA (Figures 9 and 10).

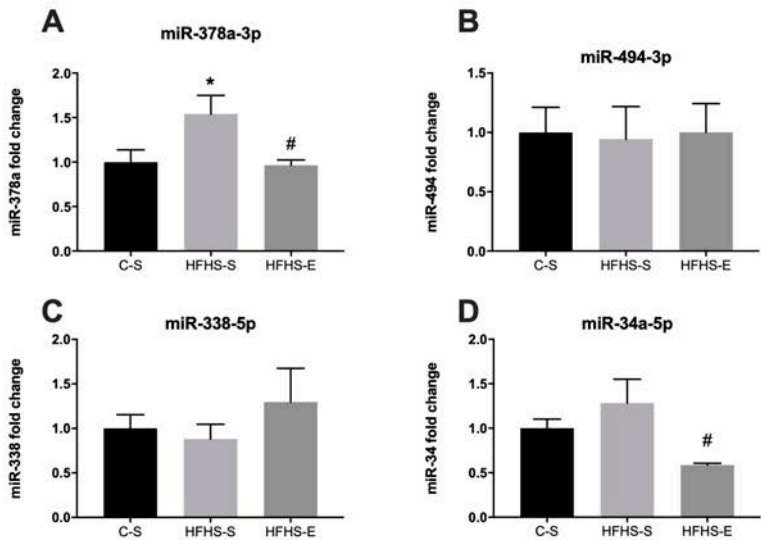


Figure 11: Expression levels of miR-378a-3p (A) miR-494-3p (B) miR-338-5p (C) and miR24a-5P (D) in Soleus muscle. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

As important players in the post-transcriptional regulation of gene expression coding for some of proteins, the expression of selected miRNAs potentially involved in mitochondrial biogenesis, fatty acid oxidation and oxygen consumption were also evaluated. Regarding SOL, the maternal diet significantly elevated the expression of miR-378a. In contrast, offspring from exercised mothers showed a downregulation of miR-378a back to control values, and decreased miR-34a expression (Figure 11A,11D).

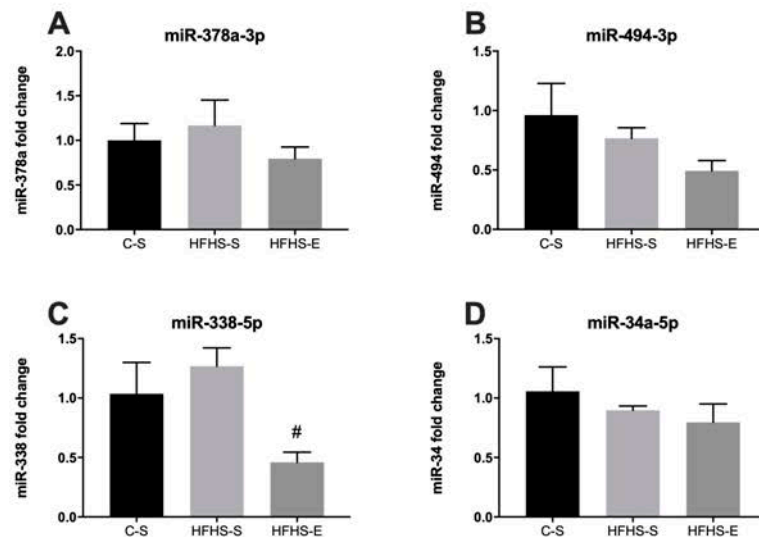


Figure 12: Expression levels of miR-378a-3p (A) miR-494-3p (B) miR-338-5p (C) and miR-34a-5p (D) in Tibialis Anterior. Offspring from control diet sedentary group (C-S); offspring from high-fat high-sucrose diet sedentary group (HFHS-S); offspring from high-fat high-sucrose diet exercised group (HFHS-E). Data are expressed as the mean $\pm$ SEM. Statistically significant differences ($p \leq 0.05$) are indicated as follows: * vs. C-S, # vs. HFHS-S.

In TA, offspring from HFHS group exhibited only a downregulation of miR-338a when compared with control group (Figure 12C). No other significant alterations were found in the expression of the remaining miRNAs in TA (Figure 12A, B, and D).

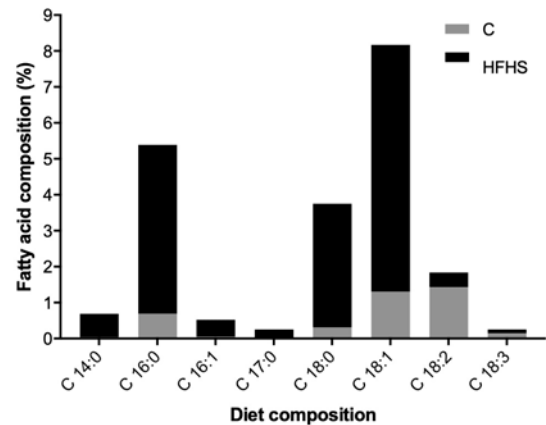


Figure 13: Proportion of fatty acid composition in maternal diet.

3. Discussion

Offspring body composition

Maternal environmental exposure during critical periods of fetal and perinatal development induces offspring physiological and metabolic changes, including the increased predisposition for the development of chronic diseases [1-3,34]. In fact, maternal HFD consumption during gestation and lactation can have a negative impact on the offspring generation and may increase the propensity to impaired glucose tolerance and insulin sensitivity, as well as the predisposition to obesity development later in the offspring life [5,6,35]. In our study, offspring from sedentary HFHS mother had higher BW at weaning, which was in line with previous works [5,26] and may be a key indicator of risk of metabolic disorders in later life [36]. Additionally, the impact of maternal lifestyle on offspring BW was maintained until the 6th week of age in contrast with other studies where offspring were fed with HFD after weaning [8,36] suggesting that post-weaning C-diet consumption could eventually prevent the obesity development later in life [20]. Interestingly, as reported before [26,37] the positive impact of GE during pregnancy on offspring BW was observed not only at weaning, but also until the 6th week of age, since the BW of the HFHS-E group was in line with the control group.

It has been reported that maternal HFD-consumption before and during gestation have deleterious consequences on offspring SM [8-10], which were associated to reductions in SM weight, muscle cross-sectional area and fiber type composition [38]. In fact, in the present study maternal HFHS-diet decreased SOL-to-BW ratio, which was restored in the offspring from exercised mothers, parallelly with an increase in TA-to-BW when compared to HFHS-S counterparts. Our results highlight the importance of maternal lifestyle role in the offspring body composition, induced by maternal diet and GE levels [26].

Skeletal muscle mitochondrial network physiology

Considering the pivotal role of mitochondrial network physiology in the SM function, several studies have recently explored the impact of maternal lifestyle on offspring SM mitochondrial network physiology [10,11,26,38,39]. Additionally, it has been reported

that maternal HFD diet induces a decrease in mRNA expression of several biomarkers of mitochondrial biogenesis and dynamics, namely PGC-1 α , NRF1, and TFAM, [10,11] associated with downregulation of subunit proteins from C-I and C-II [40]. However, here we only found a decrease in TA protein expression in C-IV subunit, while the other parameters related with mitochondrial biogenesis and OXPHOS content were not affected by maternal nutrition. The difference in our data when compared to results from previous studies may be related mainly with diet composition used in the different studies, as our diet is specifically used to induce a condition of gestational diabetes [20,41].

Interestingly, the impact of GE on the offspring SM mitochondria was evident. We found an increase in protein expression of mitochondrial biogenesis biomarkers (AMPK and PGC-1 α) in SOL as well as OXPHOS content in SOL (C-I and C-IV subunits) and in TA (C-IV subunits and ANT). Consistently, miR-338, that has been confirmed to inhibit COXIV at mRNA and protein level [42], was downregulated in TA by GE, although surprisingly not in SOL. Moreover, in accordance with data reported by Simão, et al. [43] and Lee, et al. [44], GE downregulated miR-34a, which is consistent with increased activation of SM AMPK/PGC-1 α axis and mitigates the deleterious impact imposed by maternal HFHS diet on mitochondrial biogenesis and metabolism-related markers. Being implicated in the regulation of mitochondrial metabolism of SM, miR-378a KO mice developed increased resistance to obesity induced by high fat diet and increased efficiency to oxidize free fatty acids [45]. Accordingly, and in line with miR-34a, our data showed a downregulation of miR-378a in SOL, but not in TA, mediated by GE that is consistent with improved oxidative phenotype, although an absence of alteration in SM acylcarnitine's in the offspring of exercised dams when compared to those non-exercised. In fact, when overexpressed, both miRNAs were associated with mitochondrial dysfunction and with a decrease in fatty acid oxidation and oxygen consumption [43,46], suggesting that GE prevented, at least in part, some mitochondrial impairments through epigenetic regulation. Surprisingly, in contrast with data provided by Yamamoto, et al. [47], we did not find any significant decrease in the present study both in this miRNAs and in TFAM expression, which seems to suggest that endurance exercise significantly decreases the content of miR-494 associated to TFAM expression and mitochondrial biogenesis. A clear difference in the experimental sets between these two exercise interventions might eventually explain, at least in part, the differences in the expression of this miRNA. In Yamamoto, et al. [47] work, the miRNA expression was evaluated in the SM of the exercised animals, but in our study data was obtained from the offspring of the exercised dams. Considering the lack of any other study dealing with offspring's of exercised dams and the expression of this miRNA as well as the sensitivity found in the other miRNAs, it seems that maternal exercise is not a sufficiently strong stimulus to influence the role miR-494 in the intergenerational inheritance provided to offspring SM. Differences observed between SOL and TA could be mainly due to the different SM phenotypic characteristics, as SOL expresses a prominent oxidative phenotype. Additionally, recent evidence [10] has shown that enhanced mitochondrial biogenesis is associated with increased SM oxidative capacity and with a muscle fiber type shift from glycolytic towards oxidative metabolism in offspring of exercised mothers. Moreover, acylcarnitine profiling have been used to describe alterations in mitochondrial β -oxidation in animal models of obesity [48]. Previously, we had reported [20] an increase in offspring plasma and liver acylcarnitines (C16, C18, and C18:1) corresponding to fatty acids diet composition consumed by mothers (Figure 13). Furthermore, GE was able to restore acylcarnitines back to control levels in plasma and liver. Nevertheless, as pointed out above, although we found an increase in SM long-chain acylcarnitine levels in line with maternal diet, GE despite the obvious trend was not able to revert values back to control levels (Figure 4).

Offspring maximal workload capacity

Improvements in endurance capacity, namely in maximal workload performance, induced by different training regimens [28] have been associated with enhancements in mitochondrial function [49,50], and volume density [17,51]. In addition to the metabolic implications towards a more oxidative phenotype suggested above, mice lacking miR-378 through double-KO exhibited better physical performance and improved absolute muscle strength [52]. However, the intergenerational transmission of positive physiological adaptations induced by GE to their children have been poorly described. Indeed, some evidence suggest that maternal malnutrition may have a negative impact on offspring performance [12] and may be associated to an impairment in mitochondrial network physiology [26,39]. Moreover, Eclarinal et al. [13] reported that offspring from exercised mothers had a higher propensity for PE. Nevertheless, only the work from Son et al. [10] have recently showed an improvement in maximal oxygen consumption during exercise in offspring (at weaning) of exercised mothers, as well as a higher exercise time and distance in an acute graded aerobic treadmill test. To the best of our knowledge, the present study is the first work to show an improvement in maximal workload test (MWT) in 6-week-old male offspring. Additionally, the improvements in MWT-related maximal speed and time until exhaustion were associated with markers of mitochondrial biogenesis, OXPHOS content, and decreases in miRNA-378 expression discussed before, as well as with an increase in markers of mitochondrial content and function, such as mtDNA content and CS activity in SM of offspring sired from exercised mothers. Some of the alterations found in the present study are in line with mitochondrial remodeling reported by other groups, specifically those related to mitochondrial biogenesis [26] and function [39].

Ultimately, the results from the present study suggest that GE may have a vital role in offspring's SM programming. In fact, SM offspring programming is dependent of phenotypic tissue-specific alterations that seems to be mainly associated to an improvement in the oxidative capacity. Those alterations are potentiated, at least in part, by an epigenetic modulation and consequent intergenerational inheritance.

4. Materials and Methods*Animals*

All experimental procedures involving animals were performed in accordance with guidelines for Care and Use of Laboratory Animals in Research advised by Federation of European Laboratory Animal Science Associations (FELASA) and in accordance with the European Convention for the protection of Animals used for Experimental and other Scientific Purposes (Directive 2010/63/EU). The Ethical Committee of the Institute for Research and Innovation in Health (i3S), University of Porto, and the National Government Authority (Direção Geral de Alimentação e Veterinária – No 0421/000/000/2018) approved the experimental protocol.

Seven-week-old female Sprague-Dawley rats (150–200 g) were obtained from Charles River Laboratories (L'Arbresle, France) and were randomly divided into 2 diet groups: control (C) and high-fat high-sucrose (HFHS) diets. After 7-weeks on respective diets (C: E157452–047 or HFHS: D12451(II)mod., Ssniff, Soest, Germany), HFHS dams were divided into 2 subgroups: HFHS-sedentary dams (P-HFHS-S); and HFHS-exercised dams (P-HFHS-E). C-diet group remained sedentary throughout the protocol (P-C-S). After the establishment of the 3 experimental groups (n = 6 per group), dams were mated with sedentary C-fed male rats (Charles River, L'Arbresle, France). The morning the vaginal plug was present was considered day 0.5 of pregnancy. After delivery, litter size was reduced to 3 male and 3 female pups to avoid food competition and negative effects on pups' body mass and development. After 3-weeks of nursing corresponding male offspring were weaned and identified as (n = 6 per group): C-S offspring from P-C-S, HFHS-

S offspring from P-HFHS-S, and HFHS-E offspring from P-HFHS-E (Figure 1). Thereafter, offspring were fed with standard diet and remained sedentary until euthanasia at 6 weeks of age.

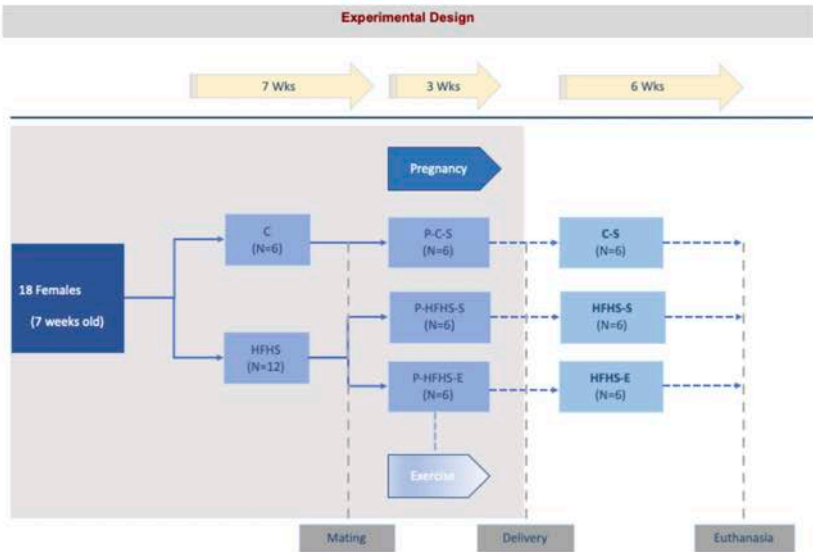


Figure 1: Schematic representation of the experimental design. Seven-week-old females Sprague Dawley rats were fed with experimental diets - control (C) and high-fat high-sucrose (HFHS) diet, and after 7 weeks were mated with sedentary male Sprague Dawley rats. After 3-weeks of nursing, male offspring were weaned and identified as (n = 6 per group): as C-S offspring from P-C-S, HFHS-S offspring from P-HFHS-S, and HFHS-E offspring from P-HFHS-E. Thereafter, offspring were fed with standard laboratory pellet chow and remained sedentary until 6-weeks of age when were euthanized.

Animals were housed in type III-H cages enriched with bedding and nesting material under controlled environmental conditions (21–22°C; 50–60% humidity), in 12-h light/dark cycles, receiving food (according to respective diet regiments) and water (pH 5.5) *ad libitum*, in a specific-pathogen free animal facility at the Institute for Research and Innovation in Health – i3S. During mating and GE protocol during pregnancy, dams were housed in polyethylene type IV cages equipped with a running wheel [perimeter=1.05 m, Type 304 Stainless steel (2154F0106-1284L0106) Tecniplast, Casale Litta, Italy)]. All the animal-related procedures during experimental protocol were blindly performed by independent animal facility technicians.

Physical exercise protocol

Exercising dams were submitted to a physical exercise protocol combining FRW and endurance treadmill running as previously established [20]. These animals had 24h access to FRW throughout the 3-weeks of pregnancy period and were exercised on a motor-

driven treadmill (LE8700, Panlab Harvard Apparatus, MA, USA) 6 days/week, 20–60 min/day during the dark cycle. The animals were adapted to the treadmill for 5 days before the exercise protocol started. In the first day, rats were placed on the immobile treadmill to adapt to the new device and in the following days, the treadmill was turned on and the speed and duration were gradually increased during the subsequent 4 days. During the first week of the protocol, GE duration was gradually increased from 20 min/day to 60 min/day until the speed of 30 cm/s was reached. In the following 2-weeks, the duration was kept at 60 min, but speed was gradually increased until 35 cm/s. Nevertheless, GE intensity was adjusted for each animal in the last few pregnancy days. Inclination was kept at 0°. Sedentary animals were placed on a non-moving treadmill and housed in cages with blocked wheels to be exposed to the same handling and environmental conditions.

Maximal workload test

The maximal workload test (MWT) was performed as previously described [28]. Briefly, offspring were individually evaluated on the treadmill at the inclination of 15°. The test started at a speed of 15 cm/s, after 5 min the speed was set to 30 cm/s, and then increased 3 cm/s every 2 min until maximal workload was achieved, where the animals were no longer able to sustain the treadmill speed. Two criteria were established to confirm maximal workload: the exhaustion of the animal and blood lactate concentration higher than 7 mmol/L. Immediately after the end of MWT and in different time points (1, 3 and 5 min post-MWT), blood samples were collected from the animals' tail until a maximal value of lactate concentration (Lactate Plus Meter, Nova Biomedical, United States) was achieved.

Animal euthanasia and muscle harvesting

All animals were fasted overnight for 12 h with access to drinking water before euthanasia. Animals were anaesthetized (induction: 5% isoflurane, 1 L/m O₂; maintenance: 2.5% isoflurane, 0.4 L/m O₂), right SOL and TA muscles were excised, weighed and immediately frozen in pre-cooled isopentane and stored at −80°C until further analysis. The blood was collected from inferior vena cava and centrifuged (3000 ×g, 10 min, 4 °C). Obtained plasma was stored at −80 °C until biochemical analysis.

Citrate synthase activity

CS activity, as a usual marker of mitochondrial content and oxidative capacity, was measured in SOL and TA homogenates as previously described [28]. Briefly, 10 mg of muscle were homogenized in 100 mL of ice-cold medium (75 mM Tris-HCl, 2 mM MgCl₂, and 1 mM EDTA (pH 7.6)). The Coenzyme A released from the reaction of acetyl-Coenzyme A with oxaloacetate was measured by its reaction with a colorimetric agent [5, 5-dithiobis (2-nitrobenzoate)]. The enzymatic activity was measured spectrophotometrically at 412 nm.

Muscle acylcarnitine levels

For SOL and TA analysis of acylcarnitine levels, an adapted protocol from Petucci et al [29] was used. Briefly, muscle tissues, homogenized in 80% methanol (1:10 ratio), were centrifuged for 5 min at 14000 rpm at 10°C and the supernatant was collected. In a 96-well plate, 140 µL of supernatant and 100 µL of methanol containing deuterated acylcarnitine internal standard solutions (Cambridge Isotope labs, USA) were added. After drying using nitrogen flow, the mixture was derivatized to the corresponding butyl esters by incubation with 60 µL of 3 N butanolic HCl at 70°C for 15 min. The mixture was then dried

with nitrogen and reconstituted with 200 µL of 80% methanol for flow injection MS/MS in an API 4000 QTRAP (Sciex, Washington, D.C., USA).

Mitochondrial DNA content

Total DNA was extracted from muscle samples (30 mg of tissue) using GRS Genomic DNA Kit BroadRange (#GK06.0100, GRISP, Portugal) following the manufacturer's instructions. Different genes were used to evaluate the relative copy number of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). For the detection of mtDNA, primers for mitochondrially encoded 16S RNA (Rnr2) were used (forward 5'-AGC-TATTAATGGTTCGTTTGT-3'; reverse 5'-AGGAGGCTCCATTCTCTTGT-3'), whereas for the detection of nDNA, primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (forward 5'-GGAAAGACAGGTGTTTGCA-3'; reverse 5'-AG-GTCAGAGTGAGCAGGACA-3') [30]. Quantitative Real-time PCR (RT-qPCR) was conducted using PowerUp SYBR® Green Master Mix (A25742, GRISP, Portugal) in a StepOnePlus™ thermocycler (Applied Biosystems) using paired reverse and forward primers with mitochondrial and nuclear products being performed separately. All reactions were performed under the same conditions 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were presented as mtDNA/nDNA ($\Delta Ct = Ct(nDNA) - Ct(mtDNA)$; relative mtDNA content = $2 \times 2^{\Delta Ct}$).

Protein semiquantification by Western blotting

Muscle tissue was homogenized in RIPA lysis buffer (20188, Millipore) supplemented with protease and phosphate inhibitor cocktails (P2714, Sigma-Aldrich; 524636, Millipore). After centrifugation (14000 × g, 10 min, 4°C), supernatants were collected and used for semiquantification by Western blotting. Equivalent amounts of muscle tissue were denatured in 2x Laemmli sample buffer and separated by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE, 12% gels) [31] and subsequently transferred to PVDF membranes [32]. Membranes were blocked with a blocking agent (non-fat dry milk or BSA in Tris-buffered saline with 0.1% Tween20) to reduce non-specific binding. After incubation with the following primary antibodies: anti-AMPK (1:1000; ab80039); anti-PGC-1α (1:1000; ab54481); anti-TFAM (1:500; sc-23588); anti-OXPHOS cocktail (1:1000; Abcam, ab110413); anti-ANT (1:1000; sc-70205); anti-Nrf2 (1:500; sc-365949) anti-UCP3 (1:1000; ab10985); and anti-GLUT4 (1:500; sc-53566), and incubation with the following corresponding secondary antibodies: horseradish peroxidase-conjugated anti-goat (1:10000; sc-2354), anti-mouse (1:10000; sc-2005), or anti-rabbit (1:10000; sc-2357), Clarity Western ECL Substrate (Bio-Rad, #1705061) was used for visualization in ChemiDoc-XRS Image System (Bio-Rad), whereas the density of protein bands was determined by using the Image Lab software 6.0.1 (Bio-Rad). For the normalization of eventual differences in protein electrophoresis or transfer to membrane, Ponceau S staining was used [33] and the final data were expressed as the percentage variation of the control values (% C-S).

Real-time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from muscles by homogenizing 15 mg of tissue using PureLink™ RNA Mini Kit (12183018A, ThermoFisher Scientific) and following manufacturer's instruction. Total RNA was then converted to cDNA using Xpert cDNA Synthesis Mastermix (#GK81, GRISP, Portugal). Real-time PCR was performed using the SYBR Green (PowerUp Sybr Green Master Mix; A25742, ThermoFisher Scientific) method in a StepOnePlus™ (Applied Biosystems). Results were normalized to 18S using fluorescence threshold cycle values (Ct) and expressed and reported as fold value of the control group C-S group. Specific PCR primer pairs for: 18S rRNA (forward 5'-

CATTGGAACGTCCTGCTAT-3'; reverse 5'-GTTTCTCAGGCTCCCTCTCC-3'); *Prkaa1* (forward 5'-AGATCGGCCACTACATCCTG-3'; reverse 5'-TCTGGATCTCTGCGGATT-3'); *Ppargc1a* (forward 5'-AAAAGCTTGACTGGCGTCAT-3'; reverse 5'-TCAGGAA-GATCTGGGCAAAG-3'); *Tfam* (forward 5'-GCTAAACACCCAGATGCAAAA-3'; reverse 5'-CGAGGTCTTTTGGTTTCC-3'); *Cox4i1* (forward 5'- CAGCAGTGG-CAGAATGTTGG-3'; reverse 5'- CCACATCAGGCAAGGGGTAG-3'); *Dnm1l* (forward 5'-CCAGGAATGACCAAGGTCCC-3'; reverse 5'- CCTCGTCCATCAGGTCCAAC-3'); *Fis1* (forward 5'- ACTTCTTCTACCCGGAGGCT-3'; reverse 5'- CTCTACAGGCATTTGGGG-3') were used.

For miRNA analysis, small RNAs were extracted from the rats' muscles (15 mg) using mirVana miRNA Isolation Kit (AM1560, Invitrogen) according to the manufacturer's protocols. The cDNA was obtained using the TaqMan Advanced miRNA Assay (A25576, Applied Biosystems) following the manufacturer's protocols. The relative expressions of miR-378a-3p, miR-494-3p, miR-34a-5p, and miR-338-5p were determined using the TaqMan Fast Advanced Master Mix (Applied Biosystem) in a StepOnePlus™ (Applied Biosystems). The primers used were assay ID rno481191_mir, rno481304_mir, rno481068_mir. Relative miRNA expression was reported as fold value of the C-S group.

Statistical Analysis

Sample size was estimated assuming $\alpha = 5\%$ and a power of 0.90 (G*Power, Düsseldorf) from previous study. Data are reported as the mean $\pm$ SEM (standard error of the mean). Statistical analyses were performed using GraphPad Prism (version 8.0). One-way analysis of variance (ANOVA) was followed by Holm-Sidak post-hoc test to compare differences between groups. In all cases, a value of $p \leq 0.05$ was considered statistically significant.

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4. Summary of the Results

4.1. Director's report

As supervisors of the present Doctoral Thesis, Doctor José Fernando Magalhães Pinto Pereira and Doctor Joan Ramon Torrella Guio hereby state that the student Jorge Manuel de Sousa Beleza has conducted his PhD thesis in the context of intergenerational inheritance induced by gestational exercise, specifically on rat liver and skeletal muscle. He has actively and decisively participated in the overall process of executing the experimental design and obtaining, analysing and discussing the results of the present report. He also had a prominent role in the diffusion and publication of the results and conclusions covered in this report, which include one book chapter review and four peer-reviewed papers, as reflected by their author order and composition.

What follows below is a concise overview of the peer-reviewed contributions included in the current Doctoral Thesis:

I. Book Chapter

Title: Targeting Mitochondria with Sweat: Improving Mitochondrial Function with Physical Activity.

Authors: **Beleza J**, Rizo-Roca D, Ascensão A, Magalhães J.

Year of publication: 2018.

Book: Oliveira PJ (ed). Mitochondria Biology and Experimental Therapeutics. ISBN: 978-3-319-73344-9. Springer: USA, pp. 379-406.

Doctoral student participation: Literature survey. Original draft. Final editing and approval.

II. Review paper

Title: Building-up fit muscles for the future.

Authors: **Beleza J**, Stevanovic J, Coxito P, Costa RC, Ascensão A, Torrella JR, Magalhães J.

Journal: European Journal of Clinical Investigation Volume: 116 Pages: 1-20.

Year of publication: 2021.

DOI: 10.1111/eci.13515

JCR Impact Factor (2020): 4.686 JCR 5 Years Impact Factor: 3.900

JCR Tercil/Field (2020): T1 (35/167) / Medicine, General & Internal.

Doctoral student participation: Conceptualization. Literature survey. Original draft. Final editing and approval. Funding acquisition.

III. Experimental Paper

Title: Self-Paced Free-Running Wheel Mimics High-Intensity Interval Training Impact on Rats' Functional, Physiological, Biochemical, and Morphological Features.

Authors: **Beleza J**, Albuquerque J, Santos Alves E, Fonseca P, Santocildes G, Stevanovic J, Rocha-Rodrigues S, Rizo-Roca D, Ascensão A, Torrella JR, Magalhães J.

Journal: Frontiers in Physiology Volume: 10:593 Pages: 1-16.

Year of publication: 2019.

DOI: 10.3389/fphys.2019.00593.

JCR Impact Factor (2019): 3.367 JCR 5 Years Impact Factor: 4.805

JCR Tercil/Field (2020): T1 (20/81) / Physiology.

Doctoral student participation: Conceptualization. Sample collection, processing and data collection. Image analysis. Original draft. Final editing and approval.

IV. Experimental Paper

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

Authors: Stevanovic-Silva J, **Beleza J**, Coxito P, Pereira SP, Rocha H, Gaspar T, Gartner F, Correia S, Martins MJ, Guimarães T, Martins S, Oliveira OJ, Ascensão A, Magalhães J.

Journal: Metabolism Clinical and Experimental Volume: 116 Pages: 1-20.

Year of publication: 2021.

DOI: 10.1016/j.metabol.2021.154704.

JCR Impact Factor (2020): 8.697 JCR 5 Years Impact Factor: 7.871

JCR Tercil/Field (2020): T1 (13/146) / Endocrinology & Metabolism.

Doctoral student participation: Conceptualization. Formal analysis. Literature survey. Original draft. Final editing and approval. Visualization. Project administration.

V. Experimental Paper

Title: Gestational exercise increases male offspring maximal workload capacity early in life.

Authors: **Beleza J**, Stevanovic-Silva J, Coxito P, Costa RC, Ascensão A, Torrella JR, Magalhães J.

Journal: International Journal of Molecular Science.

Submitted in December 2021

JCR Impact Factor (2020): 5.924 JCR 5 Years Impact Factor: 6.132

JCR Tercil/Field (2020): T1 (67/295) / Biochemistry & Molecular Biology.

Doctoral student participation: Conceptualization. Sample collection, processing and data collection. Original draft. Final editing and approval.

4.2. Summary of the Results

We start the workflow of the present PhD thesis reviewing the up-to-date knowledge about the role of PE in the modulation of mitochondrial physiology (I). In fact, the LaMetEx group has dedicated the last 20 years studying the use of PE as a non-pharmacological strategy to prevent and counteract different non-communicable diseases. Moreover, a special focus was devoted to the role of mitochondrial physiology in the progression of the disease and, in particular, the role that PE can play to prevent and revert some of the associated health complications. The contribution of LaMetEx and other groups with relevant implications in the field are detailed in the work I of the present PhD thesis. Additionally, our book chapter highlights the growing relevant role attributed to the epigenetic regulation in some of the mechanisms that can be involved in the associated protection of PE against several non-communicable diseases as well as in the mitochondrial modulation. As a consequence, we decided to further review the impact of parental exercise in offspring SM phenotypic modulation (paper II). We found some evidence about the impact of parental exercise, specifically the GE, in the SM offspring phenotypic modulation. However, the epigenetic mechanisms that can orchestrate this intergenerational offspring programming are far to be understood. Based in the literature research carried out in works I and II, we developed an experimental work, the results of which will be presented below.

Characterization of a FRW model

The experimental work of this thesis started with the characterization of a FRW model. Although, FRW has been frequently used by our group and others as an exercise regimen able to induce positive organic adaptations, doubts remained in terms of its physiological and biochemical impact, mainly due to the lack of a control in terms of intensity and duration. In this sense, we compared FRW with another forced exercise model (HIIT) from a functional, physiological, biochemical, and morphological perspective (paper III). The main objective of this experimental work was to obtain scientific robustness on the FRW model

enabling that it could later be used in the study of intergenerational adaptations induced by GE. The summary of the results is presented below.

HIIT vs. FRW

The main characteristics of the HIIT model used are summarized in Table 1 (III). Briefly, rats performed four bouts of 4 min at speeds correspondent to 85–90% of VO_{2max} interspersed by 2 min of active recovery at speed correspondent to 60% of VO_{2max} . FRW animals had free access to the running wheel 24 h/day and the running distance was recorded. Information about mean distance covered per day of animals from both models is provided in figure 4 (III). FRW animals ran a significantly higher overall day distance than HIIT. Their activity increased from the beginning of the protocol and reached a peak at the 6th week when a non-significant decrease was observed until the end of the protocol.

Maximal Workload Test

After 12 weeks of exercising, FRW and HIIT animals reached significantly higher velocities in maximal workload test (MWT) compared to sedentary (SED) animals (FRW vs. SED: 60 vs. 48 cm/s and HIIT vs. SED: 69 vs. 40 cm/s). Moreover, as shown in figure 5A (III) a significant difference was also found between HIIT and FRW (69 vs. 60 cm/s, respectively).

FRW animals showed a lower blood lactate concentration (BLC) at the end of MWT (figure 5B-III) after 12 weeks of free access to the running wheel (FRW: 11.63 ± 3.95 mmol/L vs. SED: 13.77 ± 3.35 mmol/L). In line, the animals submitted to the HIIT regimen reached also lower BLC at the end of the MWT (HIIT: 6.00 ± 1.85 mmol/L vs. SED: 13.77 ± 3.35 mmol/L).

Cardiac mitochondrial function

No alterations were found in heart mitochondrial state 3 and 4 when complex I- and II-driven substrates were used after 12 weeks of both training modalities (FRW and HIIT vs. SED), although an increase in respiratory control ratio (RCR) with complex I-driven substrates (3.89 ± 0.14 vs. 5.20 ± 0.25 and 5.42 ± 0.37) in FRW and HIIT groups was evident (figure 8A-D-III). Moreover, when complex II-

related substrates were used, only HIIT induced an increase in RCR (3.86 ± 0.17 vs. 2.98 ± 0.19) compared with SED (figure 8G-III).

Citrate synthase activity

The citrate synthase activity (CS) activity in the *soleus* (SOL) muscle was significantly higher in both exercised groups (FRW and SED) compared to SED control (figure 7A-III). However, no significant differences were observed in *tibialis anterior* (TA) after both chronic exercise regimens (figure 7B-III).

Skeletal muscle morphology

Both chronic exercise models caused alterations in skeletal muscle fiber type distribution in TA and SOL (figure 9-III). In the lateral field of TA, the FRW group exhibited an increased percentage of fast oxidative glycolytic (FOG) fibers (36.0 ± 5.0 vs. 14.0 ± 2.2 and vs. 21.9 ± 6.5), while a significant increase in the percentage of fast intermediate glycolytic (FIG) fibers was observed in the HIIT group (figure 9A-III). Moreover, both FRW and HIIT increased the percentage of FOG fibers in the TA medial field (44.9 ± 4.4 and 45.2 ± 5.3 vs. 29.7 ± 2.3), which is concomitant with the decreased percentage of fast glycolytic (FG) fibers (figure 9B-III). Regarding slow oxidative (SO) fibers, only present in the posterior field, both FRW and HIIT exercise models induced an increased percentage. Furthermore, in the SOL muscle, FRW and HIIT elicited a decrease in the percentage of FOG fibers and a corresponding increment of SO fibers (figure 9D-III).

Despite no evidence of changes in muscle to body weight ratio (Table 2-III), alterations in fiber cross-sectional area (FCSA) and fiber shape were registered (figure 10A-D-III). In TA muscle, both FRW and HIIT induced a significant increase in FCSA of FG fibers in all the muscle regions (figure 10A-C-III). This increase was also evident in FIG fibers from the medial (3781 ± 146 vs. 2966 ± 223 and $3569 \pm 193 \mu\text{m}^2$) and posterior fields (4301 ± 137 vs. 2192 ± 158 and $2796 \pm 190 \mu\text{m}^2$) in FRW animals, but not evidenced after the HIIT protocol. Both oxidative fiber types (SO and FOG) showed no significant changes in FCSAs after any exercise protocol, except for FOG fibers from the lateral field of HIIT

animals (figure 10A-D-III), in which a significant reduction in fiber size was observed (1634 ± 77 vs. 2001 ± 62 and $1881 \pm 42 \mu\text{m}^2$). In SOL muscle, a significant reduction in FCSA (figure 10D-III) was measured in SO fibers of HIIT animals (3621 ± 237 vs. 4350 ± 287 and $4893 \pm 325 \mu\text{m}^2$).

As shown in figure 11 (III), FRW and HIIT exercise protocols elicited alterations in individual capillarization of the different fiber types. FRW increased the number of capillaries per fiber (NCF) of FOG fibers in the TA lateral (6.90 ± 0.30 vs. 5.30 ± 0.29 and 6.50 ± 0.38) and posterior fields (8.10 ± 0.43 vs. 5.80 ± 0.50 and 7.10 ± 0.37). Regarding the FIG and FG fibers, the FRW model induced an increase of NCF in the TA medial and posterior fields while in the lateral field, it only increased the NCF of FG fibers (6.90 ± 0.30 vs. 5.30 ± 0.29 and 6.50 ± 0.38). Furthermore, HIIT increased the NCF of FIG and FG fibers of TA lateral and medial fields. However, in the TA posterior field, HIIT only increased the NCF of FG fibers. Regarding the SOL muscle, both exercise models increased the NCF of SO fibers (10.90 ± 0.35 and 8.40 ± 0.29 vs. 6.70 ± 0.58). Furthermore, the FRW model also induced an increase in NCF of slow oxidative (SO) fibers comparing to HIIT (10.90 ± 0.35 vs. 8.40 ± 0.29). However, in the FOG fibers, HIIT induced an increase in NCF comparing to SED and FRW (10.10 ± 0.17 vs. 6.20 ± 0.63 and 5.00 ± 0.22). Additionally, the NCF of FOG fibers decreased in the FRW.

Regarding the relative number of capillaries per $1,000 \mu\text{m}^2$ of FCSA (CCA), both FRW and HIIT increased the CCA of FOG fibers in the TA medial and lateral fields, while only the HIIT regimen induced an increase of the FOG-related CCA in the TA posterior field. Furthermore, both exercise models increased the muscle capillarization of the SO fibers from SOL (figure 11E-H-III).

Both exercise models increased CD in all the TA fields. Although this increase was not significant in the posterior region after FRW, the trend is clear. The same trend for increasing was observed in the SOL muscle in the FRW group and a significantly greater CD after HIIT protocol was noted (figure 12A-B-III).

Regarding C/F, both FRW and HIIT models significantly increased this morphological feature in the three fields of the TA and in SOL (figure 12C-D-III).

Maternal environmental exposure and intergenerational inheritance.

Results from work **III** comparing FRW and HIIT enabled to better understand some of the physiological and biochemical adaptations at different levels of cellular organization in skeletal and cardiac muscles. In fact, results showed that from a central and peripheral point of view, these two exercise regimens positively modulated animals' functional capacity to perform maximal exercise. These results provided scientific robustness to design an innovative exercise intervention model (of GE) aimed to significantly increase dams physical activity level by using a combination of FRW with an endurance training on a treadmill. Our model of GE was used as a strategy to dismiss the negative effects associated to maternal sedentarism and HFHS-diet consumption, both in maternal and offspring generation, specifically in liver and SM tissues. The results of this intervention are presented in papers **IV** and **V** and are summarized below.

Morphological characteristics and glucose homeostasis

After 18 weeks on respective diets, no differences were found in body weight between control (C) and high-fat high-sucrose (HFHS) dams (table 1-**IV**). However, HFHS induced a noteworthy 1.4-fold gestational body weight gain in sedentary dams compared to C-diet-fed or exercised HFHS-fed dams (figure 2 A **V**). Maternal HFHS-diet also increased litter size and offspring body mass by 20%. The impact of maternal HFHS-diet and the protective effect of gestational exercise on offspring body weight was prolonged until the 6th week of age (**paper IV and V**).

Prior to mating, glucose tolerance was not different between C-fed and HFHS-fed animals. However, at mid-gestation, an increased glucose intolerance and glucose are under the curve (AUC) were noticed in both HFHS groups, confirming the presence of gestational glucose intolerance characteristic for GDM (figure 2E-H-**IV**). No alterations in glucose tolerance were observed in the offspring.

Liver and skeletal muscle morphology

As shown in figure 3 (IV) GDM mothers, sedentary or exercised, had lower NAFLD score than C-fed mothers ($p = 0.56$ vs. P-C-S or $p = 0.84$ vs. P-HFHS-S, respectively), although this difference is not statistically significant. Moreover, maternal HFHS-diet increased offspring NAFLD score, while gestational exercise lessened the adverse effect of maternal HFHS-diet. Accordingly, animals from maternal generation did not differ in hepatic TG content, despite a slight decrease in HFHS groups. In contrast, offspring from HFHS sedentary mothers showed tendency towards an increase ($p = 0.26$) in liver TG content compared to their C-S offspring, whereas maternal exercise remarkably reduced ($p = 0.04$) hepatic TG levels, which are in line with the tendency observed in the NAFLD score (figure 4-IV).

SOL-to-body weight (BW) ratio was significant lower in HFHS-S group when compared to C-S, but was restored back to baseline values in offspring from exercised mothers. Still, in TA, no significant alterations associated to maternal nutrition were found (C-S vs. HFHS-S), while an increase in TA-to-BW ratio was found in HFHS-E animals compared to their sedentary counterparts (table 1-V).

Offspring Maximal Workload Test

Offspring from HFHS-E group were able to run at higher maximal speed during a longer period of time when compared to their HFHS-S counterparts. No differences between groups were found in basal and post-exercise blood lactate and glucose contents (table 2-V).

Citrate synthase activity

No significant alterations induced by maternal HFHS diet were noted in CS activity of both SM from sedentary dams (HFHS-S vs. C-S). However, offspring from exercised HFHS-fed mothers had a higher CS activity in SOL and TA compared to the offspring of their non-exercised counterparts (figure 3-V).

Skeletal muscle mtDNA content

Relative mtDNA content was not altered by either maternal exposure to HFHS diet and/or exercise in SOL, while in TA, HFHS-S offspring group had a lower mtDNA content than both C-S and HFHS-E groups (figure 5-V).

Acylcarnitine levels

The levels of plasma and hepatic acylcarnitines were not influenced by HFHS and/or gestational exercise in dams, except for C12 (figure 5-IV). Additionally, no differences were observed in SM offspring acylcarnitine levels as a response to maternal diet or exercise (figure 4-V). However, an increase in the levels of some long-chain acylcarnitines in plasma (C16, C18, C18:1) and liver (C16, C18) due to maternal HFHS-diet was significantly restored to control levels by gestational exercise.

Mitochondrial bioenergetics

HFHS sedentary dams did not show any changes in the RCR with glutamate/malate (G/M)- or succinate/rotenone (S/R)-related substrates; however gestational exercise increased the RCR, when G/M were used as substrates. The same effect of gestational exercise was noticed in respiratory rates with oligomycin (G/M and S/R). In contrast, maternal HFHS-diet and sedentarism reduced offspring RCR, an effect which was not observed in the offspring of exercised HFHS-fed mothers. Respiration with oligomycin (G/M) was greatly increased in offspring of sedentary HFHS mothers when compared to sedentary C mothers, whereas maternal gestational exercise diminished the impact of maternal HFHS. The ADP/O ratio was not affected neither by maternal diet nor by exercise in both generations (figure 6-IV).

Moreover, SOL 5' adenosine monophosphate-activated protein kinase (AMPk) protein content was significantly lower in HFHS-S offspring group than C-S, while an increase in HFHS-E vs. HFHS-S group was observed. Despite AMPk decrease in HFHS-S group, no alterations were found in PGC-1 α content for the same group. However, offspring of exercised mothers had a higher SOL PGC-1 α

protein content (figure 6-**V**). Interestingly, these alterations in SOL AMPk and PGC-1 α contents were not observed when TA was analyzed (figure 7-**V**). Mitochondrial transcription factor A (TFAM) protein content was similar in all groups for both muscles. No alterations were found in SOL, while HFHS-E group showed a decrease in the nuclear factor-erythroid factor 2-related factor 2 (NRF2) protein content of TA. Additionally, the content of oxidative phosphorylation subunits (OXPHOS) complexes I-IV subunits and ATP synthase were also determined. Relative protein content in C-I and C-IV subunits were higher in HFHS-E offspring in SOL (figure 6E-**V**). In TA, C-IV subunit content decreased in HFHS-S and was restored back to control values in HFHS-E group (figure 7E-**V**). No alterations were found in other analyzed subunits within groups from both muscles. Moreover, no statistically significant alterations were found in the expression of the selected analyzed genes (Prkaa; Ppargc1a; Tfam; Cox4i1; Dnm1l; and Fis1), neither in SOL nor in TA (figures 9 & 10-**V**).

GE increased the liver protein expression of CIII, CIV, and CV subunits in dams (figure 7A-**IV**). In the offspring, gestational exercise prevented maternal HFHS-induced decrease in CI liver protein expression and prompted an increase of CIV protein expression in HFHS-E group compared to HFHS-S (figure 7B-**IV**).

MicroRNAs expression

Maternal diet significantly elevated the expression of miR-378a in SOL. In contrast, offspring from exercised mothers showed a downregulation of miR-378a back to control values, and decreased miR-34a expression (figure 11-**V**). Additionally, in TA, offspring from HFHS group exhibited only a downregulation of miR-338a when compared with control group. No other significant alterations were found in the expression of the remaining miRNAs in TA (figure 12-**V**).

Overall findings

FRW model

- FRW induces SM, cardiac and functional adaptations similar to HIIT.

- FRW animals had an increased performance in MWT. Specifically, FRW animals reached higher velocities and ran longer periods with lower BLC.
- FRW exercise model induced improved mitochondrial respiration similar to HIIT.
- Animals submitted to FRW showed SM phenotypic adaptations towards a marked oxidative profile.

Maternal environmental exposure and intergenerational inheritance

- GE counteract negative morphological and metabolic features associated to maternal HFHS-diet and sedentarism, both in mothers and offspring.
- Gestational body weight gain was maintained in line with control group on dams submitted to GE.
- Offspring from HFHS-E dams exhibited lower body weight until 6th weeks of age.
- Offspring from HFHS-exercised dams had decreased hepatic TC content.
- GE improved SOL-to-BW and TA-to-BW ratio in SM offspring from HFHS-fed dams.
- Offspring from HFHS-E group were able to run at higher maximal speed during a longer period of time in MWT when compared to their HFHS-S counterparts.
- Offspring from exercised HFHS-fed mothers had a higher CS activity in SOL and TA compared to the offspring of their non-exercised counterparts.
- Levels of long-chain acylcarnitines were restored back to control levels in offspring from exercised mothers, in liver and plasma.
- Offspring from HFHS exercised dams exhibited improved liver mitochondrial respiratory capacity as well as SM and liver parameters of mitochondrial biogenesis and OXPHOS content.
- Improvements in mitochondrial parameters were concomitantly with the regulation of miRNAs expressions associated to mitochondrial biogenesis (miR-338; miR-34a), mitochondrial dysfunction, decrease in fatty acid oxidation and oxygen consumption (miR-378a).

5. General Discussion

In the context of the present PhD thesis, an extensive review on the current knowledge about the impact of exercise on the modulation of mitochondrial functionality from different tissues (I), as well as the impact of parental exercise on offspring phenotype, especially through the epigenetic mechanisms that may be associated with this modelling, with a special focus on intergenerational inheritance of SM modifications (II) was done.

Thereafter, in the first experimental work (III), we initially wanted to characterize the FRW model from a functional, physiological, biochemical, and morphological perspective. The FRW model has been used in rodents to attenuate the lack of physical activity associated with a sedentary way of life (Fonseca et al. 2011; Hyatt et al. 2015). In addition, it is also frequently used as a specific exercise regimen established to induce organic adaptations and thus, to positively modulate tissue phenotype (Kariya et al. 2004; Marques-Aleixo et al. 2016; Rocha-Rodrigues, Rodriguez, et al. 2017; Rocha-Rodrigues et al. 2016).

FRW model has been increasingly used in exercise-based research due to its “operational” advantages, as the wheels are placed in the housing cages and animals are allowed to freely run throughout 24 hours a day without the necessity of direct intervention of the researchers. Therefore, some of the classic constraints associated to other forced exercise models in rodents, like animals’ handling and environmental stress, may be significantly minimized (Novak, Burghardt, and Levine 2012). However, despite the advantages associated to this model, doubts remain regarding its physiological and biochemical impact mainly due to the lack of control in two very important physical exercise’s features, its intensity and duration. Moreover, when considering the used motor pattern, this model is characterized by short or middle-duration bouts of exercise performed at high speed interspersed by long-lasting pauses and periods of low intensity that, in certain sense, seem to mimic the exercise bouts performed during sessions of HIIT (Novak, Burghardt, and Levine 2012). In this sense, in study III we compared both FRW and HIIT models from a physiological, biochemical, and morphological point of view. The main purpose was to get robust knowledge about the impact of FRW allowing us to use it later in the subsequent experimental work. In fact,

this is the first research to use this comparison and, in our opinion, it provides important information to the PE field.

Using this comparative approach between FRW and HIIT we tried to better understand some of the physiological and biochemical adaptations, at different levels of cellular organization, in skeletal and cardiac muscles. Our results have shown that, both from a central and peripheral point of view, these two exercise regimens positively modulate animals' functional capacity to perform maximal exercise. In fact, the overall findings shown in **III** suggest that both protocols induced alterations in the SM fiber morphology and metabolism toward a more oxidative phenotype, as well as in cardiac mitochondria, which exhibited a more efficient energetic phenotype. Ultimately, those adaptations contributed for an increased maximal workload performance. Two different SMs were considered to study the impact of both exercise models (FRW and HIIT) on distinct SMs phenotypes: SOL, as a muscle with homogeneous and marked oxidative phenotype; and TA, as a muscle with a heterogeneous fiber-type distribution and function. Results were extremely consistent regarding the different fiber morphometrical and capillarization parameters (fiber type distribution, fiber size and shape, muscle and fiber capillarization), as well as the CS activity, suggesting a positive adaptation toward a more oxidative phenotype in both FRW and HIIT compared to the SED hypokinetic animals. Actually, TA and SOL of exercised animals (FRW and HIIT) showed an increased percentage of oxidative fibers, being these alterations in the aerobic fiber type percentage accompanied with a decrease in FCSA. This morphological muscle modifications were previously described as a favorable adaptation in conditions where the need for oxygen supply increase, with the fibers adapting through a decrease in the oxygen diffusion distance to the mitochondria (Panisello et al. 2007). On the other hand, FG fibers from TA muscle adapted to the stimulus increasing cross-sectional area in FRW and HIIT groups, suggesting that both models simultaneously induce fiber type-specific adaptations aimed to increase force production needed during short bouts of high intensity activity.

Moreover, as suggested by the reduction in circularity, both exercise models induced elongated transversal profiles in SO fibers from SOL muscle, thus

resulting in higher fiber perimeters. As it is well-known that there is a substantial proportion of mitochondria distributed in a subsarcolemmal position (Weibel 1984), longer fiber perimeters will allow more mitochondria situated at shorter diffusion distances from the capillaries in fibers still maintaining a great FCSA and, hence, a good capacity for force production. This, as has been proposed in other animal models, could be seen as a fiber morphometrical modification to facilitate the oxygen supply to mitochondria in situations of high aerobic demand (Johnston 1982; Torrella et al. 1998). All the previously described modifications were reinforced by an increased fiber capillarization (CCA) and global field capillarization (CD and C/F). Indeed, these results suggest that both FRW and HIIT protocols induced angiogenesis as reflected by higher C/F and NCF values. Since CS activity is considered a crucial biomarker of mitochondrial content and an important indicator of tissue oxidative fitness, the augmented CS activity found in both exercise groups supports the morphometrical and capillarization data. Moreover, cardiac muscle mitochondrial respiratory capacity was evaluated as an indicator of central-related training adaptations and an important hallmark reflecting cardiac metabolism and function (Abel and Doenst 2011). In fact, our results show that both exercise models (FRW and HIIT) induced beneficial cardiac mitochondrial adaptations compatible with an increase in the metabolic coupling and were in accordance with our previous studies dealing with models of forced endurance exercise (Ascensao et al. 2005; Magalhaes et al. 2013). Indeed, the results from paper III showed that cardiac mitochondria from both FRW and HIIT animals energized with complex I-linked substrates had higher RCR compared to SED counterparts, which suggests that the metabolic coupling between oxygen consumption and ATP synthesis was improved. In fact, mitochondrial RCR is recognized as one of the best general measures of mitochondrial function in isolated mitochondria (Brand M and Nicholls D 2011). Ultimately, our data suggest that physiological central mechanisms were also positively modulated by both HIIT and FRW and certainly contributed, at least in part, for the improved ability to perform maximal exercise through a more oxidative phenotype.

Finally, results of paper III showed that 12 weeks of FRW, but also of HIIT, induced an increase in the maximal speed evaluated in the MWT, a very important outcome related to animals' functional capacity, even though the maximal speed achieved by HIIT-trained animals was higher than that of FRW. These two functional-related parameters are clear indicators of the increased animals' ability to perform maximal exercise and of their metabolic response to high-levels of exercise through an improvement of the aerobic machinery at both central and peripheral levels.

Taken together, data from paper III contribute to a better understanding of the FRW model impact on skeletal and cardiac muscle and to truly clarify its relevance when compared to other more formal and forced exercise models, such as HIIT. Additionally, in the context of the present thesis, the relevant data from paper III allowed us to design an intervention exercise model aimed to increase dams physical activity levels, using a combination of FRW with an endurance training model on a treadmill (IV and V).

Maternal environmental exposure and intergenerational inheritance.

The DOHaD theory proposes that environmental conditions during fetal and early life development influence offspring health with lasting or lifelong effects on growth, structure and metabolism (Hanson and Gluckman 2014). This environmental influence during critical periods of development have been described as the *programming effect* (Barker 1998). In fact, experimental data, particularly from animal models, support the DOHaD theory and help to explain the mechanisms of chronic disease programming in utero (WHO 2001). Actually, maternal malnutrition throughout gestation or at specific periods of fetal development have been associated to impaired fetal growth and glucose tolerance (WHO 2001; Bayol, Simbi, and Stickland 2005; Pereira et al. 2015). Nowadays, sedentary lifestyle and high-caloric intake diets are contributing for an increasing rate of metabolic diseases in western societies (Saklayen 2018). These unhealthy behaviors are especially relevant during pregnancy period, for both maternal and offspring health (WHO 2001). Likewise, one of the most prevalent metabolic complications during pregnancy period is the development

of GDM (Roglic 2016). Actually, GDM condition may have a negative impact in mother and offspring health during and after pregnancy. In fact, offspring from GDM dams exhibited SM impaired glucose regulation and insulin sensitivity, higher risk for type 2 diabetes mellitus (T2DM), disrupted liver metabolome, and hepatic steatosis (Pereira et al. 2015; Patel, White, and Deutsch 2015; Bayol, Bruce, and Wadley 2014). These (patho)physiological consequences in offspring result from progressive development of less resistant phenotypes in tissues with high metabolic activity, such as SM and liver, which are key cornerstones in different important adaptive-related axis (Simar et al. 2012; Son et al. 2020; Pereira et al. 2015).

Considering the etiology of GDM, the promotion of maternal lifestyle changes, such as increased levels of physical activity, should be considered as core strategy to counteract GDM-associated health impairments for mothers and offspring (Birsner and Gyamfi-Bannerman 2020). In fact, a growing body of evidence demonstrate that promoting active lifestyle changes, such as enrolment in GE programs, induces health-related positive benefits, not only for mothers, but also for their offspring (reviewed in II) (Gilbert et al. 2012; Mottola and Artal 2016), preventing adverse effects associated to maternal environmental exposure on offspring metabolism (Laker et al. 2014; Stanford et al. 2015). Several studies demonstrated that ME improves offspring long-term glucose homeostasis (Carter et al. 2012; Mottola 2013; Harris, Baer, and Stanford 2018; Stanford et al. 2017) and contributes to improve metabolic function (Raipuria, Bahari, and Morris 2015; Rajia, Chen, and Morris 2013). However, despite the current evidence about the positive effect of GE, studies exploring its role and the associated mechanistic explanation on tissues such as liver and SM are scarce. In the context of the experimental works **IV** and **V**, we used a model where female rats were fed with HFHS-diet prior to mating in order to induce a specific metabolic environment before pregnancy that would, in the particular conditions of pregnancy, prompt the development of metabolic remodeling, characteristic of GDM. In fact, sedentary HFHS dams showed impaired glucose tolerance and a higher gestational BW gain (**IV**), both important markers for GDM (Pereira et al. 2015). Additionally, offspring from sedentary HSHS dams had higher BW at

weaning, which was in accordance with previous studies (Raipuria, Bahari, and Morris 2015; Bayol, Simbi, and Stickland 2005) and is considered a key indicator for higher risk of metabolic disorders development later in life (Shankar et al. 2008). Moreover, the negative impact of maternal lifestyle on offspring BW was maintained until the 6th week of age. Interestingly, benefits from GE were observed in mothers and offspring. Namely, gestational BW gain and offspring BW were lower in HFHS exercised dams and in offspring, respectively.

The deleterious consequences of maternal HFHS-consumption before and during gestation have been reported in offspring SM and liver tissues (Simar et al. 2012; Pileggi et al. 2016; Pereira et al. 2015; Patel, White, and Deutsch 2015). Our results showed that, from a morphologic point of view, the negative impact of maternal HFHS-diet and sedentarism was more pronounced in SM than in liver. In fact, HFHS-diet decreased SOL-to-BW ratio in the offspring from sedentary mothers (**V**). These results are in line with other studies that reported deleterious consequences on offspring SM from sedentary HFD-fed mothers (Simar et al. 2012; Pileggi et al. 2016; Son et al. 2020) and that were associated to reductions in SM weight, muscle cross-sectional area and fiber type composition (Bayol et al. 2009). Surprisingly, statistically non-significant differences were observed in morphologic parameters analyzed in liver (**IV**), such as tissue weight and histological evaluation (NAFLD score); however, a clear tendency was observed for a reduction of the NAFLD score in HFHS-fed mothers and their offspring induce by GE. This tendency was supported by a decrease in triglycerides content in offspring from exercised mothers and highlight the relevance of physical exercise against hepatic negative alterations, especially because hepatic triglycerides are considered an important marker of steatosis in rodent (Liang et al. 2014). Additionally, GE also had a positive influence in offspring SM composition inducing an increase in SOL-to-BW and TA-to-BW ratios. These results highlight the importance of maternal lifestyle impact in the offspring body composition, influenced by maternal diet and levels of physical activity (Raipuria, Bahari, and Morris 2015).

Intergenerational mitochondrial modulation

Considering the important role of mitochondria in SM and liver metabolism, several studies have recently explored the impact of lifestyle on maternal mitochondrial network physiology and the possible intergenerational transmission to their offspring (Son et al. 2020; Bayol et al. 2009; Pileggi et al. 2016; Raipuria, Bahari, and Morris 2015; Falcão-Tebas et al. 2020; Gusdon, Song, and Qu 2014; Bruce et al. 2009). In fact, maternal malnutrition, such as maternal HFD diet consumption induces a decrease in the mRNA expression of several biomarkers of mitochondrial biogenesis and dynamics, namely PGC-1 α , Nrf1, and Tfam, (Son et al. 2020; Pileggi et al. 2016) associated with downregulation of OXPHOS subunits, both in liver (Borengasser et al. 2011; Bruce et al. 2009) and SM (Latouche et al. 2014). Indeed, in the offspring from HFHS-sedentary mothers our results indicate a decrease expression of subunits from complex I in liver (**IV**) and subunits of complex IV in TA (**V**). Additionally, exhibited an impairment in liver mitochondria bioenergetics, specifically in RCR evaluation when using complex I-linked substrates (**IV**). However, as already mentioned above, PE has been proven as a non-pharmacological strategy against several pathological conditions (Fiuza-Luces et al. 2013; Pedersen and Saltin 2015), including HFD-induced health-related complications (Rocha-Rodrigues et al. 2018), being mitochondrial physiology in the center of the observed beneficial adaptations (detailed reviewed in **I**). In this sense, we aimed to explore the possible beneficial role of GE exercise in both liver and SM tissues. Interestingly, the positive impact of GE on the offspring SM mitochondria was evident. In paper **V** we showed an increase in protein expression of mitochondrial biogenesis biomarkers (AMPk and PGC-1 α) in SOL, as well as OXPHOS content in SOL (C-I and C-IV subunits) and in TA (C-IV subunits and ANT). Accordingly, recent evidence (Son et al. 2020) has shown that enhanced mitochondrial biogenesis is associated with an increase in SM oxidative capacity and with a muscle fiber type shift from glycolytic to oxidative in offspring of exercised mothers. In addition, GE-induced improvements in liver mitochondrial bioenergetics were also clear (**IV**). In fact, OXPHOS C-III to CV protein expression were upregulated in HFHS-exercised mothers, as well as C-I and C-

IV expression were upregulated in offspring. Alterations in liver protein expression were concomitantly with improvements in mitochondrial respiratory capacity, specifically when using complex I-linked substrates. Moreover, acylcarnitine profiling has been used to describe alterations in mitochondrial β -oxidation in animal models of obesity (Koves et al. 2008). We did not find alterations in long-chain acylcarnitine levels of sedentary HFHS-fed dams when analyzed in liver and in plasma (**IV**). However, an increase in offspring plasma and liver acylcarnitines (C16, C18, and C18:1) was evident, corresponding to fatty acids diet composition consumed by mothers. Conversely, statistically non-significant alterations were found in SM despite a clear tendency to increase long-chain acylcarnitine levels (**V**), also in line with different FA composition between C and HFHS-diets consumed by mothers. Our data suggest that increased accumulation of long-chain acylcarnitine in the offspring plasma and liver may be a consequence of the maternal diet. In fact, studies in humans have reported reduced placental β -oxidation in GDM (Visiedo et al. 2013) leading to triglycerides accumulation in placenta, which may result in an increased transfer of placental fatty to the fetus. On the other hand, GE showed to be an effective strategy against excessive energy supply from HFHS-fed mothers to their offspring. In fact, reduced levels of fatty acids seem to be transferred to the fetus across the placenta, positively regulating acylcarnitine levels in offspring plasma and liver from exercised mothers.

Considering the results obtained in papers **IV** and **V**, the positive effects of GE on mitochondrial SM and liver physiology were evident. However, to better understand some of the mechanisms that can explain intergenerational inheritance of GE protective effects, we evaluated the SM expression of some miRNA as markers of epigenetic regulation (**V**), able to have an impact in the biological programming of future generations. We have found that miR-338, which has been confirmed to inhibit COXIV at mRNA and protein level (Aschrafi et al. 2008), was downregulated in the SM of the offspring from exercised mothers. Moreover, in accordance with data reported by Simão et al. (2019) and Lee et al. (2010), GE downregulated miR-34a, which is consistent with increased activation of SM AMPk/PGC-1 α axis and mitigates the deleterious impact imposed by

maternal HFHS diet on mitochondrial biogenesis and metabolism-related markers. Accordingly, and in addition to miR-34a modulation, our data showed a downregulation of miR-378a mediated by GE. In fact, when overexpressed, both miRNAs were associated with mitochondrial dysfunction and with a decrease in fatty acid oxidation and oxygen consumption (Simão et al. 2019; Fulzele et al. 2019; Carrer et al. 2012) suggesting that GE prevented, at least in part, some mitochondrial impairments through epigenetic regulation.

Surprisingly, the SM expression of miR-494 was not influenced by maternal lifestyle, despite previous data suggesting that endurance exercise significantly decreases the content of miR-494 associated to the TFAM expression and mitochondrial biogenesis (Yamamoto et al. 2012). Consistently, we also did not observe alterations in TFAM expression. A clear difference in exercise interventions might eventually explain, at least in part, the differences in the expression of miR-494 obtained between our results and Yamamoto and coworkers (2012). In fact, in Yamamoto et al. (2012) work, the miRNA expression was evaluated in the SM of the exercised animals while in our study data was obtained from the offspring of the exercised dams. Considering the lack of any other study analyzing the expression of this miRNA in the offspring of exercised dams, as well as the sensitivity noted in the other miRNAs, it seems that maternal exercise is not a sufficiently strong stimulus to influence the role miR-494 in the intergeneration inheritance provided to offspring SM.

Finally, improvements in endurance capacity, namely in maximal workload performance, induced by different training regimens (III) have been associated with improvements in mitochondrial respiratory capacity (Granata et al. 2016; Jacobs et al. 2013) and volume density (detailed reviewed in I). In addition to the metabolic modulation towards a more oxidative phenotype suggested above, mice lacking miR-378 through double-KO exhibited better physical performance and improved absolute muscle strength (Podkalicka et al. 2020). However, the intergenerational transmission of positive physiological adaptations induced by ME to their offspring have been poorly described. Indeed, some evidence suggest that maternal malnutrition may have a negative impact on offspring performance (Walter and Klaus 2014) and may be associated to an impairment

in mitochondrial network physiology (Falcão-Tebas et al. 2020; Raipuria, Bahari, and Morris 2015). Moreover, Eclarinal et al. (2016) reported that offspring from exercised mothers had a higher propensity for PE. In fact, to the best of our knowledge, paper **V** was the first study to show an improvement in MWT in 6-week-old male offspring. Moreover, the improvements in MWT, in terms of maximal speed and time until exhaustion, were associated with markers of mitochondrial biogenesis, OXPHOS content, and decreases in miRNA-378 expression, as well as with an increase in markers of mitochondrial content and function, like mtDNA content and CS activity in SM of offspring sired from exercised mothers. Some of the alterations found in the present study are in line with mitochondrial remodeling reported by other groups, specifically in terms of mitochondrial biogenesis (Raipuria, Bahari, and Morris 2015) and function (Falcão-Tebas et al. 2020). Additionally, a recent work from Son et al. (2020) showed improvements in maximal oxygen consumption during exercise as well as a higher time-to-exhaustion and distance performed in an acute graded aerobic treadmill test, also in offspring from exercised mother, but immediately after weaning. Once again, our results clearly shed light on the importance of maternal lifestyle, specifically the importance of GE, on phenotypic programming effect in the offspring, namely through the protective effect on health-related metabolic state and ultimately contributing to an enhanced functional performance.

6. Conclusions

1. FRW model induced SM morphologic alterations similar to forced exercise models, such as HIIT.
2. Exercised animals engaged in a FRW program exhibited improvements in physiological central mechanisms associated, at least in part, to improvements in cardiac mitochondrial function.
3. FRW animals showed an improved ability to perform maximal exercise associated to an oxidative phenotype.
4. Gestational exercise counteracts the negative impact of HFHS maternal diet and sedentarism in offspring liver and SM morphology.
5. Offspring from HFHS exercised mothers exhibited an improvement in liver and SM parameters of mitochondrial biogenesis, OXPHOS content and respiratory capacity, associated to enhancements in maximal exercise performance.
6. Improvements in SM biomarkers of mitochondrial performance seems to be regulated, at least in part, by the role of miRNAs through epigenetic modifications inherited by the offspring generation.
7. Although performed in the animal model and should be further confirmed in humans, the overall results suggest the relevance of GE against GDM-associated metabolic impairments. Additionally, the scientific evidence from the present PhD thesis adds mechanistic knowledge that reinforces and encourages the implementation of physical exercise programs during pregnancy in the clinical counselling.

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