UNIVERSIDADE D
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EZE GREAT OGWU

EFFECTS OF EARLY PHYSICAL ACTIVITY AND RESPONSE TO LATER PHYSICAL ACTIVITY WITH A WESTERN DIET ON MITOCHONDRIAL FUNCTION IN TISSUES

VOLUME 1

Tese no âmbito do Mestrado em Biologia Celular e Molecular

orientada pela Doutora Eugénia Maria Lourenço Carvalho

e apresentada ao Departamento das Ciências da Vida

Julho de 2022

DISSERTAÇÃO

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This Master thesis project entitled: "**EFFECTS OF EARLY PHYSICAL ACTIVITY AND RESPONSE TO LATER PHYSICAL ACTIVITY WITH A WESTERN DIET ON MITOCHONDRIAL FUNCTION IN TISSUES**", would not have been possible without the help and contributions of many.

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Abstract

Mitochondrial dysfunction is associated with a wide variety of metabolic and degenerative diseases like diabetes, obesity, aging, and even Alzheimer's disease. Robust mitochondrial function is supported by physical exercise and caloric balance, and it is central for sustained metabolic health throughout life. A more consistent presentation of mitochondrial physiology will improve our understanding of the etiology of these metabolic diseases.

This experiment aims to elucidate the effects of early-in-life physical activity (PA) and the response to later-in-life physical activity on mitochondrial function in tissues (Soleus muscle and brown adipose tissue (BAT)). This animal study was carried out in male C57BL6/J mice (n=168). Following 1 week of acclimatization experiments spanned 20 weeks. Animals were obtained at 3 weeks of age, and were first divided into 2 major groups, a control group (n=84), and (PA) group (n=84). All the animals had ad-lib access to water and respective diets, mice were housed 5 per cage, except for those in the PA groups, that were housed one per cage, with running wheels attached for monitoring. Mice in the PA groups had free and voluntary access to the running wheel and daily running times and distances were recorded. The protocol for the study is described below in Figure 18. After each intervention period, 12 animals from each group were selected randomly and sacrificed. Muscle and BAT were collected for mitochondrial respiration measurements. The 1st experiment period consisted of 4 weeks of PA (two groups of animals), the 2nd experimental period consisted of 8 weeks of high-fat diet (HFD) (43% harlan fat diet) (4 groups of animals) and the 3rd and last experiment period consisted of 4 weeks of PA (8 groups of animals).

Soleus muscle and BAT were collected and processed for high-resolution respirometry using the Oxygraph O2K Oroboros technology. Our results show that under these experimental conditions, there were no significant differences across the groups, in either soleus or BAT mitochondrial function, even after exposure to a HFD after early PA and PA later in life. The animals started these experiments when they were relatively young. Thus, a more extensive exercise regimen and/or higher concentration of HF should be further tested in future analysis for possible differences.

KEYWORDS; Mitochondrial function, physical activity, high-fat diet, brown adipose tissue, soleus muscle

Sumário

A disfunção mitocondrial está associada a uma grande variedade de doenças metabólicas e degenerativas, tais como diabetes (T2D), obesidade, envelhecimento ou mesmo alzheimer, doença de Alzheimer, doença de Huntington, doença de Parkinson, atrofia muscular espinhal. Uma função mitocondrial robusta é suportada por exercício físico e equilíbrio calórico, sendo fundamental para uma saúde metabólica sustentada ao longo da vida. Um conhecimento mais aprofundado da fisiologia mitocondrial irá contribuir para uma melhor compreensão sobre da etiologia destas doenças metabólicas.

Neste trabalho pretendemos elucidar quais os efeitos da actividade física, quer no início quer ou numa fase mais tardia da vida, na a função mitocondrial do músculo Soleus e tecido adiposo castanho (TAC). Este estudo foi realizado em muganhos C57BL6/J machos (n=168), dvidios em 2 grandes grupos (grupo de control (não PA) *versus* groupo com actividade física (AF, group PA). Os murganhos do do grupo PA tinham acesso livre e voluntário à roda de corrida, sendo que os tempos e distâncias diárias de corrida eram registados. Após 4 semanas, 12 murganhos de cada grupo foram sacrificados. O músculo e o TAC foram imediatamente recolhidos para análise. Os restantes murganhos foram consequentemente divididos em quatro grupos adicionais. Sendo assim dentro de cada grupo inicial, um dos subgrupos recebeu dieta risca em gordura e o outro continuarou a receber dieta padrão. Após 8 semanas, 12 murganhos de cada grupo foram aleatóriamente sacrificados. O músculo e o TAC foram imediatamente recolhidos para análise. Os restantes animais, foram novamente transferidos para a dieta de controlo, sendo que cada subgrupo, foi subsequentemente dividido em dois novos grupos onde foi continuada ou iniciada a prática de acitivade física. Após 4 semanas, os restantes animais foram sacrificados e os tecidos recolhidos para análise.

Os nossos tecidos de interesse, o músculo Soleus e a PAB foram recolhidos e processados para respiração de alta resolução usando a tecnologia Oxygraph O2K Oroboros. Mimamos um estilo de vida regular de tal forma que quando somos mais novos, passamos por muita PA, e quando envelhecemos enquanto temos uma dieta ocidental, menos PA. Vemos que na nossa experiência, nos músculos esqueléticos e nas MTD não houve diferenças significativas entre os grupos, mesmo após a indução de uma DAF após uma DAF precoce e mais tarde na vida de PA. Assim, um regime de exercício mais extensivo e uma maior concentração de HF deveria ser testado mais aprofundadamente em análises futuras para possíveis diferenças.

PALAVRAS-CHAVE; Função mitocondrial, mitocondria, actividade física, dieta rica em gordura, TAC, músculo solitário

List of abbreviations

AA-Antimycin A

ADP-Adenosine diphosphate

AKT/PKB- Protein kinase B

ATP- Adenosine triphosphate

BAT- Brown adipose tissue

BMI- Body mass index

BW- Body weight

Ca2+ – Calcium ion

CI- Complex 1

CII- Complex 2

CMD- Congenital muscular dystrophy

CNS- Central nervous system

Con- Control

COPD - Chronic obstructive pulmonary disease

CoQ - Coenzyme Q

CoQH - Ubisemiquinone

CV - Complex V

CVD - Cardiovascular disease

Cyt c – Cytochrome c

DM- Diabetes mellitus

ET- Electron transfer

ETC- Electron transport chain

EU-European union

FA-Fatty acids

FAD - Flavin adenine dinucleotide

FADH- Flavin adenine dinucleotide+hydrogen

FCCP- Carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone

Fe-S-Iron sulfur

FFA-Free fatty acids

F-pathway – Fatty acid oxidation pathway

GDP- Guanine diphosphate

GLUT- Glucose transporter

GRP-Group

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H2O- Water

HF-High fat

HFD-High fat diet

HFDt- High-fat diet treatment

HRR – High resolution respirometry

IMF-M-Intermyofibrillar mitochondria

IQR-Interquartile range

IR – Insulin receptor

IRS1 – Insulin receptor substrate 1

MG- Malate and glutamate

NAD- Nicotinamide adenine dinucleotide

NADH – Nicotinamide adenine dinucleotide+hydrogen

No-PA-No Physical activity

OMY-Oligomycin

OXPHOS- Oxidative phosphorylation

PA-Physical activity

PCOS- Polycystic ovary syndrome

PCr- Phosphocreatine

PGC-1α-Coactivator-1α

PI3K-Phosphoinositide 3-kinase

PPARγ-Peroxisome-proliferator activated receptor γ

PTP-Protein-tyrosine phosphatase

PTP1B-Protein-tyrosine phosphatase 1B

Q1- 1st Quartile

Q3- 3rd Quartile

RCR-respiratory control ratio

ROS-Reactive oxygen species

ROX – Residual oxygen consumption

RWE-Running wheel exercise

SCR-Substrate control ratio

SD-Standard deviation

SSM-Subsarcolemmal mitochondria

SUCC-Succinate

SUIT- Substrate uncoupler inhibitor titration

T1DM-Type 1 Diabetes mellitus

T2DM-Type 2 Diabetes mellitus

TAGs-Triglycerols

TCA- Tricarboxylic acid

TG-Triglycerides

TNF α – Tumor necrosis factor α

UCP1-Uncoupling protein 1

UVR-Ultraviolet radiation

WAT-White adipose tissues

WHO-World health organisation

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7. Introduction

Aging

Worldwide, it is expected that the number of aged people ≥55 will increase exponentially by the year 2050. Humans are faced with the challenge of maintaining independence as they get older (1).

Deductions from the United Nations population projections indicate that by the year 2100 in Europe, one in seven persons will be aged 80 or over, and over 30 percent of people in three-quarters of European countries will be aged 65 or over (1). Figure 1. below shows this projection as an effective duplication of the population count of people between age 65 and above in European countries.

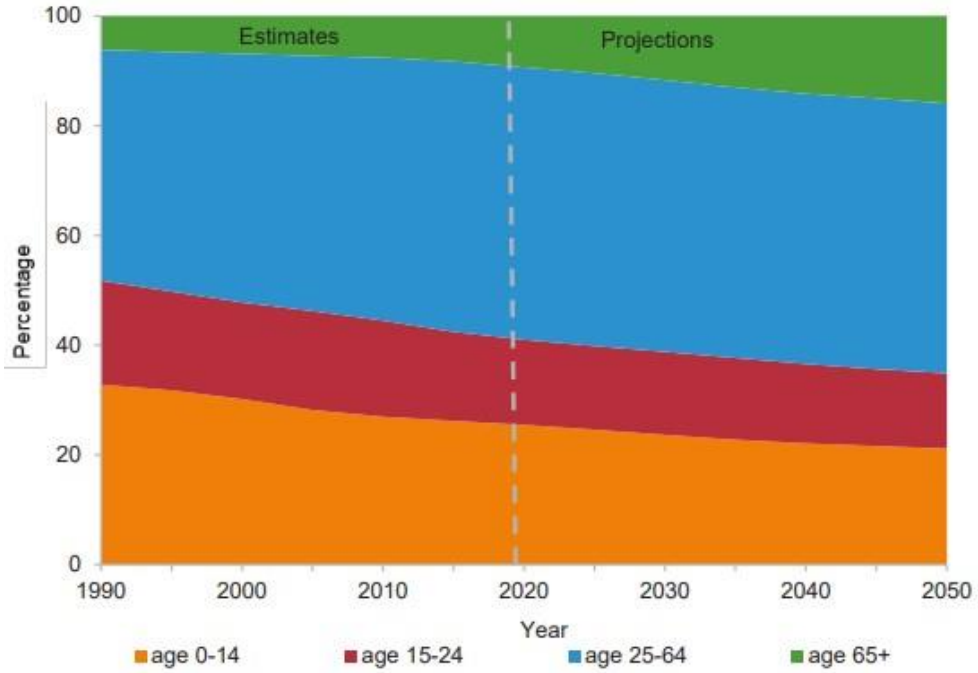

FIGURE 1. GLOBAL POPULATION BY BROAD AGE GROUP 1990-2050. ADAPTED FROM UNITED NATIONS, 2019 (1).

A lot of western nations pay more attention to policies and programs that help to achieve more stable and successful aging strategies and good market opportunities for persons living out their later life. Studies have shown that not everyone achieves success in healthy aging, and this may be caused by frailty, some persons grow tired of living later in life because of how frail they have become (2).

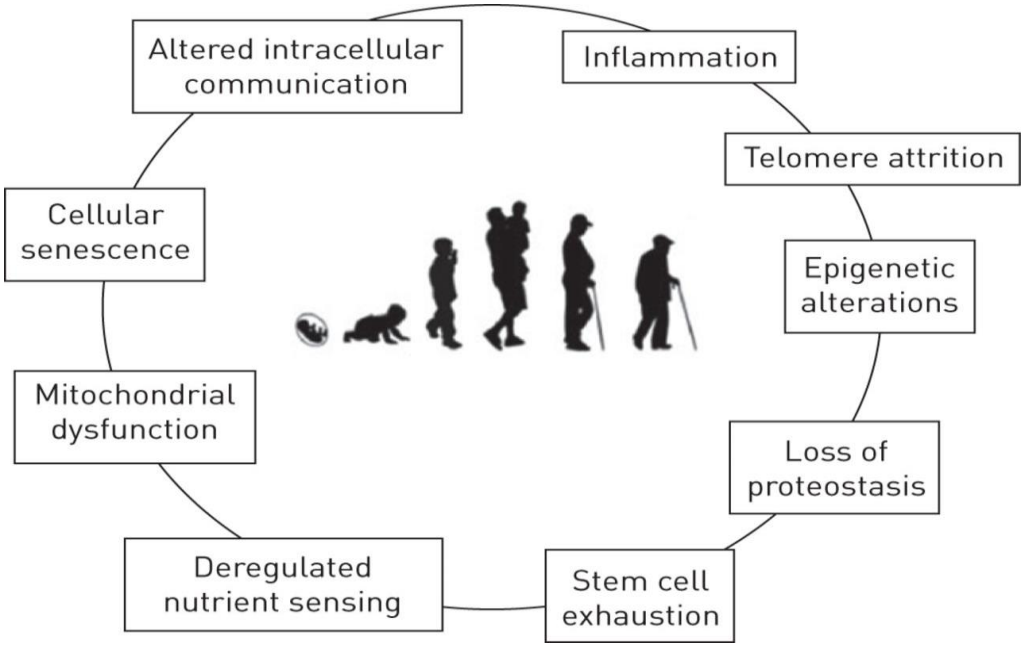

FIGURE 2. AGE DECLINE AND ITS EFFECTS IN HUMANS. ADAPTED FROM (3).

Aging is a gradual process that involves an increased, constant decline in body muscle mass, which is also known as sarcopenia (4). Sarcopenia begins from about the third to fourth decade (5) and depending on the lifestyle and diet an individual carries on with, complications that arise from sarcopenia can be seen sooner than later in life. It is usually characterized by fatigued muscles, reduced muscle abilities, and a reduction in muscle endurance ability in muscles (4). Figure 2 also shows some molecular and cellular anomalies associated with aging. Frailty comes with limitations in routine daily activities such as grooming, eating, and bathing or instrumental activities of daily living such as shopping, climbing stairs and running (3). Therefore, to be able to maintain functional independence and carry out vigorous activities, lifelong involvement in physical activities may help to provide the alertness and fitness needed without having unnecessary fatigue (6).

Aging has been known to be associated with a decrease in vasodilation and microvascular function in the muscle (7,8). Some of these complications include obesity and its complications, type2 diabetes mellitus, and cardiovascular diseases among others.

Aging also comes with negatively changed skeletal muscle characteristics, such as reduced muscle mass (9,10), and induces a reduction of the activity and effects of some mitochondrial enzymes (11,12). Variations in muscles are caused by low levels of PA as these muscles age, and a good part of these changes that occur in the muscle is believed to be a result of the biological process of aging. This is to say that the older the individual gets, the less PA they indulge in, then they become prone to changes like reduced muscle strength, muscle endurance, muscle quality, motor performance, muscle flexibility, and motor control. But some of these alterations can also occur when people age naturally with or without PA (13–17) We highlight some complications related to age decline in Figure 3 below.

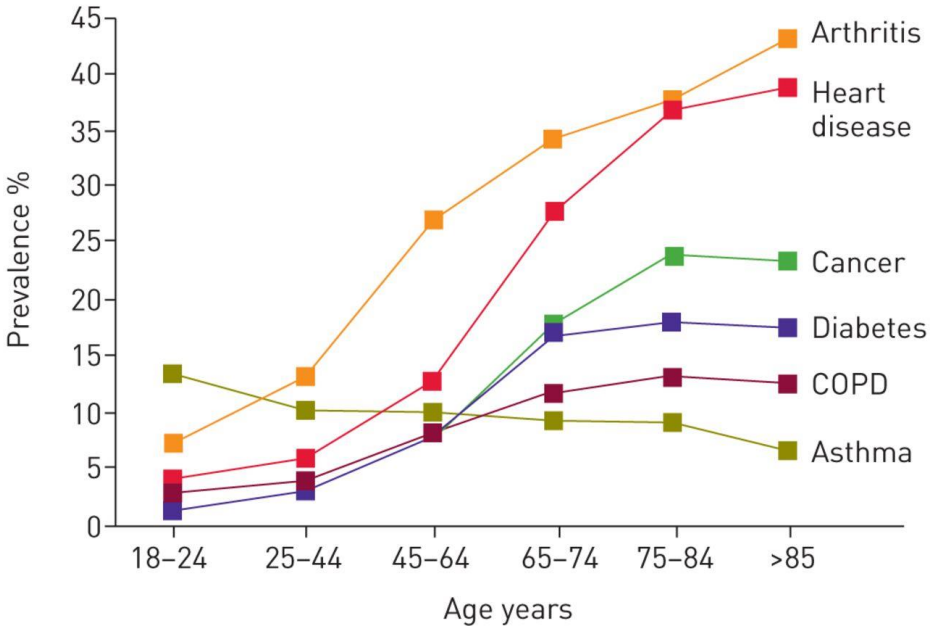

FIGURE 3. DISEASES ASSOCIATED WITH AGING ACROSS DIFFERENT AGE GROUPS. ADAPTED FROM (3)**.**

COPD: Chronic obstructive pulmonary disease

Obesity

Obesity is a condition characterized by an unhealthy and surplus amount of body fat, which increases the risk of illness and premature mortality (18). Men and women whose body mass index (BMI) ≥ 30 are considered obese and are generally more likely to have a higher mortality risk than those who are considered overweight (BMI: 25.0–29.9) (19). The risks of death associated with an increased BMI reduce with increasing age, and the BMI value associated with the lowest possible mortality is a bit higher in an older person than in younger persons (16). The occurrence of obesity is currently increasing in all age groups, including older persons (≥ 65 years). Nevertheless, absolute mortality risks synonymous with increased BMI increase with age, even up to age 75 because of increased mortality with advanced age (20).

Childhood obesity comes with a lot of risks. Obese children are at the lowest twice as likely to become obese as adults, thereby having an increased risk of cancer, premature death, and certain disabilities in adulthood (18). Children suffering from obesity have an increased risk of fractures, hypertension, insulin resistance, and other times psychological issues (21,22).

Matured adipocytes content includes single large droplets of fat that can resemble almost all the cell volume. The adipocyte's primary function is to store energy in the form of triglycerides (TG) especially when there is surplus energy and transport these lipids stored as fatty acids when energy is required (23).

Therefore, during healthy conditions fatty acids (FA) are released into the bloodstream, and they supply the peripheral tissues as energy fuels. However, when adipose tissues fail to store excess energy, it results in an increased outflow of free fatty acid from fat regions to other tissues especially skeletal muscles, a phenomenon known as intramyocellular lipid accumulation (24). This excess fat contents within the skeletal muscle induce metabolic dysregulation including insulin resistance (24). A reduction in mitochondrial fatty acid oxidation which is caused by mitochondrial dysfunction and or a reduction in the content of the mitochondria can also lead to the accumulation of enhanced levels of intracellular fatty acyl-CoA and diacylglycerol, which will inhibit insulin signaling in skeletal muscle (25). Also, the development of IR in skeletal muscles is due to an increased mitochondrial oxidant production which is a response to a

demand for excess fuel and can cause reduced insulin signaling and glucose transport through different pathways (26).

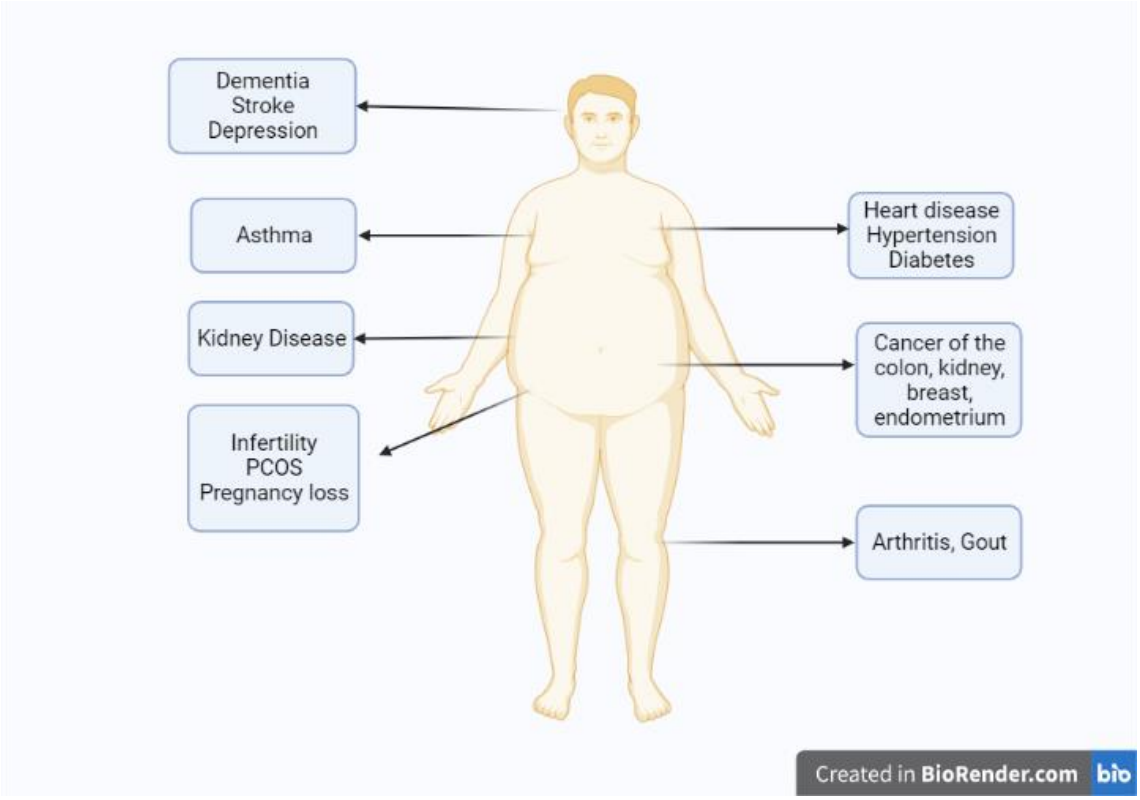

FIGURE 4. DEPICTS COMPLICATIONS OF OBESITY (26)**.**

PCOS- Polycystic ovary syndrome

Obesity can induce a lot of health complications as shown in Figure 4, which can lead to a considerably impaired quality of life and early death (21). Except for older, middle-aged and young persons, researchers have shown that diseases like hypertension, diabetes, cardiovascular diseases (CVD), and, even, osteoarthritis are associated with obesity even as age increases (27,28).

Obesity is connected to a remarkable amount of reductions in levels of physical activities and it is one of the main risk factors for a couple of diseases like osteoarthritis (18).The pronounced effect is seen on the knees but there it also occurs in hand joints, proposing an inflammatory and mechanical cause (18,29). In obesity, there is increased lower back pain which also occurs in people with low levels of PA (29).

Taking different kinds of diets like the ketogenic diet and high-fat diet (HFD) can be an effective means of weight control in the short term, but long-term results remain unclear due to difficulties with adherence (30). An excess of body fat tissue may be related not only to energy intake and energy expenditure in humans, as stated by Flatt (31).

High Fat-Diet

FIGURE 5. EXAMPLES OF HIGH-FAT DIET FOOD; HAMBURGERS, CHEESE, DOUGHNUTS, PIZZA, CHOCOLATES AND LOTS OF RED MEAT.

An HFD is a diet that consists of at least 35% of total calories consumed from fats either in the saturated or unsaturated form (32). A lot of processed foods as shown in Figure 5, and many other foods have a high-fat content including chocolate, butter, and oily fish (32). It is only when a diet produces positive energy and fat balances, thereby promoting obesity, only then can such a diet be considered an HFD (28).

HFD can lead to various metabolic alterations such as excessive eating in humans (31,33), a reduced lipolytic activity which is a metabolic pathway through which lipid triglycerides are hydrolyzed into glycerol and FA in fat tissue, reduction in leptin secretion, or sensitivity (34) which occurs due to a decreased insulin-stimulated glucose uptake into adipocytes which is also an effect of HFD and, hypothalamic neuron apoptosis which is dependent on the composition of the HFD (35% and above) and occurs as a result in the expression of cytokines like the tumor necrosis factor-alpha

(TNF-a) which can induce this apoptosis (35), mitochondrial metabolism will be impaired, insulin resistance which is an impaired response of the body to insulin which causes elevated blood glucose level thus type 2 diabetes, and obesity (28). Fat accumulations in muscle cells due to an HFD also affect insulin pathways in muscles, leading to insulin resistance which is depicted by the overcompensation of reactive oxygen species (ROS) in adult skeletal muscles (23,36). Authors suggest that HFD has also been shown to be associated with a reduced glucose transporter type 4 (GLUT-4) expression or impaired insulin action in glucose transportation in skeletal muscle (36). The exercise carried out intensely can increase lipolysis and will protect an individual from the possible effects of an HFD under certain circumstances (37).

Also, in mice fed an HFD for two days, intramuscular lipid was increased the red part of oxidative muscles, and four days after, the same increase was observed in the white part of glycolytic muscles of rats (38,39). This increase in intramuscular lipid has also been proposed to be a mechanism that promotes insulin resistance as well (39,40). HFD treatment for a long duration has shown to cause atrophy in different leg muscles in aged mice and also leads to an altered distribution of muscle fiber types in aged mice (38,41).

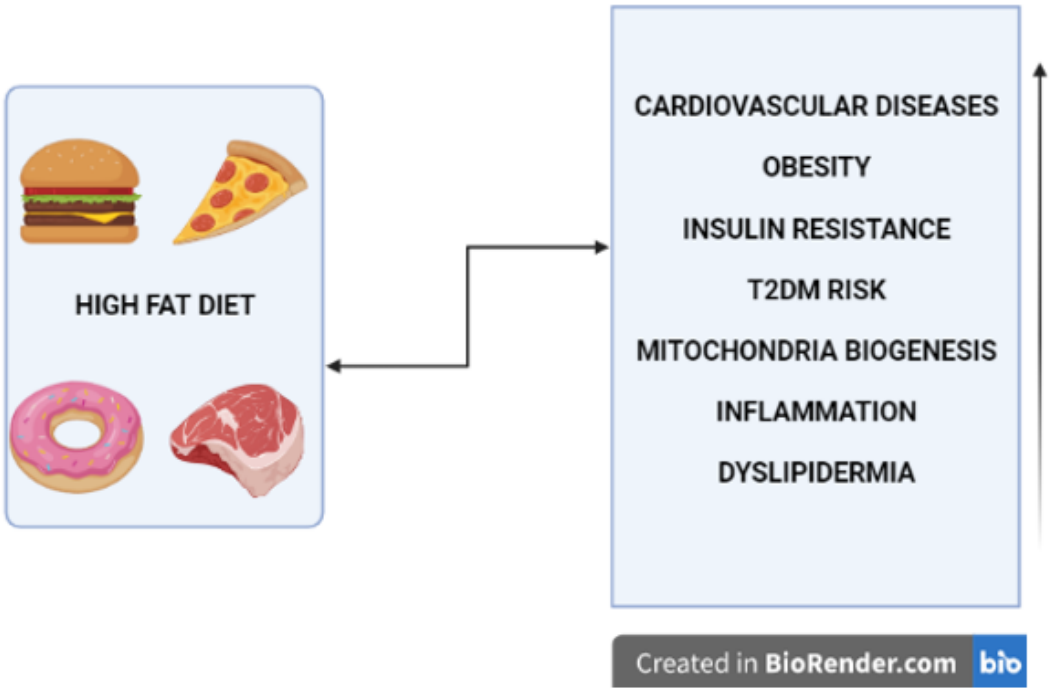

FIGURE 6. COMPLICATIONS OF A HIGH-FAT DIET. (42).

CNS - Central nervous system, CMD - Congenital muscular dystrophy, T2DM - Type 2 diabetes mellitus.

In a study by Smith et al (43), authors stated that normal-weight persons who maintained an HFD (50% of energy as fat) and either a high or low PA level had a positive fat balance on the first day of physical inactivity, which continued and increased by the day. This interprets as mice housed as individuals fed with the HFD attained a reduced body weight (10% lower) compared to the co-housed mice of their previous studies (44,45).

Dietary macro-nutrients are mainly fats, carbohydrates, and proteins and micro-nutrients are mostly vitamins and minerals (43). HFD may comprise fats, carbohydrates, and proteins but with a higher percentage of fats in the combination or just a simple combination of carbohydrates and fats with a higher percentage of fats as well (43,46). The composition of the HFD may sometimes be dependent on the hypothesis of the research, for example, to see if other nutrients and not just fats play a vital role in body metabolism, disease pathophysiology, insulin sensitivity, and so on, further complications of HFD can be seen in Figure 6.

The appropriate combination of each nutrient (fats, carbohydrates, and proteins) that make up the high-fat diet may be involved in the balance between the intake of HFD and its oxidation (30). An increase in carbohydrate and/or protein consumption is accompanied by increased oxidation rates of both nutrients (47).

An HFD can influence the fat balance produced by a day of physical inactivity more than can a high-carbohydrate diet. Studies have shown that consumption of an HFD can lead to a positive energy balance by increasing the incidence of overeating (30,46–48).

A day with PA but with HFD may have a positive impact on the storage of body fat and, therefore, could be important as a factor that induces weight gain in adults. Positive fat balances will lead to increased fat mass permanently without PA in a day, compared to carbohydrate balance acquired in the same period of (5,33,49,50). Thus, an increase in adipose mass accumulation.

Diabetes

Diabetes Mellitus (DM) can be described as a metabolic disorder that is characterized by hyperglycemia, and abnormal metabolism of macronutrients (proteins, carbohydrates, lipids) which leads to defects in the action and or secretion of insulin by the body. The effects of DM are different in the long and short term, and it includes organ and tissue failure, and chronic injury with dysfunction (51).

EPIDEMOLOGY OF DIABETES

World Health Organization and the American Diabetes Association have classified DM into 4 different types of categories. we have type 1 (T1DM), type 2 (T2DM), gestational diabetes, and secondary types of diabetes, such as pancreatic diabetes (55). The most rampant of these categories are T1DM and T2DM. Figure 7 below describes the different categories of diabetes.

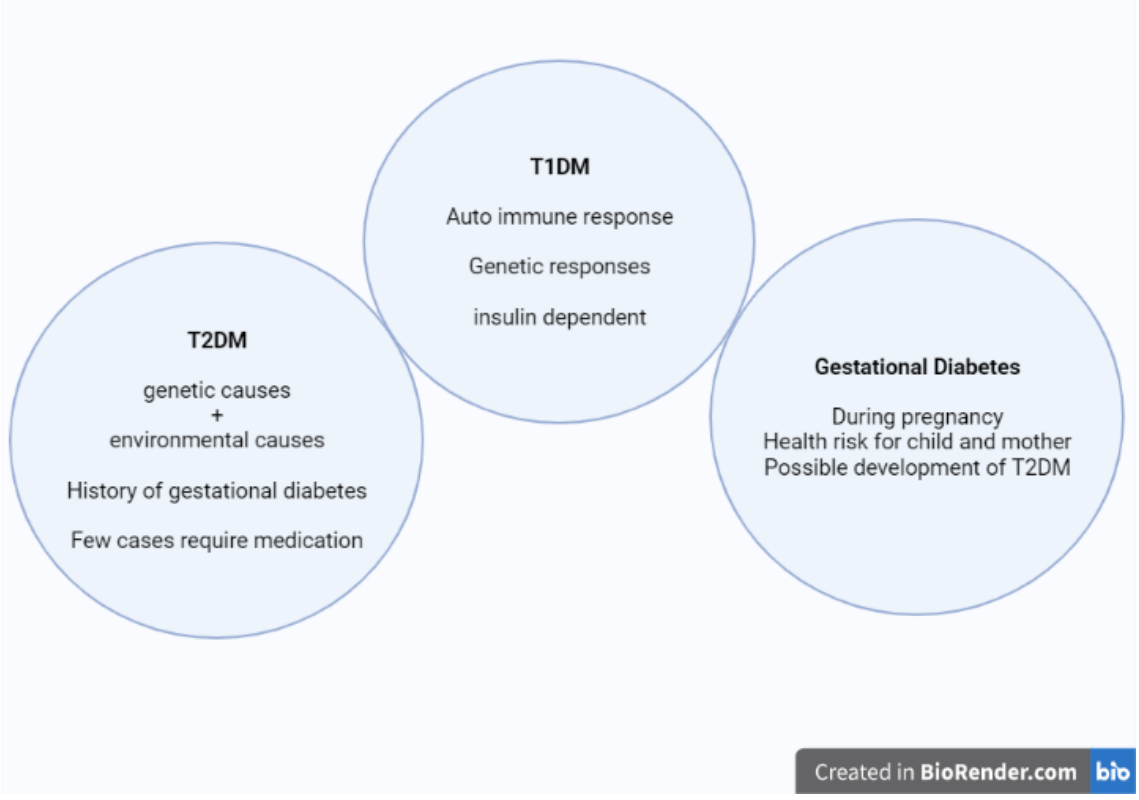

FIGURE 7. SUBGROUPS OF DIABETES AND THEIR CAUSES (52,53)**.**

T1DM – Type 1 diabetes mellitus; T2DM – Type 2 diabetes mellitus

T1DM is also known as insulin-dependent or immune-mediated diabetes. It comprises mainly of deficient insulin production which is caused by autoimmune destruction of insulin-secreting pancreatic islet beta-cells (β-cells), which is linked to other multiple genetic factors and environmental factors which are called idiopathic diabetes (52). Patients with T1DM are mostly dependent on insulin, requiring treatments based on daily insulin injections which will help to maintain adequate insulin levels and control glucose levels in the body (50). T1DM onset occurs mostly in children and young ages hence can be called juvenile diabetes but it can also appear at any age (54). T1DM represents about 10% of the total population affected by the disease while T2DM makes up the remaining 90% percent of the diabetic population (52). More recently, the number of persons with T2DM has increased exponentially, it is being considered a small epidemic.

In Portugal, T2DM has reached an epidemic level, presenting devastating consequences at personal, social, economic, and family levels. The Annual Report of the National Diabetes Observatory, the population of the Portuguese adult population living with diabetes was 12.4% in 2010, and it includes people between the ages of 20-80 years (55). And the prevalence of undiagnosed diabetes among this demographic is about 5.4%, and about 26% of the aged Portuguese population is already exposed to this disease development (55). T2DM occurs mainly among adults (persons older than 40 years). But lately, we have seen an increase in the number of younger adults and even adolescents present with T2DM, suggesting that childhood obesity may be responsible for this increase. In the last 20 years, T2DM has been seen to be prevalent among children and adolescents at a much higher rate than normal.

Obesity can cause some people to develop insulin resistance and consequent changes in blood glucose levels, mostly hyperglycemia (20). Increased glucose levels present in the blood can cause glucose intolerance. Insulin resistance can cause some obstruction of glucose into tissues and reactions occur thus increasing the hepatic glucose production by gluconeogenesis, and increasing the hyperglycemia (56).

COMPLICATIONS OF DIABETES

There are quite a couple of health complications that will arise from long-term impaired glucose regulation and insulin resistance. These alterations can be changes in glucose homeostasis and a couple of metabolic alterations like hypertension and dyslipidemia (hypertriglyceridemia and low high-density lipoproteins and cholesterol levels) (20). These changes as shown in the Figure 8 below, are a representation of clinical disorders and when linked with obesity or regional adiposity (abdominal), they can lead to diabetes (20,56).

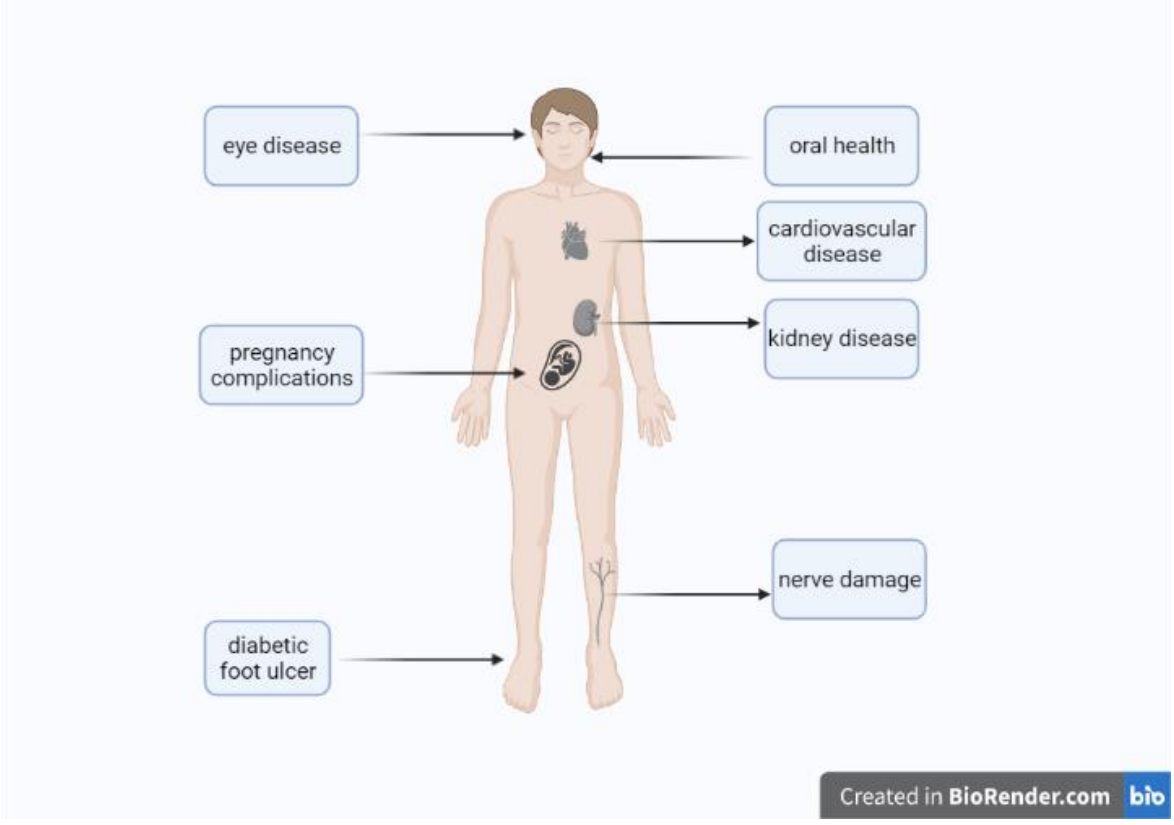

FIGURE 8. COMPLICATIONS OF DIABETES (51)**.**

Diabetes also increases the incidence of micro and macrovascular changes that lead to the development of several complication kidney diseases (nephropathy), eye disease (retinopathy) or even CVD, affecting the heart, the blood vessels, and ultimately, the entire circulatory system, leading to detrimental changes in neuronal and peripheral

blood supply peripheral including, also, the development of diabetic foot ulcers (diabetic neuropathy (52,57) as shown on Figure 8.

Physical Activity

Physical activity (PA) is any body movement that involves the muscles and the expenditure of energy. There are three main categories for PA as it could be low, high, or sedentary. In fact, PA could be measured, as simple as, the total amount of steps per day. In 2018, the EU Commission together with the world health organization recommended 10,000 steps as the average amount of steps per day for a healthy lifestyle. Figure 9 shows us a brief description of recommended time frames for optimum PA.

However, the world health organization considers that the individuals who do not partake in daily PA sufficiently to meet at least 150 mins of moderate-intense aerobic PA per week or at least 75mins of vigorous PA per week for adults and at least 60 mins of moderate-vigorous-intense daily PA for children aged 5-17 marks the prevalence of physical inactivity (6).

Recently, there has been an increase in physical inactivity globally and this increase is being associated with the advancement of technology which includes the use of mobile devices, video games, computers, and television (27,58). According to a world health report from 2019, only 42% of children between the ages of 5-11 years meet the WHO PA guidelines and about 14% of adolescents have been reported to be regularly physically inactive while 8% of 12-19-year-olds meet the recommended PA levels (59).

FIGURE 9. RECOMMENDED PHYSICAL ACTIVITY RANGES. ADAPTED FROM WHO, 2021 (60)**.**

Research on adults also showed that 30% of adults do not engage in enough PA either leisurely or in work situations (60). Figure 10 shows details of physical inactivity worldwide and we also find that, 25% of young adults (18-44years), 33% of middle-aged adults (45-64 years), 36% of older adults (65-74 years), and 53% of the elderly (75 years) are reported Low PA levels tend to have harmful and even detrimental consequences. Hence it can be inferred that inactivity prevails with increasing age (61,62). More problems arise when PA is very low or when physical inactivity becomes a lifestyle. these problems include greater dependence on others for daily living, reduced opportunity and ability for normal social interactions, and overall diminished quality of life, impaired circulation, osteoporosis, arthritis and/or other skeletal disabilities, diminished self-concept (21,27).

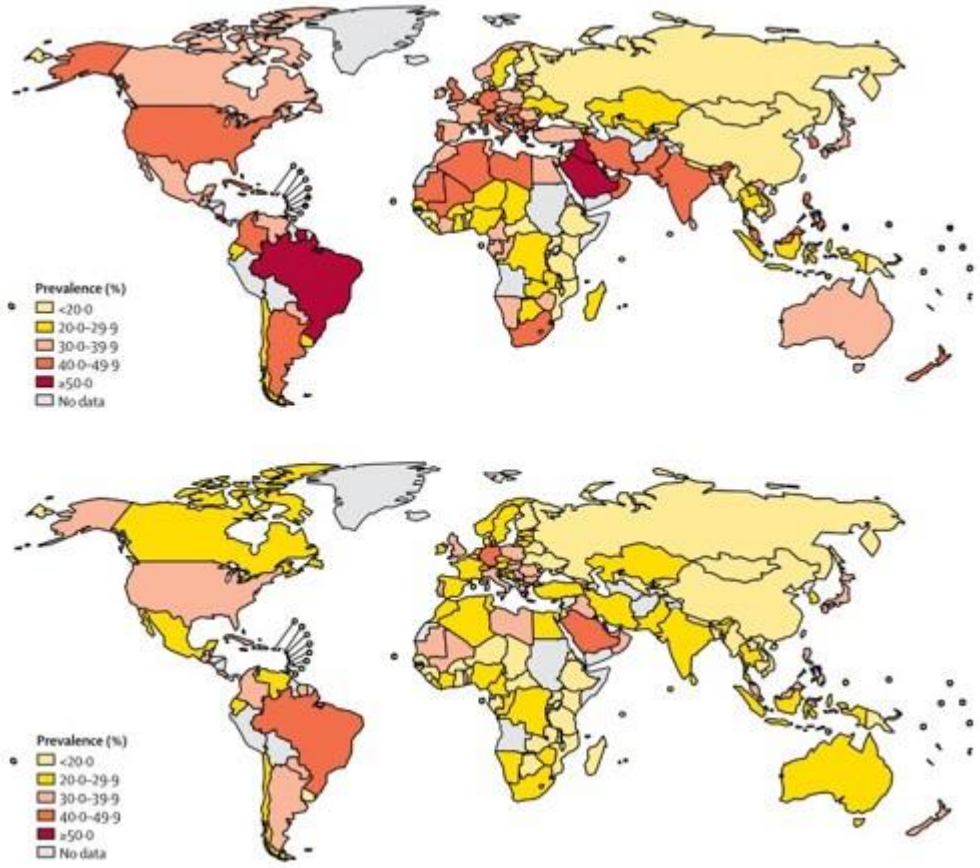

FIGURE 10. THE PROPORTION OF WOMEN (TOP) AND MEN (BOTTOM), INSUFFICIENTLY ACTIVE IN 2016. ADAPTED FROM WHO, 2018 (6)**.**

Exercise is a type of PA but a more structured, patterned, and routine PA that also involves the muscles and expends energy as shown in Figure 11. The PA routine ranges from moderate to vigorous to intense and this is also time dependent.

FIGURE 11. EXERCISE IS ONE TYPE OF PHYSICAL ACTIVITY

There are adaptations to response to PA, one of which is increased oxidative capacity in skeletal muscles (63). This is considered a classical physiological adaptation. In normal-weight young and in older adults, this response to PA is well described and quite a few studies have examined whether this adaptation in skeletal muscles occurs similarly in adults with obesity (64,65). In fact, in both men and women with obesity, there is noticeable reduced oxidative enzyme activity in muscles, including smaller mitochondria and lesser electron transport chain activity (28). This characteristic has been found to correlate in a biochemical manner with the severity of insulin resistance (66).

Some findings show impaired mitochondria function in muscles in obesity (46); therefore, it is quite important to check whether improvements can be achieved with the PA intervention together with weight loss. When such improvements fail to occur, there are possible risks for weight regain or limitations in the improvement of insulin sensitivity (21,64). There is not much information on obesity if improvement of oxidative capacity in muscles that can be induced by PA and weight loss is primarily due to an increase of mitochondrial content or an improved mitochondria function capacity (64). Moreover reduced skeletal muscle mitochondria capacity is also characteristic of aging and several experiments have suggested that mitochondria are a major factor in the etiology of sarcopenia (21,67,68) Increased levels of reactive oxygen species emission are linked to faulty mitochondria quality control as well as the activation of apoptotic pathways that are mostly thought to contribute to the pathophysiology of sarcopenia, as shown in the Figure 12 below (40,50).

The conflicting results presented above can be a consequence of a vast majority of these studies have not considered other factors that affect mitochondria like cardiovascular fitness, the subject's PA levels, or adiposity and all of which could be related to and between mitochondrial capacity and age (69–71). Also, the different methods employed in the analysis of mitochondria function including the use of isolated mitochondria that has been shown to exaggerate the observed deficit in mitochondrial function in aging (72), may contribute to the lack of clear answers. There is a reduction in exercise efficiency which means increased oxygen consumption per work performed for PA such as walking in elderly individuals (73), and this can cause activities of daily

living in older adults to be impeded potentially contributing to future risk for mobile disability. As of now, the underlying cause of low reduced exercise efficiency with aging is not known but it is believed that mitochondria energetics may be involved (74). Mitochondria energetics is the availability of reducing equivalents (hydrogen), obtained from carbs and lipids from the diet. When they were combined with oxygen to form water via mitochondrial oxidative phosphorylation determines animal energy (74). Thus, the influence of PA status, cardiovascular fitness, and adiposity on exercise efficiency in older adults has not been wholly and thoroughly elucidated.

The efficiency of exercise can be improved with endurance training in young and old (75). However, changes and or alterations in the contractile coupling, which is the connection (transduction) between the sarcolemma's action potential and the onset of a muscle contraction, are the conventional explanation for this distinction in the efficiency of exercise even though previous studies suggested that mitochondria play a role in exercise efficiency (75,76).

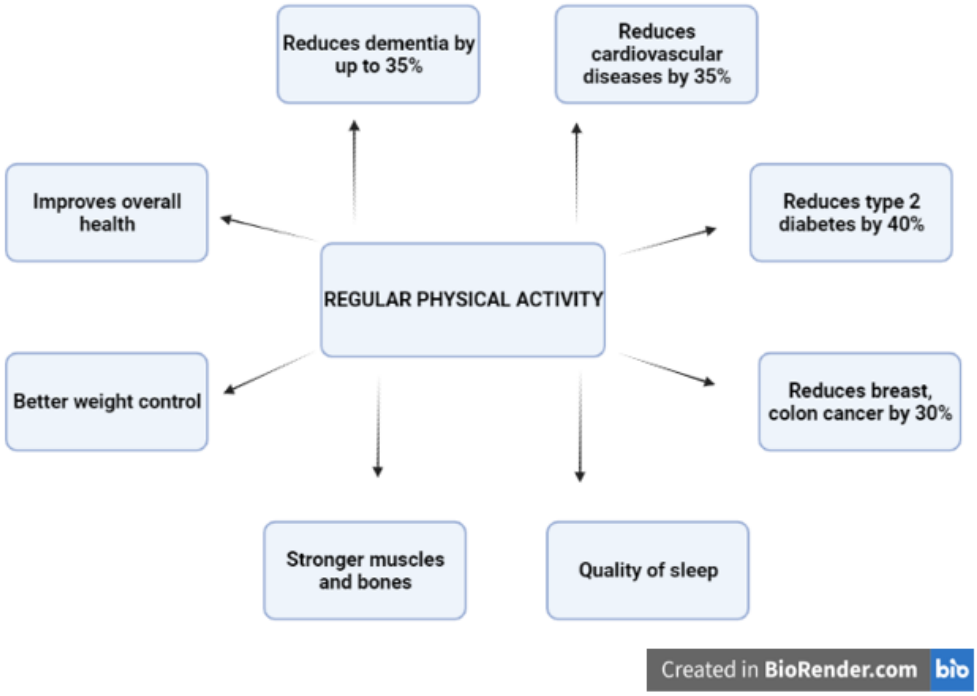

FIGURE 12**. BENEFITS OF PHYSICAL ACTIVITY** (21,77,78)**.**

Other studies have shown that dietary micronutrients like nitrites improve mitochondrial content and function and improve the function of type I fibers which are connected with exercise efficiency (50). Hence this evidence suggests that mitochondria play a role in intervening in exercise efficiency.

In the experimental test involving PA, voluntary wheel running exercise is the most widely used test to monitor the long-term and or short-term effects of exercise which can include weight control, reduced risk of cardiovascular diseases, T2DM, in rodent models. Because of their morphological, physiological, and genetic resemblance to humans, rats/mice have long been the favored species for biomedical research animal models. Rodents have a tiny size, are easy to maintain, have a short life cycle, and have a lot of genetic resources.

Singularly, PA can be good for metabolism as it improves glucose tolerance which is the ability of the body to dispose of excess glucose, and insulin sensitivity which refers to how sensitive the body's cells are in response to insulin because, in the course of PA, the body utilizes glycogen which is stored in the muscles and after PA, the muscles recover its lost glucose directly from the bloodstream (19,79). Studies have also shown that glucose utilization in the muscles during PA increases 7-20 times over the normal levels and this depends on the intensity of the activity performed (80). However. when the intensity of the activity is higher than normal PA, there will be a release of hormones that will counter insulin regulation, including glucagon and catecholamines which will cause a reduced effect on the actions of insulin which is to regulate blood glucose (24,80). On the other hand, studies have shown that PA like the running wheel activity does not affect the blood glucose in levels in mice that were about twenty-three months old, considered as very old mice (24,80).

The PA has different kinds of impacts on the body some of which are listed in figure 5. In recent animal studies, it has been shown that PA like the running wheel activity in early life in mice, increased the bone mass (21). However, the same study further explained that even though PA increased bone mass, PA could not prevent bone loss which is induced by the type of diet (HFD) in subjects exposed to PA (21). Also, studies showed that in as much as exposure to running wheel activity prevented increases in adiposity and increased energy intake, HFD-fed mice in this study showed a

reduced body weight (45). Although absolutely body mass number does not directly measure body fat. Therefore, it is possible to infer that PA is important to maintaining a good body and muscle structure (81). PA will affect a deranged fat metabolism which is induced by eating an HFD by improving lipolysis which is a metabolic pathway through which lipid triglycerides are hydrolyzed into glycerol and FA and serve as a source of energy during PA and during PA, the body requires energy and fatty acids are released from adipose cells and made available for use in a process known as fat mobilization (42,82), and FA oxidation in white adipose tissue (WAT) to use up FA instead of storing them (46,83). Other studies have shown that mice allowed running wheel access possess significantly reduced adipose tissue weights and increased food intake (this is as a result of constant running wheel activity as they utilize more energy), compared to controls fed an HFD but without PA (84).

PA may increase the bioavailability of nitric oxide, thereby lowering blood pressure (46), and increasing endothelial function (8), which has been shown to be the cardioprotective effects of PA (46,85).

During PA, exposure to ultraviolet rays (UVR) can cause the development of neuro-regulatory molecules (molecules like a corticotropin-releasing hormone, neuropeptides, β-endorphin, and corticosterone) which will impact the brain's ability to control homeostasis which is a self-regulating process that helps to maintain balance and stability while adapting to conditions that are best for its survival (84,86).

Exercise has different types of effects. Investigations have shown that a combination of resistance and endurance training can lead to gains in muscle strength and/or mass, in comparison to undergoing only endurance exercise (75,76,87). Resistance training, such as weightlifting, builds muscle strength by forcing the muscles to work against a weight or force, whereas endurance training, commonly known as aerobic exercise, includes activities like walking, running, and swimming that increase breathing and heart rate. The effects of resistance exercise, which involves loading of running wheel with weights to see if it can increase the hypertrophic potential of aging muscles have not been elucidated properly in rodents (21). However, some studies have shown that reduced body weight in trained rats for example may result from negative energy balance caused by an increased energy utilization during PA (88).

Further studies carried out on short-term voluntary running wheel for a duration of about ten weeks with the addition of resistance was enough to cause hindlimb muscle hypertrophy in the quadriceps muscle, gastrocnemius muscle, and by up to 52% in the soleus muscles of young (16 weeks) male C57BL/6J mice, and by 18% in the soleus of very old (27 months) sarcopenic mice (old) (21,67). In addition, the authors attribute that there is not so much knowledge about the effects of voluntary resistance exercise on aging skeletal muscles concerning their molecular responses either in males or females and if it poses some differences (67). This could be a novel case that should be studied.

Subjecting young and old mice, male and female C57BL/6 mice (between 15 to 23 months old) to long periods of resistant wheel exercise (RWE) prevented total body weight and abdominal fat mass gain (87). However, despite the prevention of sarcopenia in both sexes, the long periods of RWE caused cardiac hypertrophy, especially in old female mice (87).

In fact, a constant resistance wheel exercise from mid-life in mice models (15 months, before the onset of sarcopenia) is enough to prevent sarcopenia and maintain skeletal muscle mass into old age (81). The intensity of exercise can affect how enzymes respond to muscles (89). Phosphocreatine (PCr) recovery rate, an index of maximal oxidative adenosine triphosphate (ATP) synthesis is recovered in older individuals with decreased physical activity compared to young which did not show any effect (45,89). This means that the skeletal muscle energy which is lost in older individuals due to decreased PA is now being replaced by the energy produced from ATP from PCr.

Brown Adipose Tissue (BAT)

Brown adipose tissue (BAT) is a thermogenic organ that plays a role in energy expenditure and is a promising target in the fight against obesity and types 2 diabetes (90). The major substrates for BAT thermogenesis are free FA (FFA). In mice and humans, BAT activation boosts lipolysis of intracellular triacylglycerols (TAGs) and increases absorption of circulating FA and lipoproteins to fuel thermogenesis (91)(90). Mitochondrial proteins are shown to be elevated in BAT and are regulated physiologically and genetically (91). Some characteristics of BAT are shown in Figure 13 below.

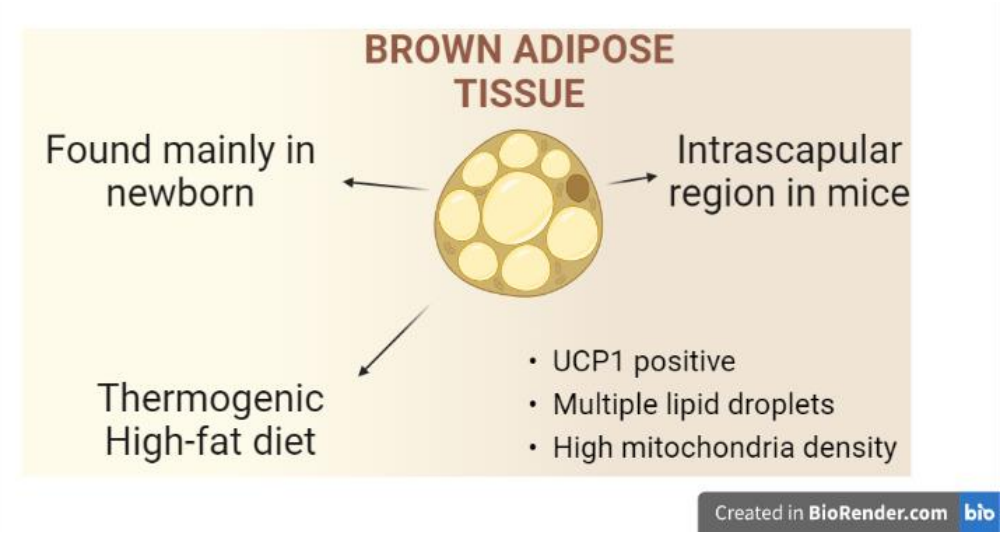

FIGURE 13. BROWN ADIPOSE TISSUE AND CHARACTERISTICS (92)**.**

UCP – Uncoupler protein 1

Several authors described that PA may promote metabolic health by improving the functional capacity of brown adipose tissue (BAT) as well as skeletal muscles by secretion of the lipokine 12,13-diHOME, due to the higher uptake of FA by the skeletal muscle, when PA is being carried out (90,93). The mechanism for the increase in FA uptake and oxidation in skeletal muscle and cells is not known, It could probably be that 12,13-diHOME activates signalling pathways that lead to translocation of fatty acid transporters (93). The effects of PA on relative aspects of BAT metabolism are not so certain, with rodent and human studies generally demonstrating reduced BAT function

(functions like glucose and lipid uptake, FA biosynthesis), and the effects of exercise on mitochondrial activity are not well described for BAT (90).

Skeletal Muscle

Skeletal muscles are muscles attached to the skeleton. It comprises thousands of cylindrical muscle fibers mostly held together by connective tissue, innervate and vascularized, and its contraction is mostly voluntary. Skeletal muscles have two ways of storing energy, glycogen, and triglycerides (TGs), which are stored in the cytoplasm(94). Glycogen can be converted into glucose swiftly when energy is required, mostly through glycolysis which is an anaerobic process, which produces 3 ATP and two lactic acid molecules (91). TGs are stored in lipid droplets and can be mobilized and oxidized by the catecholamines and mitochondria respectively (95).

Skeletal muscle is a major contributor to energy metabolism for the whole body, mostly due to its mass and it accounts for about 75% of the total body insulin-stimulated glucose and storage (23,47). As mentioned earlier, skeletal muscle is mainly responsible for insulin-stimulated glucose uptake it follows a process where insulin binds to its receptor and activates a pathway that will phosphorylate protein kinase B (AkT), which is a protein responsible for the activation of glycogen synthesis, protein synthesis, and GLUT4 translocation to the cell surface thus an increase in glucose transport (40,96). The stimulation of insulin is responsible for quite a number of tyrosine phosphorylation events, which are controlled by protein-tyrosine phosphatase (PTP), for example, the PTP1B which is expressed in all insulin-responsive tissues on the cytoplasm of the endoplasmic reticulum (95,97,98). Several reports have also shown that in cases where PTP activity is increased, insulin resistance develops through the inhibition of insulin-stimulated phosphorylation of the IR and IRS-1 (98). Metabolic complications like insulin resistance in obesity or diabetes affect glucose homeostasis in the body, FFA which are lipid species produced during lipolysis from adipose tissue and a variety of cell types can directly affect the inhibition of glucose transport as well as its phosphorylation in skeletal muscles (95,98). Dresner and colleagues were able to show that the inhibition of IRS-1 is associated with PI3K when FFA increased in circulation, also the tyrosine phosphorylation of IRS-1 was also impaired while Akt/PKB phosphorylation remained intact. Oppositely, dyslipidemia promoted by insulin resistance in muscles can occur by the stimulation of energy derived from ingested carbohydrates into hepatic de novo lipogenesis and increased VLDL production (98). An example is in mice, when the IR gene

is inactive, TGs increase in circulation, and adiposity increases as a consequence of specific insulin resistance (98).

MITOCHONDRIA BIOENERGETICS

Mitochondria have been described as the powerhouse of cells (99). They are organelles that possess a double membrane (the inner and outer mitochondria membrane) (99). As Figure 14 shows its surface characteristics in animal cells, the mitochondria have some major functions in cells that are important to understanding the pathophysiology of diseases, and some of these functions include providing energy for the cells in the form of ATP which occurs from the breaking down of carbohydrates via the Tricarboxylic acid (TCA) cycle and the oxidation of fats via beta-oxidation (100). Mitochondria are also involved in the generation and regulation of ROS, and they also mediate apoptosis through the mitochondrial permeability transition pores (101).

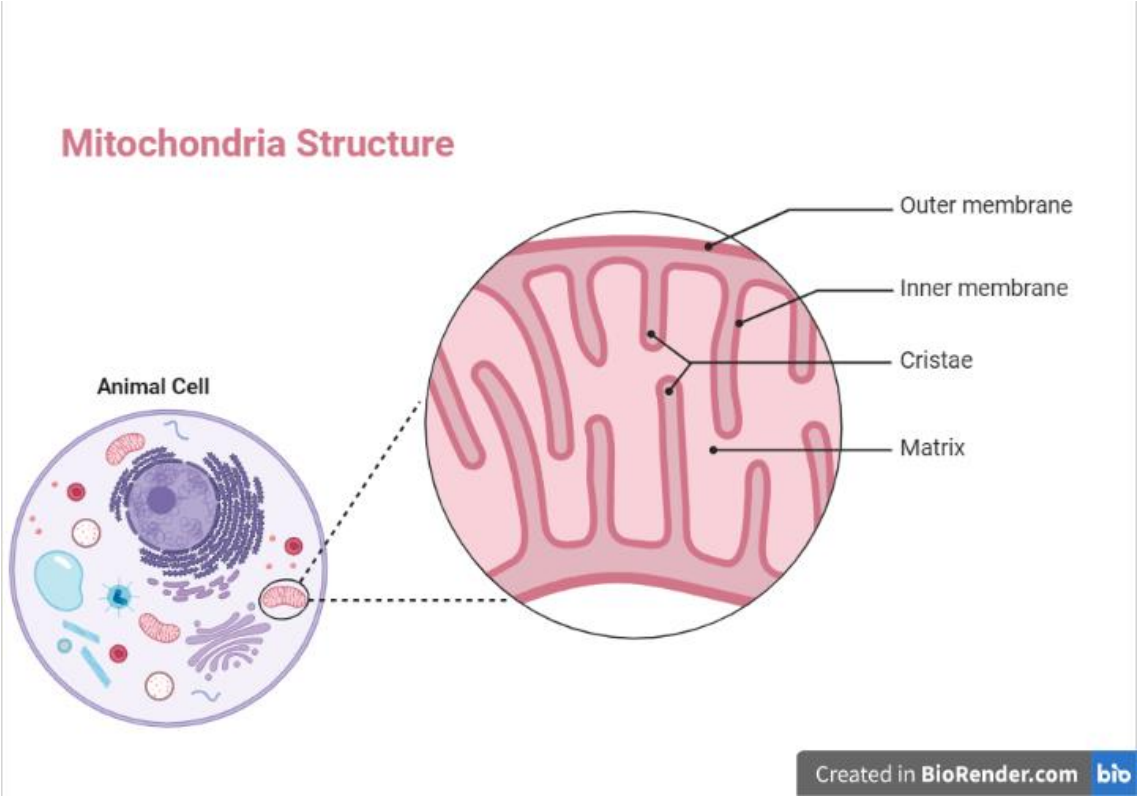

FIGURE 14. THE SURFACE STRUCTURE OF THE MITOCHONDRIA, THEIR LOCI IN THE ANIMAL CELL, AND THEIR SURFACE CHARACTERISTICS.

Mitochondria bioenergetics mainly describes a comparison between mitochondrial respiratory capacity and coupling control and this can be determined by

measuring the rate of respiration coupled to phosphorylation which is declining in adults(102). Bioenergetics is based on the availability of reducing equivalents (for example hydrogen), consumed as carbohydrates and fats, that react with oxygen to generate water via mitochondrial oxidative phosphorylation and mitochondrial respiration (103). It is a series of metabolic reactions and processes requiring oxygen, that takes place in mitochondria to convert the energy in macronutrients to ATP, which is the universal energy donor in the cell (46).

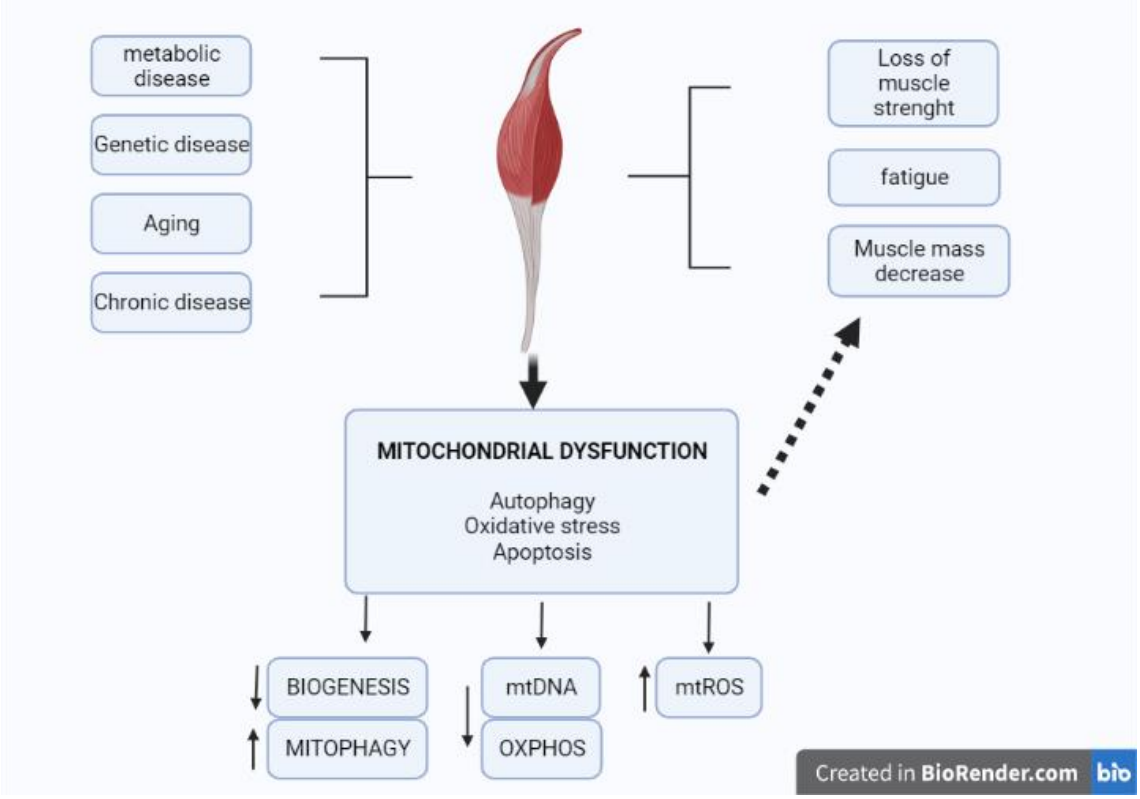

FIGURE 15**. EFFECTS OF PHYSICAL INACTIVITY ON MUSCLES AND MITOCHONDRIA FUNCTION** (50,104)**.**

MTDNA – Mitochondria deoxyribonucleic acid, MTROS – Mitochondria reactive oxygen species, OXPHOS – Oxidative phosphorylation

An important finding in recent investigations of mitochondrial energetics in skeletal muscles revealed that PA status, not chronological age, influences mitochondrial energetics (71). Physical inactivity has been shown to be associated with mitochondrial dysfunction. Mitochondrial dysfunction as shown in Figure 15 leads to a

decrease in skeletal muscle mass, muscle strength, and even rigidity as well as other comorbidities listed above (20).

Reports show that there is a reduction in mitochondrial oxidative capacity with aging (9,71) and that physically active young and old groups have been shown to possess similar protein markers of mitochondrial content and respiration (2). Of note, most of these studies do not characterize certain important aspects of the subject parameters which will most likely affect the relationship between mitochondria function and age, parameters like well-measured daily PA levels, and cardiorespiratory fitness (69,102). Researchers have also used the isolated mitochondria experimental method which served as some foundation for some of the assays of mitochondria function in aging (71). However, contradictions to studies mentioned above include experiments carried out with controlled subject phenotype that somehow failed to find age-related changes in mitochondrial respiration in permeabilized myofibers (104,105).

The mechanisms leading to age-induced muscle changes are not known, and mitochondrial dysfunction has been suggested to be a possible cause (106). Studies have shown that normal aging in mice is characterized by mitochondrial dysfunction which is characterized by a loss of maintenance of the electrical and chemical transmembrane potential of the inner mitochondrial membrane, modifications in the function of the electron transport chain (ETC), or a reduction in the transport of metabolites into mitochondria, and lower levels of mitochondrial density (107–109).

Mitochondrial Respiration

There are different techniques used in measuring mitochondria function. There is the ADP recycling methodology which measures respiration over the respiratory state (103). This method was made to keep recycling ADP for phosphorylation. The techniques involve the use of creatine plus creatine kinase, ATPase with excess ATP, and glucose plus hexokinase to clamp the ADP concentrations at a level determined by the amount of substrate added (103,110)

Also, there is the measurement of respiratory states three (3) and four (4). In this measurement, the rate of oxygen consumption in the absence of ADP or in the presence of oligomycin to block the synthesis of ATP defines the state 4 respiration and the

addition of ADP as the substrate for ATP synthesis determines state 3 respiration (103,110). There is the measurement of mitochondrial respiration in intact cells or tissues. This is where the use of the seahorse respirometer becomes relevant (103,110). It is also the same technique used in isolated mitochondria which involves the assessment of drops in oxygen content in the cell suspension (103,110). This method is hard to process as it depends on cell suspension because of either possible adherence to culture plates or the cell type is not supported by tissue matrix connections in vivo (103,110). These are some of the techniques involved in the measurement of mitochondria function.

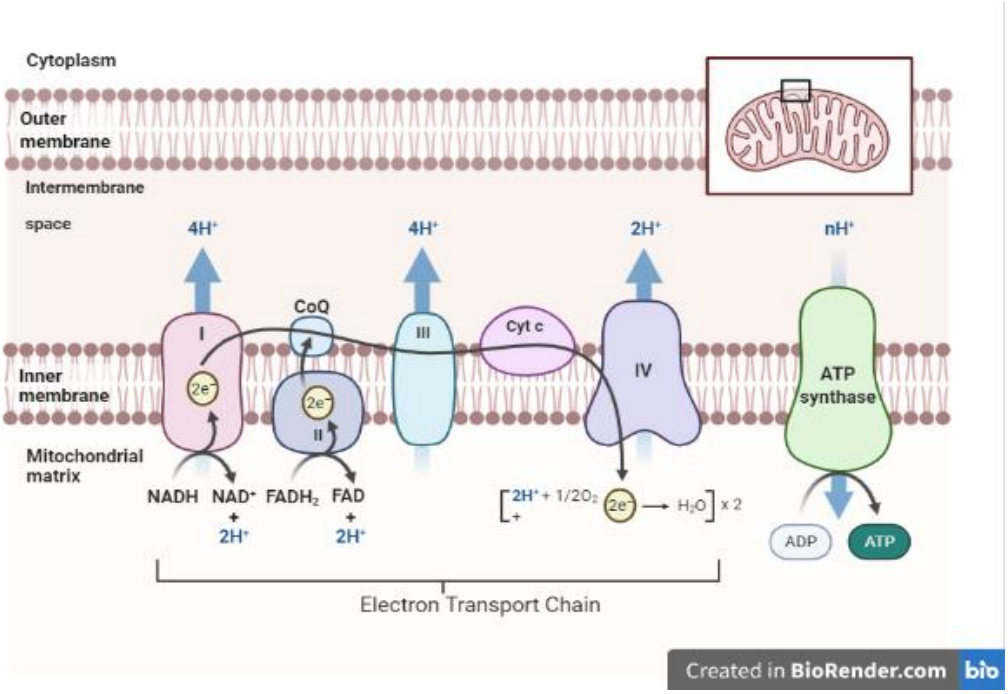

FIGURE 16. SIMPLIFIED REPRESENTATION OF THE ELECTRON TRANSPORT CHAIN LOCATED WITHIN THE INNER MITOCHONDRIAL MEMBRANE.

NADH – Nicotinamide adenine dinucleotide (NAD) + hydrogen (H), FADH – Flavin adenine dinucleotide + hydrogen, FAD – Flavin adenine dinucleotide, CYT C – Cytochrome C

Mitochondria generate energy by oxidizing hydrogen derived from our dietary carbohydrates via the TCA cycle and fats (β-oxidation) with oxygen to generate heat and adenosine triphosphate (ATP) (99,101). As shown in Figure 16, studies have shown the step-by-step transfer of electrons in the TCA cycle, two electrons are given off from

nicotinamide adenine dinucleotide (NAD)+hydrogen(H) (NADH) to the complex I and also from succinate to the complex II which is then passed on to ubiquinone (coenzyme Q) to give ubisemiquinone (CoQH). Thereafter electrons are passed on to ubiquinol (CoQH2) (99,111). Ubiquinol consequently transfers its electrons to complex III (ubiquinol cytochrome C oxidoreductase) which transfers these electrons to cytochrome c (111). Electrons move to complex IV (cytochrome c oxidase) from cytochrome c and finally to oxygen which is then converted to water (H_2O) (111,112). It is a sequential flow of electrons. These are the ETC complexes and they have multiple electron carriers (111). These complexes I, II, and III has different iron-sulfur (Fe-S) centers, but the complexes III and IV mostly use the cytochromes (b + c1 and a + a3 cytochromes) for electron transfer (111,112). The mitochondrial TCA cycle enzyme aconitase is also an iron-sulfur center protein (50,111,112).

MITOCHONDRIA AND MUSCLE TISSUE

Skeletal muscle is a tissue richly endowed with mitochondria and skeletal muscles are highly dependent on OXPHOS for the production of energy. It has been observed in human studies that have been carried out, that respiratory capacity in mitochondria is lower in older adults (102). This means that the ability of mitochondria in skeletal muscles to produce electrochemical potential and to couple this potential to adenosine diphosphate (ADP) phosphorylation is decreased as aging progress (102). Authors also showed that in their analysis of mitochondrial respiration between skeletal muscles of aged and young persons, there was a relationship between mitochondrial respiratory capacity and coupling control, this supports the notion that as respiratory ability decreases, coupling control is also lost (4,102). Several signaling pathways in skeletal muscle serve to transfer changes in PA or other extracellular signals to mitochondrial biogenesis. As shown in Figure 17, most of these pathways directly activate peroxisome-proliferator activated receptor γ (PPARγ) and coactivator-1α (PGC-1α). PGC-1α relates directly with its effector receptors and PPAR to control genes that partake in all processes of mitochondrial energy metabolism. For example, in muscles, the PGC-1 and ERR-induced regulator in muscle 1 (PERM1) is activated by exercise and for the highest activity of PGC-1α in muscle, this is a requirement. Its precise function is

exactly known, PERM1 expression in skeletal muscles also increases PGC-1α expression, mitochondrial content, and respiratory capacity.

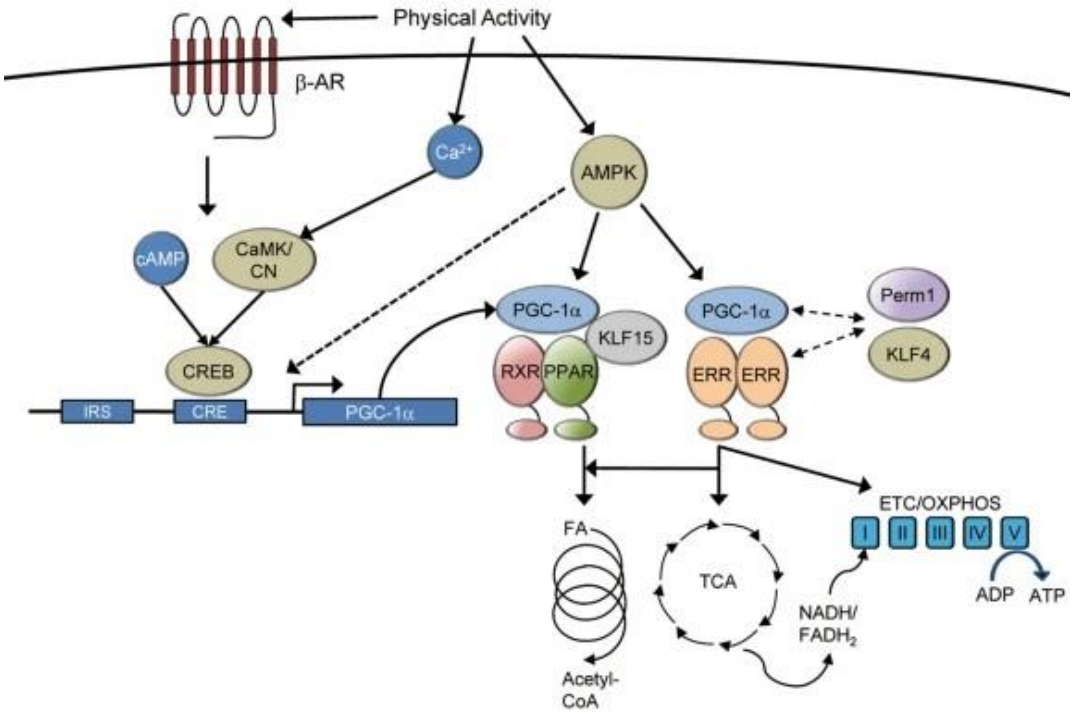

FIGURE 17. SIGNALING PATHWAYS WITH MITOCHONDRIAL BIOGENESIS IN THE MUSCLE. ADAPTED FROM (113).

AMPK: AMP-activated protein kinase; PPAR: Peroxisome proliferator-activated receptor; RXR PGC-1a: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; KLF15: Krüppel-like factor 15; ERR: Perm1 TCA: Tricarboxylic acid cycle; NADH: nicotinamide adenine dinucleotide (NAD) + hydrogen (H); FADH: Flavin adenine dinucleotide; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CREM: cyclic adenosine monophosphate-response element modulator + 1; Camp: Cyclic adenosine monophosphate; IRS: Insulin receptor substrate; Ca2+: Calcium ion.

Mitochondria are distributed differently within skeletal muscle fibers. Located closer to the cell surface are the subsarcolemmal mitochondria (SSM) and the intermyofibrillar mitochondria (IMF-M) which are located near the Z-line of muscle fibers (64). IMF-M has been observed to supply ATP to contracting myofibrils and SSM

majorly supplies energy for membrane-related cell signaling, transport of substrate, and exchange of ions, in support of surrounding nuclei (64). The skeletal muscle mitochondria comprise SSM (one-third) (64). Studies have shown that in addition to a reduction in ETC activity in muscles in T2DM and obesity, there is also an unreasonable decrease in ETC activity within the SSM fraction of skeletal muscle mitochondria (100). The SSM population of the mitochondria has been shown to be the most responsive to PA in rat muscle as well as human skeletal muscle (64).

Understanding the role of mitochondria in the health of skeletal muscles has been seen to be pivotally positive with physical activities, in as much as PA has other effects on the body, both positive and sometimes negative depending on the intensity. The importance of mitochondrial function to metabolic fitness and muscle well-being needs to be elucidated as this will be a major and less cost-effective way to lead a healthy lifestyle.

8. AIM AND OBJECTIVES

The western diet, which is a type of diet that contains quite high amounts of processed foods, high-fat dairy products, high-sugar products, high processed red meat content, and the prepacked meals, which are the foremost cause of deleterious effects on metabolism, leading to obesity, T2DM, several micro, and macrovascular complications, especially when associated to a sedentary lifestyle, that include reduced to non-physical activity. Recent studies (21,71,102) have shown the benefits of early PA to mitigate the metabolic effects of western diet consumption. However, the knowledge of the underlying mechanisms is scarce.

In the current study, we aimed to evaluate the effect of early *versus* later PA response on mitochondrial function/fitness, in the presence or absence of 8 weeks of high fat diet.

The specific aim of this study was:

- To evaluate the impact of earlier or later in life physical activity on mitochondrial function in the soleus muscle and BAT from mice that were exposed or not to high fat diet.

The study was designed to mimic the effect of both a sedentary lifestyle and the western diet, as well as the contra-effect of two physical lifestyle approaches to show the differences and comparisons across the various types of lifestyles, as shown in study protocol in Figure 19/20.

9. MATERIALS AND METHODS

Animal model and animal housing

Male C57BL/J6 mice acquired at 3 weeks of age from Jackson labs (city, State, USA.) were used in this study. All animals were maintained at normal room temperature (22-24 C), with constant humidity and lights from 6 am to 6 pm (12 hours light/12 hours dark cycle) with access to water and rodent chow diet ad libitum. After one week of acclimatization, animals were divided into two groups (control and PA). The mice in the first group (control group, n= 84 animals) were housed in polycarbonate cages, 5 mice per cage, and the other group (PA group, n= 84 animals). In the PA group, the mice were housed in individual cages designed with a monitoring device for the running wheels. The mice had free access to the running wheel.

Ethical approval

All experimental protocols were approved by the Association for Assessment and Accreditation of Laboratory Animal Care, the animal facility at the Arkansas Children's Research Institute, and experimental procedures were conducted according to the guidelines of the National and European Communities Council Directive (86/609/EEC).

Study design

Mice were obtained after winning and were exposed to 1 week of acclimatization before the start of the study. Mice were about 4 weeks of age when experiments were started and were 5 months old when the experiment was completed. Then, both groups (grp) received a control diet, but the control group (n=84) will have no PA, but the PA group (n=84) had access to the running wheels. Figure 18/19 shows the regimen implemented in this experiment.

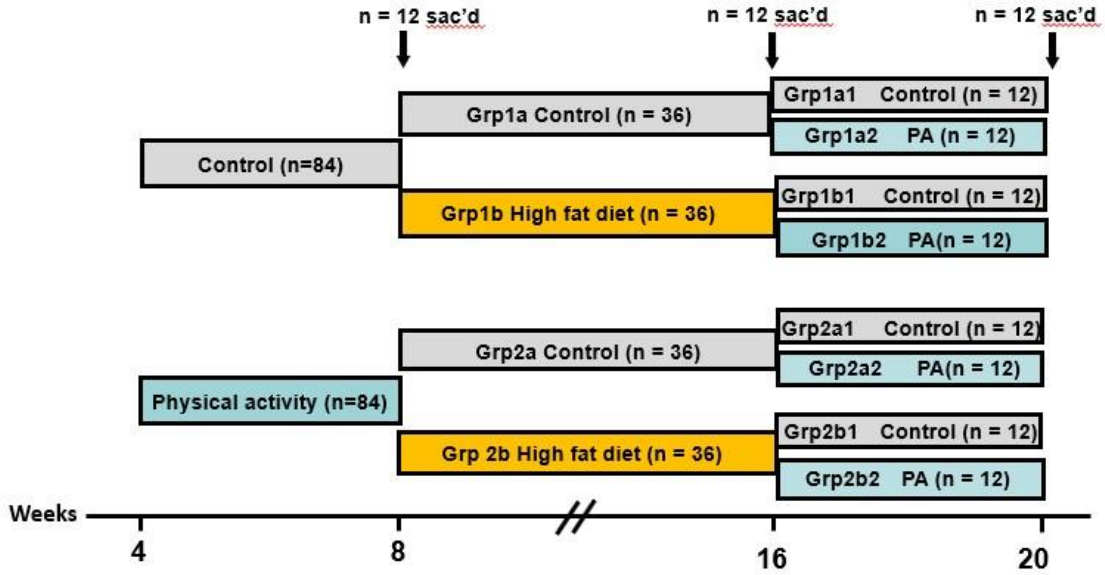

FIGURE 18. STUDY DESIGN AND GROUPS AND TIMEPOINTS FOR SACRIFICE, PA AND NON-PA AS WELL AS GROUPS HAVING A HIGH-FAT DIET AND CONTROL DIET.

PA: Physical activity; Grp: group; Sac'd: Sacrificed

1st intervention and study period: This study period lasted for a total of 4 weeks. As shown in Figure 18, during this period, mice in PA group were subjected to running wheel exercises. At the end of the 8th week, 12 mice, from both groups, were randomly selected and sacrificed using Ketamine 85mg/kg and xylazine 11mg/kg intraperitoneally and was confirmed by cervical dislocation. Sacrificing at 1st-time point (week 8) enabled us to evaluate the effects of PA and no PA across both groups in the mitochondria of soleus muscles and BAT. The first animals that were studied during this time point were about 2 months of age.

2nd intervention and study period: After the first period of PA for 4 weeks, the remaining animals of each group were further divided into 4 subgroups. The control group was divided into a group with HFD (Grp1a) (n=36) and a group with only control diet (Grp1b) (n=36). The PA group was subdivided into a group with HFD (Grp2a) (n=36) and a group on control diet (Grp2b) (n=36). The introduction of HFD into the study was to enable evaluation of the mitochondria response after PA or no PA exposure with respect to an HFD. After 8 weeks on HFD, 12 mice were selected at random from each group and sacrificed in the 16th week, in order to evaluate the effect of HFD and or PA on

mitochondrial function in soleus muscle and BAT. The animals that were studied at this time point were 4 months of age.

3rd intervention and study period: Thereafter, the remaining animals after sacrifice from the subgroups described above in Figure 18, were divided into further subgroups. Grp1a was divided into control diet and no PA (Grp1a1) (n=12) and control diet with PA (Grp1a2) (n=12). Grp1b was divided into control diet and no PA (Grp1b1) (n=12) and control diet with PA (Grp1b2) (n=12). Accordingly, Grp2a was divided into a control diet and no PA (Grp2a1) (n=12) and a control diet with PA (Grp2a2) (n=12). Grp2b was divided into control diet and no PA (Grp2b1) (n=12) and control diet with PA (Grp2b2) (n=12). Later in life, PA enabled us to investigate the mitochondrial response to effects, early life PA in the presence or absence of HFD, compared to non-PA groups. At the end of 4 weeks, all mice were sacrificed as described in the study design. The last animals to be studied here were 5 months of age.

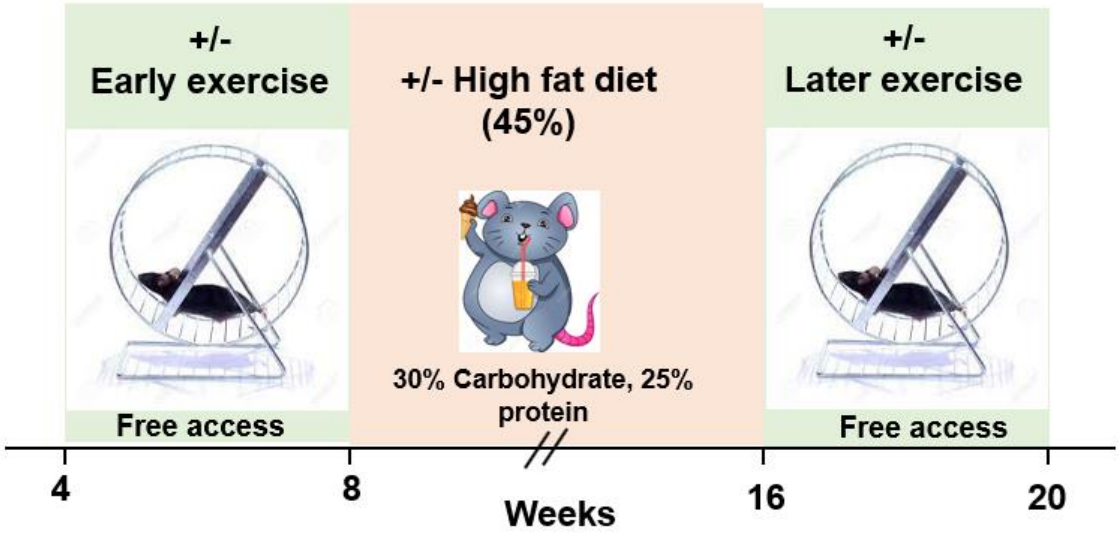

Figure 19. SHOWING THE TIMELINE OF PHYSICAL ACTIVITY AND HIGH-FAT DIET TREATMENT.

Food Intake

The diet used in this study was the normal rat chow, composed of 10% fat (Harlan diet number TD 06416). The HFD used in this study consisted of 45% fat (Harlan diet number TD 08811) 25% protein, 45% fat corn oil, and 30% carbohydrates and food intake was measured for a 48-hour period at the end of every timepoint (per cage) Mice had ad libitum access to food and water at each stage of the experiment.

Body Weight

The body weight (BW) of each group of mice was taken from the experimental start point at 4 weeks of age to the end at 20 weeks of age, using an analytical balance (KERN CB 6 K1, Germany).

Reagents

Insulin as well as Ketamine and xylazine were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethanol was purchased from MERCK (Kenilworth, NJ, USA). All other reagents used in the preparation of MiR05 and BIOPs relaxing medium, Saponin, Pyruvate, Malate, Glutamate, Adenosine Diphosphate (ADP), Succinate, Oligomycin, Carbonyl Cyanide 4-(trifluoromethoxy) Phenylhydrazone, Antimycin A., Octanolycarnitine were purchased from Sigma (St. Louis, MO, USA).

Mitochondria respiration;

Oxygraph-2k respirometer calibration

As recommended by the manufacturers of the high-resolution Oxygraph-2k respirometer instrument (Figure 20), Oroboros Instruments (Innsbruck, Austria) (114), the air calibrations were carried out before each experiment. The calibrations were

performed, at an atmospheric oxygen concentration in MiRO5 buffer solution (sucrose 110 mM; potassium lactobionate 60 mM; EGTA 0.5 mM; $MgCl_2.6H_2O$ 3 mM; taurine 20 mM; KH_2PO_4 10 mM; HEPES, 20 mM; BSA 1 g/L; pH 7.1, at 37C), under controlled temperature (37 C) and under magnetic stirring (750rpm).

FIGURE 20**. REPRESENTS AN OXYGRAPH 2K RESPIROMETRY. ADAPTED FROM** (115)**.**

Sample preparation

After tissue collection, both muscle and BAT were immediately immersed in an ice-cold relaxing solution BIOPS, (Ca^{2+}/EGTA buffer 10 mM; imidazole 20 mM; K^+/4-morpholinoethanesulfonic acid 50 mM; dithiothreitol 0.5 mM; $MgCl_2$ 6.56 mM; ATP 5.77 mM; phosphocreatine 15 mM; pH 7.1).

Muscle fibers were prepared for conducting respiration analysis by mechanically separating muscle fibers using forceps and scalpel while still in the BIOPS solution as shown in Figure 21. This is done under the view of a light microscope to avoid total shredding of the muscle tissue (112). Approximately 10mg of muscle fiber bundles were permeabilized outside the O2k chambers according to the protocol (112). Briefly, muscle fiber bundles were immediately placed into ice-cold BIOPS solution (2ml) after

separation. Thereafter, the muscle fiber bundles were permeabilized with a BIOPS/saponin solution (50µg /mL of saponin in 2mL of BIOPS and placed on a shaker (VWG analog platform shaker) for 30 minutes and then transferred to MIR05 (2ml, for 20 minutes) to wash away the saponin from the muscle fiber bundles (112).

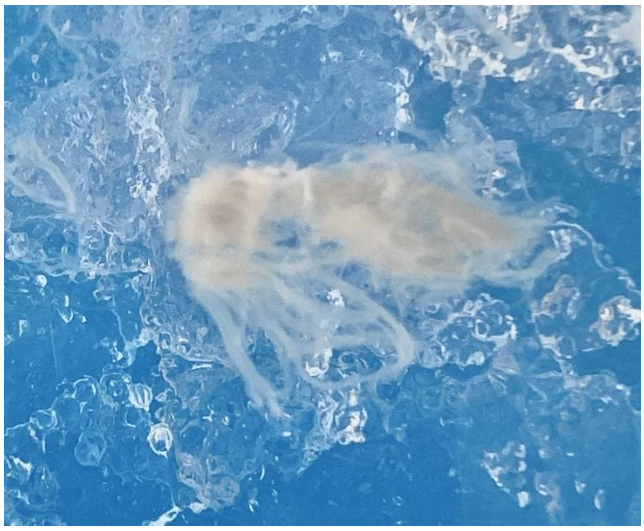

FIGURE 21. SEPARATED MUSCLE FIBERS IN BIOPS ON ICE.

The muscle fibers were then weighed to approximately 2.2mg using high-sensitivity scales (Satorius analytical balance, Model S9001, weighing capacity 220 g, precision: 0.1 mg, AC/DC input 230 V AC. After removing excess liquid by dabbing the muscle fiber using fine forceps on filter paper (112).

Brown adipose tissue may be recognized in the intrascapular region of C57BL/6 mice as an adipose mass. This area may be turned upside down after excision to expose two brown patches. HFD, for example, stimulates fat growth in brown fat patches, making the distinction between white and brown fat masses more difficult to make. Brown fat tissue explants were isolated for respiration tests, providing two 20 to 30 mg brown adipose pieces (112).

To collect enough samples for the two chambers, more than 6 mg of cleaned BAT sample was cut with a sharp pair of scissors and distributed for homogenization. After homogenization, 20 µl of homogenate containing 2mg of tissue was placed in the chamber with digitonin (1µl) for tissue permeabilization, for respirometry analysis (112).

High-Resolution Respirometry

Mitochondrial respiration was measured either in muscle fibers or in BAT into the Oxygraph-2k respirometer chamber containing 2ml of Mir05, monitored under a controlled temperature of 37 C, and under stirring (750rpm). Oxygenation of the chambers was maintained by the direct syringe injection of oxygen which is maintained between 150 and 350nmol/ml by reinjection of oxygen if necessary.

There are different types of substrate-uncoupler-inhibitor titration (SUIT) protocols that can be combined in a variety of experimental conditions designed to evaluate mitochondrial function in the coupling control states of LEAK, OXPHOS, and electron transfer (ET) capacity (116). In this work, a specific SUIT protocol was used to evaluate the effect of fatty acid oxidation and NADH-linked substrates on complex I activity.

Permeabilized muscle fiber bundles were added to the chambers and baseline respiration was recorded before the subsequent addition of substrates. For this protocol, the substrate-uncoupler-inhibitor titrations were added in the following order: Pyruvate and Octanoylcarnitine – OCT/P Complex I; Fatty Acid Oxidation, Malate and Glutamate – MG, Complex I-linked substrates; ADP Complex I linked substrate; Succinate – Complex II-linked substrate; Oligomycin – Complex V, ATPase, inhibitor; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone – uncoupler and Antimycin A – Complex III inhibitor, as described on table 1.

TABLE 1 LIST OF SUBSTRATES, UNCOUPLERS, AND INHIBITORS USED FOR HIGH-RESOLUTION RESPIROMETRY AND THEIR RESPECTIVE RESPIRATORY STATES AND CONCENTRATIONS FOR MUSCLE.

Substrate/Inhibitor	Stock concentration	Volume added (ul)	Final Concentration	Pathway control and respiratory states
Octanoyl-carnitine	0.1M	30	1.5mM	Complex I LEAK Fatty acid oxidation
Pyruvate	2M	5	5mM	
Malate	0.8M	5	2mM	Complex I LEAK state (CI L)
Glutamate	2M	10	10mM	

ADP	0.5M	20	5mM	Complex I OXPHOS (CI OX)
Succinate	1M	20	10mM	CII OXPHOS; Total (CI +CII)
Oligomycin	4mg/ml	1	2.5μg/ml	CII LEAK, CV INH
Antimycin A	5mM	5	12.5μM	CIII Inhibition Residual respiration (ROX)

ADP: Adenosine diphosphate; CI: Complex 1; CII: Complex II; CIII: Complex III; CV: Complex V; OXPHOS: Oxidative phosphorylation; CI OX: Complex I oxidative phosphorylation; CI L: Complex I leak respiration; CII LEAK: Complex II leak; CII OXPHOS: Complex II oxidative phosphorylation; ETS: Electron transport state.

For BAT, baseline respiration was recorded after the tissue homogenates were added to the chambers. BAT tissue was permeabilized in the chamber by the addition of digitonin 1 μl, followed by subsequent addition of substrates. The SUIT protocol adapted followed this sequence: Malate and Glutamate – MG, Complex I-linked substrates; GDP – guanine diphosphate was added to inhibit UCP1; ADP – adenosine diphosphate; Succinate – Complex II-linked substrate; Oligomycin – Complex V, ATPase, inhibitor; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone – uncoupler and Antimycin A – Complex III inhibitor.

TABLE 2 LIST OF SUBSTRATES, UNCOUPLERS, AND INHIBITORS USED FOR HIGH-RESOLUTION RESPIROMETRY AND THEIR RESPECTIVE RESPIRATORY STATES AND CONCENTRATIONS FOR BAT.

Substrate/Inhibitor	**Stock concentration**	**Volume added (ul)**	**Final Concentration**	**Pathway control and respiratory states**
Digitonin	8,1mM	1	8,1μM	Tissue permeabilization
Malate	0.8M	5	2mM	

Glutamate	2M	10	10mM	Complex I LEAK state, (CI L)
GDP	0.2M		1mM	UCP1 Inhibitor
ADP	0.1M	20	7.5μM	Complex I OXPHOS (CI OX)
Succinate	1M	20	10mM	Complex II; total CI+CII OXPHOS
Oligomycin	4mg/ml	1	2.5μg/ml	CII LEAK, CV INH
FCCP	1mM		0.5μM	Complex I maximum uncoupled respiratory capacity (ETS)
Antimycin A	5mM	5	12.5μM	CIII Inhibition Residual respiration (ROX)

GDP: Guanine diphosphate; ADP: Adenosine diphosphate; FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; UCP1: Uncoupler protein 1; CI: Complex 1; CII: Complex II; CIII: Complex III; CV: Complex V; OXPHOS: Oxidative phosphorylation; CI OX: Complex I oxidative phosphorylation; CI L: Complex I leak respiration.

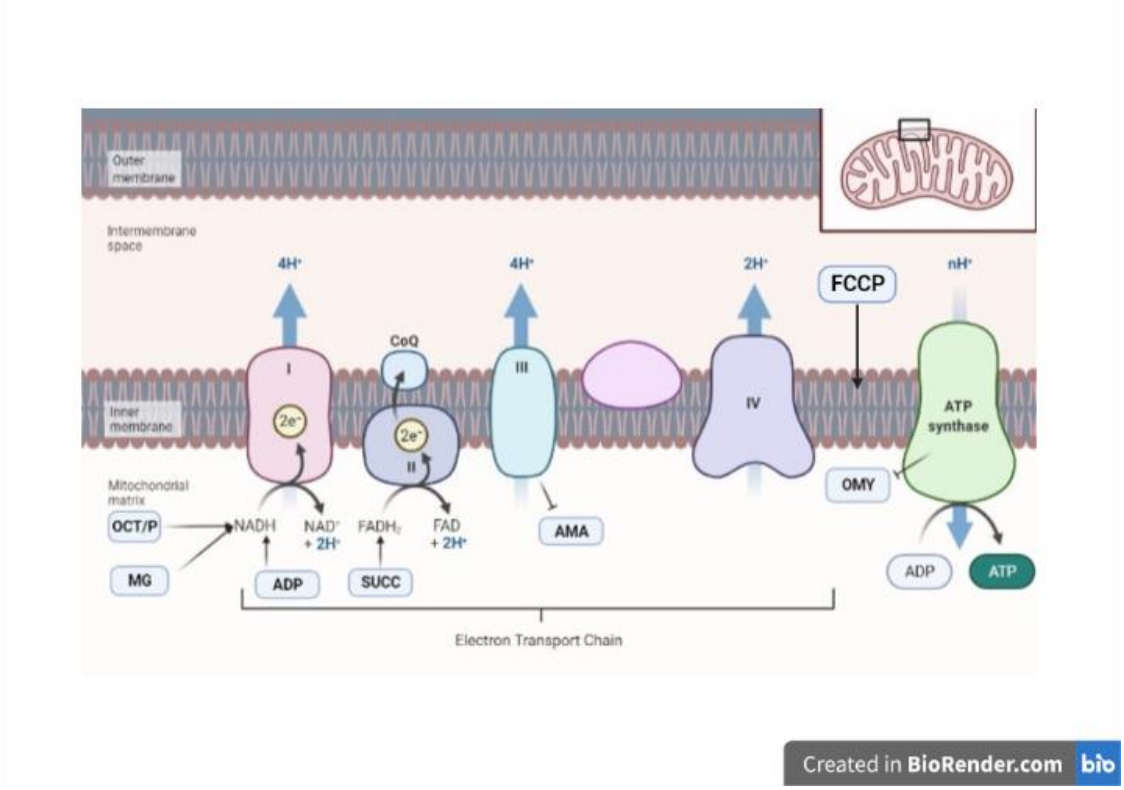

FIGURE 22 MITOCHONDRIA COMPLEXES SHOWING SUBSTRATES AND INHIBITORS AND UNCOUPLER (117)**.**

OCT/P: Octanoylcarnitine/pyruvate; MG: malate/glutamate; ADP: Adenosine diphosphate; SUCC: Succinate; AMA: Antimycin A; OMY: Oligomycin; ATP: Adenosine triphosphate; FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; NADH: Nicotinamide adenine dinucleotide+hydrogen; NAD: Nicotinamide adenine dinucleotide; FADH: flavin adenine dinucleotide+hydrogen; FAD: flavin adenine dinucleotide; CoQ: Coenzyme Q

For oxygen flux measurements, a steady-state rate of respiration was obtained after the addition of each substrate, inhibitor, or uncoupler to the chamber (DatLab version 6; Oroboros Instruments, Innsbruck, Austria). A representation of complexes where substrates, inhibitors or uncoupler affects, is shown in Figure 22. Oxygen consumption was represented as the first by-product of the oxygen concentration (nmol/ml) per time in the respiration chambers and it is known as oxygen flux [pmol/(s*mg)], corrected for pre-determined wet weight of muscle tissue mass in the chamber, according to the literature (46,115).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 9). To the normal distribution of the data, Shapiro-Wilk's normality tests were performed. Levene's test for homogeneity of variance was carried out thereafter. For the analysis of 2 independent groups, normally distributed data were expressed as mean ± standard deviation (SD) and an unpaired t-test was performed, otherwise, the Mann-Whitney non-parametric test was used, and the corresponding data were presented as median (25 percentile – 75 percentile). For comparisons between more than 2 groups, a One-Way analysis of variance (1-Way ANOVA), was used every time the assumptions were fulfilled, otherwise, Kruskal-Wallis non-parametric test was used followed by a post hoc Dunn's multiple comparison test. Data were presented as described above, for parametric and non-parametric tests. Statistical differences were considered significant when the p-value was less than 0.05 ($p < 0,05$).

10. RESULTS

Body Composition

Weekly body weights and daily food consumption were recorded throughout this experiment, as this enables us to evaluate the effects of control and western diet in the form of HFD on mice in the respective groups. Figure 23 below shows us a representation of weight distribution across the groups, from week 4 to week 20 at the end of the experimental period, from when animals were 1 month of age until the animals reached 5 months of age. We can see the differences between the control group and PA group as 24±06 and 24.1±0.4 respectively. The second groups showed weight differences of 28.7±0.6, 28.2±0.3, 36.0±1.0, and 37.0±0.7 for no PA/con, PA/con, no PA/HF, and PA/HF groups respectively.

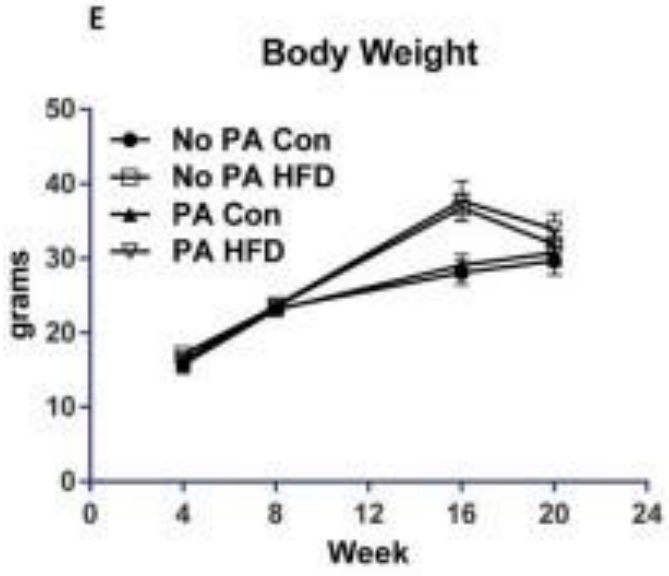

FIGURE 23. AVERAGE BODY WEIGHTS OF EACH GROUP OF MICE FROM THE EXPERIMENTAL START POINT AT 4 WEEKS OF AGE TO THE END AT 20 WEEKS OF AGE. ADAPTED FROM (21)**.**

Exercise Duration

Male C57BL/6 (n=84) mice at 4 weeks of age were housed in individual cages with free access to a wheel-running device (PA) and standard chow for 4 weeks. These mice ran an average of 9.2 km/day and 3.5 h/day. Another group (n=84) were

considered untrained controls (no PA) and were maintained in cages lacking a running wheel. The average time and distance ran are represented in Figures 24 and 25 below.

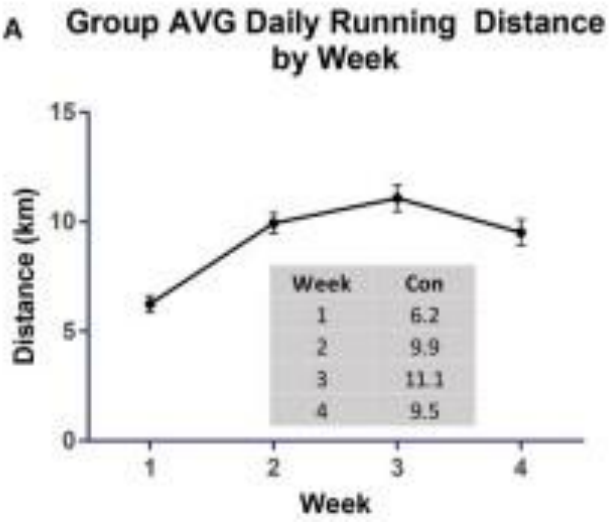

FIGURE 24. AVERAGE RUNNING DISTANCE BY MICE ASSIGNED TO PHYSICAL ACTIVITY. ADAPTED FROM (21)**.**

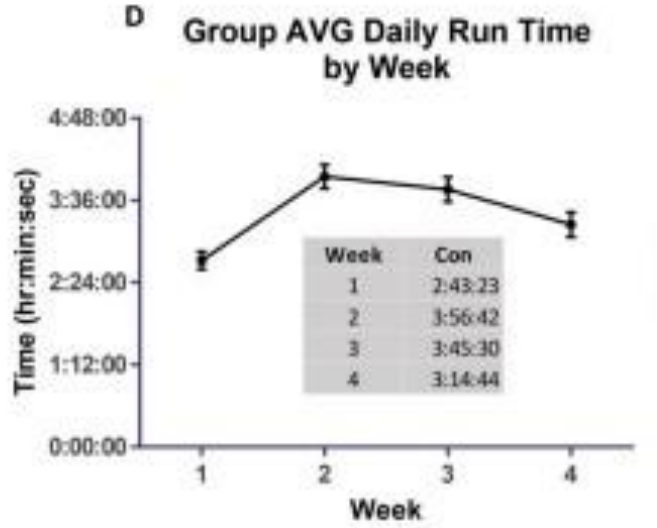

FIGURE 25. AVERAGE RUNNING TIME FOR MICE ASSIGNED TO PHYSICAL ACTIVITY. ADAPTED FROM (21)**.**

Mitochondria Respiration in Soleus Muscle

Mitochondrial respirometry analysis is important as mitochondrial oxidative activity is a critical metric for determining metabolism in health and illness and for this study, will be considering the outcome of respirometry analysis in the soleus muscle and in BAT. The soleus is a muscle prone to the development of insulin resistance due to the high mitochondrial concentration (118). For this reason, the soleus muscle was chosen for this study. The BAT was also selected for this study not just because of its high mitochondrial content, but also because of its ability to uncouple oxidation and phosphorylation of ADP, thereby reducing the production of ATP (90). Moreover, both tissues are normally directly affected by age, obesity, and PA. Figure 26 shows a representation of the respirometry protocol used for the analysis of the muscle tissues.

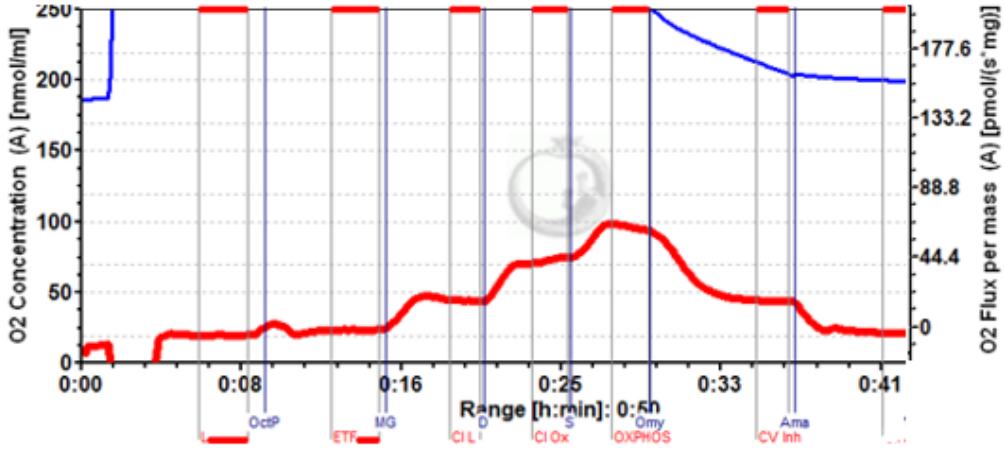

FIGURE 26 SCHEMATIC REPRESENTATION OF THE PROTOCOL USED IN THIS STUDY AND REPRESENTATIVE TRACE OF MITOCHONDRIAL RESPIRATION IN SKELETAL MUSCLE (SOLEUS)

On the left y-axis, oxygen concentration is measured in NMOL/ML and on the right y-axis, oxygen flux per mass of the mitochondrial respiratory chain is measured in PMOL/S*MG. OCT/P: Octanoylcarnitine/pyruvate; MG: Malate and glutamate; ADP: Adenosine diphosphate; SUCC: Succinate; OMY: Oligomycin; AA: Antimycin A; L: leak respiration; ETF: electron transfer flux; CIL: complex 1 leak; CI OX: complex 1 oxidative phosphorylation; OXPHOS: oxidative phosphorylation; CV inh: complex 5 inhibition.

After the first experimental period of 4 weeks the animals exposed to physical activity and the respective controls, 11 no PA and 12 PA animals were sacrificed in order to evaluate the influence of PA on the mitochondrial respiration levels for Complex 1 (CI), Complex 2 (CII), and the inhibition of mitochondrial Complex 5 (CV), on both soleus muscle fibres and BAT.

The preliminary data showed that mitochondrial respiration in the soleus muscle showed no significant difference statistically when compared between both groups, upon the addition of ADP and Complex I activation, no PA=58.31±27.23 *versus* PA=55.43±34.84; p=0.8265, as shown in Figure 27.

There was an additional substantial rise in OXPHOS when complex II was activated by succinate addition, in both no PA and PA CII respiration. As shown in Figure 27, there was an additional substantial rise by about 25pmol/s*mg in OXPHOS, when complex II was activated by the addition of succinate. However, no differences were observed in the CII respiration between the two groups, no PA=67.38±30.10 *versus* PA=68.13±41.67; p=0.9608.

Upon the addition of oligomycin, to block ATPase activity, there was a substantial decrease in respiration in both groups. However, no statistical differences between the PA and no PA groups were observed, No-PA=15.01±7.209 *versus* PA=14.20±10.46; p=0.8307, as shown in Figure 27.

As depicted in Figure 27, there was a substantial decrease in O2 flux levels by over 100 pmol/s*mg when complex V was inhibited by the addition of oligomycin across both groups.

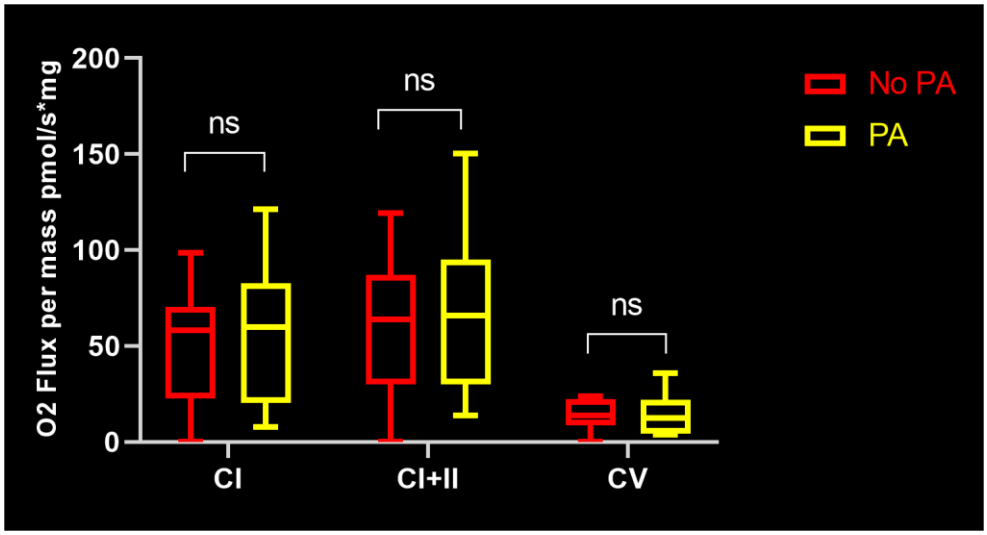

FIGURE 27. MITOCHONDRIAL RESPIRATION IN THE SOLEUS MUSCLE AFTER 4 WEEKS OF EXPOSURE TO PHYSICAL ACTIVITY

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test was applied and P<0.05 was considered significant. PA - physical activity, no PA - no physical activity, CI – complex 1, CII -complex 2, CV – complex 5

During the second experimental period that took place over two months, there was the inclusion of a western diet, for groups that had both physical activities and no physical activities, as observed in Figure 18/19. The two groups of animals from the first experimental period were now divided into four groups. Two of these groups were exposed to HFDt for 8 weeks, while the other remaining two groups were exposed to normal chow diet. Here the objective was to determine if PA from the first experimental period had any effect on mitochondrial respiration after exposing the animals to a HDF and compared to those that had no PA exposure, or no HFD exposure, as shown in Figure 18/19.

Upon the addition of ADP across the four groups, there was no statistical difference presenting a value p=0.0712. This is shown in Figure 28.

But there were statistical differences after CI energization between the PA/HF and PA/con groups: PA/HF= 111.7±37.12 *versus* PA/con= 67.45±50; p=0.0223 but no statistical difference between the no PA group: no PA/HF=99.64±29.10 *versus* no

PA/con=100.9±47.27; P=0.9998. Interestingly, the PA/con had a decrease by 45% on mitochondrial respirometry flux when compared with all the other three groups.

Also in Figure 28, upon the addition of succinate, the substrate for complex II respiration in the mitochondria, there was no statistical difference (p=0.1409) across the groups: PA/HF= 123.7±37.58 *versus* PA/con= 73±55.13; p=0.1418 or when the no PA activity groups were compared: no PA/HF=117.2± 31.02 *versus* no PA/con=114.5±39.39; p=0.9175. However, in the following CI results, the no-PA/con also presents a higher flux level of about 210 pmol/s*mg on mitochondrial respirometry flux when compared with all the other three groups.

Thereafter, the effect of Oligomycin and consequent complex V (CV) inhibition was observed. No statistical differences were observed when the PA experimental groups were compared: PA/con= 45.40±26.02 *versus* PA/HF=47.85±18.59; p=0.3263. or even when the no PA groups were compared: no PA/HF= 44.13±16.37 *versus* no PA/con= 29±23.25; p=0.9932. There was a significant reduction in flux levels as shown in Figure 28, across all groups, after the addition of oligomycin, flux levels came down to 75pmol/s*mg and below.

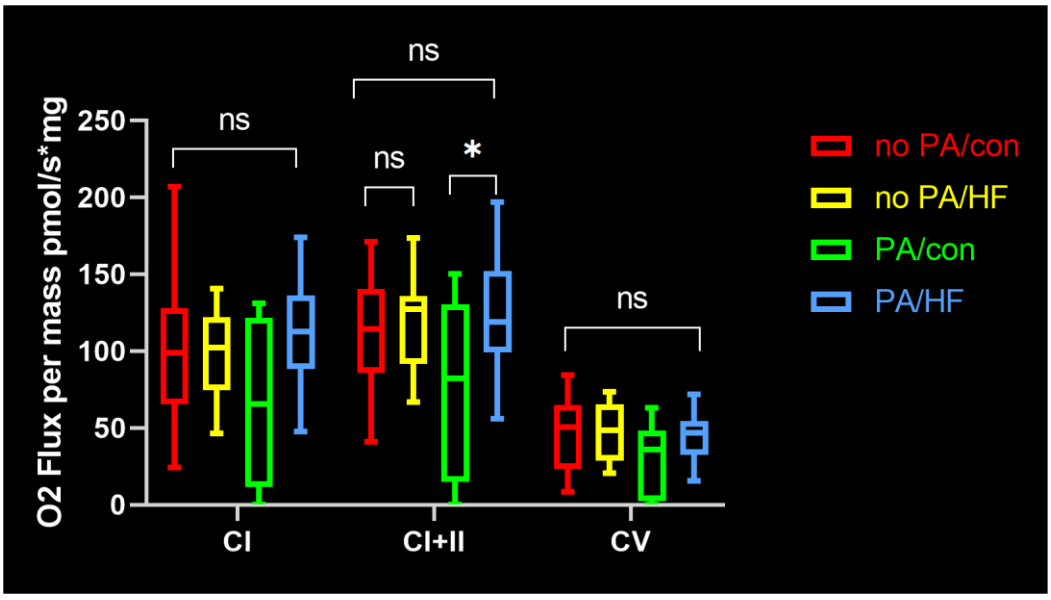

FIGURE 28. MITOCHONDRIAL RESPIRATION IN THE SOLEUS AFTER 4 WEEKS OF PHYSICAL ACTIVITY AND 8 WEEKS OF HIGH-FAT DIET.

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test

was applied and P<0.05 was considered significant. PA - physical activity, no PA - no physical activity, CON – control diet, HF – High-fat, CI – complex 1, CII -complex 2, CV – complex 5.

During the third experimental period animals were exposed to an additional 4 weeks of physical activity, ending up with 8 groups of animals, as described in Figure 18/19.

The objective of this part of the study was to evaluate whether early exposure to physical activity together with later exposure to physical activity would modulate/mitigate mitochondrial function in tissues, after exposure to 2 months of HFDt.

In Figure 29, there are no differences between the groups in regard to complex I (CI) activity. However, the group that presented the lowest respirometry flux across the groups, was the no PA HF no PA group (shown in yellow). This group was not exposed to either early or late PA, but it was exposed to 2 months of HFD, during the second experimental period.

There were no significant differences across the groups when the no PA experimental groups were compared: no PA/HF/PA= 66.17±21.15 *versus* no PA/HF/no PA= 55.68±33.43; p=0.999, no PA/con/PA=62.39±24.28 *versus* no PA/con/no PA=75.80±35.15; p=0.9993, or when PA experimental groups were compared: PA/HF/PA=59.50±35.34 *versus* PA/HF/no PA=68.59±37.30 ; p=0.5463, PA/con/PA=64.24±23.22 *versus* PA/con/no PA= 77.54±21.07; p=0.9652. Group comparisons are shown this way to compare groups that had PA later in life and those that did not.

Thereafter the addition of succinate, a substrate that energizes complex II, brought about an increased mitochondrial respirometry flux. Across these groups, there was no significant difference statistically (p=0.6439). As shown in Figure 29, when the no-PA experimental groups were compared, there was no significant differences across the groups: no PA/HF/PA=79.51 (58.67 – 91.49) *versus* no PA/HF/no PA=75.99 (52.90 – 89.37); p=0.6297, no PA/con/PA=83.24 (63.89 – 94.11) *versus* no PA/con/no PA=107.9 (70.55 – 116.9) ; p=0.1432, or when the PA experimental groups were compared as well: PA/HF/PA=75.73 (53.34 – 104.7) *versus* PA/HF/no PA=79.34 (70.64 – 102.4); p=0.5512,

PA/con/PA=78.08 (56.42 – 86.36) *versus* PA/con/no PA= 79.17 (70.64 – 104.7); p=0.4095.

Finally, with the addition of oligomycin, for the inhibition of complex V, there was a decrease in the respirometry flux across the group, when compared with CII activation. As shown in Figure 29, there was a noticeable flux reduction of over 75 pmol/s*mg. There is no significant difference statistically across the groups (p=0.5580). when the no PA experimental groups were compared, there was no statistical differences across the groups: no PA/HF/PA=26.31±7.26 *versus* no PA/HF/no PA=27.03±14.65; p=0.8814, no PA/con/PA=22.76±8.59 *versus* no PA/con/no PA=28.02±9.99; p=0.1815, or when the PA experimental groups were compared: PA/HF/PA=21.53±13.29 *versus* PA/HF/no PA=30.86±13.36;p=0.1004, PA/con/PA=23±8.35 *versus* PA/con/no PA= 27.65±9.86; p=0.2719.

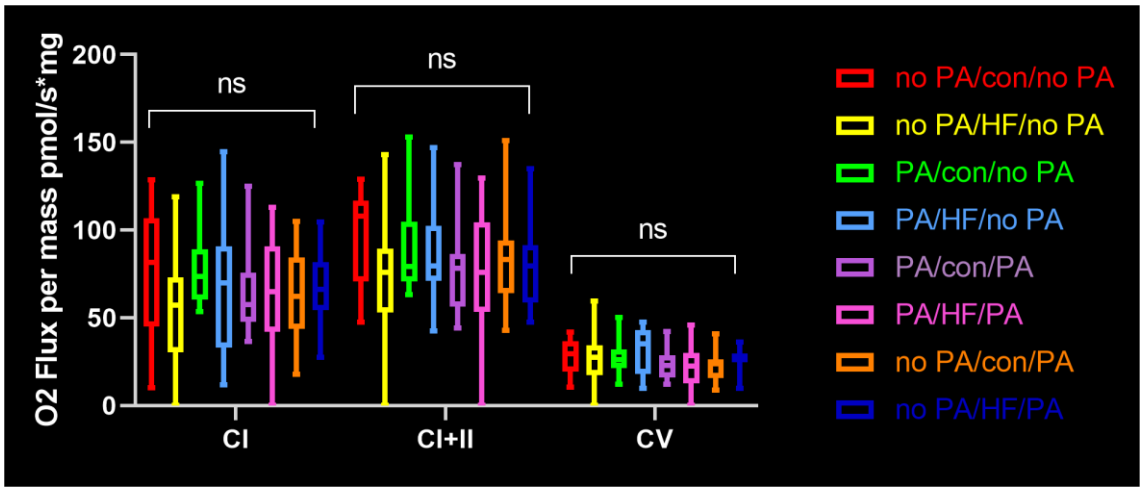

FIGURE 29. MITOCHONDRIAL RESPIRATION IN SOLEUS AFTER EXPOSURE TO 4 WEEKS OF PHYSICAL ACTIVITY, 8 WEEKS OF HIGH-FAT DIET AND ANOTHER SUBSEQUENT 4 WEEKS OF PHYSICAL ACTIVITY.

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test was applied and P<0.05 was considered significant. PA - physical activity, no PA - no physical activity, CON – control diet, HF – High-fat, CI – complex 1, CII -complex 2, CV – complex 5.

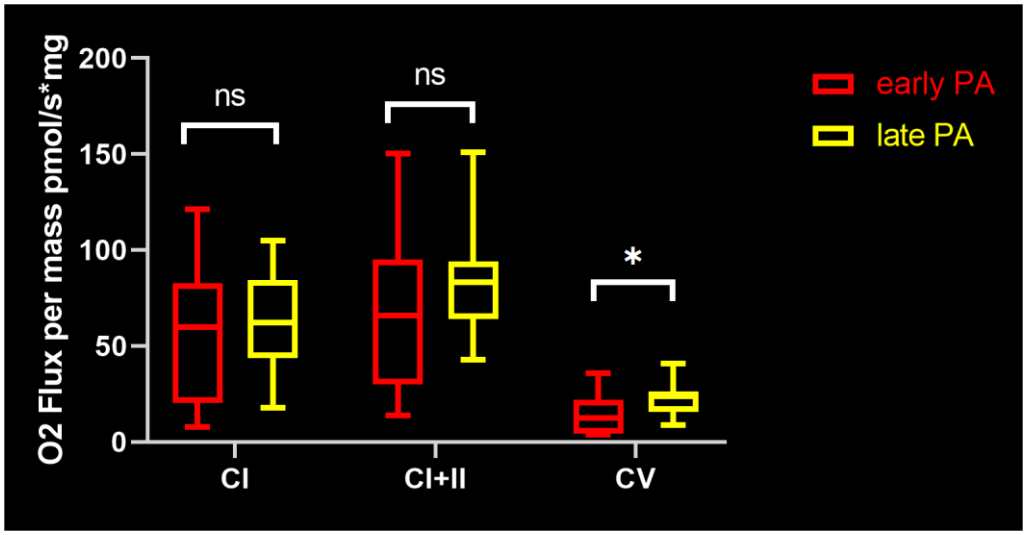

FIGURE 30 MITOCHONDRIAL RESPIRATION IN THE SOLEUS MUSCLE BETWEEN EARLY EXPOSURE AND LATE EXPOSURE TO PHYSICAL ACTIVITY.

IN RED ARE SHOWN ANIMALS THAT UNDERWENT PA AT 2 MONTHS OF AGE DURING THE 1ST EXPERIMENTAL PERIOD AND IN YELLOW ARE SHOWN ANIMALS THAT UNDERWENT PA AT 5 MONTHS OF AGE DURING THE 3RD EXPERIMENTAL PERIOD.

In red: physical activity 2-month-old. and in yellow: physical activity 5-month-old

Figure 30 shows a comparison between the early PA (2 months old) and late PA (5 months old) groups with respect to mitochondrial respiration in the soleus muscle. Analysis showed no significant difference statistically upon the addition of ADP and Complex I activation, early PA=55.43±34.84 *versus* late PA=62.39±24.23; p=0.5766, as shown in Figure 30.

Upon the activation of complex II by the addition of succinate, there was no significant differences between both groups, late PA=82.26±28.18 *versus* early PA=68.13±41.67; p=0.3426.

Thereafter, the addition of oligomycin, to block ATPase activity, there was a substantial decrease in respiration in both groups. However, a significant difference between the late PA and early PA groups was observed in CV inhibition observed, late PA=22.76±8.857 *versus* PA=14.20±10.46; p=0.0398, as shown in Figure 30. The degree of CV inhibition was significantly lower in the late PA group.

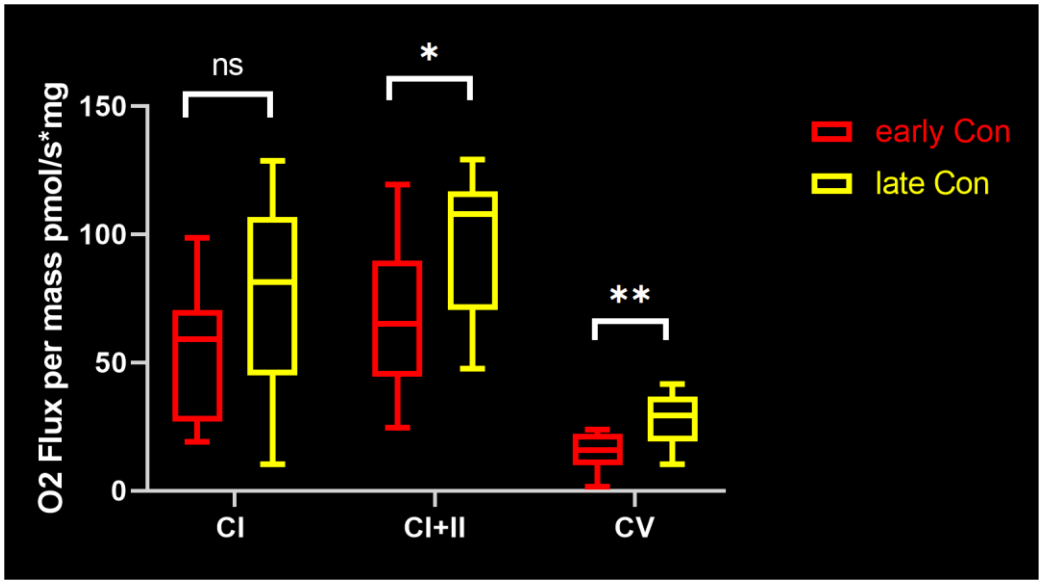

FIGURE 31. MITOCHONDRIAL RESPIRATION IN THE SOLEUS MUSCLE IN CONTROL ANIMALS.

IN READ ARE SHOWN CONTROL ANIMALS THAT WERE 2 MONTHS OF AGE DURING THE 1ST EXPERIMENTAL PERIOD AND IN YELLOW ARE SHOWN ANIMALS THAT WERE 5 MONTHS OF AGE DURING THE 3RD EXPERIENTIAL PERIOD.

In red: control 2-month-old. and in yellow: control 5-month-old

Figure 31 compares control animals at 2 months of age during the first experimental period with control animals at 5 months of age during the 3rd experimental period. The data shows no significant difference in mitochondrial respiration in the soleus muscle for Complex I activation, 2-month-old =58.31±27.23 *versus* 5-month-old =75.80±35.15; p=0.1951, as shown in Figure 31.

There was an additional rise in OXPHOS when Complex II was activated by succinate addition, for both the 2-month-old and the 5-month-old groups. As shown in Figure 31. Interestingly, there was a significant difference in the combined CI and CII respiration between the two groups, 2-month-old =67.38±30.10 *versus* the 5-month-old =96.72±26.65; p=0.0226. The older animals showed significantly higher mitochondrial respiration for the combined CI and CII activation.

Upon the addition of oligomycin, to block ATPase activity, there was a substantial decrease in respiration in both groups. However, the degree of CV inhibition was significantly lower in older control animals, there was significant differences between both groups; the 2-month-old =15.01±7.209 *versus* 5-month-old =28.02±9.992; p=0.0018, as shown in Figure 31.

Mitochondria Respiration in Brown Adipose Tissue

During the first experimental period after 4 weeks (at 2 months of age) of PA exposure, 6 no PA and 6 PA animals were sacrificed to evaluate the influence of PA on mitochondrial respiration levels for Complex 1 (CI), Complex 2 (CI+II), and the inhibition of mitochondrial Complex 5 (CV), in BAT tissue. Figure 32 shows a representation of the respirometry protocol used for the analysis of BAT.

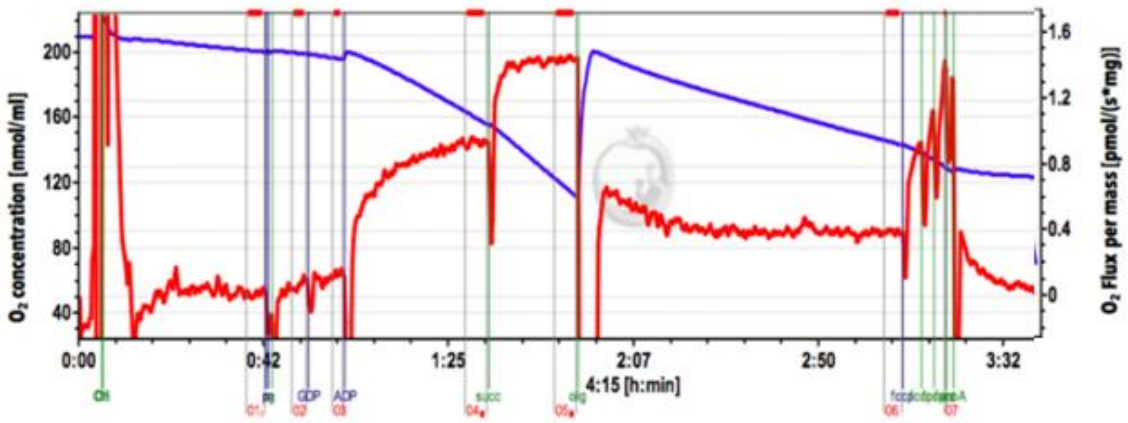

FIGURE 32. SCHEMATIC REPRESENTATION OF THE PROTOCOL USED IN THIS STUDY AND REPRESENTATIVE TRACE OF MITOCHONDRIAL RESPIRATION IN BAT

On the left y-axis, oxygen concentration is measured in NMOL/ML and on the right y-axis, oxygen flux per mass of the mitochondrial respiratory chain is measured in PMOL/S*MG. MG: Malate and glutamate; GDP: Guanine diphosphate; ADP: Adenosine diphosphate; SUCC: Succinate; OMY: Oligomycin; AA: Antimycin.

The preliminary data showed that mitochondrial respiration in the BAT showed no significant difference statistically when compared between both groups, upon the addition of ADP and Complex I activation, no PA=12.76±6.01 *versus* PA=18.16±11.20; p=0.3299. PA groups showed a 20 pmol/s*mg flux increase more than no PA groups also shown in Figure 33.

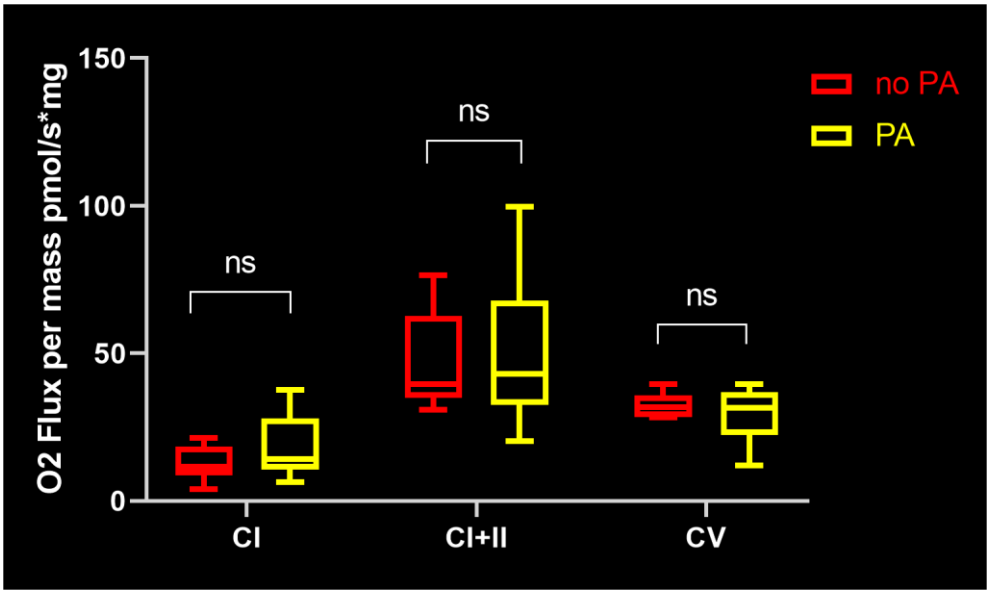

FIGURE 33 MITOCHONDRIAL RESPIRATION OF BROWN ADIPOSE TISSUES AFTER 4 WEEKS EXPOSURE TO PHYSICAL ACTIVITY.

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test was applied and P<0.05 was considered significant. PA - physical activity, no PA - no physical activity, CI – complex 1, CII -complex 2, CV – complex 5.

When complex II was stimulated by the addition of succinate, there was an increase in respirometry flux in both groups, however not significantly different. There was an increase in flux levels after the addition of succinate by over 45 pmol/s*mg, no PA=43.0 (32.61 – 67.74) *versus* PA= 31.47 (22.36 – 36.83); p=0.0931. PA groups showed a 25 pmol/s*mg flux increase more than no PA groups also shown in Figure 33.

The addition of oligomycin resulted in a significant reduction in respiration in both groups as mitochondrial CV was inhibited. There was a notable decrease in flux levels after the addition of oligomycin by over 60 pmol/s*mg. However, the PA and no PA groups had no statistically significant differences, no PA= 32.35±4.47 *versus* PA= 29.36±9.69; p=0.5140 as shown in the Figure 33 above.

Thereafter there was the inclusion of a western diet, for groups that had both physical activities and no physical activities. As previously described, mice underwent HFD for 8 weeks, to determine if the western diet in form of an HFD had any effect on BAT that had undergone physical activity.

As shown in Figure 34, there was no significant difference across the group (p=0.8852) after the addition of ADP. The no PA/con had a maximum respiratory flux of 95 pmol/s*mg, no other group had this flux level. When the no PA experimental groups were compared, there were no statistical differences across the groups: no PA/HF=26.39±14.43 *versus* no PA/con=35±86; p=0.5378, or when PA groups were compared: PA/HF=24±10.40 *versus* PA/con= 28.11±32.51; p=0.8201.

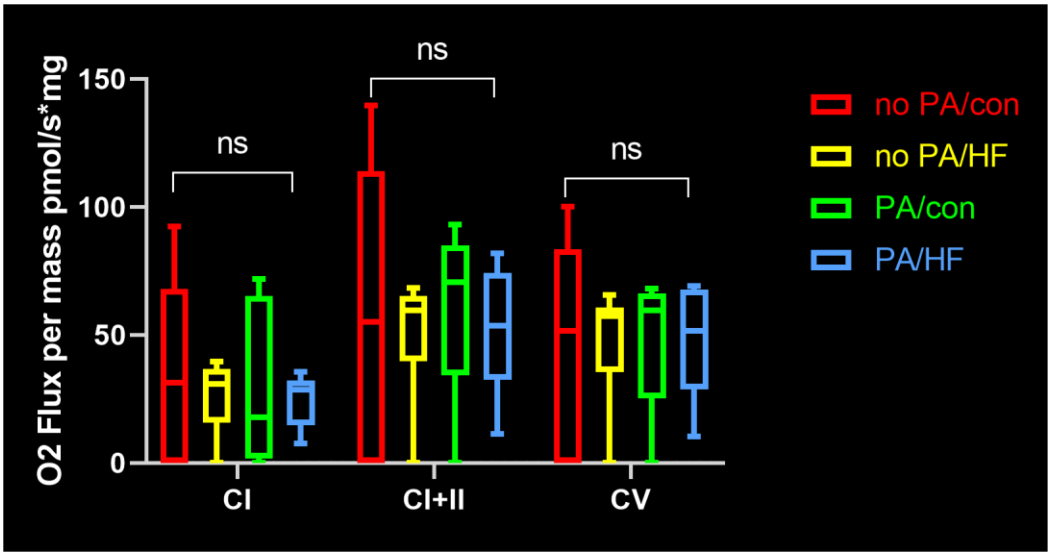

FIGURE 34 MITOCHONDRIAL RESPIRATION BROWN ADIPOSE TISSUE AFTER 4 WEEKS OF PHYSICAL ACTIVITY AND 8 WEEKS OF HIGH-FAT DIET.

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test was applied and P<0.05 was considered significant. PA - physical activity, no PA - no physical activity, con – control diet, HF – High-fat, CI – complex 1, CII -complex 2, CV – complex 5.

Upon the addition of succinate, the substrate for complex II respiration in the mitochondria, we could see in Figure 34, that there was an increase in respiratory flux levels by about 26%. However, there was no observable significant difference statistically across the different groups (p=0.9575). When the PA experimental groups were compared, there were no statistical differences across the groups: PA/HF=52.02±25.03 *versus* PA/con=60.36±33.54; p=0.6366, or when the no PA

experimental groups were compared, there was no statistical differences across the groups: no PA/con=59.22±56.62 *versus* no PA/HF=50±25.44; p=0.7506.

Thereafter we examined the effect of the addition of Oligomycin, which inhibits the respiration of mitochondrial complex V (CV). Figure 34 shows an observable decrease in respiration after the addition of oligomycin by about 24%. Also, there were no statistically significant differences across the group (p=>0.9999). When the no-PA experimental groups were compared, there were no statistical differences across the groups: no PA/HF=47.79±24.15 *versus* no PA/con=46.92±41.23; p=0.9695, when the PA experimental groups were compared, there was no statistical differences across the groups: PA/HF=47.48±22.04 *versus* PA/con=47.77±26.45; p=0.9841.

We present the results of the final experiments in BAT. All mice were reintroduced to the PA and no PA, and the figure below depicts mitochondrial flow in both groups.

As shown in Figure 35, after the addition of ADP, there was a greater respirometry flux ratio in mitochondria complex I in PA/HF/PA groups (60 pmol*smg). Statistically, there was no significant difference across the groups (p=0.6769). When the no PA experimental groups were compared, there were no statistical differences across the groups no PA/HF/PA=26.52±16.26 *versus* no PA/HF/no PA=28.55±10.07; p=0.8322, no PA/con/PA=18.28±15.34 *versus* no PA/con/no PA=19.81±12.98; p=0.8552, or when the PA experimental groups were compared, there was no statistical differences across the groups; PA/HF/PA=33.07±17.14 *versus* PA/HF/no PA=21.86±13.99; p=0.2439, PA/con/PA= 34.49±21.09 *versus* PA/con/no PA=29.84±20.11; p=0.7046.

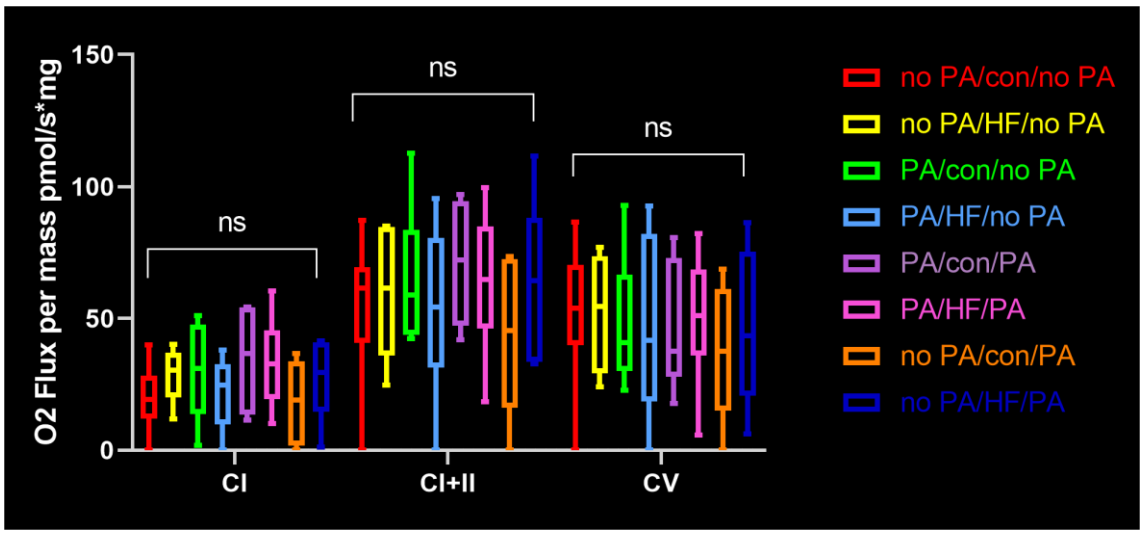

FIGURE 35 MITOCHONDRIAL RESPIRATION IN BAT AFTER EXPOSURE TO 4 WEEKS OF PHYSICAL ACTIVITY, 8 WEEKS OF HIGH-FAT DIET AND ANOTHER SUBSEQUENT 4 WEEKS OF PHYSICAL ACTIVITY.

Data are presented as median and moderate outliers, shown as circles in box-whisker plots, lie more than one and a half times the interquartile range (IQR), student's T-test was applied and $P<0.05$ was considered significant. PA - physical activity, no PA - no physical activity, CON – control diet, HF – High-fat, CI – complex 1, CII -complex 2, CV – complex 5.

Thereafter the addition of succinate, a substrate for complex II to measure the total oxidative phosphorylation, brought about an increased mitochondrial respirometry flux by more than 2-fold across the groups. Across the groups, there was no significant difference statistically (p=0.8402). As shown in Figure 35, when the no PA experimental groups were compared, there were no statistical differences across the groups: no PA/HF/PA=64.49±29.78 *versus* no PA/HF/no PA=59.40±24.13, no PA/con/PA=42.99±29.84 *versus* no PA/con/no PA=54.62±29.02, or when the PA experimental groups were compared, there was no statistical differences across the groups: PA/HF/PA= 63.77±27.45 *versus* PA/HF/no PA=53.51±32.45, PA/con/PA=70.91±24.00 *versus* PA/con/no PA=65.12±27.23

Finally, with the addition of oligomycin, for the inhibition of complex V, we observed that there was a reduction in the respirometry flux across the group compared to the addition of succinate for total oxidative phosphorylation, also seen in Figure 35. There is no significant difference statistically across the groups (p=0.9861). when the no PA experimental groups were compared, there were no statistical differences across the groups: no PA/HF/PA=43.31 (20.55 – 75.29) *versus* no PA/HF/no PA=54.47 (29.03 – 73.60), no PA/con/PA=37.57 (14.95 – 61.19) *versus* no PA/con/no PA=53.85 (39.81 – 70.31), or when the PA experimental groups were compared, there were no statistical differences across the groups; PA/HF/PA=51.02 (35.83 – 68.54) *versus* PA/HF/no PA=41.52 (18.43 – 82.13), PA/con/PA=37.55 (27.84 – 72.93) *versus* PA/con/no PA=40.77 (29.92 – 66.48) as shown in Figure 35.

11. Discussion/Conclusion

The purpose of this current pilot study was to show and compare the effects of physical activity, and its potential impact on later exposure to western diets, in the form of HFD, may affects mitochondrial function in both skeletal muscle (soleus muscle) and BAT, while factoring in early physical activity and/or later in life physical activity. Quite similar to humans that as children or even as younger adults/adolescents tend to be more active than perhaps middle-aged adults The design of this experiment mirrors therefore, the average human lifestyle from a young age to an older age. When we are younger, we tend to indulge in PA more often, and as we grow older, we tend to become more and more sedentary, especially during our middle age. As we get older, we find that we have more time and perhaps have realized we are in the need to get back in shape again, and therefore, start indulging in PA, perhaps not as rigorously and frequently as when we were younger. Previous studies have shown that undertaking exercise training activates mitochondrial biogenesis in skeletal muscles, and this supplements mitochondrial density and oxidative phosphorylation capacity by twice its original capacity (112). Muscle strength and quality in adults are known to correspond with mitochondrial phosphorylation ability. But in an interesting twist, some studies have shown that mitochondrial respiration in skeletal muscles is similar in cohorts of older men and women and young men and women (55,76)

In the current study, we evaluated mitochondrial respiration in skeletal muscle and BAT from animals that underwent PA for the first 4 weeks, using the SUIT-RP1 protocol and SUIT-RP2 protocol (slightly modified). For skeletal muscle, the SUIT-RP2 protocol was designed to evaluate the contribution of fatty acid oxidation (F-pathway) on CI and CII OXPHOS, and LEAK respiration.

In using the SUIT protocol for the measurement of mitochondrial respiration, muscle fibers are permeabilized in saponin which opens up the cellular structure of the mitochondria in the muscles without affecting all intracellular structures in the mitochondria membrane. This also helps to allow substrates like malate, glutamate, ADP and more to permeate the mitochondria thereby enabling us to evaluate the effects of substrates and inhibitors in mitochondria of this tissue.

The oxygen consumption rate (pmol/s*mg. wet weight tissue) was also measured in the current procedure in order to calculate the capacity for oxidative

phosphorylation (OXPHOS capacity) following the addition of adenosine diphosphate (ADP, 5 mM). The S-pathway was then assessed to ascertain Complex II respiration utilizing succinate as substrate. Additionally, after the addition of substrates and ADP, oligomycin (OMY; 0.2 g/ml) was added to measure proton leak respiration at complexes I and II.

PA has been shown to impact life positively and improve mitochondria function especially in young humans and mice (5,21,81,100,102,119). Porter et al (102) examined effects of PA between young and old adults and their results shows that PA modulated positively mitochondria function in young adults who underwent recreational activities. Further studies also showed that intense PA (treadmill, running wheel and resistance running wheel) in young mice showed improved mitochondria function in skeletal muscles (19,48,75,93,110). However, our results from the first group indicate no statistically significant difference (Figure 27). The respiratory flux levels were more pronounced in groups receiving PA following the addition of ADP and SUCC, respectively, to evaluate the mitochondrial complexes I and II. We also see a decreased proton leak in both PA and control groups but more decrease in the control group upon the addition of OMY. This contradicts previous experiments performed but findings from experiments carried out by Hyatt et al (78) explains why this is possible. Researchers observed that citrate synthase activity is highest in 8-week trained rat soleus muscles within 1 h after the last exercise bout and showed a significant decline in activity within 48 h post- training (78). Meaning that it is possible that our exercise time may not have been sufficient to show the differences we want to explore.

The duration of the intake of HFD and component of the HFD is important in determining mitochondria function in skeletal muscles (40). In recent studies involving short-term HFD, it has been shown that HFD has the ability to improve the mitochondrial capacity by oxidizing fatty acids without altering the mitochondrial contents, H2O2 emission, and coupling strength of skeletal muscles (40,120). Further studies have shown that mitochondria of skeletal muscles could undergo adaptations to boost their ability to oxidize fatty acids that are found in the muscle cells and the author suggested that having a long-term HFD will trigger mitochondrial biogenesis (36,39). The results of our analysis from groups undergoing HFD corroborate this when we compared groups

that had PA/HF and PA/CON as shown in Figure 28, there was a significant difference between PA groups that had HF diet and groups that had CON diet. To show if HFD had any impact to mitochondrial function in complexes I and II, in skeletal muscles; Groups that had HF diet showed an increase in respirometry flux, after the addition of ADP and SUCC, which are substrates for complex I and II respectively. Also, groups with control diet but no form of PA in the past showed an increase in respirometry flux and we can agree that this is possible due to sedentary lifestyle and possible diet content. There was a decreased proton leak across the groups but groups without HFD showed decrease for proton leak, however, not significantly different.

HFD modified mitochondrial function like alterations in mitochondrial oxidative phosphorylation activity in skeletal muscles even in the absence of changes in body weight and these modifications in mitochondrial metabolism precede the development of obesity clinically (36,39). We can therefore infer that nutrition, in our case, the HF diet plays a role in positively improving mitochondria function with a combination of PA, in skeletal muscles.

Porter et al (102), has shown that respiratory capacity of skeletal muscle mitochondria declines with advanced aging, specifically showing that respiratory capacity and the proportion of respiration coupled to phosphorylation are lower in older adults, Further studies by different group of researchers also show that PA preserves mitochondrial content in aged skeletal muscle (2,68). Our studies contradict these findings, the effects of later-in-life PA in older mice with respect to mitochondrial function in the final cohorts were then evaluated using the same protocol stated earlier. The first thing we noticed was that there were no observable differences between the groups that had early in-life PA and the later in life PA. This is in agreement with findings from previous studies (71,78,102), that age is not really a factor for mitochondrial activity but rather physical activity intensity; recall that mice ran an average of 9.2 km/day and 3.5 h/day. Also it is very possible that the duration of HFDt in older mice must have also supported mitochondria fission as shown in experiments carried out by Carvalho et al (46). We also speculate that the duration, being 4 weeks may not have been enough to improve mitochondria content to a very significant level across the

groups as shown in studies carried out by Hyatt (78) on voluntary physical activities and detraining.

A novel study carried out by researchers found that skeletal muscle mitochondria's ability to generate electrical potential and link it to ADP phosphorylation is thought to decline with age, as evidenced by studies that demonstrate respiratory capacity and the fraction of respiration coupled to phosphorylation are lower in older persons (102). In this study, however, following the addition of ADP to evaluate mitochondrial complex I, we specifically demonstrate that the proportion of respiration coupled to phosphorylation showed no significant difference between groups that did not undergo PA when compared to groups that underwent PA. This would have been an indication, that the capacity of skeletal muscle mitochondria to generate electrochemical potential and couple this to ADP phosphorylation is increased in the absence of PA, but this did not reach statistical significance. For the evaluation of complex II, after the addition of SUCC, we observed an increase, but without reaching significance, across groups that underwent both PA and ones that did not undergo PA. One of the groups that underwent PA had more phosphorylation coupled to SUCC than the rest of the groups. We also see a decreased proton leak in both PA and control groups but more decrease in the control group. One thing to consider would be that studies from previous research have shown that HFD alone when traditional complex I and II substrates like malate and glutamate and ADP for CI and succinate for complex II, were employed to fuel the electron transport chain, had no effect on mitochondrial respiration (120), but PA can also influence mitochondrial respiration. Also, a lack of increasing energy demand drives mitochondrial oxidant synthesis and emission when there is extra fuel in form of HF nutrients inside the cells (40). This can be an explanation for why groups that underwent later in life PA, but no HF plus early PA tended to have a lower respirometry flux.

Due to its nature, having a high mitochondria content, BAT was also analyzed. SUIT protocol was employed in the high respirometry analysis of mitochondria in BAT, in the presence of GDP, an inhibitor of uncoupling protein 1 (UCP1), which induces a reduction in CI OXPHOS capacity. In order to uncouple OXPHOS from ATP production, UCP1 acts as an uncoupler by increasing the rate of inner membrane proton

conductance. UCP1 mediates induced uncoupling, which is regulated physiologically (117). This protocol was designed to evaluate the contribution of substrates to the N-pathway, on OXPHOS, and electron transfer capacity.

The first result from this approach was mitochondrial residual oxygen respiration (ROX), which represents the time between the injection of the first substrate, in this case malate, and digitonin for tissue permeabilization, inside the chamber. Digitonin induces permeabilization which opens the cellular structure of the mitochondria in the muscles without affecting all intracellular structures in the mitochondria membrane. This also helps to allow substrates like malate, glutamate, ADP and more to permeate the mitochondria thereby enabling us to evaluate the effects of substrates and inhibitors in mitochondria of this tissue.

Recent research in BAT has demonstrated that voluntary wheel running for three weeks in mice reduced their mitochondrial function activity (90). Further studies has also shown that mitochondria function generally is reduced in BAT (121–123). These reduced mitochondria function is seen after the first 4 weeks of PA in BAT when compared to skeletal muscles, and a lot of factors like age, running time, and experimental procedures can be responsible for these reduced mitochondria function.

A research group has also shown that BAT has been known to be a source of circulating 12,13-diHOME, which is an exercise induced lipokine that increases fatty acid uptake(93). They further showed that 12,13-diHOME increases mitochondria respiration as 12,13-diHOME is upregulated by exercise. Our results from the first experimental time point between PA and control groups showed that in BAT, mice that underwent PA had increased mitochondrial respirometry flux after the addition of ADP and SUCC to evaluate complexes I and II with complex II showing the highest flux levels in spite of UCP1 inhibition. This could be because of the upregulation of this lipokine 12,13-diHOME due to intense PA, which could have influenced the mitochondrial flux levels in mice that underwent PA. However, there was no significant differences between these groups. We also see a decreased proton leak in both PA and control groups upon the addition of OMY, but no statistical significance was observed. However, it is important to note that more studies on mitochondrial function in BAT needs to be carried out for more understanding of this tissue.

It is important to note that in this current study, we do not have data that analyzes the mitochondria density by checking for citrate synthase activity, as this will help support results if there was an increase or decrease, across the groups for mitochondria volume. Our results showed that in BAT, groups without HF after the induction of HFDt presented a higher mitochondria respirometry flux when compared to the rest of the groups after the addition of ADP for the evaluation of complex I, and SUCC for complex II. However, there was no significant differences. Thereafter the evaluation of leak levels after the addition of OMY which inhibits the mitochondrial complex III, allowing us to evaluate proton leak from complexes I and II, respirometry flux levels were not significantly reduced across the groups (Figure 33). We also see that there were no significant differences across the groups. We can therefore infer that the western diet in the form of the HF diet did not influence mitochondria function positively in BAT. This agrees with studies carried out by researchers (124,125), who found that, long-term HFD causes BAT dysfunction that is accompanied by reduced mitochondria function, decreased insulin sensitivity, and altered thermoregulation.

PA has been shown in this text earlier as a factor that promotes mitochondrial function in skeletal muscles and when we examined the effects of later in life PA in BAT, we find out that complexes I and II respirometry flux were improved after the addition of ADP and SUCC respectively. Across all groups, the group with later-in-life PA had a higher flux level when compared to groups that had no later-in-life PA. Complex II showed a higher respirometry flux, however, peak flux levels were attained in groups that underwent PA and those that did not undergo PA but had early in-life PA and HFDt. This means that in as much as they had no PA later in life, higher respirometry flux levels could be attributed to early PA and HFDt as well as the duration of PA and how much HF diet they had. Thereafter we observed the proton leak levels in complexes I and II which can be evaluated after the addition of OMY. We could see that there was a slight decrease in the respirometry flux levels after the inhibition of complex III by OMY, groups that had PA had lower respirometry flux levels when compared to groups that had no PA, we can infer thus that PA later in life has almost no effect on complexes I and II leak respiration in BAT.

In conclusion, we have shown that animals on high fat diet increased their weight compared to those animals on chow diet. In addition, mice with access to the running wheel ad lib, run different distances and different duration times, with an average of 9.2 km/day and 3.5 h/day. Furthermore, under the present experimental conditions, we were not able to show whether early or later in life physical activity was able to modulate mitochondrial respiration in either the soleus muscle or the brown adipose tissue, after exposure or not to a high fat diet. The observed results could be in part explained due to the short running protocol of 4 weeks' time duration, and the fact that 8 weeks on this specific HFD may not have been enough. In addition, not all animals ran the exact same distances. Therefore, one important point would be to measure mitochondrial function/respiration after acute exposure to either PA or HFD or both.

12. LIMITATIONS

In this study we did not have a group of animals that was exposed to the running wheel for the 20 weeks of the study protocol, or a group that was exposed to the HFD for the entire protocol duration. There were no data about the mitochondria content measurement in form of tests for citrate synthase activity which is also a marker for improved mitochondria function in tissues. Also, to present much more significant differences across groups, it is suggested that the duration and intensity of PA be modified to a lesser time and HFDt be modified for extended long-term periods. The percentage of fat content in this study (45%), also seems to poise a lesser impact on this study. A 60% fat content may just be enough to mirror the actual representation of western diets. In addition, we did not study the time effects on mitochondrial function, as we did not compare mitochondrial respiration between mice that were 2 months of age at the end of the first experimental period with those that were studied at 4 months of age in the second experimental period or those that were 5 months of age in the last experimental period. There may have been age effects on mitochondrial respiration.

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