Effects of voluntary physical activity and endurance training in cardiac mitochondrial function of high-fat diet-fed rats.

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**KEY-WORDS:** EXERCISE; HEART; BIOENERGETICS; MITOCONDRIA; OBESITY.
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# Table of Contents

1. Introduction ........................................................................................................... 1  
2. State of art ........................................................................................................... 3  
   2.1. Obesity: prevalence and etiology ................................................................ 3  
   2.2. Obesity and Adipose Tissue ....................................................................... 5  
      2.2.1. Inflammation and endocrine function of adipocytes: adipocytokines and obesity .................................................. 5  
      2.2.2. Impact of increased adipose tissue and inflammation on insulin resistance and dyslipidemia ................................................. 8  
      2.2.2.1. Insulin resistance ........................................................................ 8  
      2.2.2.2. Dyslipidemia .......................................................................... 9  
   2.3. The relationship between obesity and cardiovascular complications ........ 9  
      2.3.1. Vascular stiffness, endothelial dysfunction and obesity-induced chronic inflammation ......................................................... 10  
      2.3.2. Obesity compromises heart structure and function .............................. 12  
      2.3.3. Adipocytokine action on the heart: the particular case of leptin .......... 14  
      2.3.4. The obese myocardium: lipotoxicity and metabolic disarrangements .... 14  
   2.4. The heart mitochondria ............................................................................... 16  
      2.4.1. Mitochondrial structure ..................................................................... 17  
      2.4.2. Mitochondrial energy production – oxidative phosphorylation ............. 17  
      2.4.3. Mitochondrial as source of oxidative stress ...................................... 19  
      2.4.4. Mitochondrial calcium homeostasis and apoptosis ............................. 23  
      2.4.5. Mitochondrial quality control ......................................................... 25  
      2.5. The cardiac “obese” mitochondria ............................................................ 27  
      2.5.1. Cardiac energy pathways and sources of acetil-CoA ........................ 28  
      2.5.2. Obesity-induced cardiac metabolic inflexibility .................................. 29  
      2.5.3. ROS involvement in obese mitochondrial-induced cell death .............. 30  
   2.6. Exercise as non-pharmacologic strategy for obesity-induced cardiac impairment ................................................................. 31  
      2.6.1. Chronic exercise and cardiac mitochondria adaptations ....................... 33  
      2.6.1.1. Exercise and mitochondrial bioenergetics ..................................... 33  
      2.6.1.2. Exercise and mitochondrial biogenesis ......................................... 34  
      2.6.1.3. Exercise and mitochondrial oxidative stress/ antioxidiant capacity ...... 35  
      2.6.1.4. Exercise and mitochondrial calcium resistance and apoptosis .......... 37  
   2.7. Relevance of the present study ...................................................................... 38  
3. Aim of the present study .................................................................................... 41  
4. Materials and methods ..................................................................................... 43
Figures

Figure 1. “Proposed mechanisms of vascular stiffness in obesity, insulin resistance, and type 2 diabetes”. ...........................................................................................................11

Figure 2. “Antioxidant enzyme system” ......................................................................22

Figure 3. Diagram summarizing the design of diet and exercise protocol ..............45

Figure 4. Typical polarographic oxygen traces obtain in Anoxia-reoxygenation model. .................................................................................................................................48

Figure 5. Food consumption (A), running distance in treadmill (Tm) and free wheel (FW) per day (B). .............................................................................................................53

Figure 6. Effect of diet and exercise treatments on heart mitochondrial oxygen consumption (A) state 3 of mitochondrial respiration, (B) state 4 of mitochondrial respiration, (C) RCR, and (D) ADP/O........................................................................................................................................59

Figure 7. Effect of diet and exercise treatments on heart mitochondria ΔΨ fluctuations (A) maximal energization, (B) ADP-induced depolarization, (C) ADP-induced repolarization and (D) ADP phosphorylation lag phase........................................................................60

Figure 8. Effect of diet and exercise treatments on heart mitochondria after Anoxia-Reoxygenation stimulation (A) state 3 of mitochondrial respiration, (B) state 4 of mitochondrial respiration, (C) RCR, and (D) ADP/O........................................................................................................62

Figure 9. Effect of exercise and diet treatment on heart mitochondria's respiratory complexes (OXPHOS): (A) Complex I; (B) Complex II; (C) Complex IV; (D) Complex V; (E) Protein loading control by Ponseau-S staining; (F) Typical immunoblots ....64

Figure 10. Effect of exercise and diet treatment on heart mitochondria's (A) MDA content and (B) MDA content in Anoxia-Reoxygenation........................................................................................................65

Figure 11. Effect of exercise and diet treatment on heart mitochondria's: (A) Reduced Glutathione content (GSH); (B) Oxidized Glutathione content (GSSG) and (C) Glutathione Ratio: GSH/GSSG........................................................................................................................................66
Tables

Table 1. Obesity-induced alterations in Adipocytokine..................................................7
Table 2. A brief resume of the mitochondrial respiratory chain complexes............19
Table 3. Anatomic characterization of animals.................................................................55
Table 4. Blood analysis after 9 and 17 weeks of treatments ........................................57
Table 5. Percentage of the calories provided by carbohydrates; fat and proteins in some typical commercial diets for rodents. .................................................................69
Resumo

No presente estudo investigamos os efeitos de duas modalidades distintas de exercício (actividade física voluntária em roda livre –FW e treino em tapete rolante – Tm) em animais alimentados com duas dietas isocalóricas diferentes na percentagem de energia proveniente de gordura e de hidratos de carbono: Dieta Standard (SD) e Dieta Gorda (HFD) na bioenergética e stress oxidativo de mitocôndrias cardíacas. Ratos Sprague-Dawley machos foram divididos em quatro grupos: dieta standard sedentário (SD+SED, n=20), dieta standard roda livre (SD+FW, n=10), dieta gorda sedentário (HFD+SED, n=20) e dieta gorda roda livre (HFD+FW, n=10). Passadas 9 semanas, metade (n=10) dos animais dos grupos SD+SED e HFD+SED foram recolocados num novo grupo de treino em passadeira rolante (8 semanas, 5 dias/semana; 60 mins/dia; SD+Tm e HFD+Tm respectivamente). Realizou-se uma avaliação ex vivo de parâmetros de funcionalidade mitocondrial, sob condições de oxigenação normais e em anoxia- reoxigenação, assim como semi-quantificação de subunidades da fosforilação oxidativa e avaliação da peroxidação lipídica e do estado redox da glutationa. 17 semanas de dieta gorda não induziram qualquer efeito na funcionalidade mitocondrial cardíaca nem no estado redox. Contudo, o treino Tm promoveu melhorias na actividade respiratória mitocondrial nas duas dietas, antes e após a anoxia- reoxigenação. Já o treino FW aumentou o conteúdo das subunidades dos complexos IV e V da OXPHOS nas duas dietas, assim como melhorou a capacidade anti oxidativa mitocondrial (GSH/GSSG), particularmente no grupo HFD. Apesar da HFD não ter induzido disfunção na bioenergética mitocondrial, o treino Tm promoveu na generalidade um efeito mais visivel no consumo de oxigénio mitocondrial, enquanto que o exercício FW pareceu modelar de forma positiva a capacidade anti oxidativa mitocondrial. Concluímos assim que o exercício crónico pode constituir uma estratégia eficiente na melhoria da funcionalidade mitocondrial cardíaca num contexto de obesidade e HFD.

PALAVRAS-CHAVE: EXERCÍCIO; CORAÇÃO; BIOENERGÉTICA; MITONDRIA; OBESIDADE.
Abstract

We here investigate the effects of two distinct chronic exercise modalities voluntary free wheel training (FW) and the endurance treadmill training (Tm) in animals feed with isocaloric diets different in energy derived from fat and carbohydrates: standard (SD) and high fat diet (HFD) on cardiac mitochondrial bioenergetics and oxidative stress. Male Sprague-Dawley rats were divided into standard-diet sedentary (SD+SED, n=20), standard-diet free wheel (SD+FW, n=10), high-fat diet sedentary (HFD+SED, n=20) and high-fat diet free wheel (HFD+FW, n=10) groups. After 9-weeks, half (n=10) of SD+SED and HFD+SED groups were engaged in a Tm program (8 wks, 5 d/wk, 60 min/day; SD+Tm and HFD+Tm respectively). Ex vivo cardiac mitochondrial function endpoints were assessed under normal oxygenation conditions and anoxia-reoxygenation. Semi-quantification of oxidative phosphorylation subunits, lipid peroxidation and the glutathione redox status were also measured. 17-weeks of HFD treatment, did not affect cardiac mitochondrial function neither the redox state (HFD+SED vs. SD+SED) Importantly, Tm exercise improved mitochondrial respiratory activity in both diet regimens before and after anoxia-reoxygenation. FW running increased OXPHOS complexes IV and V subunits in both diet treatments and improved mitochondrial anti-oxidant capacity (GSH/GSSG), particularly in HFD group. Although 17 weeks of HFD did not induced mitochondria bioenergetics impairments, Tm training had a stronger effect in overall mitochondria oxygen consumption, whereas FW exercise seems to positively modulate the antioxidant machinery. Overall, we conclude that chronic exercise may constitute an effective strategy to increase cardiac mitochondrial functionality in a context of obesity and HFD.

KEY-WORDS: EXERCISE; HEART; BIOENERGETICS; MITOCONDRIA; OBESITY.
Abbreviations and Symbols

CVD  Cardiovascular Diseases
HFD.  High-Fat Diet
ROS  Reactive Oxygen Species
AMPK  Adenosine Monophosphate-Activated Protein Kinase
ANT  Adenosine Nucleotide Translocase
ATP  Adenosine Triphosphate
Ca\(^{2+}\)  Calcium Ion
CAT  Catalase
Tm  Treadmill
FW  Free Wheel
SED  Sedentary
SD  Standard Diet
DNA  Deoxyribonucleic Acid
MRC  Mitochondria Respiratory Chain
GSH  Reduced Glutathione
GSSG  Oxidized Glutathione
OXPHOS  Oxidative Phosphorylation
BMI  Body Mass Index
GPX  Glutathione Peroxidase
WC  Waist Circumference
FA  Fatty Acids
H\(_2\)O\(_2\)  Hydrogen Peroxide
FFA  Free Fatty Acid
Ang II  Angiotensinogen II
IL-6  Interleukin-6
TNF-\(\alpha\)  Tumor Necrosis Factor alpha
IL-10  Interleukin-10
IR  Insulin Resistance
NEFAs  Non-esterified Fatty Acids
TGs  Triglycerides
LDL  Low-Density Lipoprotein
HDL  High-Density Lipoprotein
VLDL  Very Low Density Lipoprotein
NO  Nitric Oxide
PVAT  Perivascular Adipose Tissue
MnSOD  Manganese Superoxide Dismutase
mPTP  Mitochondrial Permeability Transition Pore
mtDNA  Mitochondrial Deoxyribonucleic Acid
EAT  Epicardial Adipose Tissue
OMM Outer Mitochondrial Membrane
NADH Reduced Nicotinamide Adenine Dinucleotide
NADPH Reduced Nicotinamide Adenine Dinucleotide Phosphate
nmol Nanomol
CPT1 Carnitine Palmitoyltransferase-1
NS Non-Significant
O₂ Oxygen
O₂⁻ Superoxide Radical
IMM Inner Mitochondrial Membrane
OH⁻ Hydroxyl Radical
PGC-1α Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
RCR Respiratory Control Ratio
TOM Translocase of the Outer Membrane
ADP Adenosine Diphosphate
FADH₂ Flavin Adenine Dinucleotide
SEM Standard Error Of The Mean
SOD Superoxide Dismutase
ΔµH⁺ Variation in Electrochemical Proton Gradient
H⁺ Hydron
H₂O₂ Hydrogen Peroxide
MCU Mitochondrial Calcium Uniporter
NCX Sodium Calcium Exchanger
TPP⁺ Tetraphenylphosphonium
VDAC Voltage Dependent Anion Chanel
ER/SR Endoplasmic/ Sarcoplasmic reticulum
Δψ Variation in Transmembrane Electrical Potential
Cyp D Cyclophilin D
Cyp A Cyclophilin A
Mfn 1 Mitofusin 1
Mfn 2 Mitofusin 2
OPA 1 Optic Atrophy 1
Drp 1 Dynamin- Related Protein 1
Fis 1 Mitochondria Fission 1
Mff Mitochondria Fission Factor
MID49 Mitochondrial Dynamics Protein 49
MID51 Mitochondrial Dynamics Protein 51
KD Kilodalton
MDV Mitochondria-Derived Vesicles
PHD Pyruvate Dehydrogenase
PFK-1 Phosphofructokinase-1
PDP 1 Phosphatase 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PDP 2</td>
<td>Phosphatase 2</td>
</tr>
<tr>
<td>mPT</td>
<td>mitochondrial Permeability Transition</td>
</tr>
<tr>
<td>NRF 1</td>
<td>Nuclear Respiratory Factors 1</td>
</tr>
<tr>
<td>NRF 2</td>
<td>Nuclear Respiratory Factors 2</td>
</tr>
<tr>
<td>Tfam</td>
<td>Mitochondrial Transcription Factor A</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances</td>
</tr>
<tr>
<td>AI</td>
<td>Adiposity Index</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>A-R</td>
<td>Anoxia-Reoxygenation</td>
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</table>
1. Introduction

Obesity is one of the most prevalent diseases worldwide and has been considered a global epidemic (World Health Organization, 2015b). Despite of its complex etiology, obesity is frequently related to unhealthy behavior factors, including rich carbohydrate or fat diet and sedentary life style. The interaction between these two factors leads to a positive energy imbalance that has been believed to be the central cause of obesity (Lau et al., 2015; Ogden et al., 2007). Moreover, the hyperplasia and hypertrophy of adipocytes seen in an obesity condition ultimately culminates in adiposity tissue dysfunction, which is deeply associated to Cardiovascular Diseases (CVD) (Ferranti & Mozaffarian, 2008).

Among several obesity-inducer models, a high-fat diet (HFD) consumption has been suggested to be responsible for cardiac and mitochondrial dysfunction as it is associated to increased production of reactive oxygen species (ROS), mitochondria respiratory chain (MRC) uncoupling, decreased oxidative capacity and calcium overload (Essop et al., 2016; Ferreira et al., 2015; Goncalves et al., 2016; Sverdlov et al., 2015; Sverdlov et al., 2016). Importantly, most of the studies are concerned with the effects of a high-fat and high-energy diets. The fact that not only the caloric intake but also the diet composition is responsible for inducing obesity (Estrany et al., 2011; Goyal et al., 2012), justify the need to study the effect of HFD compared with an isocaloric control diet on cardiac mitochondrial function, a topic underexplored.

Regular physical exercise has been considered a potential non-pharmacological strategy to counteract obesity, and also a potent cardio-protective therapeutic justifying its use within a clinical context (Ascensao et al., 2006; Bruun et al., 2006; Golbidi & Laher, 2012; Hafstad et al., 2013; Huang et al., 2015; Pons et al., 2013). Among the different animal exercise models, endurance training performed in treadmill (Tm) and voluntary free wheel running (FW) have been proven to be potential therapeutic and/or preventive strategies against cardiac mitochondrial dysfunction promoted by a variety of physiopathological conditions (Antonio Ascensao et al., 2011; Ascensao et al., 2006; Judge et al., 2005; Lesniewski et al., 2013). In fact, exercise training seems to positively modulate cardiac mitochondrial functionality, increasing antioxidant capacity, improving
membrane integrity and consequently improving oxidative capacity (Alleman et al., 2015; Jacobs & Lundby, 2013; Kavazis et al., 2008). However, there are still questions to be elucidated concerning Tm and FW exercise models. Differences in exercise intensity, volume, power, frequency, among other factors, can culminate in different cardiac mitochondrial adaptations.

Therefore, our aim is to analyze the effects of two isocaloric diets (differing in energy derived from fat and carbohydrates) in cardiac mitochondrial bioenergetics and redox state. Additionally, to our knowledge, no studies so far analyzed if the effects of different exercise modalities with distinct characteristics, including intensity, volume and length (FW and Tm) in cardiac mitochondria are dependent on the percentage of fat consumption. To do so, heart mitochondria respiratory parameters were measured, which included oxygen consumption under normal oxygenation conditions and in anoxia-reoxygenation, and transmembrane electric potential. Oxidative phosphorylation (OXPHOS) subunits content was also determined. Finally, markers of mitochondrial lipid peroxidation (MDA content) along with parameters of antioxidant capacity evaluated by reduced glutathione (GSH), oxidized glutathione (GSSG) were quantified.
2. State of art

2.1. Obesity: prevalence and etiology

Nowadays, obesity is one of the most prevalent diseases in developed countries, and it has started to spread through less developed countries all around the world. Moreover, dramatic increases in obesity have occurred in both children and adults (World Health Organization, 2015b), becoming a global epidemic. According to the World Health Organization (2015b), more than 1.9 billion adults were overweight in 2014. Among these, 600 million were obese, which shows how alarming this public health problem really is.

Currently, the WHO (World Health Organization, 2015b) defines obesity as an "abnormal or excessive fat accumulation that may impair health". The Body Mass Index, obtained by dividing the person’s weight (in Kilograms) by the square of his/her height (in Meters) (Bastien et al., 2014), has been largely used to classify overweight and obesity in adults. Therefore, a person with a BMI higher than 25 is considered to be overweight and a person with a BMI higher than 30 is considered to be obese. However, BMI does not take into consideration the distinction between an elevated BMI due to high level of lean mass or due to high level of fat body mass nor the body fat distribution. Indeed, several studies confirmed that the regional distribution of body fat is much more important than excess adiposity per se regarding the CVD risk associated with a given excess of body weight/fat (for ref see Despres, 2012). Comparatively, markers of absolute and relative accumulation of abdominal fat, such as Waist Circumference (WC) and Waist-To-Hip Ratio have been used to underline the importance of abdominal fat as a serious health risk (Bastien et al., 2014). The use of imaging techniques as computed tomography and magnetic resonance (MRI) represent remarkable advances in the ability to precisely and reliably quantify individual differences in body fat distribution and to selectively distinguish subcutaneous adiposity from visceral abdominal adipose tissue (for ref see Despres, 2012).

Regardless of its complex etiology, obesity is now considered to be the result from the interaction of various factors, such as behavior, environmental and genetic factors. The genetic seem to be the one that has less impact (Lau et al.,
2015), being the stem cause of this problem the energy imbalance, meaning more calories consumed than expended. Determinants responsible for the increased caloric consumption counts increased portion size, consumption of sugar-sweetened beverages, refined carbohydrates, advertising that promotes overconsumption. On the other hand, the reduced daily energy expenditure have been related to sedentary lifestyle, not only working time but also leisure time, both lacking of any sort of physical activity (Ogden et al., 2007). Therefore, the combination of energy-dense food products of poor nutritional value combined with a sedentary lifestyle has contributed to the emergence of obesity and the consequent health problems.

This excess of energy is stored in adipocytes (fat cells), leading to hypertrophy and hyperplasia (Ferranti & Mozaffarian, 2008) and resulting in a dysfunctional adipose tissue, which is associated with an increased prevalence of metabolic disorders, enlarging the risk of diabetes, CVD and mortality (Dario A. Gutierrez et al., 2009). Indeed, HFDs are not only responsible for inducing obesity but also for leading to metabolic alterations in adipose tissue, increasing levels of circulating fatty acids (FA) in the blood, which ultimately contributes for the development of metabolic syndrome and CVD (Betanzos-Cabrera et al., 2012). In accordance, the American Heart Association has published several position statements emphasizing the health hazards of obesity (Cornier et al., 2011; Klein et al., 2004; Poirier et al., 2006).

Although several strategies to control epidemic obesity have emerged, including the engagement with an active lifestyle, it has been proposed that when compared, caloric restriction and dietary fat percentage reduction, the second option can be as effective as the first one in weight reduction and even more effective in improving insulin sensitivity along with others cardiovascular risk factors (Racette et al., 2006).

The following sections will address the effects of obesity on the deregulation of adipose tissue function, as well as the link with CVD.
2.2. Obesity and Adipose Tissue

Adipose tissue is composed by adipocytes and a vascular stromal fraction incorporating macrophages, fibroblasts, endothelial cells and pre-adipocytes. Pre-adipocytes, originated from a multi-potent stem cell, hold ability to generate new fat cells during the entire human life. The main roles of adipose tissue are to store energy in shape of triglycerides, insulate and cushion the body, this means control body temperature and mechanically protect the organs. This energy store results from free fatty acids (FFA) after food intake and it can be released during the fasting state, ensuring sufficient energy status (Hajer et al., 2008).

Adipose tissue can be subdivided in fractions of subcutaneous and visceral adipose tissue with distinct functions. For instances, accumulation of intra-abdominal or visceral adipose tissue has been reported to be quite deleterious and associated with a constellation of metabolic abnormalities increasing CVD risk (Despres, 2012). Although the visceral adiposity and liver fat are common key drivers of the cardiometabolic risk associated with overweight/obesity, other ectopic fat depots may also contribute to the risk of various cardiovascular outcomes (Despres, 2012).

Nowadays, adipocytes are also known to generate peptides, hormones and cytokines, termed adipocytokines, with endocrine, autocrine or paracrine repercussion and controlling lipid and glucose metabolism, blood coagulation, inflammatory state, blood pressure and hormonal modulation. Therefore, adipose tissue is now considered to be an endocrine organ (for ref see Moura & Monteiro, 2010) with a crucial relevance on body homeostasis. The consequences of obesity-mediated adipose tissue dysfunction will be addressed below.

2.2.1. Inflammation and endocrine function of adipocytes: adipocytokines and obesity

As stated before, obesity has been established as a chronic inflammatory disease that leads to adipose tissue dysfunction, culminating in CVD and metabolic diseases (Cancello, Rouault, et al., 2005).
Adipocytokines have an important role in generating feedback action, affecting metabolism and function of organs and tissues, such as muscle, liver, vascular and brain (for ref see Hajer et al., 2008). Some of the most relevant adipocytokines are: leptin, angiotensinogen II, interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), adiponectin and interleukin-10 (IL-10) (Moura & Monteiro, 2010). However, there are, at least, twenty four adipocytokines already identified (Hajer et al., 2008).

In obesity, adipocytokines production is deregulated, leading to insulin resistance, energetic homeostasis disturbance, coagulation alterations and arterial hypertension (Arner, 2001). The table 1 summarizes the main function associated with a few relevant adipocytokines, obesity-related alterations in their production, reflected in plasma concentration, and its overall consequences at a systemic level.
<table>
<thead>
<tr>
<th>Adipocytokine</th>
<th>Main function</th>
<th>Obesity-induced [plasma] variations</th>
<th>Systemic obesity-induced alterations</th>
<th>References</th>
</tr>
</thead>
</table>
| Adiponectin  | • Anti-inflammatory marker  
• Upregulate fatty acid oxidation in muscle and liver  
• Protective role against atheroescclerosis  
• Downregulate hepatic gluconeogenesis | ↓ | • Insulin Resistance  
• Metabolic Syndrome  
• Type 2 Diabetes | Hajer et al. (2007).  
Kadowaki & Yamauchi (2005) |
| TNF-α        | • Inflammatory marker  
• Upregulate lipolysis | ↑ | • Systemic chronic inflammatory state  
• Dyslipidemia  
• Insulin resistance | Dario A. Gutierrez et al. (2009) |
| Angiotensinogen | • Vasoconstrictor  
• Arterial pressure regulator | ↑ | • Hypertension  
• Vascular stiffness | Massiéra et al. (2001) |
| IL-6         | • Inflammatory marker | ↑ | • Insulin Resistance  
• Systemic chronic inflammatory state | Wannamethee et al. (2007). |
| Leptin*      | • Regulation of Adipose Tissue, through appetite and food intake reduction  
• Upregulated catabolic paths: β-oxidation; glycolysis;  
• Mitochondrial biogenesis  
• Increase sympathetic tonus | ↑ | • Leptin Resistance  
• Increase heart rate  
• Increase blood pressure | Moura & Monteiro (2010)  
Arner (2001)  
Turer et al. (2012) |

↑: upregulated; ↓: downregulated *Leptin limits the excess calories store and blocks the development of chronic steatosis. In obesity, contrarily of what expected, the leptin production is up-regulated suggesting a possible leptin resistance, along with alteration in its signaling (for ref see Moura & Monteiro, 2010).
2.2.2. Impact of increased adipose tissue and inflammation on insulin resistance and dyslipidemia

2.2.2.1. Insulin resistance

Insulin Resistance (IR) is characterized by decreased insulin sensitivity, not only in the liver, but also in peripheral tissues. Moreover, it’s considered to be a precursor of Type 2 Diabetes Mellitus (Dario A. Gutierrez et al., 2009). As it was already mentioned, obesity is now established as a chronic inflammatory disease. This inflammatory state is assumed to contribute to IR by reducing insulin sensitivity in adipose tissue and other organs (Apovian et al., 2008).

As a result of adipose tissue dysfunction and inflammatory state, the production of pro-inflammatory adipocytokines, such as TNF-α is upregulated (Table 1). The TNF-α contributes to insulin signaling defects resulting in increased nonesterified fatty acids (NEFAs) blood concentration and their storage in liver, muscle and pancreas, directly inhibiting insulin signaling pathways and culminating in a systemic IR (for ref see Dario A. Gutierrez et al., 2009).

Besides TNF-α, other adipocytokines can also contribute to lipid homeostasis (Table 1). In fact, leptin and adiponectin play an important function. For instance, leptin can upregulate FA oxidation, reducing lipid accumulation in other organs; however, it is thought that obesity can induce leptin resistance, thereby losing its protective role against IR. Adiponectin, which has also a protective role enhancing muscle fat oxidation, is down-regulated in obesity (Unger et al., 1998).

In obesity, the increased plasma concentration of NEFAs and TNF-α, the leptin resistance and the down regulated production of adiponectin contribute to lipid accumulation in the liver, muscle and pancreas, ultimately culminating in IR (Dario A. Gutierrez et al., 2009).
2.2.2.2. Dyslipidemia

Dyslipidemia is characterized by elevated plasma NEFAs, triglycerides (TGs), low-density lipoprotein (LDL) and reduced plasma high-density lipoprotein (HDL). Obesity is considered to be a precursor of dyslipidemia, as an increased in adipocyte size can lead to adipocyte saturation and inability to store lipid excess. The uncontrolled fatty acid lipolysis from visceral adipose tissue, results in an increased delivery of fatty acids to the liver to act as substrate for very low density lipoprotein (VLDL) (Dario A. Gutierrez et al., 2009).

Furthermore, as presented in Table 1, obesity is associated with an up-regulation of TNF-α, which is able to upregulate lipolysis, resulting in an increased of circulating NEFAs and, consequently, their delivery to the liver promoting TG synthesis and VLDL secretion. Therefore, it is obvious that the increased secretion of TNFα has a major impact in dyslipidemia (Jovinge et al., 1998). Also, in table 1, is mentioned that adiponectin is down regulated in obesity. Besides having a protective role against dyslipidemia, this adipocytokine is also associated with a lower plasma concentration of TG and VLDL and a higher plasma concentration of HDL. Consequently, the downregulated production of adiponectin increases the risk of dyslipidemia (for ref see Dario A. Gutierrez et al., 2009).

The deregulated production of adipocytokines, a distinct characteristic of adipose tissue dysfunction commonly seen in obesity, is associated with the development of IR, dyslipidemia and chronic inflammation. The next sections will address the mechanisms and the consequences behind obesity-induced cardiovascular complications, including vascular stiffness, hypertension and associated myocardial complications and heart dysfunction.

2.3. The relationship between obesity and cardiovascular complications

The CVD are some of the most deadly and disability-induced diseases worldwide, affecting the heart and the blood vases. It is estimated that 31% of all global deaths are caused by CVD (World Health Organization, 2015a). They include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and
pulmonary embolism. Myocardial infarction and strokes are the ultimate outcome, mostly culminating in death. The main cause is the accumulation of fat within the blood vascular walls, known as atherosclerosis, which can cause a blockage in the blood flow, preventing blood from flowing to the heart and brain (Hobbs, 2015; World Health Organization, 2015a).

Obesity is an important cardiovascular risk factor. It is now established that adiposity not only induces adverse local effects but also has a systemic effect (Ferranti & Mozaffarian, 2008). Moreover, obesity is now considered as an independent risk factor for CVD, suggesting that the increased adiposity indices are the main drivers behind the intermediate risk factors, such as vascular stiffness, hypertension, dyslipidemia, IR, chronic inflammation, diabetes mellitus, endothelial dysfunction and atherosclerosis (Bastien et al., 2014; Strasser et al., 2015).

2.3.1. Vascular stiffness, endothelial dysfunction and obesity-induced chronic inflammation

Obesity and IR are known to interact and impair vascular function and structure, as they are linked to endothelial dysfunction, increased artery intima media thickness and increased vascular stiffness (Herouvi et al., 2013). The endothelium is a complex organ. It is responsible for the regulation of the smooth muscle cells proliferation, platelet function, vasomotor tone and thrombosis (López-Jiménez & Cortés-Bergoderi, 2011).

The first event of atherogenesis is the endothelial damage caused by various risk factors, such as arterial hypertension, hyperglycemia and dyslipidemia. The outcome of endothelial damage is actually endothelial dysfunction, which consists in an unbalance production of vasodilators agents and vasoconstrictors agents, with a decreased production of nitric oxide (NO) and an increased production of Ang II, respectively. Moreover, the production of ROS is also reported to be up-regulated (Husain et al., 2015). This contributes to an inflammatory state, along with an increase in intima membrane thickness (Figure 1).
In fact, the main driver of atherosclerosis is the excess of circulatory cholesterol, more precisely of VLDL. As the ROS production is upregulated due to the endothelial dysfunction, the VLDL molecules become oxidized. Thereafter, the oxidized VLDL connects with receptor-1, which is also upregulated in behalf of Ang II, and it is able to get through the endothelium, ending up in intima media. Additionally, the proliferation of VLDL oxidized through endothelium amplifies endothelium dysfunction, resulting in an even bigger decrease of NO production (Allahverdian et al., 2012). Furthermore, oxidized VLDL induces endothelial cells apoptosis and the expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and selectin-P, which results in endothelial damage as well as in monocyte infiltration in the intima media, boosting the inflammatory state (Apovian et al., 2008; Pasceri et al., 2000).

During the immune response and within the intima media, the macrophages (M1, M2) are not capable of fagocitating the VLDL oxidized, forming macrophage foam cells. This maladaptive immune response increases the cytokine production of TNF-α and IL6, resulting in a continuous lipid accumulation along with chronic inflammatory state (Allahverdian et al., 2012). The atheroma plaque growth is the...
result of an increased number of macrophage foam cells and lymphocyte, which up regulate the production of hydrolytic enzymes, cytokines and growth factors, leading to fibrosis and local necrosis (for ref see Allahverdian et al., 2012). At this point, atherosclerosis is settled, the blood flow is reduced due to the atheroma plaque along with the diminished endothelial vasodilatation capacity. Over time, the atheroma plaque becomes unstable, meaning an increased risk of rupture and, consequently, espousing its lipid content. This event is highly thrombogenic, ultimately resulting in myocardial infarction or in cerebrovascular accident (Apovian et al., 2008; Jia et al., 2014; Maiolino et al., 2015).

Additionally, perivascular adipose tissue (PVAT) is also a serious risk factor for the development of obesity-induced atherosclerosis (Shimabukuru, 2009). Perivascular fat serves as a structure component present in most arteries and is defined as the accumulation of fat around vascular structures, mostly in the proximity of all blood vessels and around the coronaries and aorta (Shimabukuru, 2009). This tissue is a source of molecules with varied paracrine effects, holding ability to modulate vascular responsiveness to vasoactive agents (Villacorta & Chang, 2015). In obesity, the expression and infiltration of pro-inflammatory immune cells in PVAT is increased along with a reduced expression of anti-inflammatory factors (Aroor et al., 2013), which is associated with endothelial dysfunction. Therefore, obesity is considered an important risk factor for vascular stiffness and atherosclerosis with consequently adverse effects in several organs, including the heart, kidney, liver and brain. (for ref see Jia et al., 2015). Particularly, the compromised heart function and myocardial dysfunction linked with endothelial deregulation are addressed below.

2.3.2. Obesity compromises heart structure and function

Obesity is responsible for inducing structural adaptations in cardiovascular structure and function, in order to maintain whole body homeostasis. Stroke volume augmentation, which is thought to be a compensatory adaptation to increased adipose tissue mass (Szczepaniak et al., 2007), as well as the stiffening of central arteries increase systolic pressure and decrease diastolic pressure, and culminate in increased afterload. Over time, as a response to the higher cardiac workload, the ventricle starts to get thicker (ventricular
hypertrophy) so it can overcome the new demands. Further, the decrease in
diastolic pressure is associated with reduced coronary flow during the diastole.
These alterations are associated with left ventricular remodeling and fibrosis,
leading to left ventricular-diastolic dysfunction and development of coronary
artery disease (Jia et al., 2014; Lavie et al., 2009).

Epicardial adipose tissue (EAT) is the visceral fat that is located between the
outer layer of the myocardium and visceral pericardium (Shimabukuru, 2009). In
a similar way as abdominal adipose tissue, EAT also has considerable secretory
activity. It has been reported that the increased EAT volume (commonly seen in
obesity) directly compromises myocardial metabolism (Rosito et al., 2008) and is
associated, among others, to the overexpression of pro-inflammatory TNF-α and
IL-6 (Kremen et al., 2006). Additionally, due to the proximity to coronary arteries,
EAT has been linked to increased atherosclerosis burden (Shimabukuro et al.,
2013), increasing even more the risk for cardiac structural and functional
impairments.

Alongside with vascular alterations that induced cardiac functional and structural
disarrangements, obesity also promotes fat infiltration into the myocardium
(Powell et al., 2006). Lipid over-accumulation in cardiomyocytes can result from
increased FA uptake, decreased FA oxidation or even a combination of both
(Szczepaniak et al., 2007). In obesity conditions, when adipocytes reach their
maximum storage capacity, plasma FFA concentration increases, resulting in an
ectopic accumulation of lipids in non-adipose tissues, including the myocardium
(Szczepaniak et al., 2007). Therefore, myocardial fat infiltration is associated with
several cardiac complications, including healed myocardial infarction,
arrhythmogenic cardiomyopathy, dilated cardiomyopathy and cardiomyopathy
with muscular dystrophy (Komatsu et al., 2014). In fact, this link between obesity
and the severity of myocardial disease has been demonstrated in various studies.
It has been proposed that an increased prevalence of myocardial fibrosis is
proportional to the degree of obesity and is associated with cellular degeneration
and inflammation (for ref see López-Jiménez & Cortés-Bergoderi, 2011). It is also
suggested that the longer duration of morbid obesity, the greater the left
ventricular mass and the worse the impairment of left ventricular systolic function
and diastolic filling (for ref see Szczepaniak et al., 2007). Also, the over-
accumulation of lipids in the cardiomyocytes induces steatosis of the myocardium, ultimately leading to lipotoxic cardiomyopathy (Szczepaniak et al., 2007).

2.3.3. Adipocytokine action on the heart: the particular case of leptin

Several key metabolic hormones have important central inputs besides the well known peripheral effects. Leptin is one good example of this fact, as it is known to have the ability to regulate sympathetic nervous system (Turer et al., 2012). Although the precise mechanisms by which leptin regulates autonomic nervous system (ANS) are still unclear, it is accepted that leptin plays as important role on cardiovascular regulation and that is a link between excess weight gain, increased sympathetic tone and hypertension (Bassi et al., 2015; Wang et al., 2013).

Circulatory leptin levels provide information regarding energy expenditure and amount of fat stored. This information is centrally processed by the ANS, regulating food intake and energy expenditure. Therefore, if leptin levels are elevated due to obesity, one mechanism able to increase energy expenditure is the augmentation of sympathetic tone (for ref see Barnes & McDougal, 2014). Sympathetic tone stimulation has profound impacts in cardiovascular function, including arterial blood pressure and heart rate (Correia et al., 2000). Additionally, leptin also upregulates blood pressure through increase of renal sympathetic nerve activity (for ref see Barnes & McDougal, 2014).

Additionally, Wang et al. (2013) demonstrated a relation between circulatory leptin levels and endothelial dysfunction, in which leptin enhances the effects of ang II on blood pressure. According to other studies, these effects of leptin are mediated by sympathetic nervous system activation and contribute to vascular stiffness and hypertension in obesity (for ref see Brooks et al., 2015).

2.3.4. The obese myocardium: lipotoxicity and metabolic disarrangements

Lipotoxicity can result from FA accumulation in the heart induced by excessive FA consumption. Fat infiltration in the heart leads to an alteration in substrate
preference and utilization towards FA metabolism (Hall et al., 2015). This means that the heart no longer has the ability to transit between FA metabolism to glucose metabolism, under energetically demanding conditions, which results in energy imbalance, impaired contractility, and post-translational protein modifications. This is known as a central trait of obesity-induced cardiometabolic disease (Griffin et al., 2015). This metabolic alteration is promoted by an increase in the expression of proteins involved in fatty acid oxidation, including carnitine palmitoyltransferase-1 (CPT1). As a result of this FA oxidation alteration, toxic lipids, including ceramide, are formed, contributing for cardiac dysfunction (Park et al., 2008; Slawik & Vidal-Puig, 2006).

Several studies have demonstrated that toxic lipid accumulation in myocardium, such as ceramide, is associated with an increased in the inflammatory marker TNF-α (Sharma et al., 2004) and the production of ROS leading to insulin signaling impairment, which ultimately results in cardiac IR (Murphy & Brown, 2009) and cardiac contractile dysfunction by influencing sarcoplasmic reticular calcium stores, promoting mitochondrial dysfunction and, at last, cardiomyocyte apoptosis (Listenberger et al., 2001; Turer et al., 2012).

Cardiomyocyte death, the ultimate result from myocardium fat infiltration, is recognized as a critical and direct factor in the development of heart dysfunction. Among the several molecular mechanisms associated with obesity-induced cardiac dysfunction, mitochondrial abnormalities seem to have a central role. As mitochondria integrity preservation is absolutely essential not only for maintaining metabolic homeostasis but also for survival of cardiomyocytes (Murphy & Brown, 2009), these organelles are fundamental to a proper cellular functioning. Moreover, as cardiomyocytes depend entirely on healthy mitochondria for normal heart function, mitochondria are reliable sensors of cellular homeostasis. Indeed, (dys)functional mitochondria correlates with (dys)functional cardiac tissue submitted to a variety of stimuli, including obesity. Heart mitochondrial structure and function and its relation with obesity are addressed in the following section.
2.4 The heart mitochondria

Mitochondria are essential organelles situated in the cytoplasm and associated not only with energy production, but also with ion regulation, osmotic regulation, pH control, calcium homeostasis, redox reactions, cell signaling and regulation of programmed cell death, thus assuming pivotal role in cellular function (Antonio Ascensao et al., 2011). Therefore, in the present thesis, a brief description of mitochondrial-mediated mechanisms associated with cell function will be accomplished before obesity effects on heart mitochondria.

In cardiomyocytes, there are two distinct types of mitochondria with distinct roles in cardiomyocyte physiology. Considering the energy demand of cardiomyocyte for the heart beat maintenance, the energy source is assigned by these two distinct mitochondria, depending on its final use (Pereira, 2012). There are mitochondria closer to the T tubules and to sarcoplasmic reticulum that provides energy (ATP- Adenosine Triphosphate) for the required calcium influx to the sarcoplasmic reticulum. In fact, this energy source is essential for calcium homeostasis, which is critical for the relaxation and contraction cycle. Additionally, there are also cardiac mitochondria pivotal to energy source and responsible for the release of myosin from actine and consequently for the stroke force (for ref see Pereira, 2012).

As a result of the high energy demand of the heart, a refined mitochondrial quality control is needed, this means that non-functional or damage mitochondria are removed by auto(mito)phagy and new healthy mitochondrial are formed by biogenesis. Both situations depend on fission and fusion events, and can occur in certain pathological situations. For example, very small/ fragmented mitochondria can be found in dilated cardiomyopathy and ventricular-associated congenital heart disease, or large and defective mitochondria in aged cardiomyocytes (for ref see Pereira, 2012).

Although heart mitochondria might be slightly different in morphology they both have the basics characteristics of mitochondrial from other tissues.
2.4.1. Mitochondrial structure

Mitochondria have two membranes with distinct function, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), being the space between the two membranes called inter-membrane space. These two membranes separate the cytoplasm from the mitochondrial matrix, where mitochondrial DNA (mDNA) is found. Indeed, mitochondria are the only organelles, besides cell’s nucleus, that have their own DNA (Pocock & Richards, 2006).

The OMM is highly permeable due to the presence of transmembrane channels, such as porins and protein complex known as translocase of the outer membrane (TOM), which allow the passage of proteins to the inter membrane space. (Pocock & Richards, 2006). The structure of the IMM is characterized by its folded aspect due to the presence of invaginations, formally known as cristae. These invaginations significantly increase membrane surface. In contrast to the OMM, the IMM is less permeable, which means that it is selective permeable to certain molecules depending on specific carriers (Pocock & Richards, 2006). Importantly, inserted in the inner membrane it is located the MRC pivotal for the mitochondrial energy production.

2.4.2. Mitochondrial energy production – oxidative phosphorylation

The OXPHOS is a metabolic pathway in which energy produced by oxidation/reduction reactions is used for the phosphorylation of adenosine diphosphate (ADP) to ATP (Kadenbach, 2003). The process begins with the production of reduced equivalents, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) in the Krebs cycle and beta-oxidation. The main role of these two elements is to give way electrons to the MRC (Johannsen & Ravussin, 2009).

The MRC is inserted in the inner membrane and constituted by five protein complexes (I-V) (Table 2). Some of these complexes are responsible for the transportation of protons (H⁺) from the mitochondrial matrix to the inter-membrane space, producing an electric potential that is of particular relevance for ATP producton (Δψ) (Kadenbach, 2003).
Both processes (electron transference and directional proton transportation) are intrinsically bounded. As complexes I, III and IV get oxidized (receive electrons) they pump H⁺ to the inter-membrane space, complex I pumps four H⁺, complex III also pumps four H⁺ and complex IV pumps two H⁺, resulting in the transportation of approximately ten H⁺ (Kadenbach, 2003). Although, this only happens if the first electron accepter is complex I (NADH dehidrogenase - as it receives electrons from NADH), originated in Krebs cycle, It is also possible that the first electron receptor is complex II (succinate-dehidrogenase: ubiquinone reductase - as it receives the electron pair from FADH₂) excluding complex I from the chain. If this occur, it can result in the transportation of approximately six H⁺ to the inter-membrane space, four H⁺ less than with complex I as the first point of electron entry in MRC (Johannsen & Ravussin, 2009).

The transference of electrons (oxidation/reduction – redox reactions) along the complexes (I-IV) is conducted by the decrease in their redox potential, until they reach their final acceptor, oxygen (O₂), in complex IV, forming water (H₂O) (Johannsen & Ravussin, 2009). Therefore, mitochondria produce ATP in an aerobic way, meaning in the presence of O₂.

At the end of the MRC, implicating that the electrons have already encountered O₂, the electrochemic gradient is amplified, due to the transportation of H⁺ to the inter-membrane space. This results in a returning tendency of H⁺ from the inter-membrane space to the mitochondrial matrix. Complex V (ATP- syntase) allows the protons to return to the mitochondrial matrix, originating a protomotriz force, which activates ATP synthesis (ADP+Pi) (Johannsen & Ravussin, 2009). For each four H⁺ reentering the mitochondrial matrix, one ATP will be phosphorylated, this means, in averaged, that for every electron pair provided from NADH, which results in 10 H⁺ pumped to the cytoplasm, 2,5 ATP are formed, and if the pair of electrons are provided from FADH₂ it will be formed 1,5 ATP (Hinkle, 2005).

As ATP is formed, it has to be transported to the inter-membrane space through adenine nucleotide translocase (ANT), which is also responsible for the reentry of ADP needed for ATP re-synthesis. Furthermore, the ability to ATP reach cell’s cytoplasm through the OMM is dependent of the presence of non-selective channels designated as voltage-dependent anion channel (VDAC) (Johannsen & Ravussin, 2009).
Table 2. A brief resume of the mitochondrial respiratory chain complexes

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I – NADH dehidrogenase: Ubiquinone/Coenzyme Q (CoQ) Reductase</td>
<td>First point of entry of electrons into MRC; Oxidizes NADH and reduces ubiquinol to ubiquinone; Pumps 4 H(^+) to the inter-membrane space.</td>
<td>Johannsen &amp; Ravussin (2009)</td>
</tr>
<tr>
<td>II – Succinate Dehidrogenase: Ubiquinone Reductase</td>
<td>First or second point of entry of electrons into MRC; Converses succinate to fumarate in Krebs Cycle.</td>
<td>Johannsen &amp; Ravussin (2009)</td>
</tr>
<tr>
<td>III – Ubiquinol: cytochrome-c reductase</td>
<td>Catalysis the transference of electrons from ubiquinol(oxidation) to cytochrome-c (reduction); Pumps 4 H(^+) to the inter-membrane space.</td>
<td>Johannsen &amp; Ravussin (2009)</td>
</tr>
<tr>
<td>IV – Cytochrome-c: Oxygen oxidoreductase</td>
<td>Catalysis the transference of electrons from cytochrome-c (oxidation) to O(_2) (reduction), forming H(_2)O; Pumps 2 H(^+) to the inter-membrane space.</td>
<td>Johannsen &amp; Ravussin (2009)</td>
</tr>
<tr>
<td>V – F(_0)F(_1) - ATPase</td>
<td>Uses the amplified proton gradient (protomotriz force) to synthesize ATP</td>
<td>Johannsen &amp; Ravussin (2009)</td>
</tr>
</tbody>
</table>

MRC- mitochondrial respiratory chain; NADH- nicotinamide adenine dinucleotide; O\(_2\) - oxygen; H\(_2\)O- water; ATP- adenosine triphosphate

2.4.3. Mitochondrial as source of oxidative stress

The OXPHOS in MRC is one of the major redox system in the cell and responsible for a significant amount of free radicals production (Ray et al., 2012). Free radicals are defined as any molecule with one or more unpaired electron and capable of freely exist and highly reactive in a way that they are able to “attack” any biomolecule or biological structure (Kehrer & Klotz, 2015). Free radicals are
a result of normal cellular aerobic metabolism and represent a physiological consequence of O₂ use. However, there are situations where this compound formation is deregulated, meaning that they can exist in a free form and interact with various biological structures, ultimately resulting in injury. Importantly, these molecules trigger important signaling events, both directly and through various redox-regulated transcription factors (Ray et al., 2012).

Kehrer & Klotz (2015) feature radical’s basic reaction characteristics in three categories: Initiation reactions, where the numbers of radicals increase, these reactions involve O₂ reduction; propagation reactions, involving hydrogen abstraction, electron transfer or addiction and where the number of radicals does not change; and termination reactions, resulting from the interaction of two radicals, reducing the number of radicals until they disappear.

The prevalence of oxygen in mitochondria induces oxygen-centered reactive species production, formally known as ROS (reactive oxygen species, abbreviation previously defined), which include superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (OH·), among others (Kehrer & Klotz, 2015). Besides MRC, there are other redox-related systems located in several places of the cell, including endoplasmic reticulum, peroxisomes and cytosol.

The damaging effects of ROS have been demonstrated along the years of experiments and investigation. Nowadays, there are also evidences regarding the beneficial effects of a controlled ROS production. Actually, ROS are responsible and imperative for regulating several cellular mechanisms, including mitochondrial biogenesis and apoptosis, which are pivotal processes for cell’s health maintenance, along with others cellular signaling pathways (Cadenas, 2004; Finkel, 2001). However, an additional ROS production can be implicated in several physiological and pathological conditions, including aging, DNA mutagenesis, inflammation and cell death pathways (Kehrer & Klotz, 2015). The deleterious effects of ROS in lipids, proteins and DNA are addressed below.

Lipid peroxidation has a pivotal effect in terms of ROS mediated injury, justified by lipid’s critical structural and functional role in cell’s membrane (for ref see Kehrer & Klotz, 2015). In addition, the protein oxidation is associated with age-related losses of selected biochemical and physiological functions that may be related to unrepaired damage to other macro molecules, such as DNA (Baraibar
et al., 2012). In fact, the DNA oxidation affects the integrity and regulation of genes, which may be harmful or beneficial to cells. These changes can result from direct modifications to DNA or may be due to changes in transcription factors or enzymes involved in gene expression and repair (for ref see Kehrer & Klotz, 2015). When DNA repair processes are affected, it can culminate in permanent DNA damage as it can modify DNA sequences, also known as mutations (Regulus et al., 2007).

As already mentioned, the main source of ROS in mitochondria is the MRC, more precisely, complex I, II and III (Kornfeld et al., 2015). This complexes promote \( \text{O}_2 \) reduction to \( \text{O}_2^- \) (superoxide anion), a highly reactive specie and a precursor of other ROS, such as \( \text{H}_2\text{O}_2 \) (hydrogen peroxide) and \( \text{OH}^- \) (hidroxil radical) (Ray et al., 2012). Normally, this happens in a controlled and caged form due to the presence of antioxidant molecules that are responsible for maintaining ROS production balanced. Nevertheless, there are conditions where the production of pro-oxidant molecules and antioxidant molecules is imbalanced, resulting in what is known for oxidative stress (Kehrer & Klotz, 2015). The more recent definition of oxidative stress is featured in Kehrer & Klotz (2015) work as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (for ref see Kehrer & Klotz, 2015).

Antioxidants are in permanent activity as ROS production is also permanent, as a result of aerobic metabolism. There are two different types of antioxidants, enzymatic and non enzymatic. Furthermore, they can also be classified according to their action against free radicals: scavenger antioxidants are able to turn a free radical into another less reactive free radical and quencher antioxidants able to completely neutralize free radicals (Kehrer & Klotz, 2015). Enzymatic antioxidants present in mitochondria will be addressed below.

Enzymatic antioxidant defense systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase, among others (Figure 2):

- The SOD are a family of metalloenzymes that are able to convert two superoxide anions to triplet oxygen and \( \text{H}_2\text{O}_2 \). Bearing in mind that \( \text{H}_2\text{O}_2 \) is
less reactive than O2.-, SOD’s role is simply to turn a highly reactive radical into a less reactive one. Mitochondrial SOD is manganese SOD (MnSOD) (Kehrer & Klotz, 2015).

- After superoxide dismutase, both CAT and GPx are able to cope with H2O2. CAT catalyzes the dismutation of H2O2 to water and molecular oxygen and it is essentially located in peroxisomes and its action is potentially diminished in the heart, lungs and brain, due to their reduced peroxisome presence (Kornfeld et al., 2015).

- The GPx is located in cytoplasm and in mitochondria. It catalyzes H2O2, using glutathione (GSH) as substrate. GSH transfers two H+ to H2O2, forming two water molecules. As GSH gives away electrons, it gets oxidized, becoming glutathione disulfide (GS – GS). In order to reduce GS – GS into GSH de novo, NADPH/ FADH2, from Krebs cycle, transfer electrons to GS – GS, forming GSH, this reaction is catalyzed by Glutathione reductase. Bearing in mind GPx action, as it neutralizes completely H2O2, it is acknowledged as a quencher antioxidant. GPx is present in various organs, including the heart (Herbet et al., 2015).

**Figure 2.** “Antioxidant enzyme system. The SOD convert superoxide radical into hydrogen peroxide. The CATs and GPXs convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted into the harmless product water. GPX requires several secondary enzymes (GR and G-6-PDH) and cofactors (GSH, NADPH, and glucose 6-phosphate) to function. In this scheme, GR and G-6-PDH are considered secondary antioxidant enzymes, because they do not act on ROS directly but enable GPX to function.” (adapted from Li et al., 2000).
2.4.4. Mitochondrial calcium homeostasis and apoptosis

Mitochondria are essential for the maintenance of cytosolic calcium homeostasis, as they are able to accumulate calcium through an electrochemical gradient that favors transport across the IMM (Gustafsson & Gottlieb, 2008). Mitochondrial mechanisms of calcium uptake and release have been studied along the years and include, the mitochondrial calcium uniporter (MCU) and the sodium calcium exchanger (NCX) (Carafoli, 2010). However, other pathways remain to be elucidated, such as the mitochondrial permeability transition pore (mPTP) (Bernardi & Di Lisa, 2015). Calcium homeostasis and mitochondrial permeability transition will be addressed below.

As it is already mentioned above, mitochondria play an important role in calcium homeostasis, constituting a calcium buffering system. This mitochondria role is pivotal for cell’s health since elevated calcium concentrations may be deleterious, justifying the necessity of maintaining an ideal calcium concentration in the cytoplasm. The majority of cellular calcium is stored in the endoplasmic/sarcoplasmic reticulum (ER/SR) (Gustafsson & Gottlieb, 2008), thus mitochondria positioned near calcium release sites on ER/SR are able to capture a substantial amount of calcium and to prevent the accumulation of calcium in the cytosol (for ref see Gustafsson & Gottlieb, 2008).

Mitochondrial calcium also interferes with energy metabolism. One good example is the activation of dehydrogensases in the mitochondrial matrix, culminating in increase of mitochondrial NADH/NAD ratio and leading to an increase of available energy for mitochondrial physiological processes. Furthermore, calcium present in the matrix has an effect in ATPase, boosting ATP synthesis (Balaban, 2002).

Two calcium transporters control mitochondrial calcium concentrations. The MCU is responsible for the calcium influx and NCX for calcium efflux. The first one, works in favor of the electrochemical gradient, while the second works against electrochemical gradient (Gunter et al., 2000). However, if calcium concentration in the matrix increases beyond a certain level, mitochondrial lose the ability to regulate calcium concentration, resulting in calcium overload. This condition can lead to the opening of the mPTP (Bernardi & Di Lisa, 2015; Gunter et al., 2000),
an event that have been associated with cell death, and justifies the release of proapoptotic proteins.

The mPTP is an IMM channel that is thought to be voltage and calcium dependent, cyclosporine A sensitive and conductance channel (for ref see Bernardi & Di Lisa, 2015). Besides mitochondria calcium overload, mPTP opening is facilitated through oxidative stress, mitochondrial depolarization, increased in phosphate concentrations and adenine nucleotide depletion (Bernardi et al., 2006; Rasola & Bernardi, 2011). When IMM loses its permeability (transitory permeability), allows the entrance of molecular solutes, including ions and cofactors resulting in detrimental effects that include the disruption of metabolic and ion gradients and energetic imbalance of ATP/ADP ratio between matrix and cytosol. As a consequence, mitochondrial ∆p drops and ATP hydrolyses takes place (for ref see Halestrap, 2004; Pereira, 2012). Furthermore, mPTP induces membrane depolarization, OXPHOS uncoupling, respiratory inhibition due to the loss of both co-factors and cytochrome c, and increased oxidative stress. If mPTP remains open, differences in protein concentration between cytosol and mitochondrial matrix potentiate the osmotic pressure, culminating in IMM expansion known as mitochondrial swelling, which leads to OMM disruption (Bernardi et al., 2006; Halestrap, 2004). In a situation where OMM gets ruptured, proapoptotic proteins are released, including cytochrome c, endonuclease G and apoptosis-inducing factor(AIF), resulting in cell death (Rasola & Bernardi, 2011).

The molecular nature of mPTP has been a matter of study for a long time. The multi-component composition, also known as classical model of mPTP, suggested that mPTP is formed by several proteins, including cyclophiline D (Cyp D), ANT and VDAC (Bernardi & Di Lisa, 2015). This suggests that OMM is also involved in mPTP structure as VDAC are present in the OMM. The Cyp-D is a member of cyclophilins family and has a pivotal role in mPTP regulation. It is located in mitochondrial matrix and during mPTP opening induces alterations in IMM’s channels, leading to an increase in IMM permeability (Broekemeier et al., 1989). Studies have demonstrated the existence of an interaction between Cyp-D and ANT, which can be inhibited by cyclophilin-A (CsA). In fact, CsA is one of the most studied mPTP inhibitor, as it is able to connect to Cyp-D (Galat, 1993).
However, evidences from some studies with ANT and VDAC knockout rats have called into question the truly role of these components in mPTP composition (Duchen & Szabadkai, 2010). Kokoszka et al. (2004) observed that mitochondria from ANT1 and ANT2 knockout mice still shown mPTP- CsA-sensitive activity, although triggered by an increased in calcium concentration. This suggests that ANT is not essential for pore formation but play a role in its regulation as mPTP opening is influenced by ATP and ADP concentrations (Kokoszka et al., 2004).

Recently, it has been suggested that F0 subunit from ATP synthase is also involved in mPTP opening (Bernardi & Di Lisa, 2015). In fact, several studies shown that Cyp-D binds to the F1F0 ATP synthase (for ref see Halestrap, 2014), this interaction is known to modulate mPTP formation. However, some questions remain to elucidate, which justifies the need for more research in this matter.

2.4.5. Mitochondrial quality control

Mitochondria are dynamic organelles with plastic proprieties, which means that they are capable of modifying their morphology and play important roles in maintaining cell survival, cell death and cellular metabolic homeostasis (Ni et al., 2015). These plastic properties result from a balanced interaction of fusion, fission, auto(mito)phagy and mitochondrial biogenesis events, ensuring damaged mitochondria get removed and proper organization of the mitochondrial network is maintained (Lee et al., 2004). Mitochondrial dynamics is now considered to be a pivotal cell biological process as it allows mitochondrial quality control and is fundamental for cell functioning, mPTP regulation, apoptotic signaling and, at last, for cell survival (Campello & Scorrano, 2010).

Mitochondria are responsible for cell death, including apoptotic and necrotic cell death. For this reason, mitochondria quality needs to be well controlled (Ni et al., 2015). The regulation of mitochondria quality control by several mechanisms will be addressed below with more detail.

Mitochondria’s fission and fusion events constitute one important quality control mechanism. Dysfunctional mitochondria are programmed to lose their fusion capacity by inactivating fusion and activating fission machineries. This process prevents the damaged mitochondria from incorporating back into the healthy
mitochondria (Twig et al., 2008; van der Bliek et al., 2013). Furthermore, fission and fusion are able to repair damaged components of the mitochondria. In fact, fusion events allow the exchange of portions (material) between healthy mitochondria and fission events allow the segregation of damaged mitochondria (Twig et al., 2008). Additionally, damaged mitochondria segregated by fission could be enveloped by autophagosomes and degraded in the lysosome in a process called mitophagy (Chan et al., 2011). A proper balance between fission and fusion events is possible due to the expression of various molecules responsible for regulating each process (van der Bliek et al., 2013). Although the proteins associated with the regulation of fusion and fission events are not fully understood, mitochondria fusion is mediated, at least in part, by fusion proteins mitofusin 1 (Mfn 1), Mfn 2 and optic atrophy 1 (OPA 1), whereas mitochondria fission has been described to be mediated by dynamin-related protein 1 (Drp 1), which interacts with four mitochondrial receptor proteins, fission 1 (Fis 1), mitochondria fission factor (Mff), mitochondrial dynamics protein of 49 KDa (MID49) and MID51 (for ref see Ni et al., 2015).

For instances, under oxidative stress conditions, along with other harmful events, mitochondria can be selected to mitophagy. Therefore, mitochondria-derived vesicles (MDV) are formed and damaged mitochondrial portion are expelled through fusion of MDV with lysosomes. Thereafter, within MDV, oxidized mitochondrial proteins are degraded (McLelland et al., 2014). Furthermore, mitochondrial spheroids can also be formed by damaged mitochondria through an alternative pathway for damaged mitochondria removal (Ding et al., 2012).

It is clear that mitochondria are central executioners of cell death as they release pro-cell death molecules and/or release toxic ROS. Therefore, it is absolutely necessary to remove damage mitochondria via well controlled processes, such as mitophagy, fission and fusion mechanisms or even through damaged mitochondria remodeling, forming MDV or mitochondrial spheroids (Ni et al., 2015). However, these mechanisms are not always balanced and controlled, resulting in various dramatic scenarios.

In a situation of excessive fragmentation, mitochondria suffer harmful alterations, including alterations in mitochondrial metabolism, mitochondrial membrane potential generation, in the content of complex I, IV and V subunits, excessive
DRP1-mediated mitochondrial fission and can trigger apoptotic cell death (Chan, 2006; Wang et al., 2009). Under certain pathologic conditions, excessive fission events and subsequent mitophagy can lead to an increased cell death. In fact, mitophagy can be highly increased in the myocardium to accomplish a deleterious rate of protein removal, which can culminate in the removal of vital cellular organelles and proteins, activating other proteolytic systems and inducing cell death (for ref see Smuder et al., 2013). In the majority of situations, mitophagy is triggered by energy depletion, oxidative stress, accumulation of misfolded proteins, mitochondrial depolarization and mPTP opening (Lee et al., 2012).

Cardiolipin is a phospholipid dimer and an important component of the cardiomyocyte IMM. Among several important functions under physiological conditions, cardiolipin can also act as an apoptosis inducer. Under mitochondrial damage, cardiolipin translocates to the OMM and triggers apoptosis, through the release of cytochrome c from the intermembrane space into the cytosol, leading to calcium release (Chu et al., 2013). When calcium levels reach a certain deleterious level, apoptosis is triggered. Furthermore, besides regulating apoptosis, cardiolipin is also responsible for triggering mitophagy, depending on its peroxidation status. Peroxidized cardiolipin, present in the OMM, induces cell death through apoptosis, whereas non peroxidized cardiolipin triggers mitophagy, protecting the cell from apoptosis (for ref see Ni et al., 2015).

Overall, cardiomyocytes are able to develop defense mechanisms against anomalous mitochondria before apoptotic cell death is triggered. Therefore, mitochondria have a critical role ensuring proper cardiac muscle function as they are able to self regulate and adapt to stressful situations, including obesity, modifying their own network structure and metabolism in the attempt to cell survival (Rimbaud et al., 2009).

2.5. The cardiac “obese” mitochondria

The heart is the most energetic demand organ in all body highlighting the importance of mitochondria to ensure a proper contractile function. Therefore, adequate amounts of ATP must be produced to support such energetic demands
Optimal cardiac function results from a correspondence between energy generation pathways and energy expenditure, accomplished by an adequate communication and regulation of the different metabolic pathways (Weiss & Maslov, 2004). Mostly, myocardial metabolism regulation is linked to arterial glucose substrate concentration, hormone concentration, autonomic status, coronary flow, inotropic state and nutritional status of the tissue (Stanley et al., 1997). On the other hand, obesity is known to deregulate myocardial metabolism. Bearing in mind the mechanisms by which myocardial metabolism is regulated and those cardiovascular alterations induced by obesity, it is easy to understand the relation between them. As an example, the accumulation of adipose tissue in the epicardium and consequent lipid toxicity is directly related to alterations in substrate preference and utilization towards FA metabolism (Griffin et al., 2015). Moreover, the deregulated production of adipocytokines, common in obesity, results in inflammation, vascular stiffness, increased blood pressure, over-stimulation of sympathetic tonus, among others, all leading to myocardial metabolism deregulation. The culmination of this metabolic deregulation is improper cardiac function and the loss of the heart’s ability to respond to higher energetic demanding situations, such as physical activity (Weiss & Maslov, 2004).

2.5.1. Cardiac energy pathways and sources of acetil-CoA

Fatty acids are the main substrate for cardiac mitochondrial ATP production. Mitochondrial OXOPHOS is fueled with energy from electrons that enter the MRC (detailed in sections 2.4 – 2.4.2), provided from NADH and FADH$_2$, (Weiss & Maslov, 2004). Therefore, Krebs cycle is one key step for ATP production, as it constitutes the central metabolic path for the oxidative metabolism of all nutrients. Moreover, it is characterized as a sequence of chemical reactions that produce energy through the oxidation of acetyl-CoA. The main sources of acetyl-CoA in
the heart are: lipids, thought beta-oxidation; carbohydrates, through glycolysis and subsequent pyruvate dehydrogenase (PDH) complex; and ketone bodies (Weiss & Maslov, 2004).

**2.5.2. Obesity-induced cardiac metabolic inflexibility**

Under obesity conditions, the ability to switch between fatty acid and glucose oxidation for energy production in response to alterations in substrate availability and energetic demands is impaired. This phenomenon is known as metabolic inflexibility and is thought to contribute to the development of cardiovascular disease (Crewe et al., 2013). The regulation of the various metabolic pathways is done through both the degree of expression of key metabolic proteins, such as enzymes and transporters, and complex pathway regulation controlled by allosteric regulation of enzymes and substrate/product ratio (Fell & Sauro, 1990). The process of substrate regulation is called Randle Cycle. When fatty acids are oxidized, an increase in Krebs cycle intermediate, citrate, inhibits phosphofructokinase-1 (PFK-1) which is the first intermediary and rate limiting step in glycolysis (Mor et al., 2011). PDH is also pivotal for regulating the use of glucose in relation to fatty acids use and it is regulated by several isoforms of pyruvate dehydrogenase kinase (PDK 1, 2, 3, 4) and phosphatase (PDP1 and 2). NADH and acetyl-CoA, both products of fatty acid oxidation, activate PDKs, resulting in PDH phosphorylation and inhibition (BOWKER-KINLEY et al., 1998). Therefore, when fatty acids are available, the capacity to use glucose is low (Crewe et al., 2013; Griffin et al., 2015). For instances, Crewe and coworkers (2013) have shown in mice that obesity induces an increase in PDK4 concentrations and subsequent phosphorylation and inhibition of PDH, responsible for reducing the ability of cardiac mitochondria to oxidize glucose. Furthermore, the increased production of ROS, common is obesity, also has some responsibility in metabolic inflexibility as they are capable of modulate metabolism through pos-translational alterations (Griffin et al., 2015). Besides mitochondria, peroxisomes also feature a high production of ROS in the context of metabolic inflexibility, because of their role in lipid metabolism. The chemical reactions of beta-oxidation in peroxisomes are capable of generating H$_2$O$_2$ (for ref see Griffin et al., 2015). Bearing in mind that CAT is also found in peroxisomes,
Rindler et al. (2013) showed in their study that mice fed with a high fat diet increased the expression of CAT in the heart, suggesting that excessive H$_2$O$_2$ may be implicated in metabolic inflexibility and that both peroxisomes and mitochondria cooperatively affect cellular redox state in high fat diet feeding.

The up-regulated production of adipocytokines common in obesity, is also highly related to metabolic inflexibility. Actually, leptin and adiponectin are known to regulate cardiomyocyte metabolism by increasing glucose and fatty acid uptake and oxidation (Palanivel et al., 2008). Additionally, TNF-α and IL-6 appear to impair cardiomyocyte metabolism as they inhibit PDH and MRC activity. However, over-expression of TNF-α in certain cardiac cells promote glucose oxidation by down-regulating PDK4 and reducing the expression of peroxissome - proliferator-activated receptor coactivater 1α (PGC-1α), a transcriptional co-activator of mitochondrial biogenesis and lipid metabolism genes. Therefore, taking into account these contradictory effects on cardiomyocyte metabolism, it is not yet clear how adipocytokines contribute to metabolic inflexibility (Griffin et al., 2015).

2.5.3. ROS involvement in obese mitochondrial-induced cell death

As previously mentioned, if oxidative stress is present concomitantly with alterations in cytosolic and mitochondrial calcium, the mitochondrial permeability transition (mPT) may occur with the rupture of OMM leading to the release of several apoptotic initiators usually enclosed in the intermembrane space (Ott et al., 2007).

In obesity, ROS production is amplified. Bearing in mind that obesity is related to metabolic inflexibility which signifies that ATP requirement is met by the catabolism of free fatty acids via β-oxidation, resulting in a higher ATP yield, leading to an increase in ROS production during oxidative phosphorylation (Chen & Zweier, 2014).

Among all the consequences of extensive oxidative stress in mitochondria, its involvement in apoptosis will be now clarified. As a primary target for the damaging effects of ROS, mitochondria directly influence cell viability. Therefore dependent on this impact, the cells can either repair the damage or trigger cellular
death (apoptosis) (Ligr et al., 1999). The interaction of ROS with diverse macromolecules is responsible for inducing damage and subsequent apoptosis. Mitochondrial DNA represents a critical cellular target for oxidative damage, as it is susceptible to ROS attack due to its proximity to the MRC (Ott et al., 2007). Lipid peroxidation also affects pivotal mitochondrial function including oxidative phosphorylation, IMM barrier properties, mitochondrial membrane potential, mitochondrial calcium buffering capacity (Zhang et al., 1990), and ultimately the triggering of the mPTP opening, an event profoundly connected to cell death. An example of a drastic effect of lipid peroxidation is when oxidatively modified cardiolipin losses its affinity for binding to cytochrome c, resulting in accumulation of free cytochrome c in the intermembrane space and subsequent released into the cytosol which constitutes an apoptotic stimuli (Ott et al., 2007).

The excessive production of ROS in cardiomyocytes is linked to several pathophysiological effects. In fact, the chronic exposure to ROS leads to impairment of the excitation-contraction coupling, causing arrhythmias and contributing to cardiac remodeling and fibrosis, among others potentially deathly cardiac events (Chen & Zweier, 2014).

2.6. Exercise as non-pharmacologic strategy for obesity-induce cardiac impairment

Exercise training is now considered to be a potential non-pharmacologic therapeutic strategy against several chronic diseases (Nogueira-Ferreira et al., 2016). Moreover, physical activity has been associated with the improvement of cardiac function (Ferreira et al., 2014). In fact, the beneficial effects of physical activity on the cardiovascular system are now well characterized, justifying its use as a primary prevention for cardiovascular disease. Over de last few decades, studies have support the fact that exercise induces protection against several cardiovascular diseases, including acute coronary syndromes, myocardial infarction, arrhythmia, myocardial stunning, among others (for ref see Alleman et al., 2015).

Exercise cardio-protective potential results from a multi-factorial set of systematic adaptations (morphological/biochemical alterations), such as neural, endocrine
and paracrine factors, along with autocrine signaling and adaptations of the heart itself. In a physiological context, exercise is considered as a stress stimulus that induces responses from the cells to better cope with that stressor event. Besides, it has the potential to interact with obesity-associated risk factors for cardiovascular complications, including BMI, body weight, WC, abdominal fat, blood pressure, TG levels and insulin resistance, among others (for ref see Golbidi & Laher, 2012).

In fact, evidences from animal studies using endurance running training have shown that exercise positively modulates at least the expression of 76 proteins responsible for lipid and organic metabolic processes, vasculogenesis, muscle tissue morphogenesis and tissue regeneration (Ferreira et al., 2015). Actually, one of the main characteristics of exercise induced cardiac remodeling is hypertrophy as this constitutes mechanical advantages normalizing wall stress, decreasing oxygen consumption and increasing work capacity (Frey et al., 2004). Besides the enhancement in maximal stoke, exercise is also responsible for the increase of left ventricular end-diastolic pressure along with increase in muscle mass. This adaptive cardiac hypertrophy is accompanied by angiogenesis and changes in fibrilar collagen content and organization (Boluyt et al., 2006).

Overall, exercise contributes to a better cardiovascular health, counteracting obesity-induced cardiovascular complications, as it is able to induce alterations in a structural level, but also at a molecular level. Several studies have shown that exercise is able to improve vascular endothelial dysfunction counteracting atherosclerosis, this could be due to the protective effect that exercise has against oxidative stress (Hwang & Kim, 2014). Moreover, regular exercise exerts a potent anti-inflammatory action (Sallam & Laher, 2016) in part due to the increase in energy expenditure and consequently to the reduction of body fat, which ultimately decreases the production of pro-inflammatory adipocytokines (Cancello, Henegar, et al., 2005). Besides, exercise also increases the expression of PGC-1α, an important regulator of mitochondrial biogenesis that has been associated to increase in energy expenditure (Sutherland et al., 2009). Along with all that was said above, Kawanishi et al. (2010) concluded that in obese mice, exercise training inhibited the infiltration of the inflammatory phenotype M1 macrophages while favoring the infiltration of a less inflammatory
phenotype M2 macrophages, contributing to a minor inflammatory state. Chronic exercise is also responsible for inducing the release of adiponectin from the adipose tissue (Lemos et al., 2007), which represents an anti-inflammatory molecule. However, it is important to understand that different types of exercises, differing in terms of intensity, duration and frequency may have different impacts at cardiovascular level. Epidemiological and animal studies suggest that moderate to high-intensity exercise is best for heart protection (Lee et al., 2000). Mitochondrial mediated mechanisms associated with chronic exercise-induced cardioprotection will be addressed below.

2.6.1. Chronic exercise and cardiac mitochondria adaptations

Mitochondria play a pivotal role in cell survival, being dysfunctional cardiac mitochondria deeply related to cardiac problems. Since exercise represents essential heart mitochondrial stress-induced stimuli, the resultant mitochondrial adaptations are one of the most relevant elements of exercise-induced protection against cardiovascular impairments (Alleman et al., 2015; Ascensao et al., 2006). Despite some of the mechanisms behind this phenomenon remain unclear, some are related to morphological and biochemical adaptations, including biogenesis, antioxidant production or resistance to cell death pathways (Ascensao, Ferreira et al. 2007, Kavazis, Alvarez et al. 2009, Kavazis, McClung et al. 2008). Those will be briefly addressed in the following sections

2.6.1.1. Exercise and mitochondrial bioenergetics

During exercise, as the heart energy expenditure increases, the rate of ATP production also increases along with cardiac contractility and some perturbation in redox state and in calcium homeostasis. These changes have been associated to mitochondrial remodeling induced by chronic exercise, which ultimately improve heart resistance to stress-induced stimuli, meaning a more functional and efficient heart (Antonio Ascensao et al., 2011).

Several studies with different approaches revealed exercise-induced alterations in mitochondrial bioenergetics, including parameters such as state 3 and state 4,
along with Respiratory Control Ratio (RCR- measure of dependence of the respiratory rate on oxidative phosphorylation and calculated as state 3/state 4) and ADP/O ratio of oxygen consumption in isolated mitochondria (Ascensao et al., 2007). Moreover, evidences from studies show that chronic exercise induces important metabolic adaptations on mitochondrial phosphorylative system that may result in improved ability to oxidize substrates. (for ref see Antonio Ascensao et al., 2011). In addition, under deleterious conditions as DOX treatment (Marques-Aleixo et al., 2015), diabetes (Lumini et al., 2008), aging (Golbidi et al., 2014) or high fat diet (Hafstad et al., 2013), heart mitochondria can benefit from engagement into a chronic exercise intervention, mitigating the bioenergetics impairment coupled by these pathologic conditions.

Ferreira et al. (2014) held a study in order to unveil the molecular mechanisms adjacent to the heat mitochondria beneficial effects of physical activity. They conclude that exercise training promoted mitochondria adaptations toward carbohydrate metabolism, shifting substrate utilization from fatty acids to glucose oxidation, resulting in a higher energetic efficiency and improved cardiac function. Therefore, it is possible to suggest that exercise can counteract obesity induced-metabolic inflexibility. Moreover, heart mitochondria from exercised animals showed better MRC activity and capacity, especially in chain complexes IV and V (Ferreira et al., 2014; Jiang et al., 2014) features common found to be deregulated in obese heart mitochondria.

### 2.6.1.2. Exercise and mitochondrial biogenesis

To be able to deal with stressful situations, including obesity, cardiac mitochondria should be capable of modifying their own network and structure, ensuring proper cardiac muscle function. This phenomenon is known as mitochondrial dynamics and it is characterized by balanced fusion and fission events (Chan, 2006). During pathologic conditions such as obesity, these two events are normally unbalanced in favor of fission reactions, which means that mitochondria biogenesis is down-regulated, leading to harmful effects on the heart (Liesa et al., 2009).
Evidences from animal studies have documented the beneficial effects of exercise in mitochondrial biogenesis. Tao et al. (2015) conducted a study with mice trained with a 3-week swimming protocol that were later submitted to acute myocardial infarction. Results showed an adaptive increased in mitochondrial biogenesis, justified by an increase of mitochondrial DNA replication along with activation of PGC-1α signaling in trained mice. Actually, it is now well established that physical activity increases mitochondrial content in muscles and in the heart. According to several studies, exercise induces an increase in PGC-1α protein expression, which activate nuclear respiratory factors 1 and 2 (NRF1, NRF2) and subsequent mitochondrial transcription factor A (Tfam) expression along with key mitochondrial enzymes transcription, culminating in mDNA transcription and replication (for ref see Jornayvaz & Shulman, 2010).

Given the pivotal role of mitochondrial dysfunctional in the pathogenesis of numerous diseases, including obesity, evidences provided from animal studies suggest that exercise counteracted those dysfunctions and contribute to mitochondrial biogenesis, which justify the use of exercise as a therapeutic strategy against obesity (Vettor et al., 2014; Wang et al., 2015).

2.6.1.3. Exercise and mitochondrial oxidative stress/ antioxidant capacity

Exercise and oxidative stress are linked in a very interesting way. In fact, the increased oxygen consumption resultant from higher energy expenditure during exercise naturally increases ROS production in heart MRC. This increase in ROS production after acute exercise causes undoubtedly changes in cellular redox status (LI, 1993), evidenced by alteration in antioxidant systems as well as in myocardial oxidative injury markers (Ascensao et al., 2007). However, several studies have revealed conflictive results in antioxidant enzymes production after intensive exercise, including glutathione S-transferase (GST), GPX, glutathione reductase (GR), SOD and CAT, featuring increased, decreased or unchanged activity (for ref see Ascensao et al., 2007).

On the other hand, there is growing evidence indicating that short and long duration endurance training have positive effects on gene modulation of some
enzymatic and non-enzymatic antioxidant systems (Ascensao et al., 2007). It has been suggested that regular physical activity delays the accumulation of ROS-induced cell damage by increasing the antioxidant protective mechanisms in the myocardium. More precisely, studies have shown that exercise-induced cardioprotection is related to the increased activity of MnSOD (Ferreira et al., 2015). Brown et al. (2003) demonstrated that prolonged endurance training conferred a cardioprotective effect against infarction in a region of myocardium subjected to ischemia and subsequent reperfusion, due to increased protein expression of myocardial MnSOD. Furthermore, the reduction in myocardium chronic oxidant production has also been studied. Judge et al. (2005) investigated the effects of long-term voluntary wheel running in H$_2$O$_2$ production and found a reduction in mitochondrial oxidant production. Therefore, the benefits of chronic exercise may be not only due to the increased antioxidant enzymatic system but also due to the reduction of ROS overproduction.

As obesity is strongly related to unbalanced ROS production, it is vital the understanding of how physical activity may affect oxidative stress in obesity (Huang et al., 2015). Besides, obesity also induces a pro-inflammatory state, which has also been stated to contribute to oxidative stress. Li & Reid (2000) demonstrated that TNF-α-induced skeletal muscle oxidative stress shown to be prevented by antioxidant treatment, which suggests that TNF-α may constitute an important target in correcting obesity-induced oxidative stress (Huang et al., 2015). Although the data from Li & Reid (2000) is referent to skeletal muscle, it is still relevant to this study as it takes into account the relation between obesity and oxidative stress. Additionally, several studies (Bruun et al., 2006; Johnson et al., 2007) showed that dietary caloric restriction along with aerobic exercise and anaerobic exercise promote weight loss, which is associated to improvements in oxidative stress as well as in inflammation in obesity. As an example, Oh et al. (2013) conducted a 12 weeks of moderate to high-intensity aerobic training study, demonstrating a decrease in thiobarbituric acid reactive substances, a well known lipid peroxidation byproduct and in body weight in obese individuals. Huang et al. (2015) suggested that in obese populations, exercise-induced improvements of systematic or skeletal muscle-specific oxidative stress may be
the result of intensity and/or duration of the intervention, and the weight loss may be necessary in order to modify inflammatory profiles.

However, there is a need for more studies in order to better understand these adaptive mechanisms, including against the previously mentioned cardiac oxidative stress-induced by obesity.

2.6.1.4. Exercise and mitochondrial calcium resistance and apoptosis

As it is already mentioned above, mitochondria play an important role in calcium homeostasis, constituting a pivotal role for cell’s health and survival. In case of mitochondrial calcium overload, common in several heart pathologies, mPTP may occur, resulting in loss of impermeability of the mitochondrial membranes, leading to the release of pro-apoptotic proteins, including cytochrome c and AIF, among others, and culminating in cell death (for ref see Antonio Ascensao et al., 2011).

Although there is still some controversy in this matter, reduced levels of mitochondrial-mediated apoptotic cell death markers have been observed in trained heart mitochondria, suggesting a more resistant phenotype against apoptotic stimuli. In fact, exercise is known to alter mitochondria calcium homeostasis, increasing mitochondria capacity to tolerate high calcium levels (for ref see Antonio Ascensao et al., 2011). Kowaltowski et al. (2001) proposed that the decreased in heart mitochondria ROS production as chronic adaptation to exercise may contribute to these protective effect, as increased mitochondrial oxidative stress is closely related to susceptibility to mPTP opening. Moreover, the increase in calcium retention capacity of heart mitochondria from trained rats is coincident with the lower maximal velocity of swelling observed in mitochondria of trained hearts (Kavazis et al., 2008; Marcil et al., 2006). Therefore, despite of the fact that there is still some controversy it has been accepted that exercise has a positive effect in mitochondrial calcium resistance and may help preventing apoptosis (Ferreira et al., 2015).

Accordingly, Golbidi & Laher (2011) proposed a division of beneficial mechanisms of exercise in two different categories: mechanisms that decrease oxidative stress and mechanisms of cellular repair. Among both, mitochondrial
adaptations are considered crucial in exercise-induced cardio-protection, in particular, mitochondrial calcium resistance (Kavazis et al., 2009; Kavazis et al., 2008).

Pons et al. (2013) held a study in a mouse model of obesity to understand the effects of regular exercise against myocardial infarction. Their results showed that regular exercise constitutes a powerful cardio-protective strategy. Those protective mechanisms involved cardiac mitochondrial adaptations, as it increased mitochondrial calcium retention capacity along with a better resistance to mPTP opening in obese mice. Therefore, it is accepted that regular exercise promotes beneficial effects in the heart of obese individuals, more specifically, ameliorating mitochondrial calcium resistance and cellular apoptosis prevention, which ultimately results in a protective effect against myocardial events.

2.7 Relevance of the present study
Mitochondrial dysfunction, including MRC uncoupling, increased ROS production, metabolic inflexibility related to enzymatic activity dysfunction and deficient mitochondrial content and size (mitochondria biogenesis) have been closely associated to obesity and sedentary life-style (Johannsen & Ravussin, 2009). As obesity is one of the most prevalent diseases of the century, according to World Health Organization (2015b), affecting billions of people worldwide, bringing out high costs, including economic (health care costs and job absenteeism) along with social, increasing morbidity and mortality, it is extremely important to come out with solutions to counteract this epidemic. Therefore, the relation between chronic physical activity and mitochondrial functionality in obesity is deeply relevant and worthy of investigation.

So far, a high number of animal and epidemiologic studies have been performed, revealing the benefits of regular physical activity counteracting obesity-induced overall cardiovascular complications. Based on evidences of several studies, it is suggested that a high-fat diet in both humans and mice is associated to elevated beta-oxidation and increased ROS production, among other factors, leading to mitochondrial dysfunction and cell damage. In fact, lipid overload in obese subjects compromises mitochondrial number, morphology and function (Yuzefovych et al., 2012). However, to our knowledge no studies have yet
elucidated the results of an interaction between the percentage fat consumption and exercise-induced mitochondrial adaptations.

Therefore, the relevance of the present study is mainly due to the innovator thought that different diets with different nutrient impact heart mitochondrial function differently and may differently interfere in chronic exercise-induced mitochondrial adaptations in this obesity context. The results of the present study might contribute to the understanding of whether mitochondrial adaptations to voluntary FW (as a preventive and therapeutic strategy against obesity) and to endurance Tm training (as a therapeutic strategy to mitigate obesity) may counteract the deleterious impact imposed by a high-fat diet.
3. **Aim of the present study**

The general aims of the present dissertation are:

- To compare the effects of different isocaloric diets (control with 35% energy from fat and high-fat with 71% energy from fat) on heart mitochondrial bioenergetics;
- To analyze the effects of voluntary free wheel running (as preventive strategy) and endurance treadmill training (as a therapeutic strategy) against the mitochondrial deleterious effects induced by a high-fat diet;

We can define as specific objectives of this study the analysis of the adaptations induced by both types of exercise on heart mitochondria from HFD treated animals on:

- Mitochondrial respiratory activity, before and after anoxia-reoxygenation;
- Mitochondrial electrical transmembrane potential;
- Mitochondrial respiratory chain subunits content;
- Mitochondrial lipid peroxidation and glutathione redox status.
4. Materials and methods

4.1. Animals

All experimental procedures involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS no. 123 of 18 March 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes [C (2007) 2525]. The researchers responsible for the study are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation (class C). The Ethics Committee of the Faculty of Sport, University of Porto approved the experimental protocol.

Thirty-six male Sprague-Dawley rats (aged 6 wks old) were obtained from Charles River Laboratories (L’Arbresle, France) and randomly divided into six groups (n=6 per group): Standard diet sedentary (SD+SED), standard diet treadmill endurance training (SD+Tm), standard diet free wheel voluntary physical activity (SD+FW), high-fat diet sedentary (HFD+SED), high-fat diet treadmill endurance training (HFD+Tm) and high-fat diet free wheel voluntary physical activity (HFD+FW). Only male rats were used to avoid hormone-dependent alterations. During the experimental protocol, animals were housed in collective cages (two rats per cage) and maintained in a room at normal atmosphere (21–22 °C; ~50–60% humidity) receiving water ad libitum in 12 h light/dark cycles.

4.2. Exercise and Diet protocol

The animals were fed with a standard (Lieber–DeCarli diet #710027) or with a high fat (Lieber–DeCarli diet #712031) liquid diet, different isocaloric diets (1.0 kcal/ml) purchased from Dyets Inc. (Bethlehem, USA). The standard liquid diet contained 35% of energy derived from fat, 18% from protein, and 47% from carbohydrates and the high-fat liquid diet contained 71% of energy derived from fat, 11% from carbohydrates, and 18% from protein. The quantification of food
consumption was daily obtained from graduated feeding tubes (Dyets Inc.) and calculated in accordance with manufactures instructions (1 ml = 1 Kcal).

The feeding protocol included 1 week of adaptation to the liquid diet, in which the standard diet was given to all animals, followed by 17 weeks of standard or high-fat diet according to the experimental groups (Figure 3). After the diet adaptation period, animals assigned to the free wheel voluntary physical groups (SD+FW and HFD+FW) were housed in polyethylene cages equipped with a running wheel [perimeter=1.05m, Type 304 Stainless steel (2154F0106-1284L0106) Tecniplast, Casale Litta, Italy]). The rats were allowed to exercise by having unlimited daily access to the running wheel. Running distance was recorded daily using a digital counter ECO 701 from Hengstler (Lancashire, U.K.) between 08.00 and 10.00 h.

After 8 weeks of diet consumption, the animals assigned to the sedentary groups (SD+SED and HFD+SED) remained sedentary, while the animals from treadmill endurance training groups (SD+Tm and HFD+Tm) were adapted to a LE8700 motor driven treadmill (Panlab, Harvard, USA) for 5 days at 15 m/min and 0% grade with daily increases until 30 min running was achieved. Habituation was followed by 8 weeks of endurance exercise performed 5 days/week (Monday–Friday), in the morning (between 10:00 and 12:00 AM), 60 min/day at a starting velocity of 15 m/min that was gradually increased over the training program until 25 m/min. Sedentary animals (SD+SED and HFD+SED) were placed on a non-moving treadmill 5 days/week for 60 min in order to expose the animals to the same environmental conditions without promoting any physical training adaptations.
4.3. Animal euthanasia and heart extraction

Forty-eight hours after the last Tm exercise session, non-fasted rats were anaesthetized (Ketamine 90 mg/kg and Xylazine 10 mg/kg) between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. Thereafter, the abdominal and chest cavity were opened, the organs were then perfused with NaCl 0.9% and the heart was rapidly excised, rinsed, carefully dried and weighed. Mesenteric fat deposit was excised from gastrointestinal tract nearby (from the gastroesophageal sphincter to the end of the rectum), carefully dried and weight.
4.4. Blood sampling and anatomical measurements

At 9 weeks of diet treatment, blood was collected from the tail of all animals, centrifuged (3000 rpm, for 10 min at 4 °C) and plasma was stored at -80 °C for later biochemical analysis. At the end of protocol (17 weeks) blood was collected once more but directly from the heart, centrifuged (3000 rpm, for 10 min at 4 °C) and the plasma was stored at -80 °C.

The Adiposity Index (AI) was calculated as a ratio between the sum of all visceral adipose tissue deposits and total body weight, multiplied by 100 (Novelli et al., 2007).

4.5. Isolation of heart mitochondria

Heart mitochondria were isolated using conventional methods of differential centrifugation (Bhattacharya et al., 1991). Briefly, the heart was washed and minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES (pH 7.4) and 0.1% defatted bovine serum albumin (BSA, Sigma, cat. no. A7030). The minced blood-free tissue was then resuspended in 40 mL of isolation medium containing 0.75 mg/mL protease Subtilisin A Type III (Sigma P5380) and homogenized with a tightly fitted “Potter-Elvejhem” glass homogenizer. The suspension was incubated for 1 min (4°C) and re-homogenized. The homogenate was then centrifuged at 13000 xg for 10 min, the resulting supernatants were decanted and the pellet, essentially free of protease, was gently re-suspended with a loose-fitting homogenizer. The suspension was centrifuged at 750 xg for 10 min and the resulting supernatant was centrifuged at 12000 xg for 10 min. The pellet was re-suspended in washing medium and centrifuged at 1200 xg for 10 min. The pellet was gently resuspended to obtain the final mitochondrial suspension. EGTA and defatted BSA were omitted on the final washing medium. All the procedures were performed within at 4 °C. Mitochondrial protein content was determined by the Biuret method using bovine serum albumin as standard (AG et al., 1949).
4.6. Mitochondrial respiratory activity and anoxia-reoxygenation procedure

Mitochondrial respiratory function was measured polarographically at 30ºC using a Biological Oxygen Monitor System (Hansatech Instruments, Norfolk, UK) and a Clark type oxygen electrode (Hansatech DW1, Norfolk, UK). Reactions were conducted in a 0.75 mL closed, thermostatted and magnetically stirred glass chamber containing 0.5 mg/mL of heart mitochondrial protein in a respiration buffer (50 mM KCl, 130 mM sucrose, 2.5 mM KH2PO4, and 0.5 mM HEPES, pH 7.4).

After 1 min equilibration period, mitochondrial respiration was initiated by adding glutamate/malate (G/M) to a final concentration of 5 and 2.5 mM, respectively. State 3 respiration was determined after adding ADP (150 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. Thereafter, anoxia was induced by adding an ADP pulse (1250 nmol). The anoxia period (1 min) was followed by 4 min of reoxygenation by exposing the mitochondria medium to the air (open chamber). After this period, the polarographic chamber was closed again and respiratory rates were measured once more (states 2, 3 and 4) through the addition of another ADP pulse (150 nmol/mg) (Figure 4). The respiratory control ratio (RCR) was calculated as the ratio between state 3/state 4, while the ADP/O was calculated as the number of nmol ADP phosphorylated by natom O2 consumed (Estabrook, 1967). Mitochondrial suspensions were collected and frozen (-80ºC) for lipid peroxidation measurements after anoxia-reoxygenation.
4.7. Mitochondrial transmembrane electrical potential

Mitochondrial transmembrane electrical potential ($\Delta\psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP+) using a TPP+ selective electrode prepared in our laboratory as previously described by A. Ascensao et al. (2011) and according to Kamo et al. (1979). No correction for the passive binding of TPP+ to mitochondrial membranes was performed, since the purpose of this study was to show relative and not absolute $\Delta\psi$ values. As a consequence, a slight overestimation of the $\Delta\psi$ values is anticipated. The $\Delta\psi$ was estimated from the following equation (at 30°C): $\Delta\psi = 59 \times \log (v/V) - 59 \times \log (10 \Delta E/59 - 1)$, where $v$, $V$, and $\Delta E$ stand for mitochondrial volume, incubation medium volume, and deflection of the electrode potential from the baseline, respectively.

Mitochondrial transmembrane electric potential were carried out at 30 °C in 1 mL of heart reaction buffer (50 mM KCl, 130 mM sucrose, 2.5 mM KH2PO4, and 0.5 mM HEPESpH 7.4), supplemented with 3 µM TPP+ and 0.5 mg/mL (heart) of...
mitochondrial protein. For the measurements of Δψ with complex I-linked substrates, energization was carried out with G/M (5 mM and 2.5 mM, respectively) and ADP (150 nmol) was used to produce a phosphorylation cycle. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured during the experiments. Mitochondrial suspensions were collected and frozen (-80°C) for lipid peroxidation measurements.

4.8. Western-Blotting

Heart mitochondria protein content was semi-quantified by western blotting. Samples were denatured in a lysis buffer and separated by dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) as described by Laemmli (1970). This procedure was followed by the transfer of proteins to a PVDF membrane (GE Healthcare, RPN303F) according to (Locke et al., 1990). After blotting, membranes were blocked with 5% (w/v) of non-fat dried milk or BSA in Tris-buffered saline with tween 20 (TBS-T). Membranes were then incubated with anti-ANT2/3 (1:100 dilution, goat polyclonal, Santa Cruz Sc-70205), OXPHOS antibody cocktail (1:1000 dilution, mouse monoclonal, MitoSciences/Abcam MS604). Primary antibodies were diluted in TBS-T containing 2% (w/v) of non-fat dried milk or BSA according to the manufacturer instructions. After the incubation period, membranes were washed for three times with TBS-T and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-goat (1:5000 dilution, Santa Cruz Sc2005 or Sc2354). Immunoreactive bands were visualized using ECL chemiluminescence reagents (GE Healthcare, Amersham RPN2232) and acquired by Chemidoc™-XRS image system (BioRad). Images were analyzed with Image Lab™ software 4.0 (BioRad). The Ponceau S staining was used to correct differences in protein loading or transfer, and the final data was expressed as the percentage variation of the control values (%SD+SED).

4.9. Lipid peroxidation

Before analysis, heart mitochondrial membranes of isolated mitochondrial fractions were disrupted by a combination of freeze–thawing cycles to allow free access to substrates. The extent of lipid peroxidation in heart, mitochondria was
determined by measuring MDA contents through a colorimetric assay, according to a modified procedure described previously (Buege & Aust, 1978). Suspended mitochondria (2 mg/mL) were centrifuged at 12000 xg for 10 min and re-suspended in 150 µL of a medium containing 175 mM KCl and 10 mM Tris-HCl, pH 7.4. Subsequently, mitochondria from the six groups were mixed with 2 volumes of trichloroacetic acid (10 %) and 2 volumes of thiobarbituric acid (1%). The mixtures were heated at 90 °C for 10 min, cooled in ice for 10 min before centrifugation (4000 xg for 10 min, 4 °C). The supernatants were collected and the absorbance measured at 535 nm. The amount of MDA content formed was expressed as nanomoles of MDA per milligram of protein (ε535=1.56 x 10−5 M−1 cm−1).

4.10. Gluthathione quantification

Glutathione activity in the heart was measured using Tietze (1969) modified method. For the samples preparation it was adding equal volume of perchloric acid (PCA) 10% to the homogenized samples and centrifuged at 13000 rpm for 2 min at 4 °C. The resulting supernatants were used for the total glutathione and oxidized glutathione assays.

For the determinations of total glutathione (GS), the samples, previously prepared were neutralized with potassium bicarbonate, centrifuged (1300 rpm during 1 min at 4°C) and supernatants transferred into a 96-well microplate. Proper blanks were achieved for each experiment, receiving the same treatment as samples. The next step is the incubation with NADPH solution in the microplate reader (15 min at 30 ºC). Finally glutathione reductase was added and the reaction kinetics was read in the microplate reader at 405 nm for 3 min.

For the determination of oxidize glutathione (GSSG), the samples previously prepared were incubated for 1h at 4°C with 2-vinilprinidine and neutralized with potassium bicarbonate. The samples were centrifuged (1300 rpm during 1 min at 4°C) and supernatants transferred into a 96-well microplate. Proper blanks were achieved for each experiment, receiving the same treatment as samples. Sulphydil reagent 5,52-dithio-bis (2-nitrobenzoic acid) (DTNB) was added, followed by 15 min at 30°C incubation period. Finally, glutathione reductase was
added and the reaction kinetics was read in the microplate reader at 405 nm for 3 min. GSH content was calculated by subtracting the double of GSSG from total GS content. Glutathione ratio was calculated by dividing GSH by GSSG. Protein content from heart homogenates were spectrophotometrically determined by using the Bradford method using bovine serum albumin as standard (Bradford, 1976).
5. Results

5.1. Food consumption and exercise interventions

Figure 5A represents the food consumption from all six groups since the beginning of the protocol (week 1) until the end of the protocol (week 17). The running distance on the Tm, during 8 weeks (HFD+Tm, SD+Tm) and on the FW, from the beginning to the end of the protocol (HFD+FW, SD+FW) is presented in Figure 5B. No differences were found in energy intake, free wheel running and treadmill running distances between groups. These data are valuable to better analyze and understand the majority of the results.

Figure 5. Food consumption (A), running distance in treadmill (Tm) and free wheel (FW) per day (B). Data are Mean ± SEM (n=10). Significant (p < 0.05) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT x ET) or the absence of differences (NS) is shown (adapted from Goncalves et al., 2015)
5.2. Animal characterization

Body weight, mesenteric fat, absolute and relative heart weights, heart mitochondrial protein yielding of all animal groups were measured and are shown in table 3.

After 17 weeks of HFD treatment, sedentary animals featured a significant increase in mesenteric fat and in Adiposity Index (AI) compared to SD counterparts, whereas, no differences were found in body weight and heart-to-body weight ratio between sedentary HFD and SD. Both types of physical activity successfully diminished mesenteric fat and AI not only in SD but also in HFD treated animals (SD+FW and SD+Tm vs. SD+SED; HFD+FW and HFD+Tm vs. HFD+SED). In SD treated animals, both exercise regimens induced decrease in body weight compared to their sedentary counterparts (SD+FW and SD+Tm vs. SD+SED); however, in HFD treated animals only Tm training was able to decrease body weight compared with Sedentary and FW running (HFD+Tm vs. HFD+SED and HFD+FW). Exercised animals fed with a SD showed a lower body weight than the respective groups fed with a HFD (SD+FW vs. HFD+FW and SD+Tm vs. HFD+Tm).

Although we did not find differences in heart weight among the different studied groups, it is possible to observe that both exercise modes increased heart-to-body weight ratio in SD treated animals (SD+Tm and SD+FW vs SD+SED). In HFD fed mice, only endurance Tm running intervention was able to induce heart hypertrophy (HFD+Tm vs. HFD+SED). Additionally, independently of the diet treatment, animals submitted to the Tm training showed an increased heart-to-body weight ratio compared with their FW counterparts (SD+Tm vs. SD+FW; HFD+Tm vs. HFD+FW). No significant differences were found in mitochondrial yielding neither in isolated mitochondrial protein.
### Table 3. Anatomic characterization of animals

<table>
<thead>
<tr>
<th></th>
<th>SD+SED</th>
<th>SD+FW</th>
<th>SD+Tm</th>
<th>HFD+SED</th>
<th>HFD+FW</th>
<th>HFD+Tm</th>
<th>p^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>676.8±11.5</td>
<td>612.7±15.3^a</td>
<td>559.3±10.0^a^b</td>
<td>684.3±14.2</td>
<td>710.0±10.8^b</td>
<td>592.8±7.3^A^B^C</td>
<td>DT×ET</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.8±0.00</td>
<td>1.9±0.1</td>
<td>1.9±0.1</td>
<td>1.8±0.0</td>
<td>1.8±0.1</td>
<td>1.9±0.0</td>
<td>NS</td>
</tr>
<tr>
<td>Heart / body weight (mg g⁻¹)</td>
<td>30.0±0.1</td>
<td>32.0±0.2^a</td>
<td>36.0±0.1^a^b</td>
<td>26.0±0.1</td>
<td>28.0±0.1^b</td>
<td>32.0±0.1^A^C</td>
<td>ET</td>
</tr>
<tr>
<td>Mitochondrial protein (mg ml⁻¹)</td>
<td>25.8±2.1</td>
<td>27.1±1.2</td>
<td>28.2±2.6</td>
<td>30.8±1.2</td>
<td>33.7±1.6</td>
<td>28.7±1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Heart mitochondrial isolation yield (mg protein/g tissue)</td>
<td>15.5±1.3</td>
<td>14.1±0.9</td>
<td>14.4±0.7</td>
<td>17.5±0.8</td>
<td>19.1±1.6</td>
<td>15.1±1.1</td>
<td>DT</td>
</tr>
<tr>
<td>Mesenteric fat (g)</td>
<td>10.4±0.3</td>
<td>7.8±0.8^a</td>
<td>3.6±0.6^a^b</td>
<td>15.7±0.8^a</td>
<td>11.7±0.8^A^B</td>
<td>5.5±0.2^A^B^C</td>
<td>DT×ET</td>
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<tr>
<td>Adiposity Index %</td>
<td>9.0±0.2</td>
<td>7.5±0.5^a</td>
<td>4.8±0.5^a^b</td>
<td>10.8±0.4^a</td>
<td>8.9±0.4^A^B</td>
<td>6.8±0.1^A^B^C</td>
<td>DT,ET</td>
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</table>

Data are means ± SEM, (n=10). Different letters (symbols) indicates significant differences between groups: SD+SED – standard diet sedentary; SD+FW – standard diet free wheel running; SD+Tm – standard diet treadmill running (endurance training); HFD+SED – high fat diet sedentary; HFD+FW – high fat diet free wheel running; HFD+Tm – high fat diet treadmill running (endurance training). (p<0.05) (adapted from Goncalves et al., 2015).

^a vs. SD+SED; ^A vs. HFD+SED; ^b vs. SD+FW; ^B vs. HFD+FW; ^c vs. STm ^d Significant (p<0.05) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT×ET) or the absence of differences (NS) is shown.
As it is presented in Table 4, no relevant differences were found between Tm and SED groups at 9 week in the analyzed blood parameters (SD+SED vs. SD+Tm and HFD+SED vs. HFD+Tm). After 9 weeks of HFD treatment, sedentary animals showed a higher content of cholesterol and HDL and a lower content of VLDL and triglycerides, compared to SD treated animals (SD+SED vs. HFD+SED). FW exercise influenced the content of triglycerides and cholesterol in SD animals (SD+FW vs SD+SED) and in cholesterol and VLDL in HFD animals (HFD+FW vs. HFD+SED), when compared to their SED counterparts.

Blood analysis after 17 weeks of HFD sedentary animals showed increased HDL and decreased VLDL and triglycerides plasma levels (SD+SED vs. HFD+SED). Both exercise regimens decreased triglycerides plasma levels in SD treated animals (SD+FW and SD+Tm vs. SD+SED) and only Tm running was able to decrease cholesterol levels (SD+Tm vs. SD+SED). Exercise did not influenced blood parameters in HFD groups (HFD+FW and HFD+Tm vs. HFD+SED). In SD, Tm running animals featured high levels of triglycerides and VLDL than the FW running animals (SD+Tm vs. SD+FW), whereas in HFD, FW group presented an increased cholesterol levels than theirs Tm counterparts (HFD+FW vs. HFD+Tm).
Table 4. Blood analysis after 9 and 17 weeks of treatments

<table>
<thead>
<tr>
<th>Timing</th>
<th>SD+SED</th>
<th>SD+FW</th>
<th>SD+Tm</th>
<th>HFD+SED</th>
<th>HFD+FW</th>
<th>HFD+Tm</th>
<th>p&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td><strong>Glucose (g/L)</strong></td>
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<tr>
<td>9 week</td>
<td>113.00±1.81</td>
<td>106.00±4.45</td>
<td>111.20±2.06</td>
<td>118.20±2.65</td>
<td>125.25±0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>119.10±2.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DTxET</td>
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<tr>
<td>17 week</td>
<td>228.00±18.68</td>
<td>217.00±15.59</td>
<td>246.20±12.79</td>
<td>248.30±6.90</td>
<td>235.00±12.51</td>
<td>212.10±21.27</td>
<td>NS</td>
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<tr>
<td><strong>Triglycerides(g/L)</strong></td>
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<tr>
<td>9 week</td>
<td>41.40±1.65</td>
<td>30.00±1.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.20±0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.20±1.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.00±1.27</td>
<td>26.43±1.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DTxET</td>
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<tr>
<td>17 week</td>
<td>106.50±15.58</td>
<td>57.50±5.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.50±9.30&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>30.75±2.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.20±6.99</td>
<td>22.86±2.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DTxET</td>
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<td><strong>Cholesterol (g/L)</strong></td>
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<tr>
<td>9 week</td>
<td>60.50±2.65</td>
<td>47.67±2.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.34±3.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.75±2.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.51±2.07&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>76.90±2.21&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>DT,ET</td>
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<tr>
<td>17 week</td>
<td>69.00±2.41</td>
<td>58.33±2.46</td>
<td>51.40±3.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.51±4.14</td>
<td>88.80±8.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.66±3.93&lt;sup&gt;c,b&lt;/sup&gt;</td>
<td>DT,ET</td>
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<tr>
<td><strong>HDL (g/L)</strong></td>
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<tr>
<td>9 week</td>
<td>36.60±1.28</td>
<td>32.45±0.49</td>
<td>37.00±1.52</td>
<td>52.00±1.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.76±2.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.86±1.10&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>17 week</td>
<td>35.00±2.05</td>
<td>38.72±2.05</td>
<td>34.36±1.02</td>
<td>55.50±3.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.20±4.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.41±2.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DT</td>
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<td><strong>VLDL (g/L)</strong></td>
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<tr>
<td>9 week</td>
<td>8.28±0.33</td>
<td>7.45±0.32</td>
<td>9.76±0.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.44±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.35±0.21&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.14±0.55&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>DTxET</td>
</tr>
<tr>
<td>17 week</td>
<td>17.28±1.78</td>
<td>13.13±0.51</td>
<td>19.07±0.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.15±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.04±1.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.57±0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DT</td>
</tr>
</tbody>
</table>

Data are means ± SEM, (n=10). Different letters indicates significant differences between groups: SD+SED – standard diet sedentary; SD+FW – standard diet free wheel running; SD+Tm – standard diet treadmill running (endurance training); HFD+SED – high fat diet sedentary; HFD+FW – high fat diet free wheel running; HFD+Tm – high fat diet treadmill running (endurance training). (p<0.05). HDL, high-density lipoprotein; VLDL, very low-density lipoprotein (adapted from Goncalves et al., 2015)

<sup>a</sup> vs. SD+SED; <sup>A</sup> vs. HFD+SED; <sup>b</sup> vs. SD+FW; <sup>B</sup> vs. HFD+FW, <sup>c</sup> vs. STm <sup>d</sup> Significant (p<0.05) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DTxET) or the absence of differences (NS) is shown.
5.3 Heart mitochondria oxygen consumption and transmembrane electric potential

Mitochondrial oxygen consumption and $\Delta \psi$ were measured to identify exercise and diet-dependent effects. The results are shown in Figure 6 and Figure 7.

No differences were found between diet treatment in mitochondria oxygen consumption neither in transmembrane electric potential parameters (SD+SED vs. HFD+ SED). In both diet treatments, Tm running significantly increased the coupling between oxygen consumption and ADP phosphorylation (RCR) compared to their SED counterparts (Figure.6C; SD+Tm vs. SD+SED, HFD+Tm vs. HFD+SED). Moreover, in SD treated animals Tm running significantly decreased heart mitochondrial respiratory rate in state 4 and increased RCR value when compared to FW animals (Figure 6B and 6C; SD+Tm vs. SD+FW). No significant differences were found between groups in state 3 mitochondrial respiratory rate neither in ration between ADP phosphorylated and oxygen consumption (ADP/O ratio).
Figure 6. Effect of diet and exercise treatments on heart mitochondrial oxygen consumption (A) state 3 of mitochondrial respiration, (B) state 4 of mitochondrial respiration, (C) RCR, and (D) ADP/O. Data are means ± SEM obtained from different mitochondrial preparations (0.5 mg/mL protein) for each group. Respiration was measured with G/M as substrates. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by natom of oxygen consumed. SD + SED — standard diet sedentary, SD +FW — standard diet free wheel, SD+Tm — standard diet treadmill, HFD+SED — high fat diet sedentary, HFD+FW — high fat diet free wheel, and HFD +Tm— high fat diet treadmill, n=10 per group. *SD+TM or SD+FW vs. SD+SED and HFD+TM or HFD+FW vs. HFD+SED (p ≤ 0.05); ‡SD+Tm vs. SD+FW or HFD+Tm vs. HFD+FW (p ≤ 0.05). Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E, D) or their interaction (E x D) are indicated. Non Significant (NS), p > 0.05.
As seen in Figure 7, no significant differences were found between diet treatment and exercise treatment in maximal energization (Δψ), ADP-induced depolarization (Δψ), ADP-induced repolarization (Δψ) and in ADP lag-phase.

**Figure 7.** Effect of diet and exercise treatments on heart mitochondria Δψ fluctuations (A) maximal energization, (B) ADP-induced depolarization, (C) ADP-induced repolarization and (D) ADP phosphorylation lag phase. Data are means ± SEM obtained from different mitochondrial preparations (0.5 mg/mL protein) for each group. Δψ developed was measured with G/M as substrates. Lag phase, time elapsed in the depolarization/repolarization cycle during ADP phosphorylation. SD + SED — standard diet sedentary, SD + FW — standard diet free wheel, SD + Tm — standard diet treadmill, HFD + SED — high fat diet sedentary, HFD + FW — high fat diet free wheel, and HFD + Tm — high fat diet treadmill, n=10 per group. Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E × D) or their interaction (E × D) are indicated. Non Significant (NS), p > 0.05.
5.4 Heart mitochondrial oxygen consumption in Anoxia-Reoxygenation

The results of heart mitochondrial oxygen consumption obtained after the *in vitro* anoxia-reoxygenation (A-R) experiments are shown in Figure 8. Oxygen consumption rates (state 3 and state 4), RCR and ADP/O were calculated in post-reoxygenation conditions. As seen in Figure 8, no statistically differences were found between diets (SD+SED vs. HFD+SED). In SD treated groups, Tm running significantly decreased heart mitochondrial state 4 respiratory rate and increased ADP/O after A-R (Figure 8B and 8D; SD+Tm vs. SD+SED). In HFD treated animals, Tm running significantly increased RCR (Figure 8C; HFD+Tm vs. HFD+SED). No significant differences were found between exercise groups from each diet. Neither exercise nor diet produced any effect on state 3.

Despite the differences found, is important to note that all oxygen consumption parameters before and after exposed heart mitochondria fractions to the deleterious anoxia-reoxygenation stimuli were significantly compromised independently of the diet treatment or exercise engagement (data non showed).
Figure 8. Effect of diet and exercise treatments on heart mitochondria after Anoxia-Reoxygenation stimulation (A) state 3 of mitochondrial respiration, (B) state 4 of mitochondrial respiration, (C) RCR, and (D) ADP/O. Data are means ± SEM obtained from different mitochondrial preparations (0.5 mg/mL protein) for each group. Respiration was measured with G/M as substrates. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by 1 atom of oxygen consumed. SD+SED — standard diet sedentary, SD+FW — standard diet free wheel, SD+Tm — standard diet treadmill, HFD+SED — high fat diet sedentary, HFD+FW — high fat diet free wheel, and HFD+Tm — high fat diet treadmill, n=10 per group. *SD+TM or SD+FW vs. SD+SED and HFD+TM or HFD+FW vs. HFD+SED (p ≤ 0.05). Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E, D) or their interaction (E x D) are indicated. Non Significant (NS), p > 0.05.
5.5 OXPHOS protein semi-quantification

The effects of diet and/or exercise on OXPHOS levels were evaluated by Western Blotting. As seen in Figure 9, no differences were found between diet treatments in complex I, II and IV content; however, HFD treatment significantly decreased complex V content compared to SD treated animals (Figure 9D; SD+SED vs. HFD+SED). In SD treated animals, FW running significantly increased complex IV and complex V content when compared to their SED and Tm counterparts (Figure 9C and 9D; SD+FW vs SD+SED and SD+Tm). Interestingly, also in SD, Tm running animals showed a significantly decrease in complex V content compared to their SED counterparts (Figure 9D; SD+Tm vs. SD+SED).

In HFD treatment, both Tm and FW were able to significantly increase complex IV and complex V content (HFD+Tm and HFD+FW vs. HFD+SED). In HFD treatment, FW running significantly increased complex V content when compared to their Tm counterparts (Figure 9D; HFD+FW vs. HFD+Tm). Additionally, Tm running induced higher values of complex IV and complex V in HFD animals when compared to SD counterparts (Figure 9C and 9D; HFD+Tm vs. SD+Tm). No effects of exercise or diet were found in complex I and in complex II content.
**Figure 9.** Effect of exercise and diet treatment on heart mitochondria’s respiratory complexes (OXPHOS): (A) Complex I; (B) Complex II; (C) Complex IV; (D) Complex V; (E) Protein loading control by Ponseau-S staining; (F) Typical immunoblots. Data are mean ± SEM. SD + SED—standard diet sedentary, SD + FW—standard diet free wheel, SD + Tm—standard diet treadmill, HFD + SED—high fat diet sedentary, HFD + FW—high fat diet free wheel, and HFD + Tm—high fat diet treadmill, n=8 per group. *SD+TM or SD+FW vs. SD+SED and HFD+TM or HFD+FW vs. HFD+SED (p ≤ 0.05); #SD+Tm vs. SD+FW or HFD+TM vs. HFD+FW (p ≤ 0.05); ¤SD+SED vs. HFD+SED or SD+FW vs. HFD+FW or SD+Tm vs HFD+Tm (p≤0.05). Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E, D) or their interaction (E x D) are indicated. Non Significant (NS), p >0.05.
5.3 Glutathione and lipid peroxidation measurements

Mitochondrial lipid peroxide was measured to identify whether exercise and diet treatments modulate mitochondrial oxidative stress marker before and after A-R. As seen in Figure 10, no differences were found between diet treatments (SD+SED vs. HFD+SED). Only MDA level were significantly higher in HFD treated animals engaged to Tm running compared to their SD counterparts (Figure 10A; HFD+Tm vs. SD+Tm). This difference was not found after A-R. No other differences were found between exercise regiments and diets in MDA levels and neither in MDA levels of A-R. Despite the differences found, is important to note that MDA content before and after exposed heart mitochondria fractions to the deleterious anoxia-reoxygenation stimulus was significantly increased independently of the diet treatment or exercise engagement (data non showed).

Figure 10. Effect of exercise and diet treatment on heart mitochondria’s (A) MDA content and (B) MDA content in Anoxia-Reoxygenation. Data are means ± SEM obtained from different mitochondrial preparations (2 mg/mL protein) for each group. Data are mean ± SEM. SD + SED — standard diet sedentary, SD +FW — standard diet free wheel, SD+Tm — standard diet treadmill, HFD+SED — high fat diet sedentary, HFD+FW — high fat diet free wheel, and HFD +Tm— high fat diet treadmill, n=10 per group. aHFD+Tm vs.SD+Tm or HFD+FW vs. SD+ FW (p ≤ 0.05). Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E, D) or their interaction (E x D) are indicated. Non Significant (NS), p > 0.05.
The effects of diet and/or exercise on non-enzymatic antioxidant machinery were evaluated by measuring glutathione redox status. As seen in Figure 11, in sedentary groups no significant differences were found between diets neither in reduced glutathione, in oxidized glutathione levels nor GSH/GSSG ratio (SD+SED vs. HFD+SED). No significant differences were found between exercise and sedentary groups in SD treatment. In HFD treated animals, FW running significantly increased reduced glutathione compared to their Tm counterparts (Figure 11A; HFD+FW vs HFD+Tm) and decreased oxidized glutathione levels when compared to their SED counterparts (Figure 11B; HFD+FW vs. HFD+SED). HFD+FW running significantly increased GSH/GSSG ratio when compared to Tm and SED animals (HFD+FW vs. HFD+SED and HFD+Tm Figure 11C).

**Figure 11.** Effect of exercise and diet treatment on heart mitochondria's: (A) Reduced Glutathione content (GSH); (B) Oxidized Glutathione content (GSSG) and (C) Glutathione Ratio: GSH/GSSG. Data are mean ± SEM. SD + SED —standard diet sedentary, SD +FW — standard diet free wheel, SD+Tm — standard diet treadmill, HFD+SED — high fat diet sedentary, HFD+FW — high fat diet free wheel, and HFD+Tm— high fat diet treadmill, n=10 per group. *SD+TM or SD+FW vs. SD+SED and HFD+TM or HFD+FW vs. HFD+SED (p ≤ 0.05); ²SD+TM vs. SD+FW or HFD+TM vs. HFD+FW (p ≤ 0.05); ³HFD+TM vs.SD+TM or HFD+FW vs. SD+ FW (p ≤ 0.05). Significant (p ≤ 0.05) effects of exercise treatment (E), diet treatment (D), exercise treatment and diet treatment (E, D) or their interaction (E x D) are indicated. Non Significant (NS), p >0.05.
6. Discussion

The present study provided support in order to understand the effects of two different exercise modalities (voluntary wheel running and endurance treadmill training) on mitochondrial function of rats fed with a high-fat diet.

The study sample was constituted only by male rats, as female rats were excluded due of the reported interaction between hormonal state and mitochondrial ROS production (Giglia & Bussmann, 2001). For instances, Borrás et al. (2003) showed that the expression and activity of various mitochondrial antioxidant enzymes, including SODs and GPx is higher in female rats. Two years later, Borrás et al. (2005) demonstrated an up regulation of Mn-SOD and GPx induced by low doses of oestradiol, supporting the idea of a protecting effect of the female hormonal phenotype against oxidative stress.

6.1. Diet Protocol

As a result of the increasing prevalence of obesity worldwide, along with its associated cardiovascular complications, the development of obese animal models have been essential to better understand the physiological mechanisms behind this epidemic, as well as the most effective strategies to prevent and counteract obesity induced-complications. Currently, in obesity research, scientists are resorting to obesity animal models, frequently rats. Besides genetic, obesity can also be induced by nutritional diets. Sprague-Dawley rats are highly sensitive to HFD compared to other common rat strains (Goyal et al., 2012).

Isocaloric models differ from hypercaloric models because the diet composition is the truly obesity inducer (Goyal et al., 2012). The preference for this diet model in obesity research is resultant from scientific evidences showing that not only the quantity but also diet composition is responsible for inducing an obese phenotype and the consequent obesity constrains (Estrany et al., 2011; Mann, 2002). The two most used isocaloric models are HFD or high carbohydrate diets. The present study used a HFD supported by evidences that associate this type of diet with an imbalance of energy homeostasis, ultimately leading to increases in body fat deposition, cardiovascular complications and type 2 diabetes (Grundy, 1999). Moreover, obesity and diabetes are frequently associated with nonalcoholic fatty
liver diseases, which include nonalcoholic steatohepatitis (NASH). The isocaloric diet model used in the present study was developed by Lieber et al. (2004), with the purpose of inducing obesity and NASH in animals, a protocol also used by others from our group (Goncalves et al., 2015). Since this diet protocol was created with the main purpose of inducing NASH, the SD was developed to be a control diet for NASH. Moreover, NASH is also related with an increased prevalence for developing cardiovascular complications (Bhatia et al., 2012; Lautamaki et al., 2006), justifying the use of HFD-induce NASH in the context of the present study.

Generally, independently of the obesity-induced nutritional model, the variety of diet protocols is alarming, which leads to some controversial results and makes almost impossible the comparison between studies. This methodological issue is elegantly presented by Warden & Fisler (2008). In theory, the control diet should correspond to a normal/healthy diet of the specie in study. But even if the diet protocol is perfectly controlled and adequate it is essential to question whether rats and humans have the same physiological responses to dietary changes. In fact, Borghjid & Feinman (2012) conducted a study with high-fat, no-carbohydrate diet (80% of fat and 0% of CH) in mice. The results showed that experimental mice developed fatty heart, abdominal adiposity, metabolic disruption and greater weight gain than the isocaloric control diet.

A healthy diet for human adults is composed by 45% to 65% of energy provided from carbohydrates, 20% to 35% of energy provided from fat and 10% to 35% of energy provided from protein (Institute of Medicine, 2001). The SD used in the present study (Lieber et al., 2004), with 47% of energy provided from carbohydrate, 35% of energy provided from fat and 18% of energy provided from protein is very similar to a healthy diet for human adults in accordance to the Institute of Medicine (2001). However, in accordance to the American Institute of Nutrition, a healthy diet for rats is composed by approximately 57% of energy provided from carbohydrates, 13% of energy provided from fat and 30% of energy provided from protein (Reeves PG et al., 1993). Interestingly, there are several studies that use control diets with less fat percentage (Calligaris et al., 2013; Kang et al., 2015) in diet-induced obesity studies. Therefore, the composition of healthy diets in rodents differs from a normal/healthy human diet, mostly presenting lower
fat percentage. As it can be depicted in table 5, the composition of the normal/healthy diets that can be purchased in a few companies specialized in rodents laboratory diets are different from a healthy human diet.

**Table 5.** Percentage of the calories provided by carbohydrates; fat and proteins in some typical commercial diets for rodents.

<table>
<thead>
<tr>
<th>Company</th>
<th>Carbohydrates (%)</th>
<th>Fat (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>72.4</td>
<td>8.4</td>
<td>19.3</td>
</tr>
<tr>
<td>B</td>
<td>62.7</td>
<td>13.1</td>
<td>24.1</td>
</tr>
<tr>
<td>C</td>
<td>75.9</td>
<td>9.4</td>
<td>14.7</td>
</tr>
<tr>
<td>D</td>
<td>48.0</td>
<td>4.0</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Although, as stated before, the SD used in the present study is quite similar to a healthy human diet, the unexpected lack of differences between SD and HFD namely in heart mitochondrial function evaluated parameters, could by partially explained by the differences considering rodents and human healthy diets. Despite data already published by our group (Goncalves et al., 2014) concerning the impact of the different exercise models against NASH related deleterious effects perpetrated by the high-fat diet, we could not exclude the hypothesis that the SD used in the present protocol had distinct impact in the heart compared to liver. Corroborating this assumption, SD induced a mild steathosis in rodents (Lieber et al., 2004), which is a condition not seen in humans consuming a similar diet (Institute of Medicine, 2001). Other possible explanation could be related with the sequence by which organs are affected by obesity, being the present diet protocol duration not enough to induce cardiac mitochondrial alterations. This hypothesis will be explored with detail below (6.4. The effects of high fat diet on cardiac mitochondrial bioenergetics and redox state).

There are numerous obesity studies, conducted with rodents, compare natural-ingredient chow (cereal based diet) as a control/healthy diet for rodents with HFD diets, despite differences in content of micro and macronutrients, as highlighted by Warden & Fisler (2008). Generally, chow is a high-fiber diet, composed by complex carbohydrates and fats from vegetable sources; in contrast, defined high fat diets are composed by sucrose, soybean oil or lard and supplemented with
amino-acids, minerals and vitamins, among others (Warden & Fisler, 2008). When comparing chow with a HFD, it is not possible to determine if the effects are resultant from fat percentage or from other dietary components. This important methodological question was safeguarded in the present study. We can assume that the diet protocol from the present study allows the comparison between HFD and SD groups, as both diets have the same composition in terms of micro and macronutrients, only deferring in the percentage in which these components are conjugated. Moreover, from our knowledge, this is the first study that is concerned with the effects of different diet composition, instead calories consumed, on cardiac mitochondrial function.

Although the importance of diet composition is unquestionable, the caloric (kcal/g) value of the diet or the caloric value of a daily portion (kcal/day) is extremely important and should not be forgotten. In the present study, the rats ate between 80kcal to 100kcal daily mean per week (Figure 5) and no differences were found between groups. Therefore, it is possible to say that the physiological and anatomical changes were entirely a consequence of the diet composition. Goncalves et al. (2016) showed that a hypercaloric diet (with a fat percentage of 36 % different from the regular diet, 3.1%) led to several cardiac changes, including myocardial structural and functional remodeling, cardiomyocyte stiffening and fibrosis. The protocol in the previous study used a regular diet with 0.0029 Kcal/mg and a hypercaloric diet with 0.0054 Kcal/mg. However, due to the methodological differences between this study and the present one, we still have to question if the amount of calories consumed per day by the rats of the present study corresponds to a normocaloric diet or to hypercaloric diets to rodents.

6.2. Exercise Protocol

The exercise protocol used in the present study was designed to ascertain the possible preventive and therapeutic effects of two different types of physical activity on heart mitochondrial function in a diet-induced obesity model. Firstly, it is essential to clarify the main purpose of each studied exercise model. As in voluntary (FW) run, animals started exercising at the beginning of the diet protocol and sustain this physical activity model throughout the protocol, the
discussion of the results should highlight the potential preventive and therapeutic feature of FW against diet-induced mitochondrial cardiac complications. In contrast, the endurance (Tm) trained animals started exercising after 8 weeks of diet consumption, therefore, in this case, the purpose is to analyze the therapeutic ability of endurance training against obesity-induced cardiac mitochondrial dysfunction. This set up was already used in other studies (Goncalves et al., 2015; Passos et al., 2015).

Nowadays, the public health recommendations for moderate and vigorous physical activity have been established, supporting the link between physical activity and health (Bouchard, 2001; Katzmarzyk, 2010; Physical Activity Guidelines Advisory Committee Report, 2008), including its preventive potential against cardiovascular (Alleman et al., 2015; Ascensao et al., 2007), metabolic (Goncalves et al., 2015; Hwang & Kim, 2014), mental (Farina et al., 2014), among others diseases. In accordance, an elegant review by Pedersen and Saltin (Pedersen & Saltin, 2015) provided evidence-based for prescribing exercise as a therapeutic non-pharmacological strategy against 26 different diseases, including metabolic and cardiovascular. Moreover, the American College of Sports Medicine statement position suggest that 30 minutes of moderate-intensity physical activity, 5 days per week, or more intense exercise for less time (20 minutes, 3 days per week) are recommended (Garber et al., 2011). Unfortunately, this does not seem to be accomplished by the majority of the population (for ref see Katzmarzyk, 2010).

Epidemiological studies suggest that the amount of sitting time during work time, school time, watching television or associated with other sedentary activities may constitute an independent risk factor for deleterious cardiovascular and metabolic effects. In fact, it has been suggested that the criterion to be considered sedentary is independent of whether physical activities recommendations are met (Hamilton et al., 2008; Haskell et al., 2007; Healy et al., 2008; Pate et al., 1995). Several laboratory studies have concluded that physical activity and physical inactivity (sedentary) have different and unique effects on the body. For instance, molecular processes/mechanisms that are impaired by physical inactivity can be different from those that are activated by exercise (Bey et al., 2003; Bey & Hamilton, 2003; Simonen et al., 2003; Zderic & Hamilton, 2006).
Several studies have demonstrated the beneficial effects of FW running in rats, counteracting cardiovascular complications related to obesity (Judge et al., 2005; Lesniewski et al., 2013). Likewise, there are a considerable number of studies that state the positives effects induced by endurance TM exercise in cardiac function (for ref see Ascensao et al., 2007), including mitochondrial fitness (for ref see Antonio Ascensao et al., 2011). Moreover, mitochondrial dysfunction associated with aging and several pathological conditions, including obesity, diabetes, drug administration or isquemia-reperfusion like events were prevented by endurance exercise (Ascensao et al., 2006; Brown et al., 2003; Goncalves et al., 2015; Kavazis et al., 2008; Marques-Aleixo et al., 2015).

In the present study, no differences in the running distance (daily mean per week) between the animals of the distinct diets engaged in FW running groups were found (Figure 5). This means that the differences found in cardiac mitochondria of FW animals must be related to diet treatment rather that exercise volume.

6.3 Animal Characterization

Regular physical exercise has been considered an effective strategy against obesity, promoting weight loss, visceral adiposity decrease, and lipid profile improvement, among others, leading to a decrease in obesity-induced-cardiovascular complications and risk (Bruun et al., 2006; Hafstad et al., 2013).

In the present study, no differences were found between SD and HFD in caloric intake and body weight, probably explained by the equal energetic value of the two diets, which is in accordance with previous studies (Goncalves et al., 2015; Lieber et al., 2004). However, HFD treated animals (HFD+Sed vs. SD+Sed) had increased visceral fat (mesenteric fat, Table 3) probably explained by the higher fat percentage of this diet (Goncalves et al., 2015). These results are in accordance with Estrany et al. (2011) in which a HFD led to increases in adipose tissue.

The FW was able to decrease body weigh in SD, but had no effect in HFD treated animals, which is also in accordance with Goncalves et al. (2015). On the other hand, FW had a very positive effect in visceral adiposity, decreasing mesenteric fat in both diet treated groups, also demonstrated by the decreased in AI (Table
3). This confirms findings of other authors reporting that voluntary physical activity may be an efficient strategy to prevent visceral adiposity (Levin & Dunn-Meynell, 2003). In our study, 17 weeks of FW running was enough to promote a preventive effect against visceral obesity, but promote no differences in body weight in HFD animals. On the other hand, Tm successfully decreased body weight, mesenteric fat and AI in both diet groups (Table 3), proving to be a potential therapeutic strategy against obesity (Ascensao et al., 2006; Irving et al., 2008). The increase in heart-to-body ratio in Tm running animals suggests a positive cardiovascular adaptation to this type of exercise (Ascensao et al., 2006).

6.3.1. Serum markers

Obesity has been associated with some clinical features that are recognized as risk factors for the development of cardiovascular diseases (for ref see Bastien et al., 2014). Nowadays, the development of these clinical features within an obesity context and its consequences in cardiovascular health has been previously and elegantly documented (Calligaris et al., 2013; Goncalves et al., 2016).

In contrast to some expectations, results from the present study show that 17 weeks of HFD significantly decreased TG and VLDL plasma levels, while increasing HDL plasma level (table 4). A possible explanation to these results is that the present diet protocol was designed to induce NASH, and so, these unexpected alterations, mainly the VLDL and triglycerides plasma levels decreases are resultant from a possible hepatic injury (for ref see Goncalves et al., 2015).

Concerning exercise, both interventions positively modulated TG and cholesterol levels in SD groups. However, despite the protective effect of physical activity in nonalcoholololic fatty liver disease (NAFLD) and related metabolic disorders already demonstrated by others (Bhat et al., 2012; Goncalves et al., 2013), the results from HFD animals reveal that only FW positively modulated total cholesterol and VLDL levels at 9th week. Interestingly, the same did not occur at 17th week. Furthermore, endurance Tm training did not promote any protective effect in blood lipid profile (table 4). Sullivan et al. (2012) concluded that 16 weeks
endurance training promoted a small decrease in intrahepatic TG and did not affect lipid kinetics, which suggest that endurance training may have only a modest therapeutic effect on hepatic TG and lipoprotein metabolism. In line with Sullivan et al. (2012), as Tm animals only exercised for 8 weeks, the duration of the Tm training intervention may not be enough to counteract the metabolic disarrangements induced by HFD treatment. Accordingly, Devries et al. (2008) reported the effects of endurance training on hepatic lipid content and adiposity in men and women. This research group also showed that endurance training did not lower hepatic lipid content, which suggest that in order to successfully decrease lipid content, an increase in intensity/duration and/or weight loss might be required.

6.4 The effects of high-fat diet on cardiac mitochondrial bioenergetics, OXPHOS content and redox state

Cardiac mitochondrial dysfunction induced by obesity and high-fat feeding in rats has been previously studied (Boudina & Abel, 2005; Essop et al., 2016; Goncalves et al., 2016; Kang et al., 2015; Sverdlov et al., 2015; Sverdlov et al., 2016). Most of these studies showed that a HFD induces cardiac mitochondria dysfunction, linked to, decreased of ATP synthesis and oxygen consumption, decreased on maximal membrane Δψ, mitochondria uncoupling, with increased calcium concentration, and ultimately with increases in apoptotic stimuli. Additionally, as a consequence of obesity and HFD feeding, the increase in FA oxidation in the heart could be responsible for the excessive ROS production. This seems to be explained by the increased supply of reduced equivalents (NADH and FADH₂) to the MRC without a correspondent OXPHOS capacity, that may result in the loss of electrons from the MRC and consequently generating additional ROS (Abdurrachim et al., 2014; Fillmore & Lopaschuk, 2013). Among the deleterious consequences of ROS generation, mitochondrial bioenergetics and the expression of OXPHOS proteins are negatively targeted (Boudina et al., 2005; Ge et al., 2012).

Mitochondria dysfunction induced by HFD feeding may contribute to the development of cardiac complications, such as diastolic dysfunction (Kang et al., 2015). Cole et al. (2011) conducted a study in which a group of rodents were fed
a HFD (55% of energy provided from fat) and a control group was fed a chow diet for 3 weeks. These authors demonstrated an increase in fatty acid oxidation in cardiac mitochondria accompanied by mitochondrial uncoupling, increased mitochondrial oxygen consumption and consequently decreased cardiac efficiency. Abdurrachim et al. (2014) evaluated cardiac diastolic dysfunction in HFD (45% of energy provided from fat) fed mice and concluded that HFD induced an early stage cardiomyopathy associated to lipotoxicity-associated oxidative stress, fibrosis and disturbed calcium homeostasis rather than impaired cardiac energetics. However, the variety of diet protocols used, specifically diet composition of both high fat and control diets and duration of the protocols may interfere with the results, making it difficult to compare studies. Moreover, as stated before, these studies were focused in the effects of hypercaloric versus normocaloric diets, regardless the diet composition.

In the present study, due to the differences in fat composition, a negative impact of HFD in cardiac mitochondrial functionality and redox balance was expected in comparison with SD+SED group. However, besides the significant decrease in ATP synthase in HFD (Figure 9), no other statistically difference was found between diet treatments in SED.

The observed decrease in ATP synthase content does not implicate a decrease in mitochondrial respiratory activity (Brand & Nicholls, 2011), which is supported by the lack of negative impact in mitochondria oxygen consumption and transmembrane electric potential parameters in HFD treated animals. Our explanation for the divergent results is based on the argument that increases in protein expression do not implicate increases in their activity (Vogel & Marcotte, 2012). Likewise, it can be assumed that the decrease in complex V content does not impose a decrease in their activity individually or in the overall process of mitochondrial respiration. Furthermore, Brand & Nicholls (2011) state that even moderate changes in individual complexes activity have little effects in the overall system behavior.

A study from Kang et al. (2015) concluded that HFD feeding in a mouse model for 10 weeks resulted in a decrease in complex I, NUFB5 of OXPHOS subunit and a decrease in PGC-1α in cardiac mitochondria, a potent stimulator of mitochondrial biogenesis. An interesting study from Sverdlov et al. (2015) report
that 8 months of high fat-high sucrose diet induced cardiac mitochondrial dysfunction, decreasing in 70% the activity of complex II.

As mentioned above, one of the most reported consequences of HFD is increased ROS production that, at least in part, is associated with cardiac mitochondrial impairments. Therefore, the lack of differences between diets in MDA content and glutathione redox status was not expected (Figure 10 and 11). Since the main component of mitochondria membranes are unsaturated FA that are highly prone to oxygen radical attack, the measurement of the lipid peroxidation product MDA is considered an indicator of oxidative stress. However, the use of a single oxidative damage marker and the analysis of a single antioxidant are important limitations of the present study. Hence, to better discuss the possible association between HFD and mitochondrial redox status other oxidative markers and antioxidants, including carbonyl groups and -SH, aconitase and Mn-SOD activity and measurement of the activity of proteins involved in mitochondrial oxidative stress (for instances SIRT3, p66shc and UCP2), as previously done by Marques-Aleixo et al. (2015), should be considered in future works.

A possible explanation for the lack of results is the sequence by which organs are affected by obesity. Scientific evidence available indicates that liver is generally the first organ to be affected due to the increased delivery of free fatty acids and subsequent fat accumulation, resultant from the boosted lipolytic activity in visceral fat (Abdurrachim et al., 2014). Importantly, epidemiological evidences suggest that nonalcoholic fatty liver disease (NAFLD) has been associated with an increase of coronary heart disease, abnormalities of cardiac function and structure, including left ventricular dysfunction and hypertrophy, heart failure, among others. In other words, liver diseases are a good predictor of cardiometabolic dysfunctions (Ballestri et al., 2014; Bhatia et al., 2012; Birkenfeld & Shulman, 2014; Cornier et al., 2011).

The NASH represents a marker and a mediator of increased cardiovascular risk (Bhatia et al., 2012). Among other factors, a positive association between hepatic fat and myocardial IR has been found, accompanied by reduced myocardial glucose uptake, increased plasma levels of adipocytokines and vascular adhesion molecules (Lautamaki et al., 2006). The association of increased FFA
availability and myocardial IR leads to cardiac metabolic inflexibility already explained in the introduction section, ultimately resulting in impaired cardiac function, which is demonstrated by producing less ATP per oxygen molecule consumed (ADP/O) (Bhatia et al., 2012). Furthermore, the excess of FFA supply may originate cardiac lipotoxicity, also mentioned in the introduction, which is associated with increased oxidative stress and subsequent mitochondrial dysfunction, cardiomyocyte apoptosis, and finally cardiac dysfunction (Peterson, 2006; Witteles & Fowler, 2008). Since the protocol of the present study was initially designed to induce NASH, successfully achieving it, maybe it should have been applied for a longer time in order to induce, as well, cardiac mitochondrial alterations.

6.5 The effects of exercise on cardiac mitochondrial bioenergetics, OXPHOS content and redox state

Previous studies have shown the protective effect of exercise on the heart, evidencing the preventive and therapeutic potential of a regular practice of physical exercise, expanding its’ action to a clinical context (for ref see Ascensao et al., 2007). In accordance to the literature, physical exercise has been proven to be a great strategy to improve cardiac mitochondrial functionality, increasing antioxidant capacity, improving membrane integrity and consequently improving oxidative capacity (Alleman et al., 2015; Ascensao et al., 2007; Hai et al., 2008; Jacobs & Lundby, 2013; Kavazis et al., 2008).

An important goal of this thesis was to understand whether exercise promotes improvements on cardiac mitochondrial function in animals fed a high-fat diet. Moreover the present study is also concerned with different exercise modalities frequently used in rodents studies.

In order to be able to evaluate mitochondrial function, respiratory activity along with mitochondrial membrane potential and ADP-induced phosphorylation cycle were measured. An additional approach used in the present study was the quantification of complex I, II, IV and V from MRC and mitochondrial oxidative stress and antioxidant capacity.
The cardiac mitochondrial function data obtained in the present study are, at least in part, in accordance with the cardioprotective effects induced by exercise, particularly the endurance Tm training (for ref see Ascensao et al., 2007; Antonio Ascensao et al., 2011). In animals fed a standard diet, Tm running significantly improved cardiac mitochondrial function, as it increased RCR (Figure 6). Alterations in RCR are considered a good indicator of mitochondria function/dysfunction and structural integrity, as a high RCR score is related to a higher capacity for substrate oxidation and a low proton leak (Pesta & Gnaiger, 2012). However, RCR scores depend on various factors. For instance, alterations in any aspect of OXPHOS will have an impact in RCR score (Brand & Nicholls, 2011). The literature suggests that even short-term exercise protocols have a positive impact in this important functional parameter (Kavazis et al., 2010). Unexpectedly, the increased mitochondrial function evaluated by RCR paralleled a decrease in complex V content.

Interestingly, after the anoxia-reoxygenation stimulus endurance trained animals fed with the SD revealed a protective effect demonstrated by the results in ADP/O and state 4 (Figure 8). Since state 4 significantly decreased, and as this value is mostly associated to proton leak, we can assume that Tm running had a positive effect in mitochondrial structural integrity or in maintaining a sufficient high membrane $\Delta \psi$, thus restricting electron transport (Brand & Nicholls, 2011). In accordance to Brand & Nicholls (2011), alterations in ADP/O are usually related to coupling mechanisms, for example, complexes activity and their capacity of pumping more or less protons. Though, alterations in this parameter are also related to proton leak, which suggest that endurance Tm training seems to confer a protective phenotype against an isquemia-reperfusion event. The protective effect of 14 weeks of endurance exercise against anoxia-reoxygenation was reported by Ascensao et al. (2006), using an in vitro model similar to the used in the present study.

The animals from HFD group assigned to the Tm protocol demonstrate a significant improvement in RCR before and after A-R (Figure 6 and 8). This suggests that endurance training improved mitochondrial fitness, particularly mitochondrial respiratory activity and mitochondrial ability to produce energy in a context of obesity and HFD feeding. The alterations in respiration resulting from
the endurance training may stem from increased expression of complex IV and V (Figure 9). If a long term HFD is associated with cardiac mitochondrial impairments, including OXPHOS content as seen by others (Kang et al., 2015; Sverdlov et al., 2015), Tm seems to have an important role in the cardiac protection against obesity-induced mitochondrial respiratory activity and overall dysfunction.

On the other hand, FW was ineffective modulating mitochondrial respiratory activity in both SD and HFD groups (Figure 6). As suggested by to Bishop et al. (2014), the training intensity may be determinant for inducing improvements in mitochondrial respiration in skeletal muscle, which is consistent with our results in cardiac muscle. Although we do not know the exact intensity of FW, it is presumable that a higher intensity and lower volume training characterize Tm running compared to than FW. It is however important to note that previous studies from our group reported increased cardiac mitochondrial RCR in rodents submitted to 12 weeks of voluntary running (Marques-Aleixo et al., 2015). The animals from the present study in the last 6 weeks run about 2 km per day less that the animals from the study of Marques-Aleixo et al. (2015), which may be influenced by their weight (about 100 g more). We could speculate that HFD induced-obesity may act as suppressor of voluntary running volume, and therefore cardiac mitochondria respiratory adaptations. In contrast to mitochondrial oxygen consumption, FW had a stronger effect than Tm running in complex IV and V content in both SD and HFD-related animals. Despite these results do not translate into an increase in mitochondrial functionality, it may suggest that exercise volume might constitute a stimulus to increase OXPHOS proteins expression.

The complementary study of Δψ is indispensable for a complete analysis of mitochondrial function because it reflects the basic energetic relation to cellular homeostasis maintenance. In fact, the electrochemical gradient due the pumping of protons through the inner membrane (Murphy & Brand, 1988) is indispensable to ADP phosphorylation (Stock et al., 1999). Moreover, when cytosolic concentration of calcium increases, mitochondria act as calcium buffers due its capacity to uptake and accumulate calcium (Gunter et al., 2004). It has been suggested that intramitochondrial calcium concentration, whose flow is directed
in accordance with the protomotriz gradient, has a controlling function in metabolic rate of oxidative energy production through the activation of calcium-sensitive dehydrogenases, F0F1ATPase and ANT (Glancy et al., 2013). Our results showed no alterations of maximal Δψ, depolarization, repolarization or the lag phase in cardiac mitochondria from exercise groups (Figure 7). It is however important to note that despite the inexistence of significant differences, in accordance to A. Ascensao et al. (2011) the Δψ values above −200 mV did not seem to compromise ATP synthase flow or the transport of ions and metabolites. In addition, Kaim & Dimroth (1999) suggest that ATP synthase can only reach saturation at approximately -100mV (Δψ). Our results show that the range of Δψ varies between approximately -186mV and -215mV. The necessary time to restore membrane potential after addition and consequent ADP phosphorylation, e.g., the lag phase, seems to be modulated by exercise (Marques-Aleixo et al., 2015). Unexpectedly, our results failed to show that exercise induces an increase in ADP phosphorylation.

The literature support that intimately associated with cardiac mitochondrial improvements, chronic physical exercise improve the antioxidant machinery and consequently to decrease oxidative stress markers (Ascensao et al., 2007; Antonio Ascensao et al., 2011; Starnes & Taylor, 2007). This is particularly important, as heart has lower antioxidant defenses compared with other tissues (Chen et al., 1994). Exercise neither positively impacted cardiac mitochondrial lipid peroxidation or glutathione redox status in SD groups (Figure 10 and 11). These results are divergent from literature supporting the protective effects of endurance training on antioxidant potential (Ascensao et al., 2006; Ascensão et al., 2005; Bo et al., 2008; Hai et al., 2008; Starnes et al., 2007).

Although there were no differences in MDA content between exercise groups and sedentary HFD, an increased GSH/GSSG ratio following 17 weeks of FW running suggest a positive modulation of the antioxidant machinery. Indeed, reduced glutathione (GSH) is considered to be one of the most important ROS scavengers, and its ratio with oxidized glutathione (GSSG) may be used as a marker of oxidative stress (Li et al., 2000; Rahman et al., 2006). Interestingly, FW also had a positive effect in complex IV and complex V content, and since
oxidative stress is related to lower expression of mitochondrial proteins, such as OXPHOS proteins (Boudina et al., 2005) it gives support to presume that this exercise model might constitute an efficient cardioprotective strategy in the context of HFD feeding. Corroborating with our results, Judge et al. (2005) conducted a study in which lifelong FW running decreased H$_2$O$_2$ production in cardiac mitochondria (the target for reduced glutathione) without improving mitochondrial rates of oxygen consumption or RCR.

In contrast, Tm training did not promote the antioxidant protective effect that was expected. In fact, no alterations were found in GSH/GSSG ratio compared to SED+HFD group. Considering that differences were also not found in MDA content, as previously suggested in this discussion, the assay of other markers of mitochondrial redox state should give more confidence to these results. It is important to highlight that FW running seems to better regulate glutathione redox status than Tm training group. One possible reason for the differences found in the modulation of mitochondrial redox balance by Tm and FW within a context of a HFD could be the duration of the intervention of each exercise model. Animals engaged to the FW training exercised for 17 weeks, starting at the same time as diet protocol. On the opposite, Tm animals only exercised for 8 weeks and 8 weeks after the beginning of HFD consumption.

To our knowledge this is the first study comparing endurance training effects on cardiac mitochondrial bioenergetics and redox status of animals fed with a high-fat diet. Therefore, more studies are required to elucidate important questions, more precisely the optimal training stimuli (intensity, volume, power, frequency and length of intervention) to better enhance heart mitochondrial fitness.
7. Conclusions

In summary, the data from present dissertation provides additional support about the effect of two distinct physical exercise models (voluntary physical activity and endurance training) against the deleterious impact imposed on cardiac mitochondria by a high-fat diet.

The following conclusions can be taken:

- 17 weeks of HFD treatment did not induced significant alterations on heart mitochondrial function or redox state;
- 8 weeks of endurance Tm training performed after 8 weeks of SD and HFD seems to positively modulate cardiac mitochondrial bioenergetics endpoints;
- 17 weeks of FW running performed during the course of SD and HFD resulted in increased OXPHOS subunit content and GSH/GSSG ratio

In summary, these finds suggest that exercise is likely to contribute a protective mitochondrial phenotype even in a context of HFD feeding. The knowledge of the mechanisms associated with the protective and/or therapeutic role of physical exercise in the set of physiological cardiovascular remodeling to counteract obesity side effects, should be further explored.
8. References


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