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PhD Programme in Biomedical Sciences and Biotechnology

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PhD THESIS

SKELETAL MUSCLE MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION PLASTICITY IN TWO STUDIES ON HUMANS: EXERCISE TRAINING EFFECT IN OBESE SUBJECTS AND BED REST EFFECT IN HEALTHY SUBJECTS

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La misura dell'intelligenza è

data dalla capacità di cambiare

quando è necessario (Albert Einstein).

ABSTRACT

Mitochondrion is an important organelle for cells survival being involved in many cellular processes such as energy metabolism and homeostasis, cell division and differentiation, regulation of apoptosis and autophagy, etc. Mitochondrion, due to this role, is able to respond and adapt itself quickly to any perturbation and change of conditions in the different tissues of the human body including skeletal muscle. Thus, mitochondrial plasticity is one of the mechanisms that controls modification due to different physiological conditions such as weight loss or weight gain, exercise and physical inactivity/immobility in skeletal muscle; moreover it represents the capacity of a biologic system to respond to metabolic conditions in relation to the needs of the respective tissues.

In this frame, this PhD Thesis investigated the role and adaptations of mitochondria in two different skeletal muscle pathophysiological models. Exercise is a fundamental tool known to trigger adaptations in mitochondria of skeletal muscle fibres in terms of both of exercise training and of reduced activity/immobility. The present PhD work evaluated the adaptations to exercise, or to the lack of exercise, at the level of mitochondrial oxidative phosphorylation in two conditions particularly relevant for the development of diseases. The first one investigated the effects of two different protocols of exercise training (moderate intensity continuous training and high intensity interval training) in obese patients and the second the effects of 10 days bed rest-induced microgravity and immobility in young healthy volunteers. In both studies, mitochondrial oxidative function was assayed *ex vivo* in skeletal muscle biopsies by high resolution respirometry using a substrate-uncoupler-inhibitor-titration protocol with a substrate combination .

In the first study, obese patients exhibited an increased maximal ADP-stimulated respiration and an increased maximal capacity of the electron transport system after both types of training protocol, confirming the beneficial effect of exercise.

In the second study, no impairment on mitochondrial function in terms of maximal state III respiration and coupling was observed after ten days of bed rest. No alteration in ADP sensitivity was observed after the period of inactivity.

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1.INTRODUCTION

1.1 Mitochondrion

Mitochondrion is a double-membrane organelle located in the cytoplasm of most eukaryotic cells. Mitochondria are involved in regulation of cellular bioenergetics, producing metabolic energy under the form of ATP in order to guarantee cell functions (Mitchell, 1961). Because of this main function are often called the “powerhouse” of the cell and are able to adapt their function in relation to cellular environmental changes receiving and transmitting several signals within cells. This eukaryotic organelle evolved from endosymbiont bacteria (Andersson Siv et al., 2002; Gray and Doolittle, 1982) and consequently has its own genome. The mitochondrial DNA (mtDNA) has a reduced coding capacity, being responsible in coding only a small part of mitochondrion’s proteome. The mtDNA codifies for 13 proteins which are components of the respiratory chain, for 22 transfer RNAs and for 2 subunits of ribosomal RNA. The remaining mitochondrial proteins are encoded by nuclear DNA and include components involved in different mitochondrial pathways as the tricarboxylic acid (TCA) cycle, protein import, fatty acid and amino acid oxidation, apoptosis. Mammalian mitochondrial proteome includes $\sim$ 20000 mammalian proteins with different grade of abundance and expression during the developmental stages or in different cell types (Calvo and Mootha, 2010). Mitochondrial function and structure can change in relation to cell types, but basically these organelles have a double membrane (Fig.1.1): the outer mitochondrial membrane (OMM) separates the mitochondria from the resting cellular environment, while the inner mitochondrial membrane (IMM) evolves in invaginations which are also known as cristae and many mitochondrial proteins reside on it. Protein kinases and phosphatases are on the OMM to convey signals to and from mitochondria (Lucero et al., 2019). The OMM is porous and it can be traversed by ions and uncharged molecules through porins as the voltage-dependent anion channel VDAC. The IMM acts as a diffusion barrier to ions and molecules that can traverse it only with specific membrane transport proteins. In IMM resides the oxidative phosphorylation (OXPHOS) process.

Mitochondria have other essential functions such as the production of NADH and GTP in the citric acid cycle, the biosynthesis of amino acids, heme groups and iron-sulfur clusters or the synthesis of phospholipids for membrane, calcium signalling, stress responses and apoptosis

(Kuhlbrandt, 2015). Considering all these different functions, mitochondria are considered dynamic organelles.

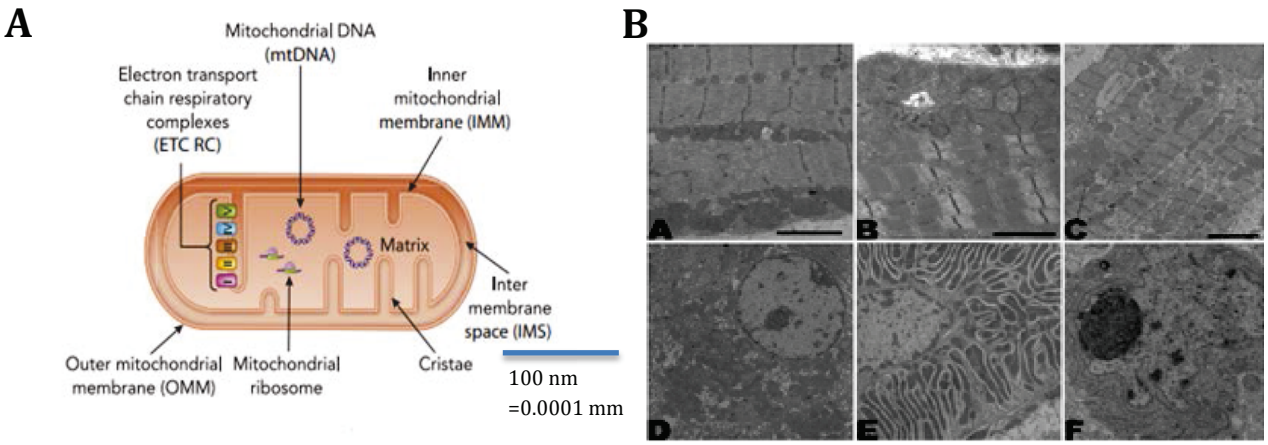


Fig.1.1. Mitochondrial structure of the double membrane-organelle. A) Representation of the mitochondrial components and their location respect to the double membrane. Image modified from Bishop et al., 2019. B) Electron microscopy images, revealing mitochondrial and cellular organizations from different tissues: a) Skeletal muscle (soleus), b) skeletal muscle (peroneus digitiquarti), c) heart, d)liver, e) kidney, f) brain. Modified from Benard et al., 2006.

1.1.1 Mitochondrial Electron Transport Chain and ATP synthase

Mitochondrion, the powerhouse of eukaryotic cells, is responsible for ATP production during oxidative phosphorylation (OXPHOS). This biological process takes place in the inner mitochondrial membrane and occurs only when there is the cooperation between the respiratory chain and ATP synthase (OXPHOS complex V). Mitochondria promote energy conversion processes in which the exergonic flow of electrons along electron transport chain (ETC) supports the endergonic pumping of protons from the matrix to the intermembrane space. This process develops the proton motive force that allows the rotation of the F_0 sector of ATP synthase leading to the synthesis of ATP in the F_1sector (Mitchell and Moyle, 1967). The OXPHOS system is composed of five inner mitochondrial membrane-embedded enzymes, 4 of the ETC and the ATP synthase. The respiratory chain is composed by four protein complexes: complex I or NADH-Q oxidoreductase, complex II or Succinate-Q reductase which directly connects the Krebs cycle to the respiratory chain, complex III or Q-Cytochrome c oxidoreductase and complex IV or citochrome C oxidase. The complexes I, III and IV are protonic pumps (Fig. 1.1.1).

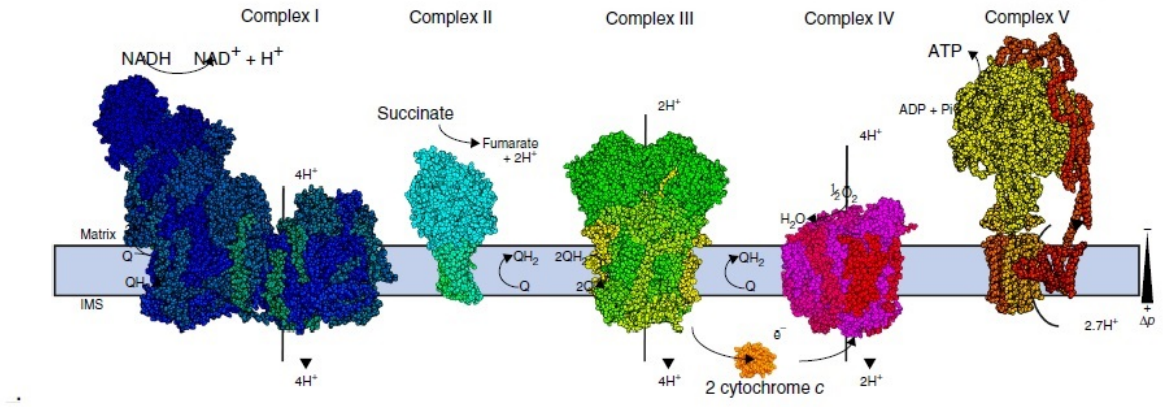


Fig.1.1.1. The OXPHOS system: The complexes of the mitochondrial electron transport chain (ETC) and ATP synthase (complex V). The electron transfer and proton pumping activity of respiratory complexes are shown. The complexes are reported in their atomic model and with different colour: CI (blue), CII (cyan and light green), CIII (green and yellow), CIV (magenta and red) and CV (yellow and red). The atomic structure of cytochrome c is shown in orange. The reactions catalyzed by the complexes are shown. Q, ubiquinone; QH2, ubiquinol. The light blue rectangle is approximate boundary of the membrane and separates mitochondrial matrix from intermembrane space (Letts and Sazanov, 2017).

Complex I is a large enzyme of ~ 900 KDa and is formed by 46 subunits. It is as a typical L-shaped molecule with the horizontal arm located in the inner mitochondrial membrane while the vertical one protrudes in the matrix. Starting from complex I, the electrons enter toward the respiratory chain. During biochemical catabolism NADH molecules are produced, representing a source of electrons. NADH molecule binds to complex I transferring two electrons to the prosthetic group of Flavin mononucleotide (FMN), which is thus reduced to $FMNH_2$. Furtherly, electrons are transferred to Fe-S centres. This electron transfer makes possible the pumping of four hydrogen ions outside the mitochondrial matrix. In this way ubiquinol is formed since coenzyme Q is reduced acquiring two protons from the matrix, and thus it leaves complex I moving through the hydrophobic zone of the membrane (Kuhlbrand, 2015; Chaban et al., 2013; Dudkina et al., 2008; Lenaz et al., 2006;).

Complex II is both a part of Krebs cycle and than of ETC. During Krebs cycle electrons are produced through reduced Flavin Adenine Dinucleotide ($FADH_2$). The electrons are first transferred to Fe-S centres and then to coenzyme Q to enter in the respiratory chain (Chaban et al., 2013; Oyedotun and Lemire, 2004,). Complex II is not a proton pump, thus transport of protons does not occur and, as a consequence, less ATP molecules are produced with respect to NADH oxidation.

Complex III is a functional dimer existing on the membrane which transfers two electrons from reduced ubiquinone to oxidize cytochrome C, pumping also two protons outside the mitochondrial matrix (Chaban et al., 2013; Kramer et al., 2004). Complex III has two types of cytochromes: b and c_1. These cytochromes use heme as prosthetic group (two for b and one for c_1). Complex III and the cytochromes have 2Fe-2S centre (Rieske centre), where the iron ions are coordinated by two hystidines in order to maximally develop the reduction potential and easily accept the electrons from the reduced ubiquinone (Kramer et al., 2004). Complex III pumps four protons into the intermembrane space following the oxidation of coenzyme Q and the reduction of cytochrome C, a small mobile electron carrier associated with the outer surface of the inner membrane.

Complex IV is a transmembrane protein complex and it consists of 13 subunits. It has a molecular weight of 200 KDa. Complex IV represents the last enzyme of the respiratory chain. It catalyses the transfer of four electrons from cytochrome C to oxygen molecules, which are the final acceptors and are reduced to water (Kuhlbrandt, 2015; Chaban, et al.,

2013; Dudkina et al., 2008). This complex makes a final contribution to the production of proton gradient across the inner membrane. In this way, all the protons are pumped from the matrix to the intermembrane space generating the proton motive force, which is used by ATP synthase (complex V) for ATP formation.

ATP synthase is a complex enzyme of large dimension formed by 15-18 subunits with a total mass of 600 KDa (Chaban et al., 2013) (Fig.1.1.1.1). Two different domains constitute complex V: F_0 domain, located in the inner mitochondrial membrane and F_1 domain, directed towards the matrix. F_0 domain contains the proton channel formed by subunits a, b, c8-10, d, f, g, CF6, A6L. The water-soluble F_1 part is the catalytic portion of the enzyme and is constituted of α3, β3, γ, δ and ε subunits. Through several atomic structures is shown that F_1 comprises 3 copies of each of the nucleotide-binding subunits α and β (Rühle and Leister, 2015; Wittig and Schägger, 2008).

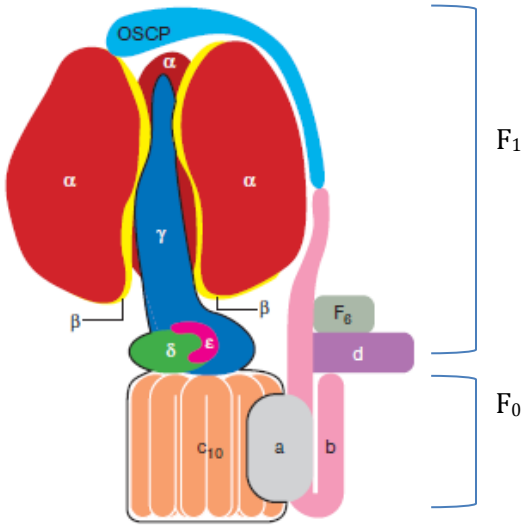


Fig. 1.1.1.1. ATP synthase structure and its subunits. Figure modified from Stock et al., 2000.

The α and β subunits constitute the majority of all complex to form a hexameric ring. Even if α and β subunits are similar, only the β subunit has catalytic nucleotide-binding sites and it catalyses the ATP synthesis/hydrolysis reaction (Gibbons et al., 2000; Boyer, 1993). F_0 and F_1 domains are connected in a central (subunits γ, δ, ε) and a peripheral (subunits b, d, F_6 and oligomycin sensitivity conferring protein OSCP) structure, known as rotor and stator. Therefore, the two parts are connected by a static peripheral stalk and a rotating central stalk. This link between the two parts generates a sequence of conformational changes from

the rotor unit in the membrane to the catalytic F_1 head (Hahn et al., 2016; Davies et al., 2011). The γ subunit contains an asymmetric coiled coil of α-helices coil that penetrates through the central axis of F_1 domain (Cabezón et al., 2003).

The ATP synthesis is an enzyme reaction that, depending on cell condition, may lead to the production or hydroxylation of ATP. This enzyme reaction uses the energy stored under the form of electrochemical proton gradient across the inner mitochondrial membrane in order to produce ATP from ADP and phosphate. The mitochondrial ATP synthase was shown to form monomers, dimers and higher oligomeric species. Wagner (Wagner, 1996) reports that the generation of normal, tubular cristae membranes depend on the oligomeric state of ATP synthase. In fact ATP synthase dimers can assemble into long filaments in the inner mitochondrial membrane. This geometrical arrangement can promote membrane bending and thus the formation of cristae tubules. Mechanisms like post-translational modification, ligand association and gene expression regulation control the function of ATP synthase. The most important physiological regulatory factor of the ATP Synthase is the Inhibitory Factor 1 (IF1). IF1, a nuclear-encoded small thermostable protein of 12 KDa, has been studied by several laboratories to understand its pathophysiological role in different tissues and illnesses (Pullman and Monroy, 1963).

1.1.2 Mitochondrial biogenesis and mitochondrial network remodelling

Mitochondria take part to a large number of essential cellular functions and their genetic or epigenetic alterations can modify and affect their function, thereby contributing to the development of several pathological conditions. The biogenesis of mitochondria is a complex cellular event and it occurs following to the expression of two physically separated genomes (Martinez-Diez et al., 2006). This process is accompanied by variations in mitochondrial size, number, DNA (mtDNA) content, etc. It occurs after an environmental cellular change (i.e. cellular stress) or as a consequence of other factors such as exercise (Coffey and Hawley, 2007; Nisoli and Carruba, 2006), caloric restriction (Liu et al., 2009; Civitarese et al., 2007), cold exposure, oxidative stress, cell division, cell differentiation (Hock and Kralli, 2009; Ventura-Clapieret al., 2008) .

Mitochondrial biogenesis is controlled by the coordinated transcription of nuclear and mitochondrial genes, which are regulated in large measure by the coactivator peroxisome

proliferator-activated receptor-coactivator 1-alpha (PGC-1α), its family members, and its isoforms (Erlich et al., 2016; Wilson et al., 2007). PGC-1α, known also as the "master regulator" of mitochondrial biogenesis, is involved in many metabolic processes, including liver gluconeogenesis, thermogenesis, and skeletal muscle fiber-type specialization (Erlich et al., 2016). PGC-1a is well known as key regulator of energy metabolism (Liang and Ward, 2006).

Mitochondria constantly undergo fission and fusion processes that exist in equilibrium, leading to a continuous remodelling of the mitochondrial network. Mitochondrial fusion is a process controlled by mitofusin 1 and 2 (MFN 1 and MFN 2) in the outer mitochondrial membrane and by optic atrophy type 1 (OPA 1) in the inner mitochondrial membrane. Instead, mitochondrial fission is mainly controlled by dynamin-related protein 1 (Drp1) and its recruitment factors fission 1 (Fis1) and mitochondrial fission factor (MFF) in the outer membrane (Ljubicic et al., 2010). An increase in fusion or a decrease in fission can lead to elongated, interconnected mitochondria, whereas a decrease in fusion or an increase in fission can lead to fragmented mitochondria (Bo et al., 2010). Mitochondrial fission and fusion processes play critical roles in maintaining functional mitochondria when cells experience metabolic or environmental stresses. Fusion helps mitigate stress by mixing the contents of partially damaged mitochondria as a form of complementation. Fission is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis processes during high levels of cellular stress (Youle and van der Bliek, 2012).

Protein aggregates, intracellular pathogens and damaged or excessive organelles, including mitochondria, can be removed via autophagy (Kim et al., 2007). Mitochondria are multifunctional cellular organelles and are the primary source of reactive oxygen species (ROS) (Simon et al., 2000; Yakes and Van Houte, 1997). ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds. When there is an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and has been implicated in different conditions such as carcinogenesis (Trachootham et al., 2009), neurodegeneration (Shukla et al., 2011; Andersen, 2004), atherosclerosis, diabetes (Paravicini and Touyz, 2006), and aging (Haigis and Yankner, 2010).

ROS involvement in the pathogenesis of disease states is not confined to macromolecular damage.

Benefical roles of ROS are also quite significant. The beneficial aspects of ROS are related to their effects on the redox state of cells and to the important role that some ROS play in signalling cascades. Redox regulation is essential for the body to maintain proper signalling processes. These redox reactions usually entail ROS interacting with the amino acid cysteine on proteins. ROS modulates cell proliferation and apoptotic pathways to ensure proper regulation of the cell cycle and programmed cell death. ROS are also involved in the angiogenesis process, and indeed vascular smooth muscle cells require ROS for appropriate cell growth. ROS have also an important role in the immune system. When there is a lack of ROS in the immune system, disease states may develop that impair an individual's ability to defend against a foreign attack (Patel et al., 2018).

The mass of mitochondria has to match the varying needs of the cell when bioenergetic and environmental changes occur (Kurihara et al., 2012). As a result, the removal of dysfunctional mitochondria and the preservation of an appropriate population of mitochondria are required for normal cellular function and cell survival (Kurihara et al., 2012; Scherz-Shouval and Elazar, 2007). Mitochondrial quantity is guaranteed via mitochondrial biogenesis and the selective clearance of damaged or excessive organelles. Damaged or unwanted mitochondria can be selectively removed by mitochondrial autophagy or mitophagy, a catabolic process of lysosome-dependent degradation, and the core mechanism of both mitochondrial quality and quantity control (Wei et al., 2015; Feng et al., 2013). The mitochondrial processes cited above are important in physiological and pathophysiological conditions in order to respond to different stimuli and perform the metabolic adaptations when necessary.

1.2. Skeletal muscle

Skeletal muscle is a tissue with great plasticity and it represents about 40% of the total body weight (Frontera and Ochala, 2015; Yang, 2014). Skeletal muscle includes 50-70% of all body proteins, accounting for 30–50 % of whole-body protein turnover. Water is the main component of skeletal muscle (75%), and proteins constitute 20% of muscle while other substances such as inorganic salts, mineral, fat and carbohydrates cover 5%. Muscle mass is dependent by the balance between protein synthesis and degradation, which are two

biological processes dependent by nutritional status, hormonal balance, physical activity/exercise, and injury or disease. Muscle is able to convert chemical energy into mechanical energy to generate force and power, allowing the physiological functions of locomotion, posture, physical activity (Frontera and Ochala , 2015).

The energy source during muscle contraction is provided by hydrolysis of ATP to ADP and Pi (Schiaffino and Reggiani, 2011; Westerbald et al., 2010). The intracellular ATP content is small (5–6 mM) and moreover, if the muscle is fully activated, ATP stores would be depleted within 2 s (Westerbald et al., 2010; Sahlin et al., 1998). Thus, during most of physical exercise ATP must be continuously re-synthetized, through the intervention of phosphocreatine (PCr) hydrolysis, anaerobic glycolysis, and oxidative metabolism (oxidative phosphorylation in mitochondria) of carbohydrate and lipid substrates. Oxidation of aminoacids can be, as a first approximation, neglected.

In skeletal muscle during submaximal exercise, oxidative metabolism utilizes as substrates carbohydrate and lipids to produce ATP (Westerbald et al., 2010; Spriet and Watt, 2003). The choice of the mechanisms to be utilized for ATP resynthesis substantially depend on the intensity and on the duration of the exercise.

The sarcolemma is the plasma membrane of muscle fiber, the cytoplasm is referred to as sarcoplasm, and the specialized smooth endoplasmic reticulum, which stores, releases, and retrieves calcium ions (Ca^{++}) is called the sarcoplasmic reticulum (SR). The skeletal muscle fibers have a striated appearance, which is due to the arrangement of the myofilaments of actin and myosin in sequential order from one end of the muscle fiber to the other. These myofilaments and their regulatory proteins**,** troponin and tropomyosin (along with other proteins) form a sarcomere. The sarcomere is the functional unit of the muscle fiber; it is bundled within the myofibril that runs the entire length of the muscle fiber and attaches to the sarcolemma at its end. As myofibrils contract, the entire muscle cell contracts. Each sarcomere is approximately 2 μm in length with a three-dimensional cylinder-like arrangement and is bordered by structures called Z-discs, to which the actin myofilaments are anchored (Frontera and Ochala, 2015).

Skeletal muscle is a heterogeneous tissue that comprises different type of fibers which have undergone, over the years, to various classifications. The most modern classification is substantially based on the presence of the myosin-heavy chain isoforms, and muscle fibers are classified in type 1, type 2A and type 2X (Bottinelli and Reggiani, 2000).

Type 1 (classically red in appearance) or slow twitch fibers are oxidative (use aerobic respiration, oxygen and glucose, to produce ATP) and are characterized by a high content of mitochondria and myoglobin, by a slow time to peak tension, by a lower force production. Type 1 fibers are surrounded by a high number of capillaries. Type 1 fibers provide ATP by oxidative metabolism and are fatigue resistant. Types 2A are oxidative and glycolytic fibers. Type 2X fibers are glycolytic fibers (white fibers in appearance), have low mitochondria content and a fast time to peak tension. In comparison to type 1 and type 2A fibers, type 2X fibers are less dependent on oxidative metabolism, providing ATP through anaerobic glycolysis and PCr breakdown, and therefore are powerful but fatigue rapidly (Bottinelli and Reggiani, 2000). The abundance of the oxygen transport protein myoglobin is reflected by the different colour of the muscle fibers, and is associated with the contribution of oxidative metabolism (Egan and Zierath, 2013); red fibers (type 1) in appearance have a high content of myoglobin, while white fibers (type 2X) have a low content of myoglobin. Myoglobin content in type 2A fibers is intermediate between those of type 1 and type 2X. In human muscles oxidative fibers contain 50% more myoglobin than glycolytic fibers (Jansson and Sylvén, 1983).

Because of its heterogeneity composition and considerable plasticity, skeletal muscle is able to adapt itself in different physiological conditions as weight loss, exercise and physical inactivity (Schiaffino and Reggiani, 2011; Maltin, 2008). This muscle plasticity is well shown by the adaptive changes occurring in response to various external stimuli (contractile activity, loading conditions, substrate supply, hormonal profile, and environmental factors) to match structural, functional, and metabolic demands (Bassel-Duby and Olson, 2006). Body size has a major role in determining the functional demands on skeletal muscles (Schiaffino and Reggiani, 2011). It is well documented that physical exercise is a potent stimulus for adaptation processes; physical exercise is known to remodel skeletal muscle to better respond to future challenges (Fluck, 2006; Hood et al., 2006). Skeletal muscle is able to rapidly adapt to exercise interventions and demonstrates remarkable malleability by changing its metabolic and contractile properties. Resistance exercise (generally encompasses short-duration activity at high or maximal exercise intensity; it increases the capacity to perform a single or relatively few repetitions of high-intensity exercise) stimulates muscle protein synthesis leading to growth of muscle fibers and hypertrophy. By contrast, endurance exercise (typically performed at submaximal intensity, with the main purpose of

progressively moving the anaerobic threshold, i.e. the beginning of anaerobic metabolism and lactate production, towards higher exercise intensity) determinates qualitative changes of muscle tissue by promoting phenotypic adaptations characterized mainly by fiber type transformation and increases in structures supporting oxygen delivery and consumption (mitochondrial biogenesis and angiogenesis), but no growth (Mounier et al., 2015).

In skeletal muscle, mitochondria can be differentiated both functionally and biochemically: intermyofibrillar (IMF) and subsarcolemmal (SS) subfractions (Fig.1.2). IMF mitochondria are located among the myofibrils, whereas SS mitochondria are proximal to peripheral myonuclei below the muscle plasma membrane. These mitochondria differ in their adaptability to a common stimulus, meaning that their location within the cell makes them differentially sensitive to an intracellular signal. Exposures to perturbations as muscle use or disuse have shown that SS mitochondria consistently adapt more than IMF mitochondria; this can be due to the location of SS mitochondria proximal to the myonuclei and also to the differential capacity of mitochondrial subfractions for protein synthesis and import (from cytoplasm) (Ljubicic et al., 2010 and reference therein).

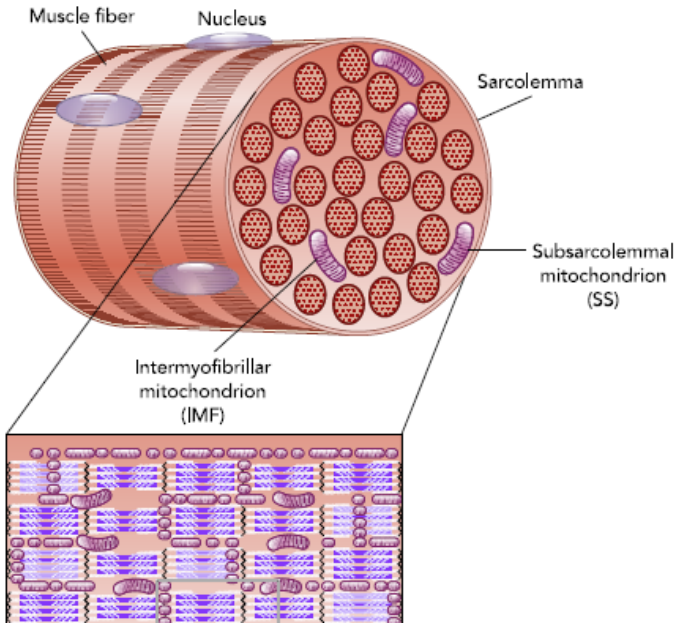


Fig.1.2. Structure of a skeletal muscle fiber with two sub-populations of mitochondria. Subsarcolemmal mitochondria (SS) and intermyofibrillar mitochondria (IMF) are shown. In skeletal muscle fiber, mitochondria create a reticulum that connects SS and IMF mitochondria for optimal energy distribution. Image modified from Bishop et al., 2019.

Mitochondrial dynamic structure forming a reticulum allows the relocation of substrates and metabolites to bioenergetically active areas. These dynamic features of the organelle, the increases in mitochondrial mass and the changes in mitochondrial morphology via fusion and fission are significantly related to the onset of metabolism-related diseases (Lee and Song, 2018).

As mentioned above, skeletal muscle is able to adapt itself in response to physiological and pathophysiological conditions. Mitochondria are subjected to modifications in presence of exercise or chronic contractile activity (Erlich et al., 2016; Wilson et al., 2007). John Holloszy was the first in 1967 to publish a remarkable evidence that exercise training promotes mitochondrial biogenesis in skeletal muscle, by showing that a strenuous program of treadmill running in rats led to increase mitochondrial protein expression and activity in skeletal muscles (Drake et al., 2016). Changes in mitochondrial biogenesis due to exercise are a result of multiple molecular events involving both nuclear and mitochondrial genomes. These pathways primarily include the activation of signalling kinases to initiate biogenesis, the induction of coactivator proteins such as PGC-1α and NRF transcription factor proteins, the transactivation of target genes, the import of precursor proteins into mitochondria, and the coordinated incorporation of both mitochondrial and nuclear gene products into an expanding organelle reticulum (Ljubicic et al., 2010). Factors which are overexpressed in biogenesis process, such as PGC-1α can increase the proportion of type 1 muscle fibers, thus contributing to the augmented endurance and resistance to fatigue (Calvo et al., 2008). Overall mitochondrial biogenesis in skeletal muscle cells can increase during the differentiation of myoblast into mature myotubes (Wilson et al., 2007).

Exercise is able to stimulate an increase in total mitochondrial protein (Scalzo et al., 2014), including enzymes involved in beta-oxidation, the tricarboxylic acid cycle and the electron transport system (Scalzo et al., 2014; Jacobs et al., 2013). Increases related to oxidative mitochondrial function as response to exercise intervention are reported (Jacobs et al., 2013).

If exercise can improve mitochondrial capacity, other physiological conditions as physical inactivity and aging can reduce mitochondrial capacity in muscle tissue.

1.3 Skeletal muscle/mitochondrial dysfunction in obesity

Obesity is a common and widespread epidemic disorder, and it can be due to the intake of a hypercaloric diet associated with poor physical activity. Different risk factors are involved in this emerging problem, also accounting for genetic and environmental factors. Obesity, in turn, is a risk factor for the onset of pathological conditions such as cardiovascular and respiratory diseases, musculoskeletal disorders and some type of cancer and Type 2 Diabetes Mellitus (De Mello et al., 2018; Dervashi et al., 2017; Williams et al., 2015; Thrush et al., 2013). In more general terms the obesity epidemics has a negative impact on human health through general pathophysiological mechanisms such as inflammation, oxidative stress, mitochondrial dysfunction and apoptosis (as in De Mello et al., and therein) (Fig.1.3). In this context, molecular processes and pathways that directly regulate energy metabolism or caloric intake appear to be possible targets for therapy.

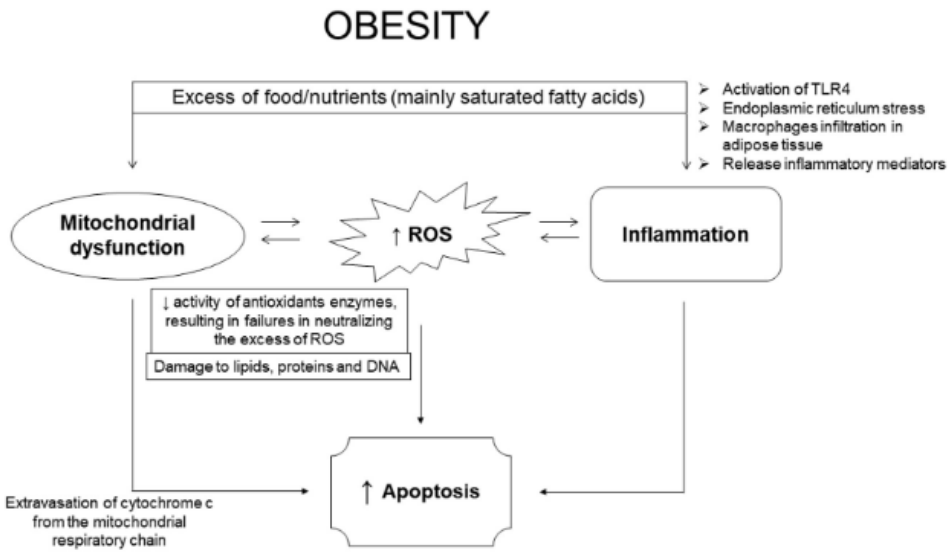


Fig.1.3. Implications of obesity in human health (De Mello et al., 2018).

Obesity is characterized by insulin resistance in major metabolic tissues such as skeletal muscle, liver and adipose tissue. The mitochondrial dysfunction in these pathological conditions is characterized by mechanisms that remain largely unknown. In obese patients the production of adipokines is altered, and this contributes the onset of metabolic syndrome and related complications such as the increased blood levels of cholesterol and

tryglicerides (Mirza, 2011). Liesa and Shirihai (Liesa and Shirihai et al., 2013) have reported evidences related to the link between mitochondrial dysfunction and excessive consumption of nutrients (as De Mello et al., 2018 and reference therein). Numerous studies have demonstrated that skeletal muscle metabolism and mitochondrial content/function are impaired in obesity, even if there is not a general agreement about the issue (Fisher-Wellman et al., 2014; Bakkman et al., 2010).

It is well documented that mitochondria in obese individuals have lower energy generation capacities, less defined internal membranes and decreased oxidation of fatty acids (Hernández-Aguilera et al., 2013), suggesting that the mitochondria of obese individuals are different from those of lean individuals. In skeletal muscles of obese mice mitochondria were small and short and mitochondrial fission increased, supporting the association between altered mitochondrial fission and mitochondrial dysfunction (Jheng et al., 2012). Altered mitochondrial function in skeletal muscle can lead to reduced fatty acid oxidation and to the inhibition of glucose-transport, and insulin-stimulated glucose transport is reduced (Hernandez-Aguilera et al., 2013). The total activity of NADH oxidase is impaired in skeletal muscle of obese compared with lean individuals (Ritov et al., 2010). Mitochondrial content (Ritov et al., 2005, Larsen et al., 2011), the levels of mitochondrial proteins (Kras et al., 2018; Wijingaarden et al., 2013) and of their (predominantly nuclear) genes (Patti et al., 2003, Mootha et al., 2003, Ritov et al., 2010, Hwang et al., 2010) have been shown to be reduced in the skeletal muscle of obese individuals, as well as in type 2 diabetes individuals, compared to lean controls. Mitochondrial dysfunction and impaired enzymatic activity of oxidative phosphorylation complexes have been confirmed more recently in obese skeletal muscle (de Mello et al., 2018; Formentini et al., 2017, Devarshi et al., 2017). In terms of oxidative mitochondrial capacity an impairment was observed in obese individuals compared to lean subjects (Vijgen et al., 2013; Bakkman et al., 2010) and this impairment is reversed when obese individuals are subjected to bariatric surgery (Coen et al., 2015; Vijgen et al., 2013). Impairments in isolated mitochondria from obese patients were observed in terms of mitochondrial phosphorylation efficiency (Konopka et al., 2015). Elevations in H_2O_2 emission rates were observed in the same patients (Konopka et al., 2015; Fisher-wellman et al., 2014). Obesity-related impairments in fatty acid oxidation were reported, with a reduction in mitochondrial content (Holloway et al., 2007), incomplete fatty acid oxidation and accumulation of intracellular fatty acids (Paran et al., 2015; Bell et al., 2010).

In terms of mitochondrial dynamics and of the regulation of mitochondrial morphology and number in skeletal muscle of obese subjects, an altered expression of optic atrophy gene 1 (OPA1) and a decreased expression of mitofusin 2 (Mfn2) (Putti et al., 2015; Zorzano et al., 2009) have been described, while mitofusin-1 was reduced in the skeletal muscle of obese rodents and humans along with smaller mitochondria and fragmentation of the mitochondrial network (Bach et al., 2003).

Liu and colleagues (Liu et al., 2014), have shown an altered mitochondrial respiratory function and a decreased ATP content in the muscle tissue of obese mice undergoing a high-fat diet (HFD). Moreover the authors confirmed that MFN1 and MFN2 were decreased while proteins, as Fis1 and Drp1, involved in the fission process, increased when compared to normal-weight high-fat diet mice.

Taking into account that, as already mentioned, skeletal muscle mitochondria are defined as subsarcolemmal (SS) and intermyofibrilar (IMF) based on their localisation, in obese patients Kras et al observed decreased amount of mitochondrial OXPHOS complexes mainly in IMF compared to lean controls (Kras et al., 2018).

The skeletal muscle comprises ∼40% of body mass and serves as one of the major regulators of body energy homeostasis, as described above. Under severely obese conditions, however, skeletal muscle accounts for ∼25% of body mass only and remains metabolically active to a certain extent.

Obesity leads to a sedentary lifestyle and it is reaching epidemic proportions in developed countries. This obesity condition is accompanied by increased fat and lean mass, but the fat mass increases at a larger scale, resulting in a smaller lean muscle to fat ratio. The increase of body mass in obese individuals also leads to skeletal muscle overload, resulting in greater leg and trunk muscle strength, whereas handgrip or arm strength are unchanged. Individuals who exercise regularly gains more muscle mass; have better muscle function, and lower risks of onset of obesity (Lafortuna et al., 2005). In contrast, physical inactivity is associated with a decrease in muscle mass, an increase of visceral adiposity, macrophage infiltration, chronic systemic inflammation, insulin resistance, obesity, and T2DM (Pedersen et al., 2012). As discussed in the previous paragraph, fiber type may affect skeletal muscle function and weight loss success in obesity. Type 2a fiber expression is positively correlated with the weight gain response to overfeeding (Sun et al., 2002). Since type 2 fibres have a reduced oxidative capacity, these fibers may also have a reduced capacity to control oxidative stress

(Anderson and Neufer, 2006). Mitochondrial dysfunction helps to explain a number of the common signs and symptoms of obesity: low energy expenditure, chronic food intake in excess of expenditure, and markers of low-grade systemic inflammation (Rogge, 2009). Investigations have demonstrated that the reduced muscle mitochondrial content and functional capacity in obesity is reversible with moderate weight loss combined with physical activity (Toledo et al., 2007, 2006; Menshikova et al., 2007, 2005; Goodpaster et al., 2003). Therefore, biological factors, metabolic and behavioural adaptations would contribute to changes in energy expenditure, leading to the weight loss success. This represents a possible therapeutic approach to adopt in this metabolic condition.

The beneficial effects of exercise are known and are adopted in several diseases and physiological conditions. Exercise is a potent and non pharmaceutic intervention for management and treatment of a wide spectrum of lifestyle-related diseases (Lee and Song, 2018) such as pulmonary, cardiovascular, muscle, bone, joint diseases, cancer and depression (Vina et al., 2012). Exercise is a mean to improve physical performance and overall health, preventing multiple chronic diseases, to a large extent as a consequence of the adaptations occurring in skeletal muscle (Drake et al., 2016). Obesity, immobility and muscle atrophy are conditions that lead to mitochondrial dysfunction in skeletal muscle causing metabolic inflexibility. In this regard exercise training is well known to enhance mitochondrial function, leading to improvements in whole-body metabolic homeostasis. Exercise activates signalling networks that control coordinately mitochondrial remodelling, including mitochondrial biogenesis, dynamics and mitophagy (Gan et al., 2018) (Fig.1.3.1). In obese subjects exercise training (i.e. high intensity/high volume interval training) has determined improvements in aerobic capacity and exercise performance (Boyd et al., 2013). Sprint interval training improves circulatory function and increases VO_2 max in sedentary, overweight/ obese women (Trilk et al., 2011). Exercise training leads, in obese patients, to enhanced rates of mitochondrial fatty acid oxidation and to glucose tolerance (Bruce et al., 2006). Moreover, as showed by Gerritis and colleagues (Gerritis et al., 2010), different degrees of weight loss success reveal differences in skeletal muscle gene expression profiles, in structural and metabolic characteristic and in muscle fibers composition. Induction of weight loss by bariatric surgery also determines an improvement of mitochondrial functions, in terms of oxidative capacity (Vijgen et al., 2013).

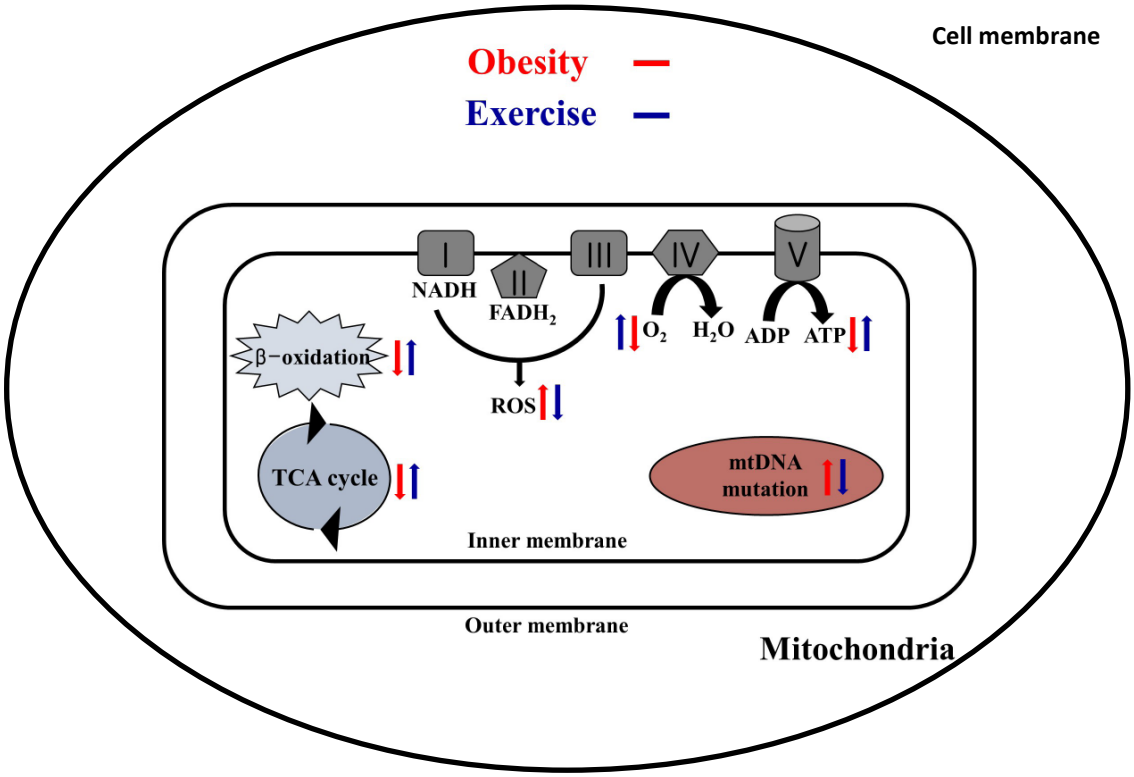


Fig.1.3.1. Effects of obesity and exercise training on mitochondrial dysfunction. Exercise training protects against obesity-induced mitochondrial dysfunction (e.g., O_2 respiration, ATP production, ROS emission, β-oxidation, markers of TCA cycle, mtDNA mutation) in skeletal muscle (modified from Heo et al., 2017).

1.4 Skeletal muscle/mitochondrial dysfunction in microgravity/inactivity

Skeletal muscle, as described previously, is a dynamic tissue able to support different conditions of training. Several pathological conditions as disuse and muscle atrophy can modify muscle in its molecular structure and function, impairing the overall quality of life. Specifically, disuse and muscle atrophy are common events occurring with prolonged bed rest, casting, spaceflight, aging, caloric restriction, physical inactivity. What actually occurs at the molecular level during a period of disuse/immobility has not yet been fully clarified and understood. For this reason bed rest studies are often utilized as experimental models and as attempt to understand consequent changes of prolonged muscle disuse and unloading occurring in skeletal muscle (Pavy-Le Traon et al., 2007). In this respect bed rest studies are

also of interest to space agencies, since they allow to simulate the effects of reduced gravity ("microgravity") on different organs and physiological functions (Pavy-Le Traon et al., 2007). Physiological adaptations to microgravity comprehend, among others, loss of body weight, tissue fluid redistribution and cardiovascular adaptations, hormonal changes, muscle atrophy, loss of muscle strength and power, profound deconditioning, bone loss, alterations of the immune system, altered sensory inputs within the body (Hargens and Richardson, 2009) (Fig.1.4). Pavy-Le Traon and colleagues (Pavy Le-Traon et al., 2007) describe in detail several physiological adaptations associated with bed rest, which are very similar to those occurring in space. Bed rest, ranging from several hours to several weeks or months, has been the model most frequently used to simulate the physiological effects of weightlessness, in particular on the cardiovascular system and body fluid regulation. Bed rest induces a fluid shift from the lower to the upper part of the body. This fluid shift results in a transient increase of plasma volume as more fluid moves into the vascular compartment from the lower body than that is filtered out of capillaries into the upper body (Atkov and Bednenko 1992; Gharib et al., 1988; Greenleaf, 1984). This thoraco-cephalic fluid shift together with the plasma volume expansion stimulate central volume carotid, aortic and cardiac receptors inducing an increase in diuresis and natriuresis and a decrease in plasma volume (Pavy-Le Traon et al., 2007). The reduction in plasma volume leads to a set of cardiovascular changes including changes in cardiac performance and baroreflex sensitivity and reduced aerobic capacity. As it occurs in spaceflight, cardiovascular deconditioning characterised by orthostatic intolerance and reduced exercise capacity is observed at the end of bed rest. Calcium excretion is increased during the bed rest leading to a sustained negative calcium balance, while calcium absorption through the gut is reduced. Bodyweight, muscle mass, muscle strength are reduced during immobility and also bone density and bone architecture are altered. Some alterations can involve circadian rhythms. Bed rest can also reduce the production of erythropoietin, lowering red cell mass. Both in bed rest and spaceflight subjects complain of poor and disturbed sleep even though EEG records appear to be generally normal. On return from space or reambulating after bed rest period, subjects show a high incidence of orthostatic hypotension, balance problems, wider short steps and foot dragging occasionally, poor coordination. All changes described depend on neural, neurohumoral and humoral mechanisms that are affected likewise in both bed rest study and spaceflight (Pavy Le-Traon et al., 2007). Furtherly, immobilization, as in bed rest, can modify

energy requirements, protein metabolism, insulin resistance, changes in the humoral regulating mechanisms and others. Indeed, during bed rest, energy requirements are reduced to the basal metabolic rate plus a small percentage of calories because of the energy expenditure generated by the residual small amount of movements. These activities include those of turning around in bed and being awake.

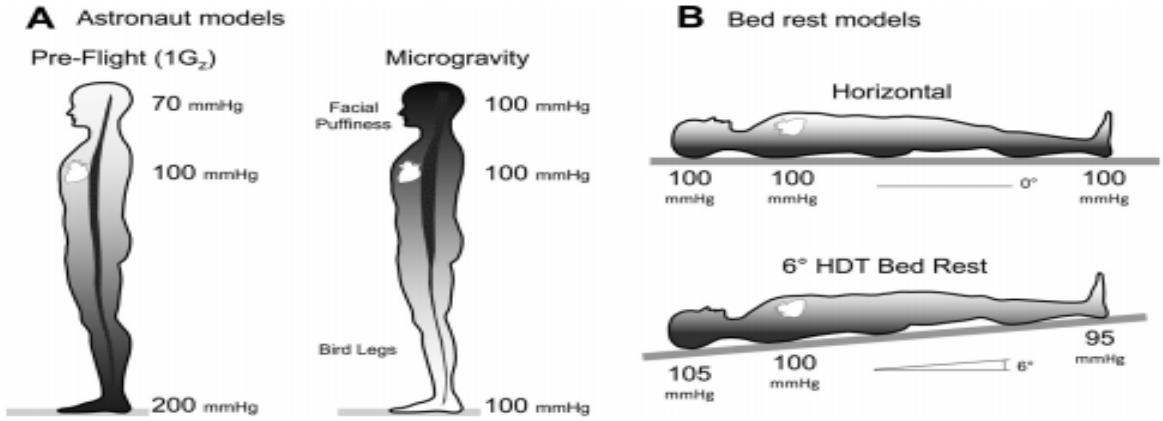


Fig.1.4. Adaptations to microgravity. The adaptation to microgravity is a potential mechanism that includes loss of body weight, tissue fluid redistribution, loss of hydrostatic pressures and decreased sensory inputs within the body A) Astronaut models and B) horizontal bed rest (top) and 6 degrees nead-down-tilt bed rest (bottom) (Hargensen and Vico , 2016).

Generally, in healthy individuals bed rest or spaceflights induce skeletal muscle atrophy, characterized by reduced muscle strength, lower protein synthesis and higher protein degradation rate, protein carbonylation, shift in muscle fiber type from slow type 1 to fast type 2A and 2X increased oxidative stress, development of insulin resistance, and intramuscular fat deposits. During atrophy/disuse, skeletal muscles can be infiltrated by immune cells and adipose tissue.

At molecular level, a decrease in protein synthesis and an accelerated proteolysis during periods of prolonged inactivity lead to muscle atrophy (Rennie et al., 2010; Sandri, 2008). Protein degradation process occurs in muscle fibers and allows to recycle and remove damaged proteins. Protein synthesis rates decrease as result of a decreased transcriptional activity, increased degradation of transcripts (e.g.mRNA stability), and decreased assembly of translational machinery (i.e., translation). The PI3K/Akt/mTOR pathway is a key regulatory pathway involved in processes as protein synthesis in skeletal muscle and it is a down-regulated in skeletal muscle during prolonged periods of inactivity (Kelleher et al., 2015,

2013; Haddad et al., 2006). There are different proteolytic systems able to interact each other and to maintain proteostasis in the muscle by removing damaged structures. Autophagy, ubiquitine proteasome system (UPS), calpain, and the caspase-3 proteolytic systems contribute to protein degradation during disuse muscle atrophy (Romanello and Sandri, 2016; Powers et al., 2012; Jackman and Kandarian, 2004). Importantly, the UPS plays a large role in the protein degradation that occurs in skeletal muscle atrophy (Bilodeau et al., 2016). Atrogin-1/MAFbx and muscle ring fingerprotein-1 (MuRF-1), are two muscle specific E3 ligases acting in UPS mediated protein degradation in skeletal muscle wasting (Rom and Reznick, 2016). The calpain and caspase-3 systems are allosterically regulated by Ca^{2+} and act as critical proteolytic enzymes required when it occurs a condition of inactivity induced atrophy (Talbert et al., 2013). Damaged and dysfunctional mitochondria can also release mitochondrial-specific signalling molecules as the mitochondrial proteins apoptosis inducing factor (AIF) and CytC into the cytosol, and this leads to the activation of proteolytic systems as caspase-3 which triggers myonuclear apoptosis, lowering the transcriptional capacity of myofibers (Adhihetty et al., 2005). An important transcriptional regulator playing a role in muscle wasting is Forkhead box O (FoxO) (Sandri et al., 2004; Gomes et al., 2001); it induces the transcription of UPS related genes (e.g. Atrogin-1 and MuRF-1) as well as autophagy related genes (e.g. microtubule-associated proteins 1A/1B light chain 3b (LC3), BCL2 interacting protein 3 (Bnip3), and cathepsin L (Mammucari et al., 2007). The autophagic system can be considered a proteolytic system involved in inactivity-induced muscle atrophy (Smuder et al., 2018). During autophagy proteins can be degraded but also organelles; the autophagic process involves the formation of an autophagosome engulfing damaged protein structures and in order to do it, it needs the fusion of the autophagosome with lysosomes.

The effects of a prolonged bed rest were also described as decline of VO_2 max (Ried-Larsen et al., 2017), muscle mass loss and impairment of oxidative function (Salvadego et al., 2018, 2016, 2011; Dirks et al., 2016; Porcelli et al. 2010, Bergouignan et al., 2011, 2009). Prolonged period of bed rest leads to impairments in oxidative function at several levels including pulmonary function and cardiovascular O_2 delivery to peripheral O_2 utilization (Ade et al., 2015). Bed rest studies, carried out on young subjects, observed a significant functional impairment of skeletal muscle oxidative metabolism evaluated in vivo following 10 days

(Salvadego et al., 2016), 21 days (Salvadego et al., 2018), and 35 days (Salvadego et al., 2011; Porcelli et al., 2010) of bed rest conditions.

Mitochondrial function following immobilization is subjected to a downregulation including oxidative phosphorylation, TCA cycle, fatty acid metabolism, mitochondrial transcription and translation, and solute and protein transport (Abadi et al., 2009). Mechanisms and factors involved in mitochondrial impairment during muscle inactivity have not been fully clarified. Mitochondria undergo decreases of respiratory capacity and coupling, morphological changes and increased mitochondrial ROS emission (Picard et al., 2015, 2012; Abadi et al., 2009; Kavazis et al., 2009; Muller et al., 2007) (Fig.1.4.1).

Ten days of exposure to inactivity showed an impaired skeletal muscle oxidative function in vivo without affecting mitochondrial oxidative phosphorylation function (respiration) ex vivo and mitochondrial content (Salvadego et al., 2016), while a period of twenty days to exposure bed rest revealed changes of mitochondrial respiratory function and the degree of mitochondrial coupling (Salvadego et al., 2018). Furthermore, the decrease of mitochondrial respiration was seen in association with decreased insulin sensitivity and energy expenditure following 21 days of bed rest (Kenny et al., 2017). In individuals subjected to 14 days of bed-rest a reduction key regulators of mitochondrial biogenesis/remodelling, namely PGC-1α and Sirt3 and also in single OXPHOS complexes protein content (Buso et al., 2019) was observed. Other investigations observed that one week of bed rest strongly reduced muscle mass, strength, and physical performance but also protein content of all complexes of OXPHOS (Dirks et al., 2016). Changes in mitochondrial proteins during immobilization-induced muscle atrophy/limb can induce a coordinate downregulation of mitochondrial pathways (Abadi et al., 2009). Moreover, pathways involved in mitochondrial biogenesis are downregulated during skeletal muscle inactivity (Brocca et al., 2012; Chen et al., 2007). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a key regulator of mitochondrial biogenesis, is shown to be significantly decreased during prolonged muscle inactivity (Kang et al., 2013), negatively affecting mitochondrial function and structure (Fig.1.4.1). PGC-1α is able to regulate signalling and transcriptional events, also outside of mitochondria. For example TFAM is a downstream target of PGC-1α and it regulates the transcription of mitochondrial DNA (mtDNA) encoded genes. TFAM protects mtDNA from damage and degradation, and decreases in TFAM during muscle atrophy may expose mtDNA

to a possible damage, and, consequently, lead to mitochondrial signalling events that activate atrophic pathways (Theilen et al., 2017).

The fission mitochondrial protein, dynamin related protein 1 (DRP1) is localized in the cytosol and in order to became active and translate to mitochondria is phosphorylated at specific-serine sites, Ser616, thereby inducing mitochondrial fission during muscle atrophy (Chou et al., 2012; Chang et al., 2010; Taguchi et al., 2007). In regard to mitochondrial fusion, levels of MFN1, MFN2 and OPA1 protein content decrease during muscle inactivity (Cannavino et al., 2015). A decrease in fusion process along with a concomitant increase in mitochondrial fission leads to a mitochondrial fragmentation during muscle inactivity (Fig.1.4.1).

An important component of mitochondrial dysfunction-induced muscle atrophy is the role of mitochondrial ROS emissions during inactivity (Powers et al., 2012); high levels of ROS production can negatively affect the pathways involved in maintaining and controlling muscle mass, even if mitochondrial ROS emissions has a role of signalling molecules in homeostatic regulation of muscle fibers (Powers et al., 2016).

As explained, several mitochondrial impairments during muscle inactivity were observed through different studies, revealing areas of research to investigate in order to establish the possible mechanisms of protection of skeletal muscle mitochondria against inactivity-induced dysfunction.

The cytoprotective role of exercise against muscle atrophy is well recognized. More specifically, exercise increases skeletal muscle capacity to resist to stresses (Wiggs, 2015). Tunner and colleagues (Tunner et al., 2015) have shown that exercise rehabilitation restored bed rest-induced deficits in lean mass, strength, muscle protein synthesis in older adults. Furthermore, exercise rehabilitation improved mitochondrial pathways which were downregulated by the bed rest period (Buso et al., 2019). The recovery of muscle mass and function in obesity and immobility/atrophy depends on the exercise protocol.

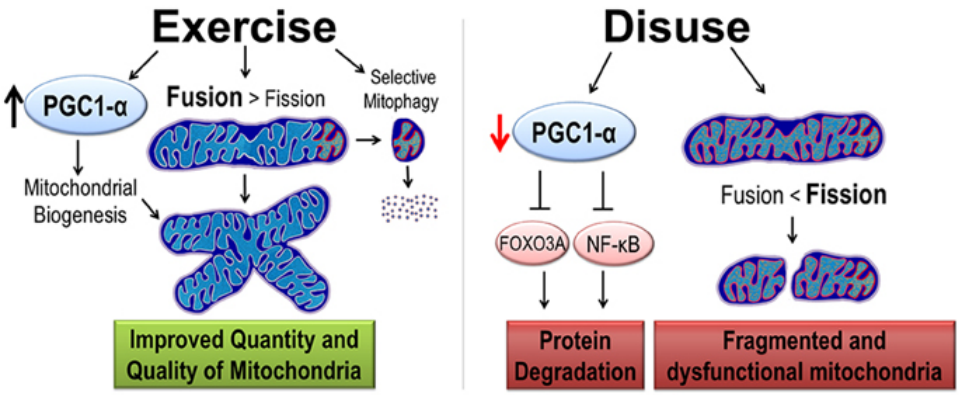


Fig.1.4.1. Exercise and disuse effects on mitochondrial quality and quantity. (Wiggs, 2015)

2. AIM OF THE WORK

As mentioned in the Introduction, skeletal muscle is highly dynamic tissue which, apart for force and movement production during exercise, is deeply involved in whole-body homeostatic adjustments to different physiological and pathological conditions. Mitochondria, the intracellular organelles responsible for ATP production by oxidative phosphorylation, are responsible for a significant portion of the muscle plasticity mentioned above. One of the main environmental stressors known to trigger adaptations in mitochondria and skeletal muscle fibers is exercise, in terms both of exercise training and in terms of reduced activity/immobility. The aim of the present PhD work was to evaluate the adaptations to exercise, or to the lack of exercise, at the level of mitochondrial oxidative phosphorylation function in two conditions particularly relevant for the development of diseases, that is obesity (study n. 1: obese patients undergoing different protocols of exercise training) and bed rest-induced microgravity and immobility (study n. 2, carried out on young healthy volunteers).

3. EXPERIMENTAL STUDIES

3.1 Study n. 1: Mitochondrial adaptations to exercise training in obese patients

3.1.1 Introduction

Obesity is a common health problem and it represents a significant risk factor for several pathological conditions. Success in obesity treatment programs is highly variable, and it is related in part to compliance and program characteristics (e.g., type and duration of hypocaloric diets, educational components, exercise-associate energy expenditure, type of exercise). Numerous studies have demonstrated that skeletal muscle metabolism and mitochondrial content/function are impaired in obesity (de Mello et al., 2018). Mitochondrial alterations could be involved at several levels in the pathogenesis of the disease (see General Introduction above).

If and to which extent these mitochondrial alterations can be affected by therapeutic interventions, and particularly by those associated with exercise training, is not known. . We hypothesize more pronounced effects following HIIT vs. those determined following MICT.

The study was performed at the Mountain Sports Study Centre, Gemona Hospital (Italy) and it was supported by the Department of Medicine of University of Udine under the supervision of Professor S. Lazzer with the collaboration of a research group from the University of Ferrara (Prof. Angela Passaro).

3.1.2 Aim

The aim of this project was to evaluate mitochondrial oxidative ohosphorylation function in obese individuals undergoing to one of two types of exercise training, MICT or HIIT. Data obtained on mitochondria were evaluated in association with those related to functional variables related to oxidative function at the whole-body level (although these

data will not be specifically discussed in the present thesis). High-resolution respirometry in permeabilized fibers is considered by many authors (see e.g. Picard et al., 2011) the "gold standard" in the functional evaluation of mitochondrial function. Since high-resolution respirometry was rarely used in obese individuals undergoing exercise training, the present study provides an evaluation of mitochondrial function following two types of physical intervention, moderate intensity continuous training (MICT) and high intensity interval training (HIIT) (Gibala, 2009), a relatively new training method which has recently gained a lot of attention also in patients populations.

3.1.3 Materials and Methods

Participants and Study design

Thirty-two healthy obese volunteers, 17 men and 15 women (age 38± 8.5 years, BMI 35.5±4.2 kg/m^2) were recruited from the Exercise Physiology Laboratory of the University of Udine where they underwent a medical and dietetic evaluation. The inclusion criteria were: age between 18 and 50 years and body mass index (BMI) $\geq$ 30 kg m^{-2}. Subjects who had previously participated in weight management programs, had cardiovascular, respiratory, neurological, muscular-skeletal, metabolic and/ or endocrine diseases or those who were taking any drugs known to influence energy metabolism and cardiorespiratory adjustments to exercise were excluded. No subject was taking beta-blockers. The Ethics Committee of the Friuli-Venezia-Giulia Region approved the study. Before the study began, the purpose and objectives were carefully explained to each subject and written informed consent was obtained. A physical activity questionnaire was administered to exclude potential volunteers who engaged in any continuous activity longer than 20 minutes than once a week, indicative of a moderate physical activity level (IPAQ-SF) (Craig et al., 2003).

After the first inclusion visit, subjects were admitted to 3 months of multidisciplinary weight-management program including lifestyle education, physical activity and dietary follow up. Control tests including assessment of body composition, physical capacities, fat oxidation rate, physical activities and dietary habits were performed during two weeks before the beginning (PRE) and immediately after completion the weight-management program (POST). At the same time, skeletal muscle biopsies of the vastus laterals muscle were taken for measurement of *ex vivo* mitochondrial respiration. In addition, indexes and physical

capacities were monitored monthly during the program, in order to adjust food allowances and physical activities individually**.**

Physical activity

During the 3 months-weight-management period, subjects followed a physical training program including three training sessions per week under supervision. The subjects were split randomly in two groups, one group following a moderate-intensity continuous training (MICT, n =16) and the second group following high intensity interval training (HIIT, n= 16). All subjects completed 34 ± 0.14 sessions of physical training. The intensity of MICT on the treadmill was set at a heart rate (HR) corresponding to 60% of the initial $V'O_2$peak, the duration of the training session was 44 ± 8 min. HIIT consisted of 10 min of warm up (50 % of $V'O_2$peak) followed by 3 to 7 repetitions of 3 min bouts of high-intensity walking (100 % of $V'O_2$peak), interspersed by 1.5 min walking at low intensity (50% of $V'O_2$peak) and followed by 5 min of cool down (50% of $V'O_2$peak); the duration of the training session was 33 ± 4 min. Exercise intensity was set up by adjusting the slope of the treadmill. The amounts of energy expended during the training sessions were similar for both groups: 20 kJ per kg of fat-free mass (FFM), which corresponds to about 1.5 MJ per session.

Research assistants and physical trainers were responsible for verifying that each subject participated to each training session, performed the exercises correctly, and completed at least 90 % of the exercise sessions. All subjects were also advised to practice leisure physical activities during the weekend and holidays.

During the 4-months follow up the same training suggestions were given to all subjects. The suggestions consisted of three training session per week covering the full intensities range: one high intensity (90% HR peak and less than 30 min), one medium intensity (~70-80% HR peak and 30-50 min) and one low intensity (<70% HR peak and more than 60 min). Training during the follow-up period was not supervised and compliance was checked by a questionnaire (Craig et al., 2013).

Anthropometric characteristics and body composition

The medical history and a physical examination of subjects were taken at the time of admission to the weight-management program. Body mass (BM) was measured to the nearest 0.1 kg with a manual weighting scale (Seca 709, Hamburg, Germany) with the subject dressed only in light underwear and no shoes. Stature was measured to the nearest 0.5 cm on a standardized wall-mounted height board. BMI (Nuttal, 2015; Wells and Fewtrell, 2006) was calculated as BM (kg) x stature^{-2} (m). Body composition (Wells and Fewtrell, 2006) was measured by bioelectrical impedance (BIA, Human IM Plus; DS Dietosystem, Milan, Italy) according to the method of Lukaski et al. (Lukaski et al., 1986). Fat mass (FM) and fat-free mass (FFM) were calculated with equations derived either in obese people of different ages and BMI (fat-specific formulae), by utilizing a two-compartment model (Gray et al., 1989).

Biopsies and Samples Collection

Skeletal muscle biopsies were obtained from the *vastus lateralis* muscle of individuals by percutaneous biopsy after an overnight fast. Muscle biopsies were taken in all subjects one week before the exercise (PRE) and one week after the end of exercise (POST). Considering the methodological problems, which will be described in detail later, is reported the selection of subjects in relation to two groups before (MICT) and after (HIIT) the physical intervention: N=6 for PRE and N=8 for POST in MICT group, N=6 for PRE and N=7 for POST in HIIT group. Biopsies were obtained using a microneedle (Tru-cut Histocore, 12 G, Biomed Instrument & product DmbH, Germany), after anesthesia of the skin, using lidocaine (2%) and a small incision to penetrate skin and fascia. For each subjects two specimens were collected which were put at 4°C in BIOPS solution (imidazole 20 mM, MES 50 mM, DTT 0.5 mM, EGTA-calcium buffer 10 mM – free Ca^{2+} concentration 100 nmol/L, $MgCl_2$ 6.56 mM, ATP 5.77 mM, taurine 20mM, phosphocreatine 15 mM; pH 7.1) (4°C) containing 10% (wt/vol) fatty acid-free BSA and 30% (vol/vol) DMSO (4°C), and then immediately frozen in liquid nitrogen and stored at -80°C until the moment of analysis (Kutznetsov et al., 2003). Experimental measurements were set up within 3 months after the collect of muscle biopsy.

High Resolution Respirometry Analyses

High-resolution respirometer Oxygraph-2k (Oroboros-2K, Innsbruck, Austria) was used to evaluate mitochondrial respiration ex vivo of permeabilized *vastus lateralis* skeletal muscles fibers obtained by biopsy (see above). This procedure permeabilizes the plasma membrane in order to allow the entrance into the fibers of the substrates during the experiment. The permeabilization is obtained by incubation with saponin, a mild cholesterol-specific detergent that selectively permeabilizes the sarcolemmal membranes while keeping mitochondrial membranes, which contain little cholesterol, completely intact (Kuznetsov et al., 2008).

Mitochondrial activity was evaluated by measuring O_2 consumption polarographically by high-resolution respirometry (Pesta and Gnaiger, 2012). The muscle sample (~ 10-20 mg of wet weight) was quickly thawed and immediately placed in BIOPS containing 2 mg / ml (w/v) of BSA, to remove any residual DMSO from the tissue. Fiber bundles were cleaned as much as possible from the connective tissue and fatty tissue excess and separated with sharp-ended needles under magnification (50 x) (MC170 HD, Leica Microsystems, Switzerland, LTD) leaving only small areas of contact. The mechanical separation of the fibers with sharp-needle was carried out before the respirometry analyses. The excessive of tissue adipose as shown in Fig.3.1.3 C, D, E was not always easy to remove thus, the possibility of damage to muscle fibers were increased by the isolation procedure. Therefore, many muscle specimens had lipidic component which was difficult to remove in order to avoid the disruption of muscle fibers and to reach homogeneity of the tissue samples for the experimental analyses. The mechanical separation of specimen (Fig.3.1.3 F, G) in isolated fibers under magnification was difficult and required specific attention because of the morphology of muscle fibers. Indeed muscle fibers specimens had a small length because of the microneedle used during the biopsy procedure (Fig 3.1.3 A, B). Muscle biopsies were taken by utilizing a microneedle and not by the classic Bergstrom approach (Fig.3.1.3.1 A, B) for safety and ethical reasons. The small length of muscle fibers specimens made them more exposed to a risk of a rigor state induced by ATP, and for this reason we have added Blebbistatin, a myosin II ATPase inhibitor to the respiration medium (see below) (Hughes et al., 2015). Blebbistatin prevents the shortening of the muscle fiber due to spontaneous contractions during the measurements (Ebrahim et al., 2013).

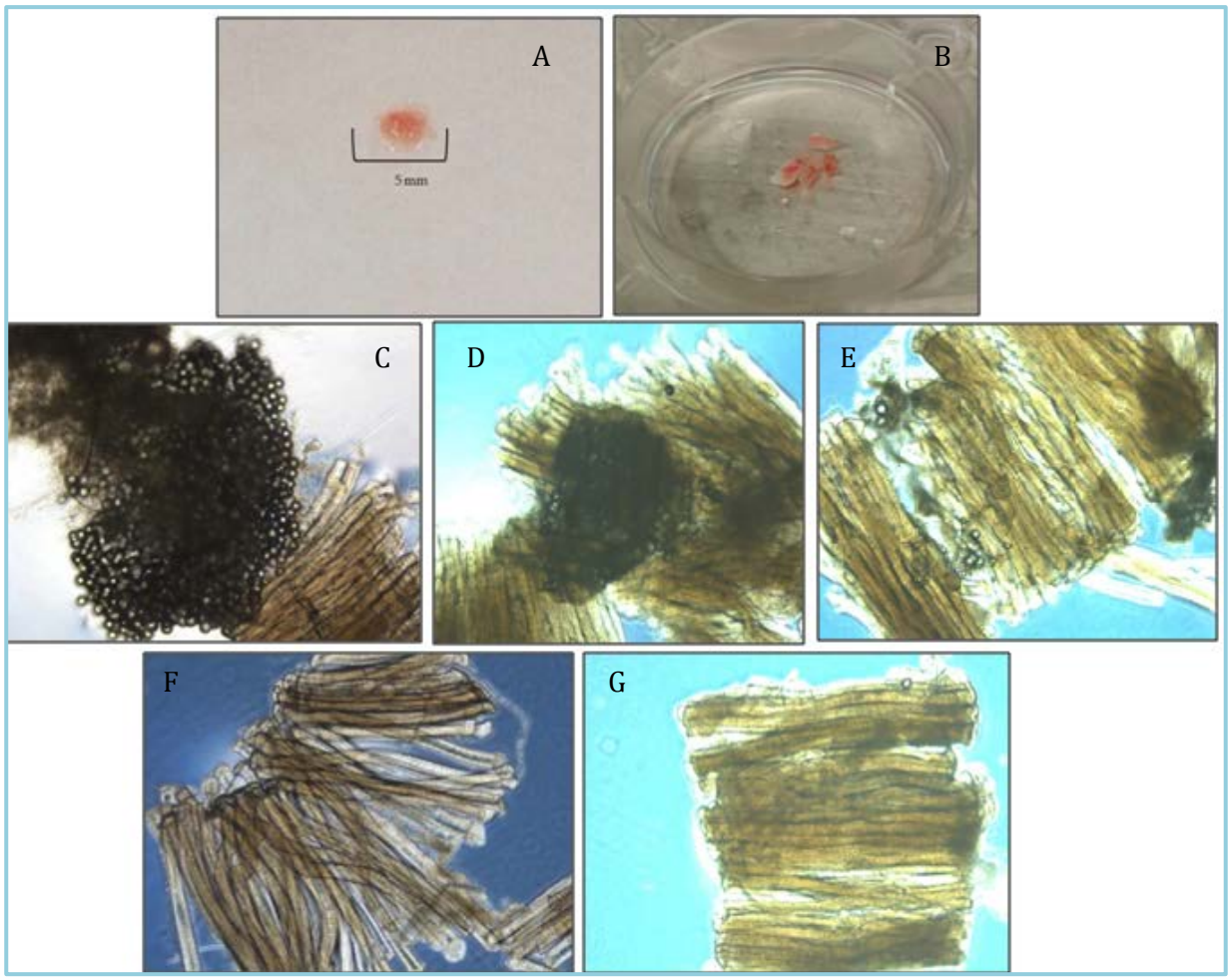


Fig.3.1.3. Preparation of permeabilized muscle fibers from biopsy of human vastus lateralis taken from obese individuals. (A, B) Length and size of typical muscle; (C, D) Amount of adipose tissue (dark brown); (E) Lipidic component located between the fiber bundles. (F, G) Fiber bundles after mechanical separation with sharp-needle.

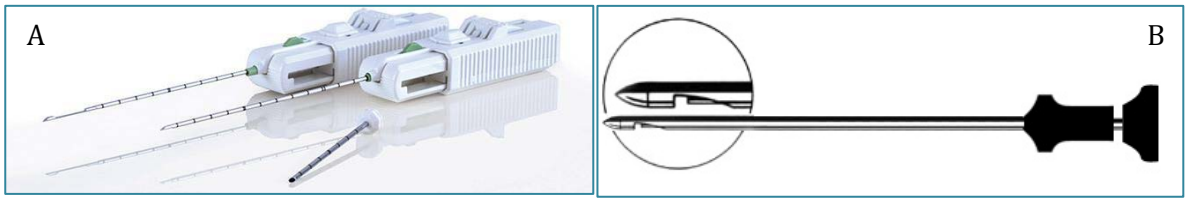


Fig.3.1.3.1. Images of two type of needles used for muscle biopsies. A) Microneedle and B) Bergstrom needle for muscle tissue removal.

Specimens were carefully analysed and evaluated in terms of outer mitochondrial membrane intactness. Lesions of this membrane could be the result of the biopsy procedure itself, of the freezing/thawing sequence, and/or of the mechanical manipulation of the fiber before the actual measurement.

Fibers were then incubated in 2 ml of BIOPS (4°C) containing 20 µg/ml (w/v) saponin for 30 min with continuous gentle stirring to ensure complete permeabilization. After being rinsed twice for 10 min in a respiration medium (MiR05; Oroboros Instruments: EGTA 0.5 mM, potassium lactobionate 60 mM, $MgCl_2 \cdot 6H_2O$ 3 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, sucrose 110 mM, and BSA 1 g/l, pH 7.1), permeabilized fibers were measured for wet weight and immediately transferred into the respirometer (Oxygraph-2k) chambers for O_2 consumption analysis. The instrumentation allows for O_2 consumption measurements with small amounts of sample in closed respiration chambers containing 2 ml of air-saturated respiration medium (MiR05 plus 280 U/mL catalase) at 37°C (Pesta and Gnaiger, 2012). 2–4 mg of muscle fibers were used for the analysis. Standardized instrumental and chemical calibrations were performed to correct for back-diffusion of O_2 into the chamber from the various components, leak from the exterior, O_2 consumption by the chemical medium, and by the sensor O_2 (Pesta and Gnaiger, 2012). The O_2 concentration in the chamber was maintained between 300 and 450 µM (average O_2 partial pressure $PO_2 \sim 250$ mmHg) to avoid O_2 limitation of respiration. Intermittent reoxygenation steps were performed during the experiments by adding 3 µl of 0.3 mM hydrogen peroxide solution into the medium containing catalase (Pesta and Gnaiger, 2012). All respirometric analyses were carried out in duplicate.

A substrate-uncoupler-inhibitor-titration protocol with a substrate combination was applied (Pesta and Gnaiger, 2012). Measurements were run in the presence of 25 µM blebbistatin to prevent ADP-induced contraction (rigor), particularly evident in small length biopsies such as those obtained by microneedle (Hughes et al., 2015). Non-phosphorylating resting mitochondrial respiration was measured in the presence of malate (4 mM) and glutamate (10 mM), and in the absence of adenylates, so that O_2 consumption was mainly driven by the back leakage of protons through the inner mitochondrial membrane (Complex I state 2, or “leak” respiration). Saturating ADP (5 mM) was then added to measure Complex I respiration in phosphorylating condition (Complex I state 3 respiration). Succinate (10 mM) was added to support convergent electron flow into the Q-junction through Complexes I and II, thereby achieving the maximal ADP-stimulated mitochondrial respiration sustained by Complex I and II (Complex I+II state 3 respiration), as verified by further addition of 5 mM ADP. Cytochrome C (10 µM) was added to test for mitochondrial outer membrane integrity and only samples demonstrating < 10% increase in respiration were considered good for the

analysis. Maximal electron transport system (ETS) capacity was then evaluated by stepwise addition of the chemical uncoupler protonophore carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Afterward, Rotenone (1 µM) was added to inhibit Complex I and to evaluate ETS sustained by Complex II (rotenone-insensitive) and by Complex I (rotenone-inhibited). Finally, antimycin A (2.5 µM) was added to inhibit also Complex III, providing a measure of residual O_2 consumption, indicative of non-mitochondrial O_2 consumption. Mitochondrial respiration was then corrected for O_2 flux due to the residual O_2 consumption. Data were digitally recorded using DatLab4 software (Oroboros Instruments). The respiration parameters were normalized by citrate synthase (CS) activity and expressed as pmol $O_2 \cdot s^{-1} \cdot mU^{-1}$. The degree of oxidative phosphorylation coupling for a specific substrate supply (glutamate and malate in this case) was determined by calculating the ratio between Complex I+II state 3 respiration minus Complex I leak respiration and Complex I+II state 3 respiration [(state 3 – leak)/state 3] (Gnaiger, 2014, Salvadego et al., 2018, 2016).

Citrate Synthase Activity Assay in Obese Study

Upon completion of the measurements, muscle fibers were taken away from the chamber and underwent to a motor driven homogenization in a pre-cooled 1 ml glass-glass potter (Wheaton, USA). The muscle specimen was suspended 1:20 w/v in a homogenization buffer containing sucrose (250 mM), Tris (20 mM), KCl (40 mM) and EGTA (2mM) with 1:50 v/v protease inhibitor cocktail (P8340-Sigma). The specimen was homogenised in an ice-bath with 20 strokes at 500 rpm. The homogenate was centrifuged at 600 x g for 10 minutes in order to discard cellular debris. The supernatant was used to evaluate protein concentration according to the method of Lowry (Lowry et al., 1951). 10 µg of protein were added to each well of a 96-well-microplate along with 100 µl of 200 mM Tris-Triton X-100 (0,2% v/v), 20 µl of 1 mM 5,5'-dithiobis-2-nitrobenzoate (DTNB) freshly prepared, 6 µl of 10 mM acetyl-coenzyme A (Acetyl-Co-A) and mQ water to achieve a final volume of 190 µl. A background ΔAbs, to detect any endogenous activity by acetylase enzymes, was recorded for 90 seconds with 10 seconds interval at 412 nm at 25°C by an EnSpire 2300 Multilabel Reader (PerkinElmer). This ΔAbs was subtracted from the one given after the addition of 10 µl of 10 mM oxalacetic acid that started the reaction. All assays were performed at 25 °C in triplicate

on homogenates. Activity was expressed as mUnit (nanomoles/min) per mg of protein. This protocol was modified from (Spinazzi et al., 2012; Srere, 1969).

Statistical analyses

Collected data were analysed with different statistical methods, according to data subdivision, number of groups and clinical intervention on the studied group.

Statistical analyses were performed using SPSS 20.0 software (IBM, Chicago, USA), with significance set at $p<0.05$. All results were expressed as means and standard error (SE). Normal distribution of the data was tested using the Shapiro–Wilk test. Sphericity was verified by Mauchly's test. When the assumption of sphericity was not met, the significance of the F-ratios was adjusted according to the Greenhouse–Geisser procedure.

Respirometric analyses data were analysed with a generalized linear mixed, multilevel, growth model, fit by maximal likelihood, which accounts for random effect due to subjects and intercept and fixed effects due to group (MICT *vs* HIIT), gender, time, and interaction Group x Time, taking in account the correlation of the data. Since the gender distribution of the subjects was balanced in the two groups, and no gender differences and no interaction between groups were found in the parameters studied, then male and female subjects were considered together.

3.1.4 Results

Thirty-two healthy obese volunteers have taken part in this investigation, their main physical characteristics at baseline and after the training intervention are shown in Table 1.

Before the exercise no significant differences were found between MICT and HIIT in anthropometric characteristic and body composition. After the exercise (POST), the subjects showed a decreased body mass, BMI, waist circumference, hip circumference, FM decreased, while FFM did not change significantly in MICT and HIIT groups, without differences between groups.

Before the training intervention, no significant differences were found between MICT and HIIT for HRpeak and $V'O_2$peak (Table 1). After the exercise, HRpeak decreased in MICT and HIIT groups, without difference between groups (Table 1). Absolute $V'O_2$peak increased in MICT and HIIT groups, respectively; with a significantly lower increase in MICT than HIIT (+6% and 16%, $P<0.001$). $V'O_2$peak normalized by FFM increased in MICT and HIIT groups,

respectively, with a significantly lower increase in MICT than HIIT (+8%, and +16%, P<0.001, Table 1).

	MICT		**HIIT**	
	PRE (N=16)	**POST(N=16)**	**PRE(N=16)**	**POST(N=16)**
Body mass (Kg)	107.1 ± 4.4	101.2 ± 4.5*	103.5 ± 2.7	97.8 ± 2.5*
BMI (Kg *m^{-2})	36.1 ± 1.3	33.9 ± 1.2*	35.1 ± 0.9	33.2 ± 1.0*
Waist (cm)	113.0 ± 3.5	109.4 ± 4.0*	114.1 ± 2.2	108.8 ± 2.1*
Hip (cm)	123.1 ± 2.8	118.0 ± 2.9*	120..5 ± 1.8	116.1 ± 2.2*
FFM (Kg)	69.4 ± 3.9	68.6 ± 4.1	65.1 ± 2.9	64.7 ± 2.7
FM (Kg)	37.7 ± 2.7	32.4 ± 2.3*	38.4 ± 2.1	32.9 ± 2.5*
Hrpeak (bpm)	180.1± 0.9	177.2 ± 1.1*	181.0 ±0.9	176.0 ±0.5*
V'O_2peak (L* min^{-1})	3.02 ± 0.05	3.19 ± 0.05*	2.88± 0.04	3.35 ± 0.05*
V'O_2peak (mL* min^{-1} * Kg^{-1} FFM)	43.58 ± 0.39	46.80 ± 0.039*	44.26 ± 0.45	51.51 ± 0.40*

Table 1. **Anthropometric characteristic, body composition characteristics and physical capacities of the obese subjects.** Data are expressed as mean ± SEM before (PRE) and after 3-months (POST) of weight- management program in Moderate Intensity Continuous Training (MICT) and High Intensity Interval Training (HIIT) groups. BMI: body mass index; FM: Fat Mass; FFM: Fat free Mass;*: significantly different from PRE, P<0.05.

Mitochondrial oxidative phosphorylation function was evaluated before the effect of the exercise (PRE) and after the effect of exercise (POST) by high resolution respirometry.

32 muscle samples were collected by biopsy and immediately frozen. 5 samples were not considered in the analyses as a consequence of an incorrect freezing procedure.

In the present study, high-resolution respirometry measurements could not be carried out immediately on fresh biopsies because of the wide number of biopsies obtained in the same day and for other the logistic issues. Our group has repeatedly performed high-resolution respirometry measurements on frozen samples (Salvadego et al., 2018, 2016; Tam et al.,

2016; Cannavino et al., 2015). Accurately controlled procedures proposed by Kuznetsov et al (Kuznetsov et al., 2003) were followed.

Intactness was evaluated during the respirometric measurements by adding cytochrome C (10 µM) in the O2K chamber. If the outer mitochondrial membrane is damaged the respiration is stimulated by cytochrome C. On the contrary if the membrane is intact the response to the substrate is null. We considered for our analyses only the samples with an increase in mitochondrial respiration following administration of cytochrome C within the limits allowing the exclusion of significant damage of the outer mitochondrial membrane (<10% increase of cyt.C-induced respiration) (Fig.3.1.4). Thus, unfortunately, we were unable to analyse mitochondrial respiration in all samples. We had to exclude 23% of respirometric measurements, obtaining four numerically homogeneous populations (PRE-MICT N=6, POST-MICT N=8, PRE-HIIT N=6, POST-HIIT N=7).

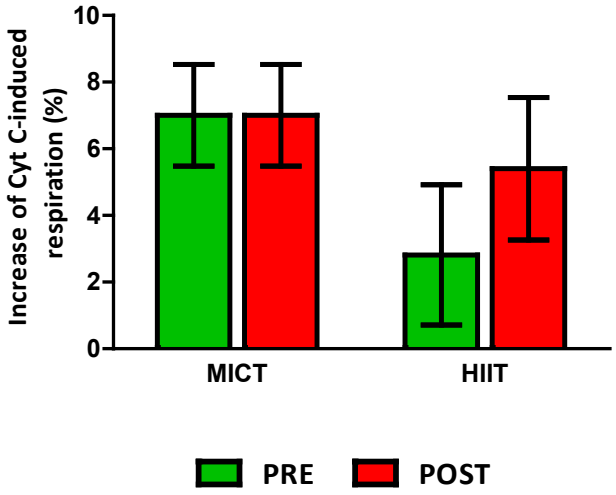


Fig.3.1.4. Cytochrome C percentage increase in four group analysed. The percentage increase of Cytochrome C was calculated with the ratio between value of Cytochrome C and the previous value corresponding to last titration of ADP, in PRE-MICT N=6, POST-MICT N=8 , PRE-HIIT N=6, POST-HIIT N=7**.** All data are represented as mean ± SEM.

In the Fig3.1.4.1 a representative trace of high resolution respirometry (HRR) including the protocol of substrate-uncoupler-inhibitor-titration used in the study.

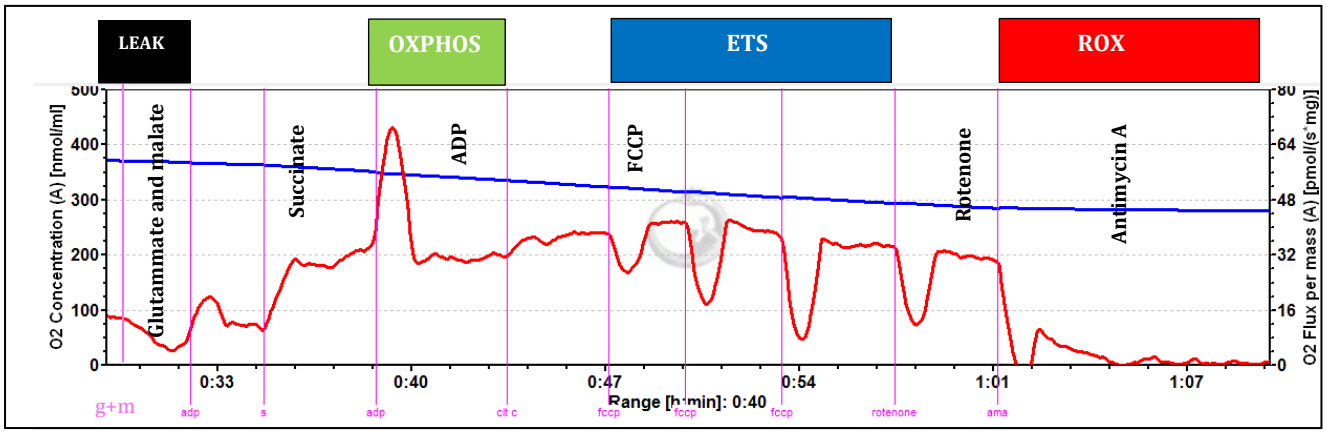


Fig.3.1.4.1. Representative HRR trace. The blue trace represents the oxygen concentration (in nmol/ml) in the chamber and the red one the oxygen consumption (pmol/s*mg wet weight). The magenta and dark blue marks represent the substrates/inhibitors addition in the chamber. It is represented on the top of the graph the investigated conditions during the protocol used.

The collected data were expressed per mg of wet weight and then normalized by CS activity, taken as an estimate of mitochondrial mass (Larsen et al., 2012a). CS activity did not change before and after the exercise in the two groups examined (Fig.3.1.4.2), indicating that the mitochondrial content of fibers was not modified by the training protocol.

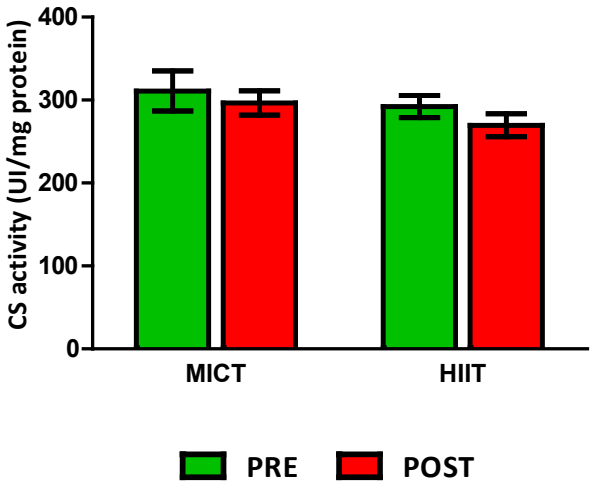


Fig.3.1.4.2. Citrate synthase activity. Citrate synthase activity measured before (green column) and after weight-management program (red column); N=6 for PRE and N=8 for POST in MICT group, N=6 for PRE and N=7 for POST in HIIT group. Values are means ± SEM.

The main results of mitochondrial respiration *ex vivo*, obtained by high-resolution respirometry, are presented in Figure 3.1.4.3. Mitochondrial leak respiration (Complex I state

2 respiration) or non-phosphorylating resting mitochondrial respiration sustained by Complex I was not affected by both type exercise interventions, when normalized by CS (Fig. 3.1.4.3).

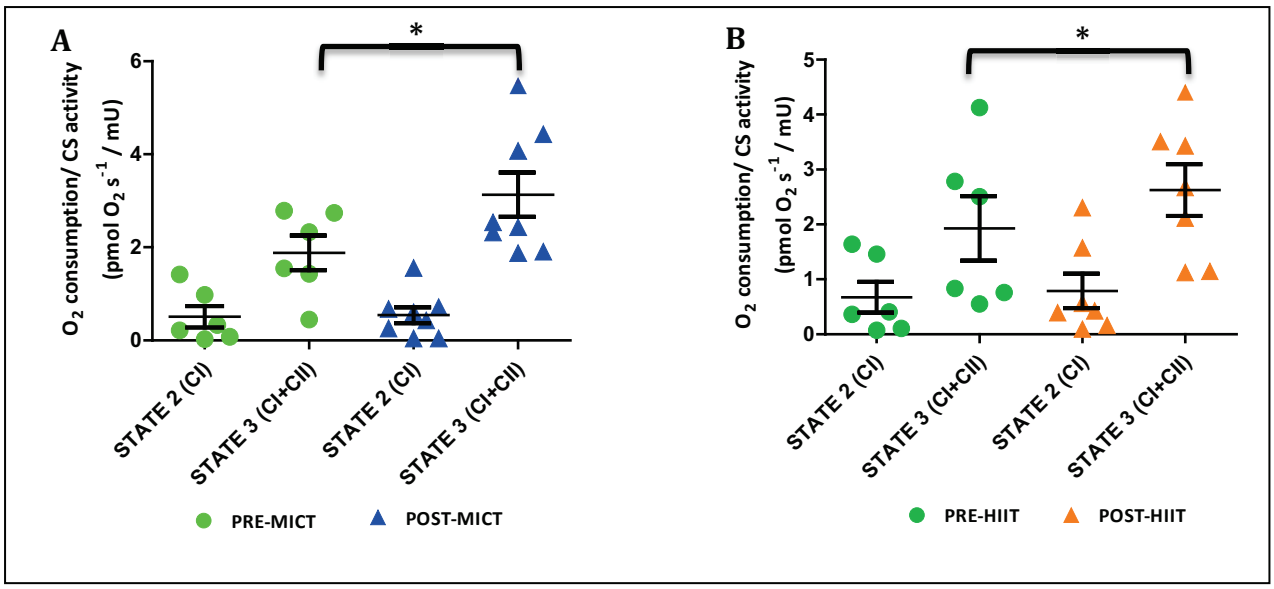


Fig.3.1.4.3. Mitochondrial leak respiration (Complex I state2 respiration) and Maximal ADP-stimulated mitochondrial respiration (Complex I+II state 3 respiration). In panel a data are reported for MICT group, in panel B for HIIT group. Leak resting respiratory rate was determinated in the presence of glutamate and malate (without ADP), while Maximal ADP-stimulated mitochondrial respiration was determined with 5 mM ADP and glutamate, malate, succinate as substrates. Data are expressed by CS activity. Values are means ± SEM; N=6 for PRE and N=8 for POST in MICT group, N=6 for PRE and N=7 for POST in HIIT group. Statistical significance is reported as * if p<0.05.

Maximal ADP-stimulated respiration (CI+CII state 3 respiration) increased significantly after the weight-management program (POST) in both MICT and HIIT groups (P: 0.042) with respect to the data obtained before training (Fig.3.1.4.4). Although the difference in the increase between the two groups was not significant (P: 0.403), percentage-wise the increase following MICT (+67%) was more pronounced compared to the increase observed following HIIT (+36%). Values before training were not significantly different in the two groups.

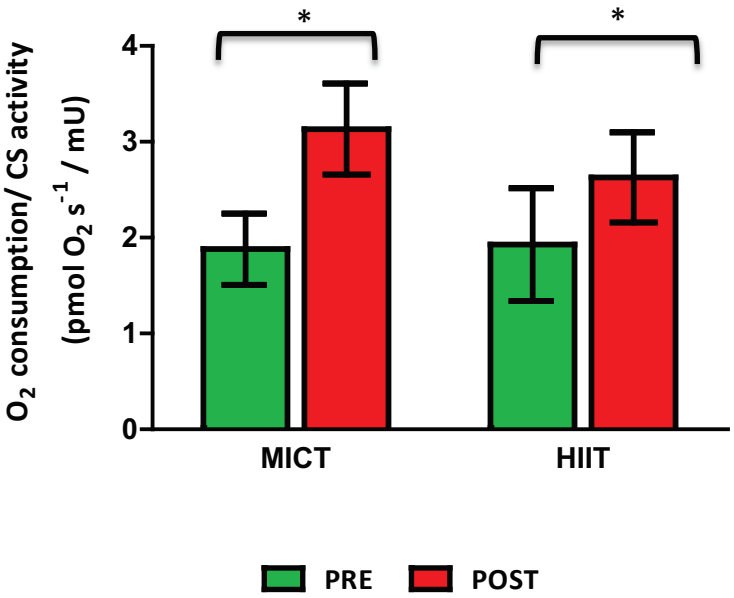


Fig. 3.1.4.4. Maximal ADP-stimulated mitochondrial respiration (CI+CII state 3 respiration) measured before and after the weight-management program in MICT and HIIT groups. Data are normalized by citrate synthase (CS) activity, expressed as mU/mg protein. Values are means ± SEM; N=6 for PRE and N=8 for POST in MICT group, N=6 for PRE and N=7 for POST in HIIT group. Statistical significance is reported as * if $p<0.05$.

The data dealing with oxidative phosphorylation coupling at a specific substrate supply (glutamate and malate), calculated as ratio [(State 3-Leak)/ State3] and reported in Fig.3.1.4.5, show that two training protocols did not affect oxidative phosphorylation efficiency. At baseline the values of the ratio were within 0.77-0.80 and did not change significantly after the weight management program, for both MICT and HIIT.

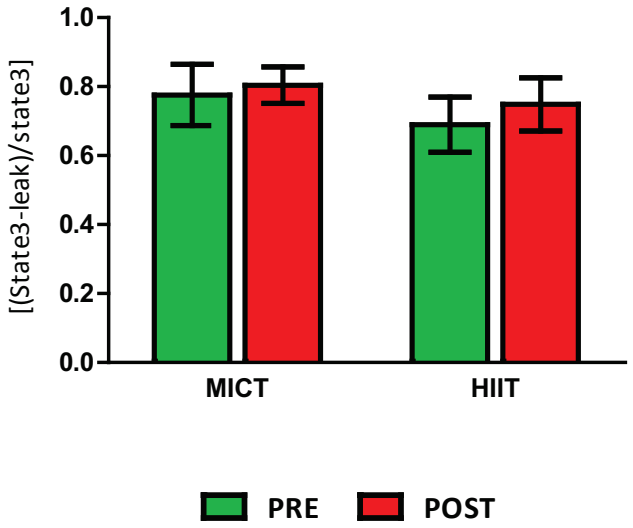


Fig.3.1.4.5. Oxidative phosphorylation coupling. The degree of oxidative phosphorylation coupling was measured before (green column) and after (red column) the weight-management program in MICT and HIIT groups. Values are means ± SEM.

The maximal capacity of the electron transport system uncoupled from the phosphorylating system (complex I+II ETS), evaluated trough titrations of the chemical uncoupler FCCP, increased significantly (P:0.042) as compared with baseline in MICT (+45%) and HIIT(+61%), without significant difference between the two interventions (Fig.3.1.4.6). Either rotenone-sensitive or-insensitive ETS exhibited a pattern similar to that of complex I+II ETS, although the increase of these variables in comparison to the baseline values did not reach statistical significance (Fig. 3.1.4.6).

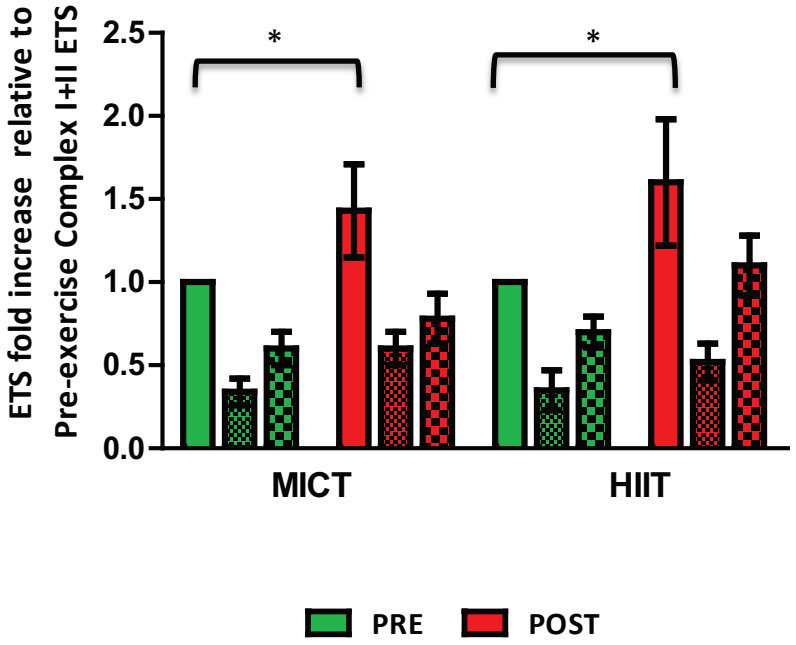


Fig.3.1.4.6. Maximal capacity of the electron transport system. It is shown maximal capacity of ETS sustained by Complex I (rotenone-sensitive ETS, stripes) and by Complex II (rotenone-insensitive ETS squares) expressed as fractions of the electron transport system capacity, i.e. ETS sustained by both Complex I and II (full coloured). Data are normalized to the pre-exercise Complex I+II ETS. Values are means $\pm$ SEM; N=6 for PRE and N=8 for POST in MICT group, N=6 for PRE and N=7 for POST in HIIT group. Statistical significance is reported as * if $p<0.05$.

3.1.5 Discussion

Obesity is a common disease associated with mitochondrial dysfunction, even if the pathophysiological mechanisms are not completely understood. It is already stated that success in obesity treatment programs, including physical intervention, is highly variable, and it is related in part to compliance and program characteristics, althought the benefical effect of exercise training on mitochondrial function is recognized and it can not be neglected.

The study described in this thesis was performed on obese individuals exposed to two types of training (within a weight-reduction program) carried out for 3 months: moderate intensity continuous training (MICT) and high intensity interval training (HIIT). The hypothesis was that HIIT would have a greater efficacy than MICT at several levels, both systemically (body mass and fat mass, fat oxidation rate, peak pulmonary O_2 uptake [VO_2peak]) and at the level of mitochondrial respiration. In terms of systemic variables, the hypothesis was confirmed: body mass and fat mass decreased more following HIIT vs. MICT, VO_2peak increases were more pronounced following HIIT vs. MICT, and fat oxidation rate increased only after HIIT. These data (Vaccari et al., 2019 submitted) are not specifically mentioned in the present thesis.

As discussed above, mitochondrial respiration was evaluated *ex vivo* by high-resolution respirometry on permeabilized skeletal muscle fibers obtained by biopsies carried out on the vastus lateralis muscle. The functional evaluation was carried out by determining the following variables (Pesta and Gnaiger, 2012; Salvadego et al., 2016): Complex I "leak" respiration (state 2 respiration, O_2 consumption not associated with the phosphorylation of ADP but with the leaking of protons across the inner mitochondrial membrane); maximal ADP-stimulated mitochondrial respiration (state 3) through respiratory complexes I + II (also known as "oxidative phosphorylation capacity"); ETS respiration evaluates the maximal capacity of the electron transport system uncoupled from the phosphorylating system. Rotenone allows to discriminate the contribution of Complex I and II to the "ETS capacity". Oxidative phosphorylation coupling is an index of the degree mitochondrial coupling at a specific substrate supply (glutamate and malate), calculated as the ratio [(State 3-Leak)]/ State 3. All data were normalized by CS activity, considered a valid estimate of mitochondrial content (Larsen et al., 2012a).

Our results revealed an improvement of maximal ADP-stimulated respiration and maximal ETS capacity after both MICT and HIIT, without changes in oxidative phosphorylation coupling, suggesting that oxidative phosphorylation capacity, but not its efficiency, was enhanced by exercise training. Although the increase in maximal ADP-stimulated mitochondrial respiration was, percentage-wise vs. baseline values, more pronounced following MICT than following HIIT, this difference did not reach statistical significance. CS activity, taken as an estimate of mitochondrial mass, did not change after both training interventions. This observation is in contrast with data obtained in non-obese subjects (Bartlett et al., 2012), documenting that some markers of mitochondrial biogenesis increase in high-intensity interval running more than in moderate-intensity continuous running. Thus, we may infer that the increases of mitochondrial function we observed in obese subjects after the two training interventions were due to OXPHOS complexes activity/assembly regulation, or remodelling of mitochondrial inner membrane. Menshikova et al., 2007 suggested a similar hypothesis in sedentary obese individuals undergoing moderate-intensity physical activity combined with weight loss. In particular, the authors observed an improved enzymatic capacity for oxidative phosphorylation without a significant change in mtDNA content, hypothesizing a mitochondrial cristae remodelling. Our data showing an increase in maximal capacity of both Complex I and Complex II may be in accordance to such hypothesis.

Overall, our results prompt us to propose exercise training as a good strategy to counteract the alterations of mitochondrial proteome observed in skeletal muscle of subjects with obesity, with proteins forming the TCA cycle being increased and those forming protein complexes of the oxidative phosphorylation decreased (Kras et al., 2018). Indeed, such proteomic profile has an increased capacity to produce reducing equivalents of NADH and $FADH_2$ in an impaired electron transport chain, thereby generating oxidative stress. Moreover, we formulate the hypothesis of OXPHOS complexes activity/assembly regulation or remodelling of mitochondrial inner membrane, supported by a variety of literature data regarding mitochondrial dysfunction in obesity, specifically documenting alterations in mitochondrial oxidative phosphorylation function (Vijgen et al., 2013; Bakkman et al., 2010), mitochondrial content (Ritov et al., 2005) and protein expression of OXPHOS complexes (Kras et al., 2018; Wijingarden et al., 2013).

In our study, despite both training modalities improved mitochondrial oxidative phosphorylation function, only after HIIT the whole body capacity to oxidize lipids during exercise improved (Vaccari et al., 2019 submitted). It should be considered that mitochondrial oxidative capacity widely exceeds systemic O_2 delivery (Boushel et al., 2011), and does not seem to be related with total body fat oxidation. Looking at our results, the improvement in fat oxidation in HIIT was not associated with changes in CS activity (Vaccari et al., 2019 submitted). This suggests that at least for 3 months of training, the improvement of fat oxidation is not due to mitochondrial adaptations, but to other factors, like improvements in O_2 muscle supply, capillary density and O_2 diffusion. Indeed, endurance athletes, compared with untrained individuals, have higher whole body maximal fat oxidation which however does not correlate with mitochondrial fat oxidation (Nordby et al., 2006). This further suggests that in the obese patients of the present study higher O_2 availability might be the main factor increasing whole body fat oxidation. A similar type of reasoning could be made for pulmonary VO_2peak, which in the present study increased more significantly following HIIT *vs.* MCIT.

Although the values range for maximal ADP-stimulated respiration obtained for this study is rather low, it is seems to be in agreement with other studies carried out *ex vivo* by high resolution respirometry (Layec et al., 2018; Warren et al., 2017; Park et al., 2014), even on fresh muscle samples. Conflicting opinions can be found in the literature on the possibility of freezing and thawing the samples undergoing high-resolution respirometry. The accurately controlled procedure proposed by Kuznetsov et al (Kuznetsov et al., 2003) was used in the present study, as in several previous studies by our group and by others (Salvadego et al., 2018, 2016; Tam et al., 2016, Cannavino et al., 2015, Wüst et al., 2012). On the other hand, according to Meyer et al., 2014 and Larsen et al., 2012b, cryopreservation of samples can result in an underestimation of maximal ADP-stimulated mitochondrial respiration. So, it cannot be excluded that the freeze-thaw procedure used in the present study led to some underestimation of maximal ADP-stimulated mitochondrial respiration values. Even if this occurred, however, the effect was present both before and after training, leaving the comparison between the two conditions substantially “safe”. However, the same authors (Meyer et al., 2014; Larsen et al., 2012b) observed a good correlation (r^2 = 0.82) between respirometry measurements in fresh and cryopreserved samples. Moreover, in the studies

that raised doubts about the possibility of cryopreserving the samples (and Meyer et al., 2014; Larsen et al., 2012b) significant damage to the outer mitochondrial membrane was frequently present, as suggested by the substantial increase in respiration following the administration in the measurement chamber of cytochrome C. As mentioned in the methods, as in our previous studies on cryopreserved samples (Salvadego et al., 2016, 2011) also in the present one very stringent criteria on cytochrome c increases in respiration were adopted.

3.1.6. Conclusions

In conclusion, this study reveals functional improvements in mitochondrial function in obese individuals exposed to two types of training (MICT vs HIIT). This may have impact in the pathophysiology of obesity, which is strongly related to mitochondrial dysfunction. Whereas HIIT determined greater improvements vs. MICT in systemic variables of oxidative function (fat oxidation rate, peak O_2 uptake), this difference was not observed in terms of mitochondrial function. Factors “upstream” of mitochondria are presumably more sensitive to HIIT.

3.2 Study n.2: Mitochondrial adaptations to bed rest

3.2.1 Introduction

Skeletal muscle is a dynamic tissue able and its whose plasticity allows to support different conditions of training/inactivity. Disuse is a major feature that can shape muscle molecular and systemic structure and function and is a very common condition that can be caused by many situations, such as a cast leg/arm, absence of gravity as during space flights, or ageing. The molecular pathways and phenomena that occur during a period of disuse/immobility are not completely characterized and understood yet. In order to study these changes in skeletal muscle are often used bed rest studies. This approach simulates a reduced level of physical activity as well as microgravity. Bed rest conditions lead to skeletal muscle hypotrophy and/or atrophy that consent to evaluate systemic and molecular changes experienced. The effects of a prolonged bed rest were also observed as impairment in mitochondrial function (Salvadego et al., 2018), but mechanisms and factors involved in mitochondrial impairment during muscle inactivity have not been fully clarified.

3.2.2 Aim

The aim of this project was to evaluate mitochondrial oxidative phosphorylation function in subjects undergone to 10 days of bed rest. Ten healthy young men were recruited with this aim and the oxidative function in these subjects was evaluated before the bed rest (PRE-BR) and after ten days of bed rest (POST-BR) by high resolution respirometry. The main attempt was not only evaluate mitochondrial oxidative phosphorylation function but also possible changes in ADP sensitivity after bed rest period.

3.2.3 Materials and Methods

Participants and Study Design

Ten healthy young men (aged 18-30 years) were recruited for the study carried out under a condition of horizontal bed rest. All participants underwent medical examination and routine blood and urine analysis. Basic anthropometric parameters of the group were: age

(years) → 23 ± 4.61 (mean ± SD); stature (m) →1.81 ± 0.03; body mass (kg) →77.51 ± 10.03; body mass index (kg/m^2) 23.56 ± 2.45. Exclusion criteria were: regular smoking; chronic disease requiring clinical treatment, habitual use of drugs, blood clotting defects, history of deep vein thrombosis (TVP) with D-dimer values > 500 µg/l; acute or chronic skeletal, neuromuscular, metabolic and cardiovascular disease conditions, previous history of embolism, inflammatory diseases, psychiatric disorders, epilepsy, participation in sports at a competitive level, ferromagnetic implants. Participants were informed of the purpose, procedures and potential risk of the study before signing the informed consent.

The study was conducted in controlled medical environment at the General Hospital of Izola, Slovenia. The participants were housed in standard air conditioned hospital rooms and were under constant surveillance with 24-hour medical care. For 10 days, the participants performed all daily activities in bed and received standard hospital meals three times a day. The study was approved by the Local Ethical Committee under the acronym: MARS-PRE BED REST SBI 2019; it was funded by the Italian Space Agency (ASI) and was coordinated by Science and Research Centre Koper. Oure research group from the University of Udine has carried out the functional assessment measures of oxidative bioenergetics metabolism in collaboration with Biomedical Technology Institute of National Research Council (CNR) of Milan (Italy).

Biopsies and mitochondrial respiration analyses

Two biopsies were taken from *vastus lateralis* muscle of each subject: one before starting bed rest for baseline data collection (BDC), one after the bed rest period (BR9). Samples were obtained from the mid-region of the right *vastus lateralis* muscle. Biopsy was done after anaesthesia of the skin, subcutaneous fat tissue, and muscle fascia with 2 ml of lidocaine (2%). A small incision was then made to penetrate skin and fascia, and the tissue sample was harvested with a Rongeure-conchotome (GmbH & Co, Zepf Instruments, Dürbheim - Germany). The samples were put at 4°C until the experimental analysis. Fiber bundles were trimmed from the connective and fatty tissue excess (if present) and separated with sharp-ended needles under magnification (70 x) (Stereomicroscope CRYSTAL-PRO, Konus-optical & sports systems, Italy) leaving only small areas of contact.

Fibers were then incubated in 2 ml of BIOPS (4°C) containing 20 µg/ml saponin for 30 min with continuous gentle stirring to ensure complete permeabilization. After being rinsed twice

for 10 min in a respiration medium (MiR05), permeabilized fibers were measured for wet weight and immediately transferred into the respirometer (Oxygraph-2k) chambers for O_2 consumption analysis, as described in 3.1.3 paragraph.

Analyses were performed in duplicate, by processing separately two specimens from each biopsy (Fig.3.2.3). The tissue samples were placed onto a pre-cooled Petri dish on ice, and were cut diagonally into small portions. Subsamples used for replicates were placed quickly in ice-cold BIOPS and connective tissue was removed (when it was necessary). Then fiber bundles were separated mechanically using a sharp-needle. The mechanical separation did not require in this case the difficulties seen during the manipulation of muscle samples taken from obese patients. Indeed, samples were not frozen, were free of adipose tissue, and were collected using a Rongeure-conchotome (Fig.3.2.3.1), a surgical instrument that not altered the morphology and the integrity of muscle fibers. Nevertheless, visual inspection of the samples under a microscope revealed an inhomogeneous geometry of the bundles.

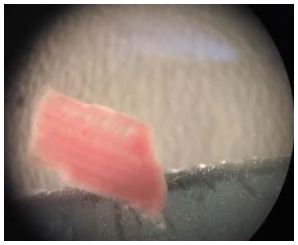


Fig.3.2.3. Biopsy of human *vastus lateralis* taken from individuals subjected to 10 days of bed rest. It is shown small muscle specimens after the collecting procedure.

According to experimental procedure, muscle specimens were carefully evaluated in terms of outer mitochondrial membrane intactness. Intactness was evaluated during the respirometric measurements adding cytochrome C in the O2K chamber (as described in paragraph 3.1.4). We considered for our analyses only the samples with an increase in mitochondrial respiration following administration of cytochrome C within the limits allowing the exclusion of significant damage of the outer mitochondrial membrane (<10% increase of cyt C-induced respiration). Since high-resolution respirometry measurements were carried out immediately on fresh biopsies, we were able to analyse mitochondrial respiration in all the recruited subjects.

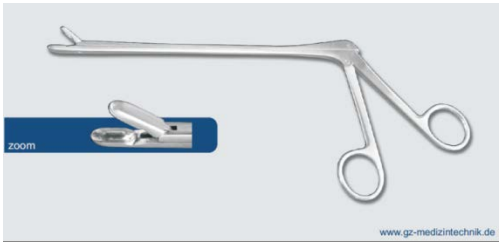


Fig. 3.2.3.1. Image of Rongeure conchotome used to muscle biopsy.

Apart from the ADP sensitivity evaluation (see below), the sequence of substrates was the same described in 3.1.3 and in previous papers of our group (see e.g. Salvadego et al. 2016, Salvadego et al. 2018). For ADP-stimulated respiratory kinetics, malate (4 mM), glutamate (10 mM) and succinate (10 mM) were added before ADP titration (25 µM-10 mM) in order to determine the apparent Km (Holloway et al., 2018) of a Michaelis-Menten kinetics, allowing to evaluate ADP sensitivity of mitochondrial respiration. The apparent Km for ADP was determined through the Lineweaver-Burk plot, fitting the model [x = $\frac{1}{[S]}$; y= $\frac{1}{JO2}$] where [S] is [free ADP] (ADPf) and JO_2 is the O_2 flux at [free ADP].

Cytochrome C (10 µM) was added to test for mitochondrial outer membrane integrity and only samples whose increase in respiration was < 10% were considered suitable for the analyses demonstrating. Data were digitally recorded using DatLab4 software (Oroboros Instruments). The respiration parameters were normalized by citrate synthase (CS) activity and expressed as (pmol $O_2 \cdot s^{-1} \cdot mU^{-1}$). The degree of oxidative phosphorylation coupling for a specific substrate supply (glutamate, malate, succinate in this case) was determined by calculating the ratio between Complex I+II state 3 respiration minus Complex I+II leak respiration and Complex I+II state 3 respiration [(state 3 – leak)/state 3] (Salvadego 2018, 2016; Gnaiger, 2014). At the conclusion of each experiment, muscle samples were recovered from the chamber, immediately frozen in liquid nitrogen and then stored at -80°C.

Citrate Synthase Activity Assay in Bed Rest study

In order to carry out citrate synthase activity, muscle samples were thawed and underwent a motor driven homogenization in a pre-cooled 1 ml glass-glass potter (Wheaton, USA). The muscle specimen was suspended 1:50 w/v in a homogenization buffer containing sucrose (250 mM), Tris (20 mM), KCl (40 mM) and EGTA (2mM) with 1:50 v/v protease (P8340-Sigma) inhibitors. The specimen was homogenised in an ice-bath with 20 strokes at

500 rpm, but before the last hit, Triton X-100 (0.1% v/v) was added to the solution. After this, the sample was left in ice for 30 minutes. The homogenate was centrifuged at 13000 rpm for 10 minutes in order to discard cellular debris. The supernatant was used to evaluate protein concentration according to method of Lowry (Lowry et al., 1951). 5-10-15 µg of protein extracts were added to each well of a 96-well-microplate along with 100 µl of 200 mM Tris, 20 µl of 1 mM 5, 5'-dithiobis-2-nitrobenzoate (DTNB) freshly prepared, 6µl of 10 mM acetyl-coenzyme A (Acetyl-Co-A) and mQ water to a final volume of 190 µl. A background ΔAbs, to detect any endogenous activity by acetylase enzymes, was recorded for 90 seconds with 10 seconds interval at 412 nm at 25°C by an EnSpire 2300 Multilabel Reader (PerkinElmer). The ΔAbs was subtracted from the one given after the addition of 10 µl of 10 mM oxalacetic acid that started the reaction. All assays were performed at 25 °C in triplicate on homogenates. Activity was espressed as mU (nanomoles/min) per mg of protein. This protocol was modified from (Spinazzi et al., 2012; Srere, 1969).

Statistical analyses

Data were expressed as mean values $\pm$ standard deviation. The normality of data was tested with the Shapiro-Wilk test. Student *t*-test was used in order to verify when two datasets, normally distributed, differed significantly from each other. This test was used for paired and unpaired data. The level of significance was set at $p \leq 0.05$. Statistical analyses were carried out by a commercial with software packages (GraphPad Prism 5.0, GraphPad Software).

3.2.4 Results

Ten healthy subjects have taken part to the bed rest study and their anthropometric characteristics are shown in Table 2. After bed rest intervention body mass and BMI were modified significantly in the recruited subjects for the study.

	PRE-BR (N=10)	**POST-BR (N=10)**
Body height/ cm	181.18 3.90	181.72 3.85
Body mass (Kg)	77.51 $\pm$ 10.03	75.86 $\pm$ 9.47*
BMI (Kg *m^{-2})	23.56 $\pm$ 2.45	22.93 $\pm$ 2.40*

Table 2. Anthropometric characteristic before (PRE) and after (POST) the bed rest. All values are presented as mean ± SD. BMI: body mass index; *: significantly different from PRE, P<0.05.

Mitochondrial oxidative phosphorylation function was evaluated *ex vivo* by high resolution respirometry in 10 individuals before (PRE-BR) and after (POST-BR) 10 days of inactivity. Muscle samples were collected by biopsy from *vastus lateralis*. Respirometry analyses were performed in controlled medical environment at the General Hospital of Izola (Slovenia) and carried out immediately after the collecting of muscle biopsies from the individuals. In Fig.3.2.4. is shown a representative trace of the protocol used in this study. The averaged data are shown in the plots in Fig.3.2.4. Unfortunately, we decided to exclude one of the subjects due to a high difference of the values of the two replicates, maintaining two numerically homogeneous populations as: PRE-BR (N=9), POST-BR (N=9).

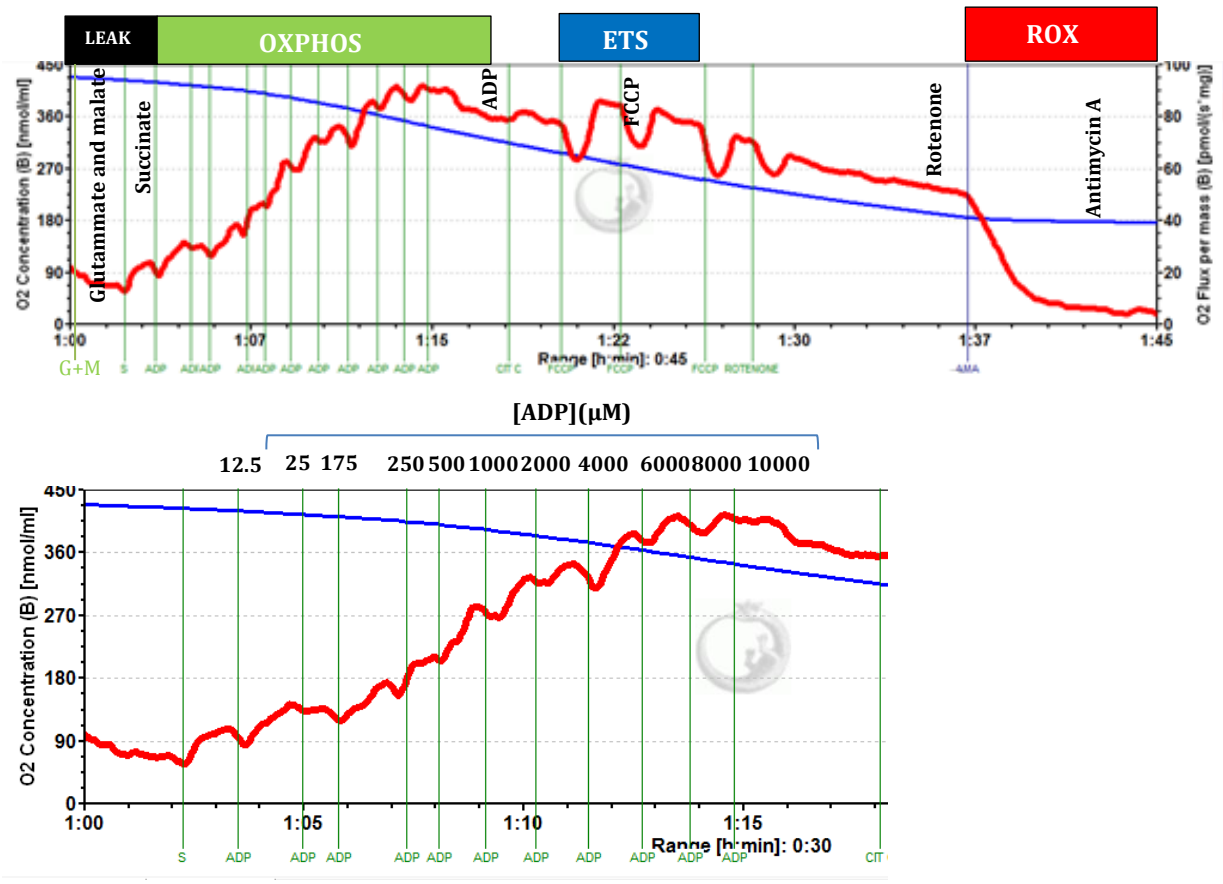


Fig.3.2.4. Representative HRR recording. In upper panel the blue trace represents the oxygen concentration (in nmol/ml) in the chamber and the red one the oxygen consumption (pmol/s*mg wet weight). The green marks represent the substrates/inhibitors addition in the chamber. On the top of the graph is shown a summary of the investigated conditions during the protocol used. Lower panel shows a magnification of the first part of the trace representing ADP titration, in the presence of 12.5, 25, 175, 250, 500, 1000, 2000, 4000, 6000,10000 µM ADP, to assess the ADP sensitivity of respiration.

CS activity was carried out on muscle specimens, which were immediately frozen after the recover from the chamber at the end of the respirometric measurements. CS activity was not modified in two group examined (137.62 ± 21 PRE-BR) (132.13 ± 16.4 POST-BR), indicating that physical inactivity did not change mitochondrial content of fibers. Data are shown in Fig.3.2.4.1.

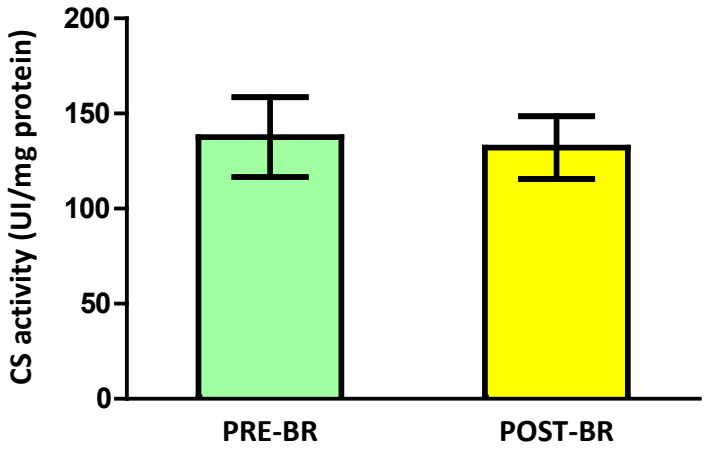


Fig.3.2.4.1. Citrate synthase activity. Citrate synthase activity measured before (light green column) and after bed rest period (yellow column); N=10 for PRE-BR and N=10 for POST-BR. Values are means ± SD.

The main results on mitochondrial respiration *ex vivo*, obtained by high-resolution respirometry under conditions of saturating ADP, are shown in Fig.3.2.4.2. The collected data were expressed per mg of wet weight, as well as per CS activity in order to evaluate the intrinsic mitochondrial respiration. This also allowed us to correct the results for possible inaccuracies in fibers wet weight measurement. Mitochondrial leak respiration (Complex I+II state 2 respiration), which represent the non-phosphorylating resting mitochondrial respiration sustained by Complex I and Complex II, was not affected by period of bed rest when normalized by mg of wet weight and also when normalized by CS (Fig.3.2.4.2). Further, maximal ADP-stimulated respiration (CI+CII state 3 respiration), which represent the phosphorylation capacity, was not affected after bed rest period. No change was observed in comparison to the values observed before bed rest considering data reported per unit of tissue mass (wet weight) and when were normalized by CS activity. The maximal capacity of the electron transport system (ETS) uncoupled from the phosphorylating system did not decrease significantly after the bed rest period (Fig.3.2.4.2), but it was observed a trend of decrease around 11%. In PRE-BR subjects ETS capacity was slightly but significantly higher than phosphorylation capacity (P: 0.099, paired data t-test), indicating that the phosphorylating system exerts a control over coupled respiration (Pesta and Gnaiger, 2012) under physiological condition of mobility. Intriguingly, this control was not seen after immobility condition.

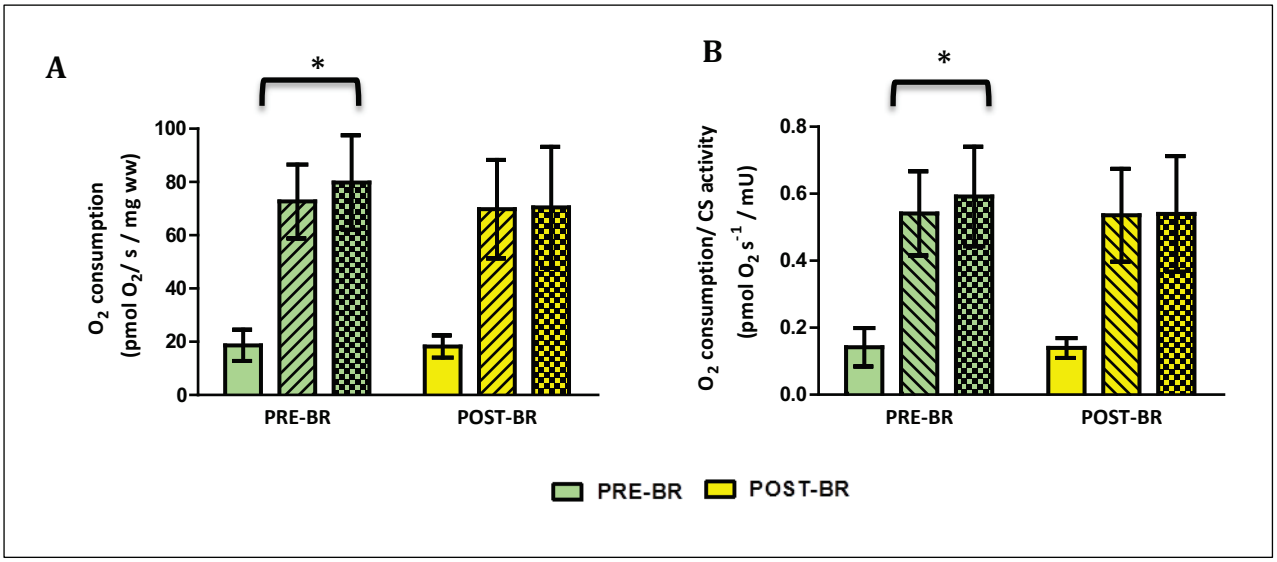


Fig.3.2.4.2. Parameteres of mitochondrial respiratory function measured by high-resolution respirometry in permeabilized muscle fibres. Leak or resting respiratory rate in the presence of glutamate, malate and succinate without ADP (full coloured), maximal ADP-stimulated respiration (stripes) and electron transport system (ETS) (squares) before (light green column) and after bed rest period (yellow column). In panel A data are expressed per mg wet weight, in panel B data are normalized by citrate synthase (CS) activity, expressed as mU/mg protein. Values are means ± SD; N=9 for PRE-BR and N=9 for POST-BR.

The data dealing with oxidative phosphorylation coupling [(state 3 – leak/ state 3)] are reported in Fig.3.2.4.3 and at baseline the values of the ratio were 0.88-0.86 for both groups, meaning that ten days of bed rest did not alter oxidative phosphorylation efficiency.

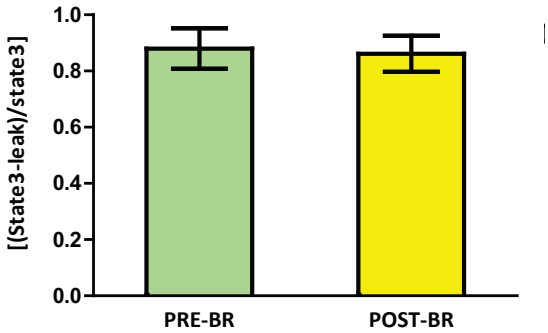


Fig.3.2.4.3. Oxidative phosphorylation coupling. The degree of oxidative phosphorylation coupling was measured before (light green column) and after bed rest period (yellow column) in PRE-BR (N=9) and in POST-BR (N=9) groups. Values are means ± SD.

As mentioned above, the maximal capacity of electron transport system did not change before and after exposure to ten days of bed rest. Either rotenone-sensitive or insensitive

ETS in both cases did not display any type of change. This suggests that the muscle inactivity following the bed rest period did not reveal an impairment in the respiratory function of complex I and II (Fig.3.2.4.4).

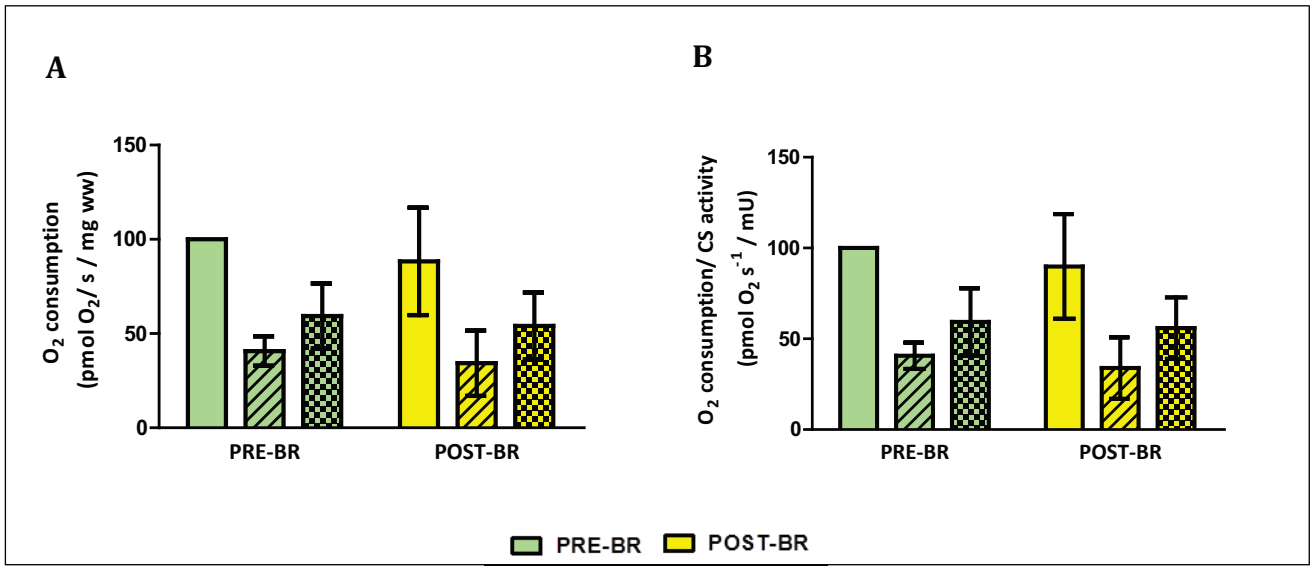


Fig.3.2.4.4. Mitochondrial electron transport system capacity sustained by Complex I (rotenone-sensitive ETS, stripes) and by Complex II (rotenone-insensitive ETS squares) expressed as fractions of the electron transport system capacity, i.e ETS sustained by both Complex I and II (full coloured). In panel A data are expressed per mg wet weight, in panel B data are normalized by citrate synthase (CS) activity, expressed as mU/mg protein. Values are means ± SD; N=9 for PRE-BR and N=9 for POST-BR, where PRE-BR = before bed rest and POST-BR = after bed rest period.

Finally, high-resolution respirometry was used to assess ADP sensitivity of respiration by ADP titration**,** in the presence of 12.5, 25, 175, 250, 500, 1000, 2000, 4000, 6000,10000 µM ADP**,** in the presence of glutamate, pyruvate and succinate. It was considered to be more physiological to assess the apparent ADP-affinity with a combination of substrates, which leads to electron transfer through both complexes I and II. In order to evaluate the apparent ADP Km, the collected data were analysed according to Michaelis-Menten kinetics and elaborated with Lineweaver-Burk equation (Barbour and Chan, 1981). The apparent Km ADP values from the two replicates are reported in Table 3. Unfortunately, based on MAD values and due to some experimental difficulties, it was possible to analyse only 9 subjects (PRE-BR) and 7 subjects (POST-BR).

PRE-BR				
Km (µM)				
	Replicate 1	Replicate 2	Replicate Mean	MAD
Subject 1	157,2	200,5	178,85	21,65
Subject 2	17,24	Instrument instability		
Subject 3	66,6	64,3	65,45	1,15
Subject 4	144,8	37,1	90,95	53,85*
Subject 5	58,8	60,7	59,75	0,95
Subject 6	36,4	39,8	38,1	1,7
Subject 7	59,2	Hard dissection of fibers		
Subject 8	25,6	21,7	23,65	1,95
Subject 9	18,27	Increase Cit c >10%		
Subject 10	479	753,5	616,25	137,25

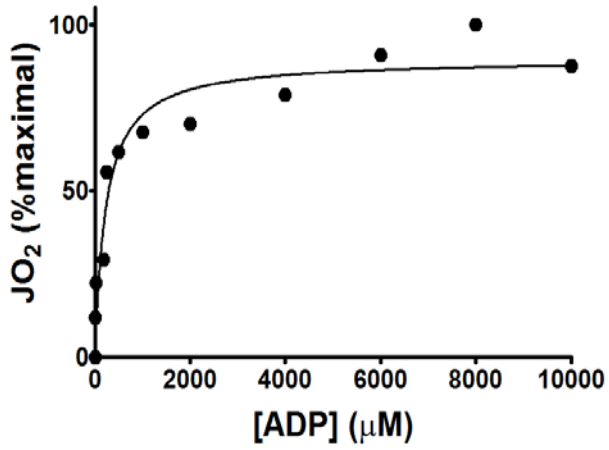
POST-BR				
Km (µM)				
	Replicate 1	Replicate 2	Replicate Mean	MAD
Subject 1	27,1	320	173,55	146,65*
Subject 2	Higher leak	31,88	31,88	
Subject 3	Low response to substrates	100,9	100,9	
Subject 4	61,5	49,1	55,3	6,2
Subject 5	187,1	93,9	140,5	46,6*
Subject 6	101,3	89,6	95,45	5,85
Subject 7	111,8	59,1	85,45	26,35*
Subject 8	33,6	55,7	44,65	11,05
Subject 9	110,1	63,1	86,6	23,5
Subject 10	40	74,8	57,4	17,4

Table 3. Tables with values of Km obtained with Lineweaver-Burk equation. Each subject was analysed in duplicate and it is reported a value of Km for each replicate. It is shown mean of the two replicates and Mean Absolute Deviation (MAD). *values excluded from the analysis due to very high MAD.

In Fig.3.2.4.5 are shown a representative Michaelis-Menten curve (panel A) and the corresponding reciprocal values used for Lineweaver-Burk analysis (panel B). Linear regression analysis of reciprocal values of the data obtained from the indicated subjects documented good values of linear correlation coefficient (ρ) (panel C, D). In Fig.3.2.4.6 are shown the whiskers and box plots of the apparent Km ADP values for such subjects, analysed using a t-test with unpaired data (P:0.4918).

The apparent Km ADP was similar in the two groups (PRE-BR and POST-BR), indicating that ten day of exposure to bed rest condition did not affect significantly ADP sensitivity of mitochondrial respiration.

A



B

[ADP] (μM)	JO_2	1/[ADP] (μM^{-1})	$1/JO_2$
12.5	11.91	0.080	0.140
25	22.37	0.040	0.075
175	29.27	0.057	0.057
250	55.71	0.004	0.030
500	61.58	0.002	0.027
1000	67.75	0.001	0.025
2000	70.23	0.0005	0.024
4000	78.92	0.00025	0.021
6000	90.90	0.00016	0.018
8000	100.00	0.00012	0.017
10000	87.55	0.00010	0.019

C

PRE-BR		
Linear regression coefficient (ρ)		
	Replicate 1	Replicate 2
Subject 1	0.97	0.98
Subject 2	0.86	
Subject 3	0.99	0.97
Subject 5	0.98	0.97
Subject 6	0.92	0.97
Subject 7	0.95	
Subject 8	0.86	0.81
Subject 9	0.75	
Subject 10	0.96	0.97

D

POST-BR		
Linear regression coefficient (ρ)		
	Replicate 1	Replicate 2
Subject 2		0.86
Subject 3		0.96
Subject 4	0.98	0.97
Subject 6	0.99	0.96
Subject 8	0.91	0.90
Subject 9	0.90	0.97
Subject 10	0.90	0.94

Fig.3.2.4.5 Assessments of ADP Sensitivity of Mitochondrial Respiration. In panel A is shown a representative Michaelis-Menten curve; panel B reports the values used for Lineweaver-Burk analysis corresponding to the curve in A. In panels C and D tables show linear regression coefficient (ρ) of two replicates Libeweaver-Burk analysis of different subjects.

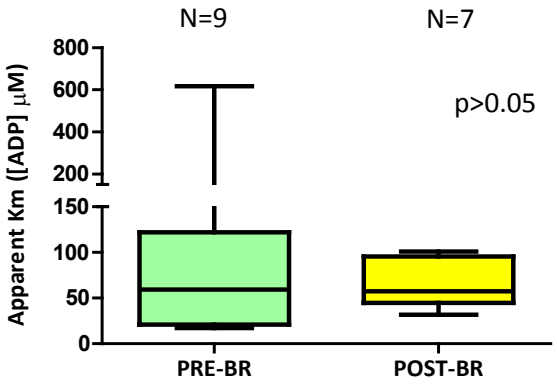


Fig.3.2.4.6. Whiskers and box plot of the apparent Km ADP. Minimum, 25th percentile, 75th percentile, and maximum values are represented along with the medians inside the box N=9 for PRE-BR and N=7 for POST-BR.

3.2.5 Discussion

The bed rest studies are performed to evaluate physiological adaptations in skeletal muscle during immobility causing muscle atrophy. This study investigates the effect of ten days of immobility in young healthy individuals. The data of the study reported in the present PhD thesis deal with a functional evaluation of skeletal muscle mitochondrial oxidative phosphorylation, *ex vivo*. As explained above, mitochondrial respiration was evaluated by high-resolution respirometry on preparations of permeabilized non-contracting skeletal muscle fibres obtained from biopsies carried out on the *vastus lateralis*. As expected (Salvadego et al.,2016), none of the respirometric parameters, assayed in the presence of saturating ADP levels and oxygen availability, was modified by 10 days of bed rest. It should be inferred that oxidative phosphorylation capacity and efficiency, as well as the maximal capacity of the Electron Transport System (ETS) uncoupled from the phosphorylating system, remain substantially unaltered after brief period of inactivity in healthy young individuals. Intriguingly, in such subjects only before immobility ETS was slightly but significantly higher than oxidative phosphorylation capacity. This suggests that i) the phosphorylating system (i. e. ATP synthase, ANT and Pi carrier) exerted a control over coupled respiration only under physiological conditions of mobility, and ii) after immobility mitochondrial oxidative phosphorylation missed the capacity to act in response to stress-linked energy request. Further investigations are needed to clarify the possible causes of such deficit.

Concerning the experiments aimed at detecting the mitochondrial respiration ADP sensitivity (apparent Km ADP), it should be considered that such measurements using permeabilized fibres are very critical because the fibres are in bundles and they may assemble on the top of the stirrer during the respirometry recordings. This may restrict the diffusion of ADP, whereas a more thorough dissection of the fibres to overwhelm this problem may cause mechanical damage. As a consequence, the apparent Km of the bundles obtained in this study varied strongly between our experiments, ranging from 616.25-23.65 µM, with the average value being ~ 60 µM. The reason might be the variance of effective diffusion distance for metabolites inside the bundles, which depends on the mechanical separation procedure (see METHODS), leading to the existence of large ADP diffusion gradients between mitochondria and the surrounding medium. Diffusion paths depend on the permeabilized cells in the bundle and the unstirred water layers surrounding the bundle. In additions, differences in intra-fibre ATP-consuming activity might also affect such ADP diffusion gradient mainly at low concentrations required for detection of ADP sensitivity.

The apparent Km ADP values were obtained in this study by applying the Lineweaver-Burk analysis, within its limits. No significant differences in apparent ADP Km were observed after ten days of bed rest. There is a wide range of Km values reported in literature (Holloway et al.,2018; Perry et al., 2011), because of the different experimental conditions and mathematical elaborations adopted. Indeed, internal comparisons of effects of treatments on Km ADP values *vs.* controls are reliable irrespective of the different procedures employed in each laboratory.

3.2.6 Conclusions

The findings obtained reported in this study showed no alteration of mitochondrial respiration and ADP sensitivity after ten days of bed rest inactivity of healthy young individuals outcomes align with a previous work carried out over a comparable period of time (Salvadego et al., 2016). Anyway, showing how longer bed rest periods elicited an impaired mitochondrial function, without decrease in mitochondrial mass, in terms of maximal ADP-stimulated and ETS respiration, as well as an increased leak respiration documented an impaired oxidative phosphorylation efficiency (Salvadego et al., 2018). No data are available relative to ADP sensitivity following longer bed rest, which remains to be assessed.

4. CONCLUDING REMARKS

In this PhD thesis work two different studies were carried out in order to evaluate skeletal muscle mitochondrial adaptations as consequence of exercise training or reduced activity/immobility.

In the first study, though the results should take into account of the limitations of experimental procedure and of the methodological difficulties, an improvement of maximal ADP-stimulated respiration and ETS capacity after two different types of training (MICT and HIIT) were observed in obese subjects, suggesting how exercise training is able to enhance oxidative phosphorylation capacity. The improvement of mitochondrial function after the two training interventions probably involves the remodelling of mitochondrial inner membrane or the OXPHOS complexes activity/assembly regulation determined a greater improvement in the systemic variables of oxidative function (fat oxidation rate, peak O_2 uptake) with respect to MICT suggesting the involvement of factors "upstream" of mitochondria, presumably more sensitive to HIIT. Overall, it cannot neglect the beneficial role of exercise training on the mitochondrial function.

In the second study the physiological condition of muscle disuse/atrophy (i.e. 10 days of bed-rest) was investigated at the mitochondrial level. No change in mitochondrial variables was observed with respect to pre-immobility conditions, with the exception of ETS capacity. This parameter resulted significantly higher than state III respiration before bed-rest and not after it, highlighting how the system is able to respond to energy request only in the pre-immobility condition. Obviously, this finding needs to be further investigated.

The relevance of both these studies is represented by their scientific contribution to the wide bio-medical literature regarding mitochondrial plasticity in response to different cellular energetic requests or in conditions of limited/unlimited substrates and oxygen supply.

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6. PUBLICATIONS

24th Annual Congress of the EUROPEAN COLLEGE OF SPORT SCIENCE 3 - 6 July 2019, Prague - Czech Republic

Conventional Print Poster CP-PM09. Molecular biology

ENDURANCE TRAINING IMPROVES SKELETAL MUSCLE MITOCHONDRIAL FUNCTION IN OBESE SUBJECTS

MAGNESA, B[1]., COMELLI, M[1]., MAVELLI, I[1]., VACCARI, F[1]., PASSARO, A[2]., LAZZER, S[1]., GRASSI, B[1].

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INTRODUCTION: The aim of this study was to evaluate the effect of the exercise on skeletal muscle mitochondrial function in obese subjects, considering the recognized alteration of mitochondria in obesity and the beneficial effects of exercise on oxidative and energy metabolism.

METHODS: Skeletal muscle biopsies were obtained from the vastus lateralis of healthy obese volunteers (age 38.03±8.56 years, BMI 35.57±4.29 kg/m2). Subjects (sedentary) underwent a supervised protocol consisting of 3 months of two different type of exercise training walking on a treadmill: i) low intensity endurance training, ET (60% VO2 peak) and ii) high-intensity interval training, HIIT (100% VO2 peak). Before and after the training period, biopsies were

obtained using a microneedle (Histocore, 12 G), immediately frozen in liquid nitrogen and stored at -80°C until the moment of analysis by high-resolution respirometry (Oroboros-2k oxygraph). To measure mitochondrial respiration, a substrate-uncoupler-inhibitor-titration protocol was applied (1). Upon completion of the measurements, muscle fibers were immediately homogenized and analyzed for citrate synthase (CS) activity by a spectrophotometric method (2). Further, the subjects performed an incremental test on the treadmill to obtain the whole body V'O2 peak. The V'O2 was measured breath by breath through the metabolimeter CPET, COSMED. RESULTS: Maximal ADP-stimulated respiration sustained by complex I and II, of biopsies from twenty-seven subjects (13 men and 14 women) were measured and normalized for either wet weight or CS activity. Data showed a statistically significant improvement with respect to controls before exercise only in ET group. ADP-stimulated mitochondrial respiration resulted higher in ET group (7.83±3.36 pmol O2· s-1·IU-1) with respect to the controls (4.76±2.87 pmol O2· s-1·IU-1), while HIIT group was not significantly different from the controls (6.57±3.11 pmol O2· s-1·IU-1). Intriguingly, CS activity was not affected by neither exercise protocols. In fact, mean data from controls were 0.31±0.05 IU/mg protein, while after exercise were: 0.27±0.05 IU/ mg protein in ET group and 0.26±0.05 IU/ mg protein in HIIT group. The whole body V'O2peak is improved in both groups but significantly more in HIIT compared with ET (respectively 16% and 6%).

CONCLUSION: Overall, these data suggest that ET induced an improvement of mitochondrial function (ADP-stimulated respiration), while HIIT did not. No mitochondrial bioegenesis was hypothesized based on unchanged CS activity, suggesting that OXPHOS complexes activity/assembly regulation, or remodeling of mitochondrial inner membrane, could be triggered by ET in obese subjects. The whole body V'O2 peak is improved more in HIIT, suggesting a contribution of factors linked to central circulation more in HIIT than in ET beside mitochondrial factors.

(1) Pesta and Gnaiger, Methods Mol Biol 810, 25-58 (2012)

(2) Spinazzi et al., Nature Protocols 7, 1235–1246 (2012)

Abstract- event "Conoscenza in festa" (May 2019)

Moderate-intensity Endurance training improves skeletal muscle mitochondrial function in obese subjects

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Endurance training improved mitochondrial function (ADP-stimulated respiration) in obese subjects, not due to mitochondrial biogenesis. This effect suggested an OXPHOS complexes regulation or remodelling of mitochondrial inner membrane.

Submitted article

Effect of 3-month High Intensity Interval Training vs. Moderate Endurance Training and 4-mounth follow-up on fat metabolism, cardiorespiratory function and mitochondrial respiration in obese adults.

Filippo Vaccari, Angelina Passaro, Andrea D'Amuri, Juana Maria Sanz, Francesca Di Vece, Eleonora Capatti, Benedetta Magnesa, Marina Comelli, Irene Mavelli, Bruno Grassi Federica Fiori, Giulia Bravo, Alice Avancini, Maria Parpinel, Stefano Lazzer.

frontiers in Physiology

ORIGINAL RESEARCH
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Mitochondrial Adaptations in Elderly and Young Men Skeletal Muscle Following 2 Weeks of Bed Rest and Rehabilitation

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The aim of the study was to evaluate the expression levels of proteins related to mitochondrial biogenesis regulation and bioenergetics in *vastus lateralis* muscle biopsies from 16 elderly and 7 young people subjected to 14 days of bed-rest, causing atrophy, and subsequent 14 days of exercise training. Based on quantitative immunoblot analyses, in both groups a reduction of two key regulators of mitochondrial biogenesis/remodeling and activity, namely PGC-1α and Sirt3, was revealed during bed-rest, with a subsequent up-regulation after rehabilitation, indicating an involvement of PGC-1α-Sirt3 axis in response to the treatments. A difference was observed comparing the young and elderly subjects as, for both proteins, the abundance in the elderly was more affected by immobility and less responsive to exercise. The expression levels of TOM20 and Citrate Synthase, assayed as markers of outer mitochondrial membrane and mitochondrial mass, showed a noticeable sensitivity in the elderly group, where they were affected by bed-rest and rehabilitation recalling the pattern of PGC-1α. TOM20 and CS remained unchanged in young subjects. Single OXPHOS complexes showed peculiar patterns, which were in some cases dissimilar from PGC-1α, and suggest different influences on protein biogenesis and degradation. Overall, exercise was capable to counteract the effect of immobility, when present, except for complex V, which was markedly downregulated by bed-rest, but remained unaffected after rehabilitation, maybe as result of greater extent of degradation processes over biogenesis. Phosphorylation extent of AMPK, and its upstream activator LKB1, did not change after bed-rest and rehabilitation in either young or elderly subjects, suggesting that the activation of energy-sensing LKB1-AMPK signaling pathway was "missed" due to its transient nature, or was not triggered under our conditions. Our study demonstrates that, as far as the expression of various proteins related to mitochondrial biogenesis/remodeling, adaptations to bed-rest and rehabilitation in the two populations were different. The impact of bed-rest was greater in the elderly subjects, where the

pattern (decrease after bed rest and recovery following rehabilitation) was accompanied by changes of mitochondrial mass. Modifications of protein abundance were matched with data obtained from gene expression analyses of four public human datasets focusing on related genes.

Keywords: mitochondria-related proteins, immobility, aging, exercise, skeletal muscle, western blot, *in silico* gene expression data mining

INTRODUCTION

Skeletal muscle is a very plastic tissue that responds and adapts quickly to inactivity or exercise. Around the fourth decade of life, skeletal muscle mass and functional performance, including oxidative metabolism, inevitably decline (Short et al., 2005; Salvadego et al., 2011; Dirks et al., 2014; Wall et al., 2014). Such decline accelerates with aging (Hughes et al., 2001) and it is usually associated with a decreased physical activity that can be deleterious for skeletal muscle, cardiovascular function, metabolic control and several other systems of the body (Brower, 2009). Physical inactivity in elderly people is a growing problem in western countries, also due to the impact of hospitalization. In fact, injuries in the elderly are very common and even a brief period of immobilization can result in a great loss of muscle mass and function, hard to restore even with rehabilitation interventions (Hvid et al., 2010).

Because of these clinical implications, the research on elderly subjects and their response to physical inactivity has been very active in the last few years. In this context, bed-rest studies are often employed, in order to simulate profound inactivity as well as microgravity. Bed-rest studies carried out by our group on young subjects observed a significant functional impairment of skeletal muscle oxidative metabolism evaluated *in vivo* following 10 days (Salvadego et al., 2016), 21 days (Salvadego et al., 2018), and 35 days (Porcelli et al., 2010; Salvadego et al., 2011) of bed-rest conditions. After 21 days, but not after 10 days of bed-rest, the functional impairment *in vivo* was associated with an impaired mitochondrial respiration evaluated *ex vivo*.

We discussed in detail in a recent publication (Pišot et al., 2016) previous bed-rest studies from different laboratories on elderly populations. In short, these studies often lacked a control group of young individuals, did not consider a rehabilitation phase, or did not comprehend periods of inactivity long enough to induce significant changes also in the young controls. These considerations prompted us to design a bed-rest study in which young (Y) and elderly (E) participants were exposed to precisely the same protocol of inactivity in bed for 14 days, and to a subsequent rehabilitation with high-intensity interval training for another period of 14 days. The details of the study, as well as some results about systemic variables, are given in Pišot et al. (2016) and Rejc et al. (2018). Briefly, adaptations to bed-rest and rehabilitation in the two populations were different. Interestingly, the impact of bed-rest on muscle mass and function (muscle force and power, fiber strength, and $V'O_2$peak) was greater in E compared to Y, as well as the rehabilitation was slower and/or less complete in E.

There is still debate about which mechanisms are involved in the loss of muscle mass and function after inactivity. During the last few years interest has arisen around the role played by skeletal muscle mitochondrial function and biogenesis following inactivity and aging (Carter et al., 2015), as well as in the pathogenesis of inactivity-related diseases (Booth et al., 2012). Proteomic and gene expression analyses documented decreases in expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and OXPHOS complexes as consequence of induced disuse, in association with a reduction in mitochondrial biogenesis and an overall impairment of energy metabolism (Chen et al., 2007; Alibegovic et al., 2010a; Brocca et al., 2010, 2012; Ringholm et al., 2011). "Upstream" of PGC-1α, AMPK/LKB1 energy sensor signaling pathway has been reported to be involved (Ringholm et al., 2011; Brocca et al., 2012). AMPK, a serine/threonine protein kinase, has emerged as a master sensor of cellular energy balance in mammalian cells, including skeletal muscle, (Hardie and Sakamoto, 2006; Kjøbsted et al., 2018) and one of the upstream activators of AMPK signaling pathway is LKB1. In the context of mitochondrial activity-regulated signaling "downstream" of PGC-1α, Sirt3, has emerged as the major regulator of mitochondrial protein deacetylation (Lombard et al., 2007; Menzies and Auwerx, 2013).

The aim of the present study was to evaluate the changes occurred as response to inactivity and rehabilitation by E people,

Abbreviations: AMPK, AMP-activated protein kinase; ATP5A1, ATP synthase F1 subunit alpha, mitochondrial complex V; BDC, baseline data collection; BSA, bovine serum albumin; CaMK, calcium/calmodulin-dependent protein kinase; CaMKKs, calcium/calmodulin-dependent protein kinase kinases; COA4, cytochrome c oxidase assembly factor 4 homolog; COA7, cytochrome c oxidase assembly factor 7; COX IV, cytochrome c oxidase subunit 4, mitochondrial complex IV; COX7C, cytochrome c oxidase subunit 7C; CS, Citrate Synthase; DEG, differentially expressed genes; FBXO32, atrogin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPD2, glycerol-3-phosphate dehydrogenase 2; LKB1, Liver kinase B1; NDUFA8, NADH:ubiquinone oxidoreductase subunit A8; NDUFAF6, NADH:ubiquinone oxidoreductase complex assembly factor 6; NDUFB8, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial complex I; NDUFC1, NADH:ubiquinone oxidoreductase subunit C1; OXPHOS, oxidative phosphorylation; PGC-1α, Peroxisome proliferator activated receptor-γ coactivator-1α; PVD, polyvinylidene fluoride; ROS, reactive oxygen species; SDHB, succinate dehydrogenase [ubiquinone] iron-sulfur subunit B, mitochondrial complex II; Sirt3, Silent mating-type information regulation 2 homolog sirtuin 3; TIMM23, translocase of inner mitochondrial membrane 23; TIMMDC, translocase of inner mitochondrial membrane domain containing 1; TMEM70, transmembrane protein 70; TOM20, mitochondrial import receptor subunit TOMM20 homolog; TOMM40L, translocase of outer mitochondrial membrane 40 like; Tris, tris(hydroxymethyl)aminomethane; UCP3, uncoupling protein 3 - mitochondrial proton carrier; UQCC3, ubiquinol-cytochrome c reductase complex assembly factor 3; UQCR11, ubiquinol-cytochrome c reductase complex III subunit XI; UQCRC2, cytochrome b-c1 complex subunit 2, mitochondrial complex III.

compared to Y, in the expression levels of the proteins above mentioned, playing a key role for mitochondrial biogenesis and function. In no previous studies, the expression levels of such proteins were determined in Y and E subjects undergoing the same duration of inactivity in bed followed by the same rehabilitation protocol, as in the present study. We hypothesized a pattern of changes in the protein expression similar to that described for systemic variables directly related to mitochondrial function (muscle mass and peak aerobic power) in the same subjects exposed to the same environmental stimuli, i.e., a more pronounced decrease during bed-rest in E vs. Y and a slower/incomplete rehabilitation.

Furthermore, with the aim to validate our protein expression data, we performed some *in silico* analyses of public human gene expression datasets, focusing in particular on mitochondria-related genes involved in skeletal muscle responses to immobility and rehabilitation in young adult individuals, or demonstrated to change in association with aging. Combining data from bioinformatics analyses of gene expression with those of protein abundance from our bed-rest study encouraged us to hypothesize possible molecular mechanisms implicated in the effects observed in Y and E subjects.

MATERIALS AND METHODS

Participants

Twenty-three healthy men, of which 7 young (Y; aged 18–30 years) and 16 elderly subjects (E; aged 55–65 years) were recruited for the study. All participants underwent medical examination and routine blood and urine analysis. Basic anthropometric parameters of the two groups and exclusion criteria are reported previously in Pišot et al. (2016). Participants were informed of the purpose, procedures and potential risk of the study before signing the informed consent. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and was approved by the National Ethical Committee of the Slovenian Ministry of Health on April 17, 2012 under the acronym: IR-aging 1200.

Study Design

The study was conducted in concomitance with the study of Pišot et al. (2016) and Rejc et al. (2018) in a controlled medical environment at the Valdoltra Orthopedic Hospital, Slovenia. The participants were housed in standard air-conditioned hospital rooms and were under constant surveillance with 24-h medical care. For 14 days, the participants performed all daily activities in bed in the horizontal position and followed an individually controlled eucaloric diet. Such conditions are denoted as bed-rest (BR). Dietary energy requirements were designed for each subject by multiplying resting energy expenditure by factors 1.2 and 1.4 in BR and during the rehabilitation period, respectively (Biolo et al., 2008). The macronutrient food content was set at 60% of carbohydrates, 25% fat and 15% of proteins. Energy balance was checked weekly by fat mass assessment performed with bioelectrical impedance analysis (tetra-polar impedance-meter, BIA101, Akern, Florence, Italy), using the software provided by the manufacturer, as in Rejc et al. (2018). After the BR participants underwent a rehabilitation protocol (R+14) that consisted of 2-week supervised multimodal exercise program with 3 sessions per week as described in details in Rejc et al. (2018). In each session, participants performed 12-min warm-up, 15–20 min of balance and strength training and 20–30 min of aerobic training (high-intensity interval training).

Three different biopsies were taken from *vastus lateralis* muscle of each subject: one before starting bed-rest for BDC, one after the bed-rest period (BR14), the last after the rehabilitation protocol (R+14) and specifically 4–5 days after the final training session during the period where the subjects completed the *in vivo* performance tests.

Procedures

Muscle Biopsies

Samples were obtained from the mid-region of the left *vastus lateralis* muscle. Biopsy was done after anesthesia of the skin, subcutaneous fat tissue, and muscle fascia with 2 ml of lidocaine (2%). A small incision was then made to penetrate skin and fascia, and the tissue sample was harvested with a purpose-built rongeur (Zepf Instruments, Tuttlingen, Germany). The samples put in cryopreservation solution were immediately frozen in liquid nitrogen, and stored at −80°C until the analyses (Kuznetsov et al., 2003).

Immunoblot Sample Preparation

Just thawed biopsy samples were rapidly washed in PBS solution, dried, weighted and placed in a cooled 2 mL glass Teflon Potter-Elvehjem (Wheaton, IL, United States), in a suspension 1:4 w/v with PBS containing 0.32 M sucrose, P8340 – Sigma protease inhibitors (1:50 v/v) and 10 mM NaF + 1 mM Na_3VO_4 as phosphatase inhibitors (Mavelli et al., 1978). Samples were homogenized with 40 motor driven strokes (ForLab AT120, Carlo Erba, Italy) and aliquots were withdrawn and stored at −80°C. Further 1:1 v/v dilution of the residual homogenates was made with RIPA buffer 2× (300 mM sodium chloride, 2% NP-40, 1% sodium deoxycholate, 0.2% SDS, 0.8 mM EDTA and 100 mM Tris, pH 8.0), followed by other 40 motor driven strokes, to obtain a better membrane protein solubilization, and 30 min of incubation. All processes were carried out on ice-bath. Homogenates were centrifuged at 10,000 × *g* for 10 min and the extracts were stored at −80°C until using for the assays. Protein concentration was tested with Lowry assay (Lowry et al., 1951) using BSA as a standard.

Extracts of H9c2 cell line (ATCC® CRL-1446™) were obtained by resuspension of cellular pellet (10^6 cells/ml) in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.4 mM EDTA and 50 mM Tris, pH 8.0), containing protease (P8340 - Sigma) and phosphatase (10 mM NaF and 1 mM Na_3VO_4) inhibitors. After 30 min of incubation at 4°C, the samples were centrifuged at 14,000 × *g* for 15 min at 4°C. Extracts were used as internal standards (IS) for quantification of immunoblot results.

Quantitative Immunoblot

Separation of sample's different proteins was obtained by electrophoresis in denaturing conditions (SDS-PAGE), following Laemmli's method (Laemmli, 1970) with Tris-glycine running buffer, using 8–16% polyacrylamide gradient precast resolving gels (Thermo Fisher Scientific, Waltham, MA, United States). After separation, analysis of the protein expression levels was carried out by immunoblotting. After the proteins were electro-transferred from the gel to a PVDF membrane, the membrane was divided based on the molecular weight of the single proteins according to the molecular weight markers. Each part of the membrane was blocked in a Tris-Buffered Saline (TBS) solution, containing 0.1% Tween 20, 2.5% BSA solution for 1 h and then incubated overnight with the proper antibodies. Specifically, the membranes were probed with antibodies vs. AMPK and p-AMPK^{Thr172} 1:500 (Santa Cruz, Dallas, TX, United States, catalog numbers sc-74461 and sc-33524, respectively), GAPDH 1:10000 (Santa Cruz, sc-32233), CS 1:10000 (AbCam, Cambridge, United Kingdom, ab 129095), PGC-1α 1:5000 (AbCam, ab 722301), Sirt3 1:1000 (CST, Danvers, MA, United States, 5490S), LKB1 and p-LKB1^{Ser482} 1:1000 (Phosphoplus Duet CST, 5132) and TOM20 1:7000 (Santa Cruz, sc-17764). We also probed the membranes with AbCam Ab Cocktail (ab 110413) vs. OXPHOS complexes (1:5000) for complex I (mitochondrial NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 – NDUFB8), complex II (succinate dehydrogenase [ubiquinone] iron-sulfur subunit B – SDHB), complex III (Cytochrome b-c1 complex subunit 2 – UQCRC2), complex V (CV subunit α – ATP5A1). For complex IV a single antibody against cytochrome c oxidase subunit 4 – COXIV (Abcam, ab 110272, 1:10000) was used due to its greater efficiency. Thereafter, the membranes were incubated in the presence of the proper secondary antibody (rabbit-anti-mouse [61–6520] or goat-anti-rabbit [32460], Thermo Fisher Scientific) conjugated with horseradish peroxidase (HRP).

For quantification purposes, each gel was loaded with 11 samples along with molecular weight markers (Bio-Rad Laboratories, Berkeley, CA, United States) and IS (prepared as described above) in order to normalize the results from the single gels with those of replicates or different samples. 20 μg of proteins were loaded for each sample and 40 μg of IS. Samples from both Y and E subjects were assayed together matching the conditions (namely all BDC or BR14 or R+14 samples).

The protein bands were visualized by an enhanced chemiluminescence method using ChemiDoc (Bio-Rad Laboratories) and quantified with ImageQuant TL program (GE Healthcare, Little Chalfont, United Kingdom). Quantification was made based on "Adjusted Volume Intensity," i.e., the volume given by the sum of the intensities of the pixels inside the boundary volume corrected for the background. The intensity of each band was normalized on total bands revealed by Coomassie-staining of PVDF membrane, considered as appropriate loading and transferring control (Welinder and Ekblad, 2011). Each sample was tested in triplicate and values were expressed as Arbitrary Units (AU).

In separate experiments, the single subjects were also analyzed individually by loading each gel with samples of the three investigated conditions (BDC, BR 14, and R+14). Gels were loaded with scalar amounts (5, 10, 15, and 20 μg) of Y or E samples, to verify the linearity of the band intensity vs. the loaded protein amount. As an example, see representative immunoblot images denoted as (4) of **Figure 1**.

Statistical Analysis

Continuous variables were summarized as mean ± standard deviation. Data were tested for normal distribution using Shapiro–Wilk test. Equality of variance was assessed using Levene test. The significance of differences of expression levels of analyzed proteins between groups (Y vs. E) throughout different conditions (BDC or BR 14 or R+14) was explored using the Linear Mixed Models for Longitudinal Data. Comparisons between groups for each condition were performed using *t*-test. Comparisons among the different conditions within each group were achieved using paired *t*-test. Bonferroni correction for multiple comparisons was applied.

Gene Expression Analysis

Human microarray datasets were downloaded manually from public repositories, ArrayExpress (Parkinson et al., 2007) and GEO (Barrett et al., 2011); the data were all related with Bed-rest or atrophy due to clinical-associated disuse, as well as with muscle and aging. The raw files were downloaded when available. All the CEL files were processed together by using standard tools available within the affy package in R (Gautier et al., 2004). An UniGene ID centered Chip Description file (CDF) was used in order to have only one intensity value per gene. CDF was downloaded from the Molecular and Behavioral Neuroscience Institute Microarray Lab[1] (Dai et al., 2005). All annotation information were downloaded from the same website. The normalization step was done with the standard RMA algorithm (Irizarry et al., 2003). For determination of the DEG Standard *t*-test was performed. Lists of the top DEG are in **Supplementary Tables S1–S4** where the genes were selected based on fold change ($>+1.5$ or <-1.5; $>+1.3$ or <-1.3) and $p < 0.05$. *Gene enrichment* analysis on each DEG list was performed using DAVID 6.8 software (Sherman et al., 2007).

As concern data related with muscle and aging, the Spearman's correlation analysis was also performed making minimal assumptions about the relationship between the two diverse variables. Spearman's Rank correlation coefficient was used to evaluate the strength and direction (negative or positive) of a relationship between two variables. Genes with a significant negative strong correlation were selected, correlation coefficient < -0.6, Benjamini–Hochberg

[1]http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp

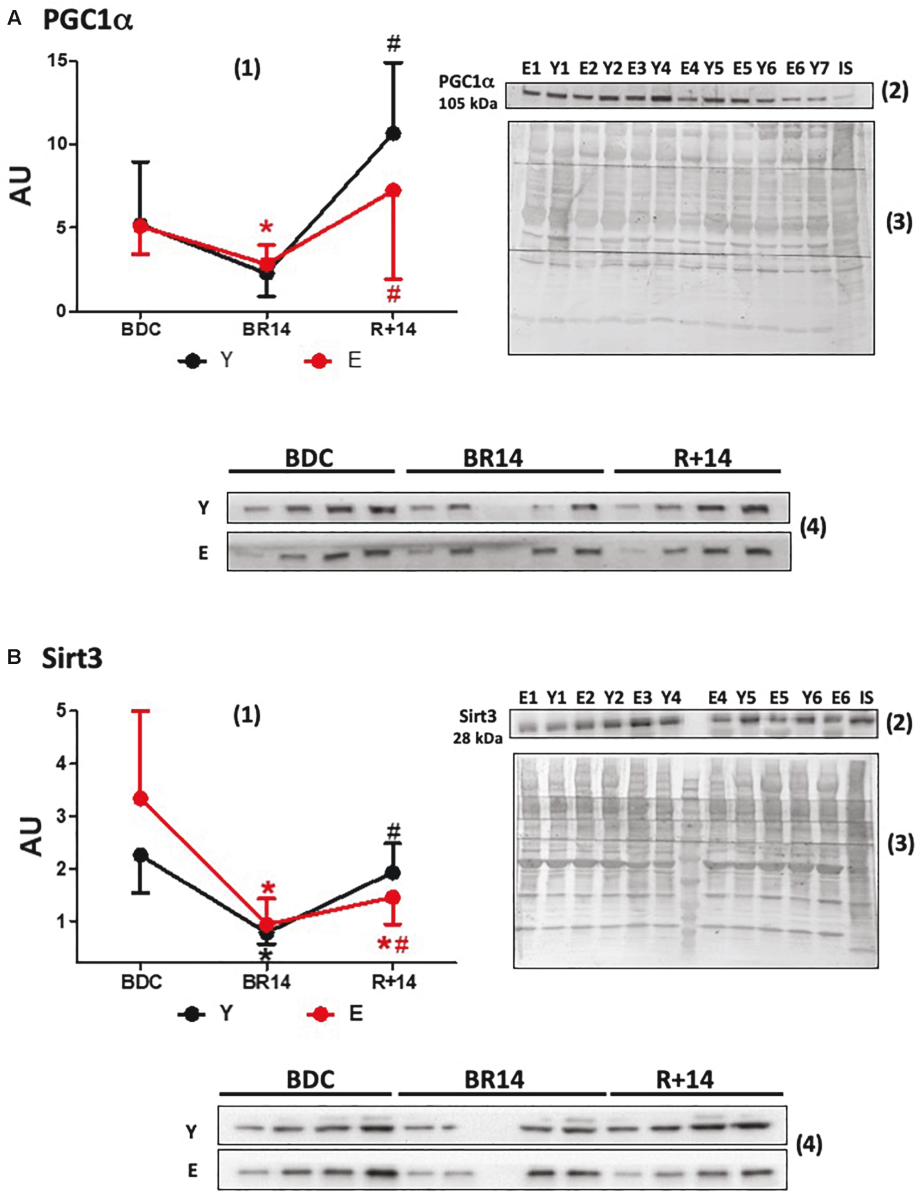

FIGURE 1 | Changes in expression levels of PGC-1α **(A)** and Sirt3 **(B)** in *vastus lateralis* muscle biopsies from young and elderly subjects after bed rest and rehabilitation. Panels **(A,B)** graphs denoted as (1) represent immunoblot data of 7 young (Y; aged 18–30 years) and 16 elderly (E; aged 55–65 years) subjects, under the three conditions: BDC, baseline data collection before bed rest; BR14, after 14 days bed rest; R+14, after 2-week rehabilitation. Gels were loaded with samples from Y and E subjects (20 μg of proteins) along with IS (40 μg), prepared as described in Section "Materials and Methods." ImageQuant TL values for single immunoreactive bands normalized to Coomassie staining of PVDF membrane and corrected for IS are expressed as arbitrary units (AU). Quantitative data are means ± SD of three different assays for each subject. Red line: Elderly group; black line: Young group. *Represents statistical significance ($p < 0.05$) vs. BDC condition, # between BR14 and R+14 conditions. Immunoblot images denoted as (2) are representative of experiments with samples from both Y and E subjects loaded on each gel matching the conditions (namely all BDC or BR14 or R+14 samples), and images denoted as (3) are representative of the corresponding Coomassie-stained whole PVDF membranes, used as loading and transferring measurement. Immunoblot images denoted as (4) are representative of experiments where each subject was analyzed individually by loading gel with samples of the three investigated conditions together (BDC, BR 14, and R+14). Gels were loaded with scalar amounts (5, 10, 15, and 20 μg) of Y or E samples to verify the linearity of the band intensity vs. the loaded protein. The central lane of the gel was loaded with molecular weight markers.

corrected $p < 0.05$ (**Supplementary Table S5**). Rule of thumb for interpreting the size of a correlation coefficient was used. *Gene enrichment* analysis was then performed using DAVID 6.8 software.

RESULTS

All participants were able to comply with the study protocol. No dropouts and no medical complications occurred

(see Pišot et al., 2016 for more details). Anthropometric, metabolic and muscle function data of the cohort were described in that publication (Pišot et al., 2016).

Key Proteins of Mitochondrial Biogenesis and Function

Data about the expression of PGC-1α, a master regulator of mitochondrial biogenesis and structural/functional integrity, are given in **Figure 1A**. At the BDC, before the bed-rest campaign, PGC-1α protein levels were not different in E vs. Y. Both in E and in Y bed-rest (BR14) induced a remarkably similar decline in PGC-1α expression levels, although significance was not reached in the Y group. The subsequent rise following rehabilitation was less pronounced in E (R+14 values: 2.6 times vs. BR14) than that observed in Y (R+14 values: 4.7 times vs. BR14). Both in Y and in E PGC-1α levels "rebounded" after rehabilitation attaining in R+14 values higher than those observed at BDC. This rebound was more pronounced in Y (2 times vs. BDC levels compared to 1.4 in E).

The expression levels of Sirt3, the most characterized sirtuin in mitochondria, declined during bed-rest following a pattern similar to that of PGC-1α (**Figure 1B**), in accordance with the concept that they are controlled by PGC-1α in the nucleus (Brenmoehl and Hoeflich, 2013). No rebound to values over BDC was observed following rehabilitation in Y or in E. The recovery to the BDC levels after rehabilitation was complete in Y, whereas it was only partial in E.

The effects of bed-rest and rehabilitation on OXPHOS complexes protein expression are shown in **Figure 2**. The behavior of the different complexes was rather heterogeneous. The respiratory chain carriers CII, CIII, and CIV showed both in Y and in E a similar general pattern, although in some cases the differences did not reach statistical significance. Namely, a decrease at BR14 and a recovery (in CII, CIII, and CIV) with a rebound (only in CII and CIII) at R+14 were observed, resembling the pattern described above for PGC-1α. At BDC, protein abundance of CII was significantly greater in E vs. Y, whereas for the other complexes no significant differences between groups were observed. Protein abundance of the respiratory chain carrier CI did not significantly change in any condition, both in Y and in E. Lastly, OXPHOS Complex V (CV) showed a unique pattern: both in Y and in E, the expression decreased at BR14 and did not recover at R+14.

In order to estimate mitochondrial mass we determined the expression levels of TOM20, a component of the translocase of the outer mitochondrial membrane, and CS, an enzyme of the mitochondrial matrix. The patterns of TOM20 (**Figure 3A**) and CS (**Figure 3B**) protein abundance were similar and appeared to be in agreement with those of PGC-1α and Sirt3 only in E, showing a decrease following bed-rest with a restoration following rehabilitation. Indeed, in E the levels were lower in BR14 than BDC and increased after rehabilitation (higher values in R+14 vs. BR14). The recovery vs. BDC was complete (no significant differences between R+14 and BDC). Conversely, in Y no statistically significant changes were observed.

The expression levels of the key glycolytic enzyme GAPDH are shown in **Figure 3C**. It should be pointed out that the change from BDC to BR14 in E was less pronounced than Y and did not reach the statistical significance. Nevertheless, in both groups the pattern was similar: the levels increased during bed-rest (suggesting a shift from oxidative to non-oxidative metabolism) and returned to BDC levels during rehabilitation.

Finally, we also investigated the activation of the energy sensor AMPK, in order to evaluate if the observed changes were associated/driven by a condition of energy stress, and if AMPK signaling pathway, upstream of PGC-1α, was triggered in concert with the PGC-1α-Sirt3 axis. With this aim, we assessed the p-AMPK^{Thr172}/AMPK ratio. Intriguingly, no significant changes were observed in the ratio (**Figure 4A**), as well as in the total AMPK protein levels (data not shown), in both groups across conditions. We also assessed the p-LKB1^{Ser482}/LKB1 ratio considering that LKB1 is one of the upstream activators of AMPK signaling. No significant changes were observed even for such variable (**Figure 4B**), supporting the unexpected absence of AMPK activation.

Gene Expression Data Mining From Public Datasets

In an attempt to validate our protein expression data, we performed some *in silico* analyses of public human gene expression datasets, focusing on mitochondria-related genes relevant for our bed-rest study. As no public databases were available relative to elderly people in immobility conditions, our analysis was focused on GEO-included datasets of genes involved in skeletal muscle responses to aging or to immobility/rehabilitation in young adult individuals.

Firstly, we analyzed GSE24215, which is one of the most complete dataset regarding the inactivity-induced responses of gene expression in adult healthy subjects along with the effects on exercise rehabilitation (Alibegovic et al., 2010b). Our DEG analysis focused on genes relevant for skeletal muscle structure/function with particular attention to mitochondria and energy metabolism, and showed that a number of such genes were significantly downregulated following bed-rest conditions resembling those of our study (list of the top DEG following inactivity in **Supplementary Table S1**). We present our results of *gene enrichment* analysis in **Table 1** reporting the significantly enriched categories/terms, and summarize in **Table 2** the DEG relative to such categories/terms (see gray cells) for the genes most relevant for our bed-rest study. Notably, among the downregulated mitochondrial genes, 2 are subunits of TIM and TOM complexes, 8 are subunits of OXPHOS Complexes I, II, IV, and V, and 1 is a subunit of permeability transition pore PTP. Interestingly, PGC-1α also results downregulated (PPARGC1A gene is included in category/term "hsa04920:adipocytokine signaling pathway"). Concerning the genes contained within the category/term "muscle proteins" (not shown in **Table 2**), it should be underlined that, along with 8 genes downregulated, 6 genes are upregulated including two myosin heavy chain in accordance to the recognized switch from slow to fast muscle fibers after

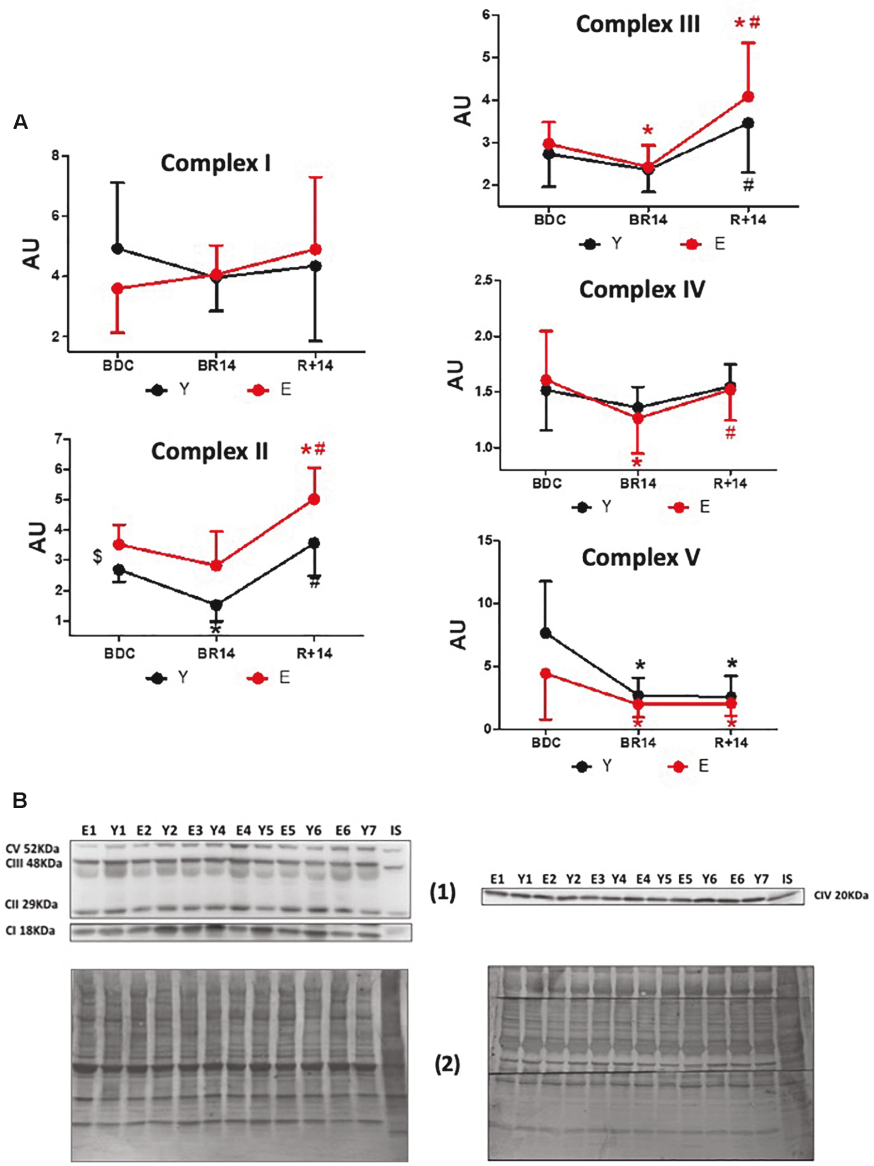

FIGURE 2 | Changes in OXPHOS complexes' expression levels in *vastus lateralis* muscle biopsies from young and elderly subjects after bed rest and rehabilitation. **(A)** Graphs represent immunoblot data (means ± SD) of OXPHOS complexes I, II, III, IV, V for the Y and E subjects, under BDC, BR14, and R+14 conditions. Red line: Elderly group; black line: Young group. **(B)** Immunoblot images denoted as (1) are representative of experiments with samples from both Y and E subjects loaded on each gel matching the conditions, along with IS, and images denoted as (2) are representative of the corresponding Coomassie-stained whole PVDF membranes used as loading and transferring measurement. All details of the analysis and quantification are as in **Figure 1**.

immobility (Schiaffino and Reggiani, 2011; Lynch et al., 2015). After rehabilitative exercise training a number of mitochondria-related genes were found significantly upregulated with respect to immobility (list of the top DEG following post-inactivity exercise in **Supplementary Table S2**). Among these genes, much corresponding to those downregulated by bed-rest, there are subunits of mitochondrial proton-transporting ATP synthase Complex V and PTP, as well as of Complex IV, Complex II, TIM and TOM complexes. All are comprised within the 41 genes included in category/term "Mitochondrion" of *gene enrichment* analysis reported in **Table 1** and are summarized in **Table 2**. Intriguingly, the categories/terms "hsa00190:Oxidative phosphorylation," "GO:0005753~mitochondrial proton-transporting ATP synthase complex," and "hsa04920:adipocytokine signaling pathway" were not significantly enriched after the exercise training. This might be due to the higher number of genes upregulated after rehabilitation with respect to those downregulated after immobility (386 vs. 252). Of note, the category/term "Energy production and conversion" and GPD2 gene comprised within, appeared significantly downregulated after exercise, in agreement with a switch toward OXPHOS (**Tables 1**, **2**). Indeed, GPD2

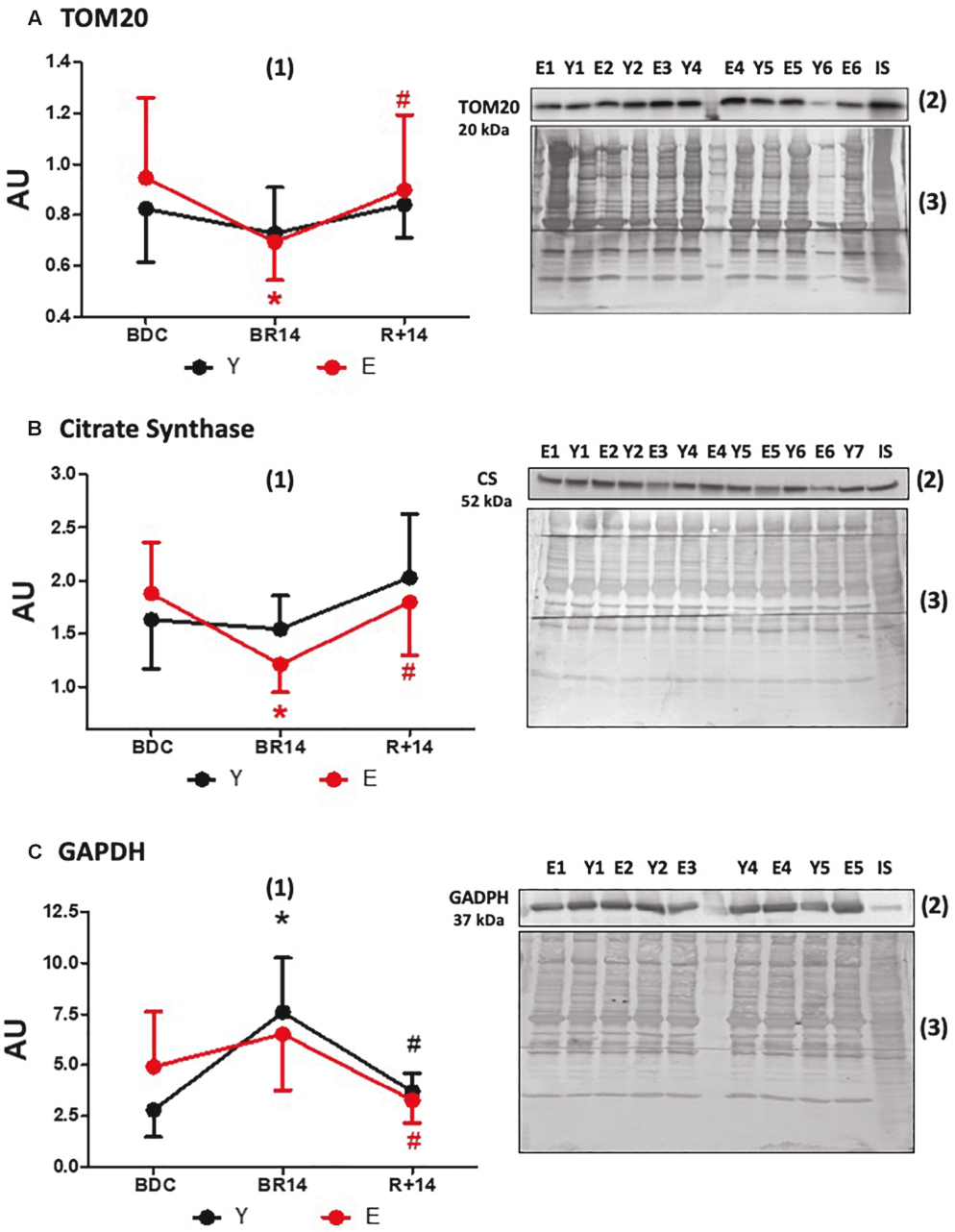

FIGURE 3 | Changes in TOM20 **(A)**, Citrate Synthase **(B)**, and GAPDH **(C)** expression levels in *vastus lateralis* muscle biopsies from young and elderly subjects after bed rest and rehabilitation. Panels **(A–C)** graphs denoted as (1) represent immunoblot data (means ± SD) for the Y and E subjects under BDC, BR14, and R+14 conditions. Red line: Elderly group; black line: Young group. Immunoblot images denoted as (2) are representative of experiments with samples from both Y and E subjects loaded on each gel matching the conditions, along with IS, and images denoted as (3) are representative of the corresponding Coomassie-stained whole PVDF membranes, used as loading and transferring measurement. All details of the analysis and quantification are as in **Figure 1**.

encodes for GPD2, which catalyzes the conversion of glycerol-3-phosphate using FAD as acceptor of reducing equivalents within the inner mitochondrial membrane.

Remarkably, negligible changes were observed for CS either after bed-rest or after post-immobility exercise (**Table 2**), in accordance with the results of protein abundance obtained for the young group of our bed-rest study, where changes were observed for PGC-1α – Sirt3 and single OXPHOS complexes, but not for markers of mitochondrial mass.

Finally, consistent with the idea that protein turnover could be affected *via* alteration of breakdown pathways together with biogenesis, we also searched for genes involved in ubiquitin-proteasome pathway. Results of DEG analysis revealed that gene expression for FBXO32 (Atrogin-1) was significantly up- and

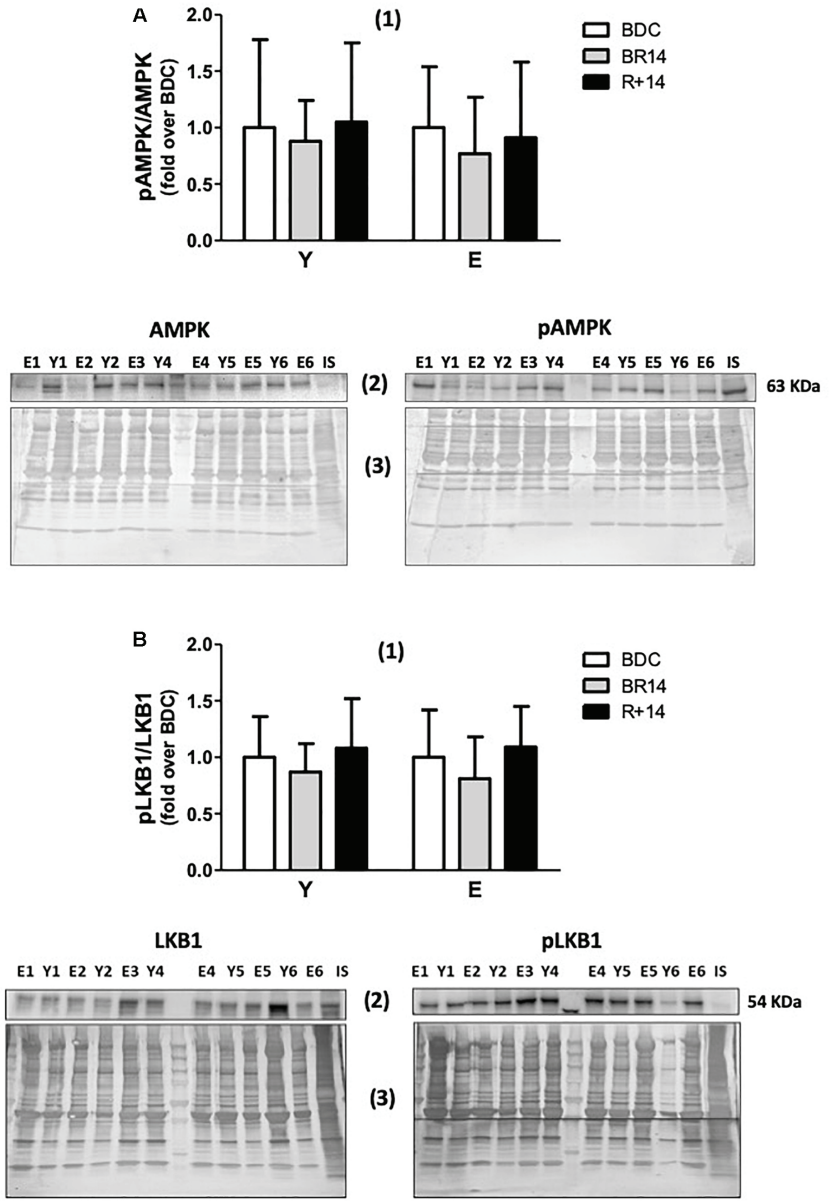

FIGURE 4 | p-AMPK/AMPK **(A)** and p-LKB1/LKB1 **(B)** ratios in *vastus lateralis* muscle biopsies from young and elderly subjects after bed rest and rehabilitation. In both panels **(A,B)**, histograms denoted as (1) represent the fold-increase (means ± SD) of the normalized ratio between phosphorylated and total proteins from immunoblot analyses for Y and E subjects under BDC (empty columns), BR14 (gray columns), and R+14 (black columns) conditions. Immunoblot images denoted as (2) refer to total (on the left) and phosphorylated (on the right) proteins and are representative of experiments where samples from both Y and E subjects were loaded together on each gel matching the conditions, along with IS. Images denoted as (3) are representative of the corresponding Coomassie-stained whole PVDF membranes, used as loading and transferring measurement. All details of the analysis and quantification are as in **Figure 1**.

down-regulated by immobility and rehabilitation, respectively, although just below the threshold chosen to define the top DEGs (**Table 2** and **Supplementary Tables S1, S2**). Intriguingly FBXO2, that is another component of the ubiquitin E3 ligases playing important roles in the ubiquitin-proteasome protein-degradation pathway, resulted more markedly upregulated by immobility, but not affected following exercise.

We further analyzed the GEO dataset GSE8872 (Chen et al., 2007), including data from *medial gastrocnemius* muscle of adult subjects (around 30 years old) undergoing disuse atrophy due to shorter (5 days) immobilization attained using a short leg cast with the ankle in a neutral position.

From DEG analysis, performed with the aim to see if there were changes in mitochondria-related genes' expression as an early response to muscle inactivity, resulted some interesting data, which we considered relevant for our bed-rest study (list of the top DEG in **Supplementary Table S3**). Not many subunits of only two OXPHOS complexes were downregulated

TABLE 1 | Differentially expressed genes after inactivity and subsequent exercise for muscle- and mitochondria-related proteins in *vastus lateralis.* GSE24215 dataset.

Category	Term	Count	%	*p*	Benjamini	Fold enrichment
Gene enrichment analysis of downregulated genes after inactivity						
UP_KEYWORDS	Mitochondrion	49	20.94	1.5E-16	3.14E-14	4.00
UP_KEYWORDS	Muscle protein	8	3.42	5E-06	0.00036	11.74
KEGG_PATHWAY	hsa00190:Oxidative phosphorylation	8	3.42	0.00303	0.03573	4.12
KEGG_PATHWAY	hsa04920:Adipocytokine signaling pathway	7	2.99	0.00049	0.01087	6.84
GOTERM_CC_DIRECT	GO:0005753~mitochondrial proton-transporting ATPsynthase complex	4	1.71	0.00200	0.05541	15.62
Gene enrichment analysis of upregulated genes after inactivity						
UP_KEYWORDS	Muscle protein	6	1.78	0.00296	0.08000	6.10
Gene enrichment analysis of downregulated genes after exercise						
COG_ONTOLOGY	Energy production and conversion	4	3.15	0.00086	0.00257	18.33
Gene enrichment analysis of upregulated genes after exercise						
UP_KEYWORDS	Mitochondrion	41	11.39	7.3E-06	0.00031	2.15

Category, original database/resource where the term orient; term, enriched terms associated with the gene list; count, genes involved in term; %, percentage of genes involved/total genes; p, modified fisher exact p-value, EASE Score; Benjamini, statistical correction; fold enrichment, down (up) regulated genes in a specific category/term over total down (up) regulated genes (%)/number of genes in that category/term over total number of genes (%). Lists of the top DEG in ***Supplementary Tables S1, S2****. For DEG analysis, see Supplementary Table legends.*

significantly (Complex I NDUFS4/B3/B5 and Complex V ATP5G3/L/C1: ctrl/immobility FC = 1.49–1.62 around the threshold, $p < 0.05$). Notably, among the few genes resulted to be significantly upregulated there are UCP3 (uncoupling protein 3 – mitochondrial proton carrier), showing a ctrl/immobility FC = −1.51, $p = 0.0055$, as well as MT1X and MT2A, codifying for metallothioneins 1X and 2A (ctrl/immobility FC = −1.83 and −1.78, $p = 0.014$ and 0.001, respectively).

As a final point, to explore the expression of mitochondria-related genes involved in response to aging we focused on two GEO datasets comprising human microarray data from *vastus lateralis* biopsies of subjects with different age. Specifically, we analyzed the dataset GSE9103 (Lanza et al., 2008) for gene expression changes between young (18–30 years) and aged (58–76 years) sedentary people, as well as the dataset GSE47881 (Phillips et al., 2013) in order to perform a correlation analysis with age taking advantage from data of tree different groups of sedentary subjects (age 20–28, 45–55, and 64–75 years). For both datasets, the ranges of age analyzed included the age of the subjects of our bed-rest study (E: 55–65 years vs. Y: 18–30 years).

From DGE analysis of GSE9103 (list of the top DEG in **Supplementary Table S4**) emerged that some of the genes significantly downregulated in elderly subjects are relevant in the context of the proteins analyzed in our bed-rest study. Specifically, a marked decline was observed for the expression of TOMM40L (FC = −1.54; $p = 0.0016$), a gene encoding for the channel-forming subunit of the translocase of the outer mitochondrial membrane, which might be indicative of reduction of mitochondrial mass/biogenesis in elderly people. Conversely, negligible changes were observed for all OXPHOS complexes, while for PPARGC1A the decline was just below the threshold selected (FC = −1.3989; $p = 0.0080$). Finally, though of different extent, the downregulation of gene expression for TMEM70 (FC = −1.73; $p = 0.0402$) and COA7 (FC = −1.39; $p = 0.0061$), is also interesting with respect to our bed-rest study, as such genes are required for assembly of mitochondrial ATP synthase complex V and respiratory chain complex I and complex IV, respectively.

Notably, the results of the correlation analysis with age of mitochondria-related genes' expression from the second dataset GSE47881indicate that there is a negative strong correlation with age of the expression of a number of mitochondrial genes relevant in the context of the proteins analyzed in our bed-rest study. The results of *gene enrichment* analysis are recapitulated in **Table 3**, while DEG are summarized in **Table 2** (list of the top DEG in **Supplementary Table S5**). Specifically, negative strong correlation with age emerged for some subunits of respiratory carriers and assembly factors, as well as for some subunits of TIM and TOM complexes, which are included within the three categories/terms reported in **Table 3** as highly significantly enriched. In addition, PGC-1α (PPARGC1A) showed a weak but significant negative correlation with age, while no correlation resulted for any subunits of mitochondrial ATP synthase complex V (**Table 2**).

DISCUSSION

The main aim of the present study was to evaluate variables related to mitochondrial biogenesis and function in young (Y) and elderly (E) subjects undergoing 14 days of profound inactivity (bed-rest), followed by 14 days of rehabilitation by a multimodal exercise program with an aerobic phase consisting in high-intensity intervals training. More specifically, we intended to compare the changes of the expression levels of key proteins related to the regulation of mitochondrial energy metabolism with those of "systemic" variables of functional evaluation, determined in the same subjects and recently published (Pišot et al., 2016; Rejc et al., 2018). The study was conducted under strictly controlled conditions and the period of inactivity was long enough to induce a marked muscle atrophy in both groups. Indeed, a significant decrease in quadriceps

TABLE 2 | A summary of results obtained for mitochondria-related genes from DEG, correlation analysis, and *gene enrichment* of two public datasets.

	SYMBOL	GSE24215 immobility (FC)	GSE24215 exercise (FC)	GSE47881 correlation with age (*r*)
Complex V	ATP5C1	↓↓	↑↑	* NS
	ATP5E	↓↓	↑↑↑	
	ATP5F1	↓↓↓	↑↑	* NS
	ATP5G1	↓↓↓	↑↑↑	* NS
	ATP5G3	↓↓↓	↑↑↑	* NS
	ATP5L	↓↓	↑↑	**
Complex IV	COX7A2	↓↓↓	↑↑↑	**
	COX7B	↓↓↓	↑↑	**
	COX7C	↓	–	***
	GPD2	↑↑↑	↓↓↓	–
Complex I	NDUFA8	↓↓	↑↑	***
	NDUFB10	↓↓↓	–	**
	NDUFB3	↓↓↓	↑↑	* NS
	NDUFB5	↓↓	–	* NS
	NDUFC1	↓	–	***
	NDUFS4	↓	↑	**
	CS	↓	↑ NS	
Complex II	SDHB	↓↓	↑↑	***
	SDHC	↓↓↓	↑↑↑	–
Complex III	UQCR11	↓↓	↑	***
TIMM	TIMM23	↓	–	***
	TIMM8A	↓↓↓	↑↑↑	–
	TIMMDC1	↓	↑	***
TOMM	TOMM40L	↓↓↓	↑↑↑	**
	TOMM7	↓	–	***
	UCP3	↓ NS	↓↓↓	–
PTP	PPIF	↓↓↓	↑↑↑	* NS
Assembly factors	UQCC3			***
	COA4			***
	COA7	↓ NS	–	*
	NDUFAF6			***
	TMEM70	↑ NS	–	–
Ubiquitin-proteasome	FBXO2	↑↑↑	–	* NS
	FBXO32	↑↑	↓↓	* NS
Metallothioneins	MT1X	–	–	–
	MT2A	–	–	* NS
PGC1a	PPARGC1A	↓↓↓	↑↑↑ NS	*

(↑↑↑) FC > 1.5; (↑↑) 1.30 < FC < 1.49; (↑) 1.20 < FC < 1.29; (–) FC < 1.19.
(↓↓↓) FC < −1.5; (↓↓) −1.49 < FC < −1.30; (↓) −1.29 < FC < −1.20; (–) FC > −1.19.
*(***) negative strong correlation; (**) negative moderately strong correlation; (*) negative weak correlation; (–) r < −0.2 (no correlation). Empty cells: values not present in the array. The gray cells indicate data referred to results of the gene enrichment analyses (**Tables 1**, **3**).*

TABLE 3 | Mitochondria-related genes negatively correlated with age in *vastus lateralis.* GSE47881 dataset.

Category	Term	Count	%	*p*	Benjamini	Fold enrichment
Gene enrichment analysis of genes negatively correlated with age (Spearman correlation analysis)						
GOTERM_CC_DIRECT	GO:0005743~mitochondrial inner membrane	32	7.27	5.5E-09	1.99E-06	3.42
UP_KEYWORDS	Mitochondrion	50	11.36	1.97E-07	2.19E-05	2.24
UP_KEYWORDS	Transit peptide	26	5.91	0.0000819	0.00679	2.43

*Rule of thumb for interpreting the size of a correlation coefficient was used. Genes with a significant negative strong correlation were selected (correlation coefficient −0.6 ≤ r ≤ −0.8), Benjamini–Hochberg corrected p-values < 0.05. Category, original database/resource where the term orient; term, enriched terms associated with the gene list; count, genes involved in term; %, percentage of genes involved/total genes; p, modified Fisher Exact p-value, EASE Score; Benjamini, statistical correction; fold enrichment, down (up) regulated genes in a specific category class over total down (up) regulated genes (%)/number of genes in that category class over total number of genes (%). Lists of the top negatively correlated genes in **Supplementary Table S5**.*

muscle volume occurred in elderly (−8.3%, $p < 0.001$), and the same trend was observed in the young controls (−6.1%, $p = 0.052$) (Pišot et al., 2016). We expected that the expression of the investigated proteins would be at the base of the functional adaptations occurring in skeletal muscles following inactivity and subsequent rehabilitation, supporting the role of mitochondrial regulation in muscle plasticity even in older individuals. The general finding of the previous study (Pišot et al., 2016) was that "systemic" variables of functional evaluation were often affected by inactivity more profoundly in E, in whom the rehabilitation was also less complete vs. that of Y, or did not occur. A similar pattern was observed for some variables determined in the present study. The main difference between the results of the two studies relates to baseline values, which in the present study were, in most cases, not significantly different in E vs. Y. As an example, the similar values observed for PGC-1α protein expression levels are in accordance with previous reports (Lanza et al., 2008; Irving et al., 2015). What observed for the systemic variables by Pišot et al. (2016) is in sharp contrast. For example, peak pulmonary O_2 uptake ($V'O_2$peak), a variable estimating maximal aerobic power, which should be related to mitochondrial function, was at baseline about 30% lower in E vs. Y (Pišot et al., 2016). In other words, a clear dissociation was present at baseline between systemic and mitochondrial variables related to oxidative metabolism, confirming the concept that mitochondrial factors are not the main determinant of systemic maximal aerobic power, and that factors "upstream" of mitochondria (mainly cardiovascular O_2 delivery) are more relevant in this respect (Lundby et al., 2017). In the present study, most variables related to mitochondrial oxidative metabolism decreased following bed-rest. For some variables the decrease was more pronounced (or was statistically significant only) in E. A complete restoration was observed in Y for most mitochondrial variables; the restoration was incomplete in E in some cases. Thus, we infer that mitochondrial adaptations occurring under conditions of inactivity-induced atrophy and after rehabilitation went substantially in parallel with changes of systemic variables related to mitochondrial function.

PGC-1α and Sirt3

In accordance with a decreased need of new mitochondrial proteins and energy, we observed that inactivity led to a diminished expression levels of PGC-1α, a master regulator of mitochondrial biogenesis and structural/functional integrity both in physiological conditions and during pathophysiological processes of muscle atrophy and aging (Finck and Kelly, 2006). Likewise, we observed a decrease of protein levels for Sirt3, a NAD^+-dependent protein deacetylase localized solely inside mitochondria (Scarpulla, 2002; Brenmoehl and Hoeflich, 2013), that is known to be a main mitochondrial activity regulator with a prominent role in skeletal muscle (Jing et al., 2011, 2013; Vassilopoulos et al., 2014). Following rehabilitation, the levels of PGC-1α and Sirt3 protein expression rose, consistently with the increased energy needs, and PGC-1α levels reached values even beyond the baseline. Our results are in line with previous data on PGC-1α expression (protein and mRNA) obtained in young subjects (Brocca et al., 2012; Wall et al., 2014). As for Sirt3 data, this is the first study that examined the protein expression levels in relation to bed-rest and subsequent rehabilitation in both Y and E people. Nevertheless, exercise training was documented to upregulate the expression levels of Sirt3 (and PGC-1α) in skeletal muscle by several studies (Lanza et al., 2008; Hokari et al., 2010; Irving et al., 2015). The similar trend exhibited by PGC-1α and Sirt3 in the present study was expected based on the following considerations. (i) PGC-1α in the nucleus, when active, is recognized to regulate Sirt3 expression (Brenmoehl and Hoeflich, 2013); (ii) contractile activity during exercise is documented to trigger signaling pathways leading to Sirt3 induction by PGC-1α (Ventura-Clapier et al., 2008 and references therein); (iii) the overexpression/knockdown of Sirt3 or PGC-1α is reported to elicit in muscle similar effects and to promote the activity of several enzymes involved in oxidative and energetic metabolism (Kong et al., 2010). In addition, Sirt3 can enhance in a positive feedback system PGC-1α expression and the subsequent regulation of mitochondrial related proteins (Palacios et al., 2009; Kong et al., 2010; Hokari et al., 2010; Brenmoehl and Hoeflich, 2013). In line, PGC-1α-Sirt3 signaling pathway triggered by contractile activity is documented to result in both mitochondrial biogenesis and activation of several enzymes of oxidative and energetic metabolism (Palacios et al., 2009; Hokari et al., 2010).

The changes in the expression of such proteins observed in the present study were paralleled by systemic changes (Pišot et al., 2016), with the decrease in response to bed-rest being more pronounced (or statistically significant only) in E and exercise-restoration complete in Y but incomplete in E. Thus, we infer that mitochondrial adaptations occurring under conditions of immobility-induced atrophy, and after exercise training, were associated to PGC-1α-Sirt3 signaling pathway and linked with changes of systemic variables related to mitochondrial function.

OXPHOS Complexes

Taken as a whole, our data document that the various OXPHOS complexes show diverse patterns of expression following inactivity and rehabilitation, each of them very similar in both E and Y. The patterns of respiratory chain complexes CII, CIII, and CIV, similar to that described for PGC-1α, are in line with studies using a protocol of 2 weeks of one-leg immobilization (Gram et al., 2014). The dissimilar behavior from that of PGC-1α, observed in the cases of CI and CV, may be considered in contrast with the well- known regulation by PGC-1α of the expression of mitochondrial- and nuclear-encoded subunits of OXPHOS (Scarpulla, 2002). Nevertheless, as the observed steady state-levels of proteins are in principle the end-result of biogenesis and degradation, this divergence might be attributed to different responses to immobility and rehabilitation by the degradative pathways of OXPHOS complexes, compared to the expression regulatory pathways linked to PGC-1α. Indeed, it is recognized that disuse muscle atrophy is accompanied by activation of multiple catabolic pathways beside inhibition of protein synthesis (Powers et al., 2012 and references therein – Brocca et al., 2012; Bonaldo and Sandri, 2013; Cannavino et al., 2015). Moreover, PGC-1α might elicit different regulation of single proteins involved in energy production, thereby controlling mitochondrial remodeling rather than biogenesis.

Such an effect was described in several reports providing evidence that PGC-1α in skeletal muscle may selectively and differently control the expression levels of several mitochondrial proteins (Chan and Arany, 2014 and references therein).

As for the peculiar behavior exhibited by CV in the experimental conditions of the present study (expression decreased at BR14, but not recovered at R+14), it should be considered that CV is recognized to be finely regulated at post-transcriptional level and to be expressed in large excess with respect to the working molecules. In this context, CV is reported to be a main target of Sirt3, undergoing a deacetylation-mediated activation in several models (Ahn et al., 2008; Bao et al., 2010; Jing et al., 2011; Wu et al., 2013; Lin et al., 2014), and specifically in skeletal muscle in response to exercise-induced stress (Rahman et al., 2014; Vassilopoulos et al., 2014). In this scenario, as Sirt3 expression levels in the present study were documented to be more abundant after rehabilitation both in E and Y, it might be hypothesized that exercise triggered a Sirt3-mediated deacetylation of CV, enhancing the enzyme activity in the presence of unchanged protein expression. These aspects need further investigations.

Variables Estimating Mitochondrial Mass

The patterns of the expression levels of the mitochondrial matrix protein CS, and of the outer mitochondrial membrane protein TOM20, both usually recognized as reliable mitochondrial mass markers, reveal a marked difference between E and Y subjects, as in these latter no changes were observed. Based on this behavior apparently conflicting with the pattern of PGC-1α, we may hypothesize specific effects on protein turnover as occurring in Y, rather than modulation of mitochondrial biogenesis. Regardless of the mechanism involved in the effects observed, the data of CS and TOM20 expression levels, taken as a whole, are in line with a less pronounced susceptibility to immobility of Y, with respect to E.

Glycolytic Marker GAPDH

The glycolytic marker GAPDH increased during bed-rest and decreased after rehabilitation in both groups, with a more pronounced effect in Y, suggesting an up-regulation of glycolytic metabolism during bed-rest, possibly as a compensatory response to the mitochondrial impairment, and a subsequent return to a more oxidative metabolism following rehabilitation. These results are part of the still open debate on glycolytic and oxidative metabolism in muscle atrophy and inactivity. In fact, there is not agreement on this topic in literature, likely due to dissimilar protocols applied by diverse authors. In accordance with our data are various bed-rest studies documenting an increased reliance on glycolysis (Acheson et al., 1995; Fitts et al., 2000; Stein and Wade, 2005). Conversely, other reports (Alibegovic et al., 2010b; Moriggi et al., 2010; Ringholm et al., 2011; Brocca et al., 2012) showed a downregulation of both glycolytic and oxidative metabolism during disuse.

LKB1-AMPK Signaling Pathway

During cell stress events, one of the upstream activators and inducers of PGC-1α expression through phosphorylation is AMPK (Ringholm et al., 2011; Brocca et al., 2012). AMPK is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells, including skeletal myocytes (Hardie et al., 2012), and is recognized to be upregulated by several endogenous stimuli leading to energy impairment, including exercise/muscle contractile activity (Kjøbsted et al., 2018 and references therein). The regulation of AMPK activity is quite complex and, in addition to an allosteric regulation by the [AMP]/[ATP] ratio, it involves also increased phosphorylation by upstream kinases and decreased de-phosphorylation by protein phosphatases. LKB1 appears to be the primary AMPK upstream activating kinase in skeletal muscle under conditions of high-energy stress (Kjøbsted et al., 2018 and references therein). Thus, considering that Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs), also key activators of AMPK, were documented to be not considerably expressed in skeletal muscle (Hardie and Sakamoto, 2006), and that CaMKKb was reported to be involved to a lesser extent than LKB1 (Kjøbsted et al., 2018), in the present study we examined the activation of the LKB1-AMPK axis. We determined p-AMPK^{Thr172}/AMPK and p-LKB1^{Ser482}/LKB1 ratios, and we did not observe any significant change in both groups.

These results suggest that during bed-rest no energy stress (increased [AMP]/[ATP]) was likely present. This is not difficult to conceive. Indeed, skeletal muscle energy turnover in resting conditions is very low, and the energy charge only rarely challenged (Kjøbsted et al., 2018). In addition, the marked decrease in skeletal muscle energy demand during the profound inactivity associated with bed-rest could have played a role even in the presence of downregulated mitochondrial biogenesis and activity. Our results are in accordance with data from another bed-rest study in young subjects (Brocca et al., 2012).

On the other hand, the lack of activation of LKB1-AMPK axis by the rehabilitation intervention could be considered, at a first sight, rather unexpected. According to Combes et al. (2015), high-intensity intervals training (representing the aerobic component of the exercise training regimen adopted in the present study) should elicit pronounced AMPK signaling pathway. This activation is only transient, however, likely as consequence of downregulation or de-phosphorylation of LKB1/AMPK after exercise (Combes et al., 2015). Thus, AMPK activity decreases after exercise to levels observed in resting muscle typically within 3–7 h (Kjøbsted et al., 2018 and references therein). By our protocol, therefore, we might have "missed" the activation of this signaling pathway, due to the interval between the last exercise bout and the muscle biopsy.

In any case, we cannot exclude that the changes of PGC-1α expression, and the resulting modulation of mitochondrial biogenesis/remodeling, observed in the present study were driven by mechanism(s) not linked to LKB1-AMPK axis, among the multiple signaling pathways appearing to converge on regulation of PGC-1α (Gan et al., 2018). One might hypothesize that the decrease in PGC-1α levels observed following inactivity involved Ca^{2+}-dependent signaling and was due to diminished intracellular Ca^{2+} levels, which might be counteracted by exercise (Irrcher et al., 2003; Kusuhara et al., 2007; Kang et al., 2012). If this is the case, the greater sensitivity to inactivity

observed in E might be explained by the tendency of Ca^{2+} concentration to decrease during aging in skeletal muscle cells (Berchtold et al., 2000 and references therein). A validation of this hypothesis would require additional studies, as alterations of intracellular Ca^{2+} concentration during immobility are still matter of debate due to contrasting reports (Ingalls et al., 1999; Fraysse et al., 2003).

Gene Expression Analysis

Several studies have tried to comprehend through gene expression analyses the molecular mechanisms involved in skeletal muscle responses to immobility and rehabilitation in humans, as well as associated with aging.

Overall, data emerged from our analysis of GSE24215 gene expression dataset, focused on mitochondria- and OXPHOS-related genes (OXPHOS complexes, PGC1-α, CS) in young adult populations, are in accordance with the results of protein abundance obtained for the young group of our bed-rest study. This support two main messages: (i) decline of the steady-state levels of mitochondria-related proteins in atrophic muscle and recovery after rehabilitation for most of them, (ii) mitochondrial remodeling rather than biogenesis at the basis of mitochondria modulation. Nevertheless, with regard to some discrepancies observed, it should be noted that difference between mRNA and protein stability might be diverse under different conditions. Moreover, the data of gene expression are not always related to the same protein subunits which were analyzed by immunoblot for the single OXPHOS complexes. In the case of complex I, intriguingly, despite NDUFB3 and NDUFB10 gene expression was downregulated by immobility, the absence of changes for NDUFB8, a nuclear DNA-encoded subunit integral to the assembly of complex I, is in accordance with our immunoblot data.

Interestingly, from DEG analysis of GSE24215 dataset we also observed an up- and down-regulation by immobility and exercise training of gene expression for FBXO32 (Atrogin-1) and FBXO2, two essential components of ubiquitin-proteasome pathway. FBXO32 (Atrogin-1) is a specific constituent of muscle playing a critical role in mediating the loss of muscle protein (Lecker et al., 2006). Intriguingly, a more marked upregulation was seen after immobility for FBXO2, which binds to high mannose glycan-containing glycoproteins, and is a gene known to be expressed specifically in the brain. Indeed, as a member of F-box associated family, it displays divergent binding to glycan and glycoproteins, and tissue-specific distributions reflecting differences in glycoprotein distribution (Glenn et al., 2008). In this scenario, upregulation induced by immobility in skeletal muscle is an apparent divergence with respect to tissue specificity of FBXO2. Thus, it is tempting to hypothesize that such rise might reflect a variation of the need for regulation of the myocyte glycome. As mentioned above, increased expression of genes/proteins ascribed to ubiquitin-proteasome pathway is common in atrophic muscle (Reich et al., 2010; Powers et al., 2012 and references therein), and there are data supporting the idea that degradative pathways are enhanced depending on length of immobility (Brocca et al., 2012). Based on these considerations, we may infer that the effects observed in our bed-rest study on the steady-state levels of mitochondria-related proteins might be ascribed, at least in part, to regulation of the expression of ubiquitin-proteasome pathway components.

On the other hand, one must consider also that data from diverse studies should be compared with caution, due to the multiple protocols of immobility and rehabilitative exercise training operated in different laboratories.

From our analysis of GSE8872 dataset emerged that a gene expression downregulation early occurred (5 days of leg cast immobilization) for a number of genes (such as some muscle proteins as MYH3 and MYL12A), including, however, only few mitochondrial OXPHOS subunits. For some of such genes (i.e., NDUFB3, ATP5G3, and MYL12A), a downregulation of the expression resulted also from GSE24215 analysis along with a number of connected genes. GSE8872 data are from *medial gastrocnemius* muscle and comparison between the two datasets was based on similarity to *vastus lateralis* as concerns fiber composition (about 50% fast twitch and 50% slow twitch fibers). The combined data suggest that the immobility effects on mitochondria-related genes appeared to augment with the timespan of the immobility and/or severity on the protocol (leg cast immobilization vs. bed-rest). This finding may be considered in line with our previous reports documenting an impaired mitochondrial respiration, evaluated *ex vivo* in *vastus lateralis* muscle, after prolonged bed-rest conditions (21 days) (Salvadego et al., 2018).

Of note, very few genes from GSE8872 resulted to be significantly upregulated by immobility, among which there is UCP3. An upregulation of muscle UCP3 protein was demonstrated as well, but following prolonged muscle unloading (Mazzatti et al., 2008). In accordance, our recent report proved that after 21 days of bed-rest an enhanced leak respiration (i.e., dissipation of the proton gradient across the inner mitochondrial membrane) occurred associated with a reduced efficiency of OXPHOS (Salvadego et al., 2018). On this basis, we may infer that upregulation of UCP3 gene expression should be an early event in the atrophy program provoked by immobility, in face of the evidence for higher levels of UCP3 protein at later time points upon prolonged immobility conditions (Mazzatti et al., 2008; Salvadego et al., 2018). The relevance of such hypothesis is, in our opinion, in the possibility that uncoupling provoked by immobility would protect the cells against an excessive mitochondrial ROS generation, although at the price of an increased energy dissipation. Indeed, a marked reduction in skeletal muscle energy demand is expected during the profound inactivity of the bed-rest regimen. Comparison with data from GSE24215 dataset showed a significant downregulation of UCP3 gene expression by exercise training, although in this case no effect by immobility was seen.

From another point of view, the finding that MT1X, MT2A appeared also among the few genes, which resulted as significantly upregulated from our analysis of GSE8872 dataset is worthy of note, though not directly linked to mitochondria-related proteins investigated in our bed-rest study. Indeed, metallothioneins are a group of genes associated with muscle

atrophy in humans (Lecker et al., 2004) and their increased expression in muscle undergoing atrophy may be necessary to detoxify metals released by metal-containing compounds, such as myoglobin and mitochondrial cytochromes, during muscle protein degradation. In partial accordance with the data emerged from our analysis is an earlier article (Urso et al., 2006) documenting a gene expression upregulation for numerous metallothioneins in *vastus lateralis* muscle after 48 h of knee immobilization. The authors suggested that this may play a role in the initiation of the atrophy program, and inferred that the atrophy program in humans might be denoted by an early transcriptional response for metallothioneins, maybe as consequence of elevated levels of metals and ROS generated in immobilization. Searching in GSE24215 dataset, collecting data from prolonged immobility conditions similar to our bed-rest study, we observed no changes in metallothioneins gene expression in line with the hypothesis that upregulation should be transitory.

Unfortunately, there was not enough tissue remaining from our bed-rest study with which to perform additional assays, but future directions should include measurements for the expression levels of UCP3 and metallothioneins proteins following prolonged immobility, to evaluate their possible involvement in muscle disuse atrophy.

In summary, combining data from the analysis of gene expression of two different datasets with those of protein abundance from our bed-rest study support the idea that immobility and exercise can affect mitochondria-related protein expression levels by both gene expression regulation and protein degradative pathways.

With regard to the expression of mitochondria-related genes involved in aging, by analyzing the GSE9103 dataset (subjects' age 18–30 and 58–76 years) we focused in particular on certain genes/proteins in *vastus lateralis* relevant for our bed-rest study and obtained some evidence for gene expression downregulation. Specifically, to explain the decline observed for TOM40 and PGC-1α, although it was lower for this latter, it should be postulated that a reduced physical activity by the aged people examined had a crucial role. Indeed, in elderly sedentary subjects exercise was reported to restore PGC-1α protein levels to the ones of young people (Koltai et al., 2012). Furthermore, we have taken advantage from the correlation analysis with age (20–28, 45–55, and 64–75 years) made with data from GSE47881 dataset to obtain information about the behavior of the expression of key mitochondria-related genes. It should be emphasized that the data obtained from the analysis of the two datasets are consistent each other with regard to some, but not all, of such key genes/proteins, specifically, the weak negative correlation with age observed for PPARGC1A should be considered in line with the low decline of PPARGC1A gene expression emerged from GSE9103 analysis. This is also in accordance with the results of our bed-rest study, where at baseline PGC-1α protein abundance was similar regardless of the subjects' age. It should be considered also that the subjects of the elderly group in our study were moderately active, differently from sedentary people whose data of gene expression were used to create both GSE9103 and GSE47881 datasets. Concerning gene expression for OXPHOS complexes' subunits, negligible changes were observed in GSE9103 dataset, in apparent conflict with the results emerged from the correlation analysis made with GSE47881 data. This may suggest that a more advanced age should be needed to elicit appreciable downregulation of such genes. In accordance with this hypothesis are data obtained at baseline in our bed-rest study where the OXPHOS protein abundance investigated was similar regardless of the subjects' age, except for complex CII that was higher in the elderly subjects. However, the downregulation of gene expression for TMEM70 and COA7 observed in GSE9103 dataset reminds to the strong negative correlation of NDUFAF6, COA4, and UQCC3 emerged from GSE47881 dataset analysis. Downregulation of such genes during aging should be taken into account when one evaluate the similar protein expression levels for single subunits of complexes I, III, IV, and V observed at baseline in our bed-rest study for E and Y groups. Indeed, such genes encode for assembly factors of ATP synthase complex V and of respiratory chain complexes I, III, and IV; thus their decline might be responsible to reduce in elderly subjects the assembly of whole complexes in membrane. However, future studies will need to address this.

In conclusion, data from our analyses of the effects of aging on expression of a number of mitochondria-related genes relevant in the context of the proteins analyzed in our bed-rest study prompt us to highlight that immobility should be more critical for mitochondrial efficiency and energy production in case of people more aged than our elderly subjects. Indeed, we might infer that the trend of the effects documented by our bed-rest study should go on with age.

Study Limitations

The limited number of subjects enrolled in our study may weaken its outcomes. Nevertheless, this is a consequence of logistical limitations, which are intrinsic to this type of studies. Specifically, this is a very complex study performed with two populations of subjects (Y and E) and three muscle biopsies per subject, one of them obtained after 14 days of bed-rest and another one after 14 days of supervised exercise program. From a statistical standpoint, limitations are mainly related to the uneven sample size of the groups (7 Y and 16 E subjects) and particularly to the more restricted size of Y group that impact on statistical power to detect differences at some points.

In addition, only limited amounts of tissue specimens from the muscle biopsies were available for our experiments, due to the numerous participants to the bed-rest campaign where our study was comprised. This reduced the number of proteins we decided to assay by quantitative immunoblot analyses in order to achieve accurate quantifications. Most important in this respect, we did not determine the expression levels of key components of ubiquitin-proteasome pathway despite our hypothesis that a reduction of mitochondrial-related proteins in atrophic muscle might be ascribed, at least in part, to regulation of the expression of such pathway. Our hypothesis is validated based on our gene expression data mining from human public datasets, as well as on solid literature.

CONCLUSION

In summary, based on the expression levels of key proteins related to mitochondrial biogenesis regulation and bioenergetics in *vastus lateralis* muscle, our study confirms a crucial role of mitochondrial biogenesis/remodeling in muscle plasticity following inactivity and exercise rehabilitation. The heterogeneous patterns of the expression levels observed for some proteins are indicative of different responses to the treatments by the respective degradative ways compared to the biogenesis regulatory pathways. Furthermore, our study provide evidence that responses to bed-rest causing atrophy, as well as adaptations to rehabilitation, in E and Y populations were of different extent and qualitative diverse. Namely, the impact of bed-rest of most proteins was greater, and the rehabilitation recovery was less complete in the elderly subjects, where the changes observed were associated with modifications of mitochondrial mass. Results on protein expression levels are reinforced by data obtained from *in silico* analyses of four public human gene expression datasets, focusing on mitochondria-related genes affected in skeletal muscle responses to disuse and rehabilitation of adult individuals, or declined in association with aging.

DATASETS ANALYZED IN THE STUDY

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ETHICS STATEMENT

This study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and was approved by the National Ethical Committee of the Slovenian Ministry of Health on April 17, 2012 under the acronym: IR-aging 1200.

AUTHOR CONTRIBUTIONS

BG and IM designed the research. DS, BŠ, RadP, and JR organized the bed-rest campaign. AB and BM performed the experiments, prepared figures, and references' list. AB and MC analyzed the data. RafP performed the gene expression analyses. MI performed the statistical analysis. AB, MC, and IM interpreted the results of experiments. IM, MC, AB, and BG edited and revised the manuscript. All authors approved the final version of manuscript.

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SUPPLEMENTARY MATERIAL

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