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Steroid Myopathy: Understanding the pathogenesis.

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ABSTRACT

Muscle plasticity is a key element in human health and disease. Exercise is an important element that leads to many positive adaptations, which improve survival and quality of life. Conversely, muscle atrophy is a condition found in many chronic diseases. Atrophy is the outcome of an imbalance between the processes that lead to protein synthesis (MPS) and the processes that lead to muscle protein breakdown (MPB) resulting in net muscle mass loss.

Chronic administration of glucocorticoids causes steroid myopathy, characterised by muscle weakness, fatigue and atrophy. The primary pathogenetic phenomenon causing such condition is still unknown. The present study aims to identify the molecular phenomena involved in triggering the myopathic process.

To achieve such goal, the adapations of intracellular signalling pathways, which have been previously shown to be potentially involved in steroid myopathy, were studied. A single dose of dexamethasone (DEX) was administered intravenously to healthy subjects. Muscle biopsies were taken from vastus lateralis muscle 1h, 4h and 8h after DEX injection. Western blot and real time PCR were used to assess the adaptations of markers related to the ubiquitine-protesome degradation pathway (UPS), protein synthesis, autophagy, muscle metabolism, redox status and mitochondrial remodelling.

Results suggest that DEX induced increased gene expression of *Atrogin1*, mitochondrial dysfunction and impairment of oxidative metabolism. The latter phenomenon would cause redox imbalance. Redox imbalance could further stimulate muscle MPB. This vicious loop results in an increased activation of the autophagy pathway. The activation of the autophagy process together with the activation of the pathway of protein degradation would finally lead to muscle atrophy.

The ability by two-week intake of a mixture of branched chain aminoacids to counteract the effects of DEX on intracellular pathways have been also tested. Preliminary data are reported.

CHAPTER 1

INTRODUCTION

SKELETAL MUSCLE

The skeletal muscle contributes to the performance of many activities thanks to the characteristics of plasticity and dynamism that distinguish it. It is composed by 75% of water, 20% of proteins and the remaining 5% by carbohydrates, minerals, inorganic salts and fat. Muscle mass is related to the balance between synthesis and protein degradation, influenced in turn by hormonal factors, nutritional status, physical activity and pathological condition. Skeletal muscle accounts for 40% of the total body weight and contains about 50-75% of the total body proteins, which perform precise structural, regulatory and contractile functions contributing to the ability to move and exercise, as well as maintaining muscle health.

1. Myogenesis

Skeletal muscle originates from the paraxial layer of the mesoderm, which leads to the formation of the somites. The somites initially have a spherical shape and are composed of a wall of epithelial cells and a nucleus of mesenchymal cells. Subsequently, the somites are subdivided into sclerotome, from which the rib and vertebral cartilage originates, and dermomiotome. This in turn is formed by the dermatome, which generates dermis and brown fat, and the myotome from which the skeletal muscles of limbs and trunk originate [1].

In the dermomiotome there are the pluripotent Progenitor Cells of Muscle (MPC), whose delamination is the primary event of the formation of the myotome. The maturation of the myotome is a multiphase process that primarily involves the formation of the primary myotome, which expands in the subsequent development phase.

Delamination, that is the subdivision of a cellular lamina into two or more laminae, occurs initially starting from the dorso-medial region of the epiaxial dermomiotome and then from the ventrolateral portion of the hypoaxial demomiotome. In fact, the myotome is formed through several waves of migration in which MPC are located under the dermomiotome [2], express MyoD and Myf5 and differentiate in myoblasts.

Later these cells express Myogenin and differentiate into multinucleated myotubes. Only in the later stages of embryonic development, the cells present in the central portion of the dermomiotome migrate and become part of the myotome constitution. Some of these cells express MyoD and Myf5, they differentiate and they merge into multinucleated myotubes that evolve at times into myofibres. The remaining cellular subpopulation, on the other hand, proliferates and constitutes a reserve of cells for growth during development or for muscle regeneration in adults [3] (Fig.1).

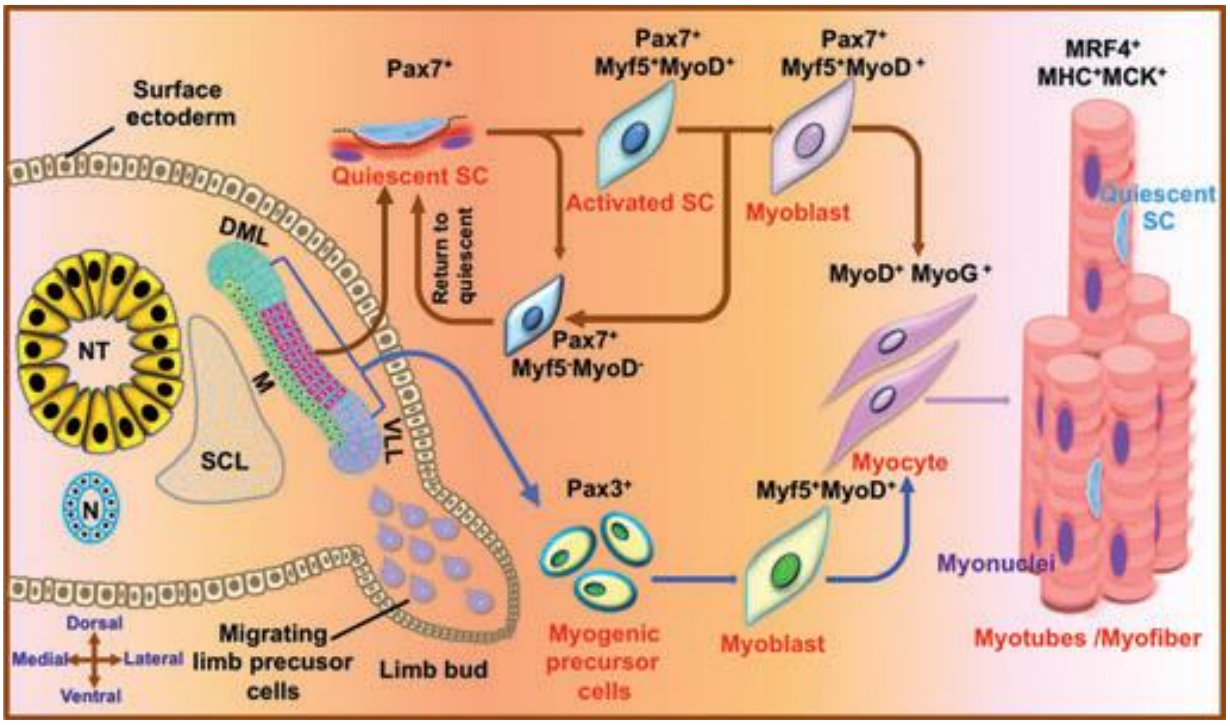


Figure 1: A schematic representation of embryonic and postnatal skeletal myogenesis*.DM: dermomyotome. N: notocorda. NT: neural tube. SCL: sclerotomo. DML: dorsomedial lip. VLL: ventrolateral lip. SC: satellite cells.*

The extracellular signalling molecules that regulate the differentiation of the dermomiotome are multiple and among them are Wnts and Shh. In particular, Wnt1 and Wnt3a together with Shh determine the myogenic development of the epiaxial region, Wnt7 of the hypo axial region [4]. Other factors of myogenic determination are Myf5 and Mrf4, and together with MyoD, induce the expression of Myogenin [5]. Finally, Pax3 and Pax7, markers of MPCs, are fundamental transcription factors for myogenesis [6]. Pax3 is expressed in somites, is implicated in the early stages of myogenesis, induces the expression of Myf, while Pax7 is essential in post-natal myogenesis, and is expressed by satellite cells [7].

2. Structure

From a structural point of view, the skeletal muscle is composed of muscle fibres, a set of multinucleated cells formed by the fusion of mononuclear myoblasts [8]. Adult skeletal-muscle fibres have a cylindrical structure, a diameter between 10 and 100 μm and can even reach a few centimetres in length. The absence of proliferating myoblasts during adult life makes that in case of damage of the individual muscle fibres the only restoration function is covered by the so-called satellite cells [9], that will undergo differentiation into new muscle cells [10]. However, this mechanism (hyperplasia) doesn't often occur and a great deal of compensation occurs through hypertrophy of the surviving muscle cells that will replace the loss of function. Muscles are usually linked to bones by bundles of collagen fibres known as tendons, which are located at the end of each muscle (Fig. 1) [11].

Single muscle fibres are surrounded by a cell membrane named sarcolemma and coated by the endomysium, a thin layer of connective tissue. The sarcolemma has a key function in the health status of individuals. Many proteins connect the sarcolemma to internal myofilament structures such as the actin present in the thin filament. The dysfunction and partial or complete absence of these bridging proteins, can in turn result in muscular dysfunction, atrophy and weakness. A relevant example of these proteins is the protein Dystrophin which is at the basis of pathologies such as Duchenne and Becker muscular dystrophies [12].

Single muscle fibres are organized into fascicles surrounded by the perimysius and another more robust connective membrane, the epimysium, delimits the whole muscle. Each fibre is composed of thousands of myofibrils and contains a large number of myofilaments, which are assembled going to form the sarcomere that represents the functional unit of skeletal muscle [13] (Fig. 2).

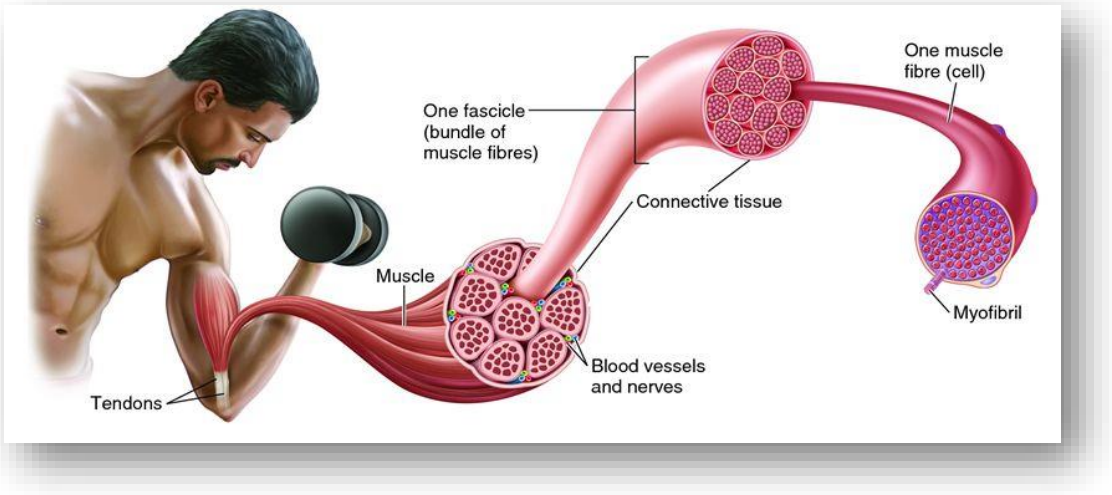


Figure 2: *Structure of skeletal muscle.*

Each sarcomere is a highly organized unit given by the repetition of thin actin filaments and thick myosin filaments, that give the characteristic streaked appearance of skeletal muscle [14]. The striation observed under the microscope is due to the different refractive indices of the various parts of the fibre, ie the light and dark bands [15]. The dark bands are called anisotropic bands (A-bands) and cover the entire length of the thick filaments, including the overlapping zones with the thin filaments, and are divided in half by the H band which presents the dark line M in the middle. The light bands are called isotropic bands (I-bands), are composed only of thin filaments, and are divided into two parts by Z discs that delimit the sarcomere. The Z dics connect adjacent sarcomeres and performs many other important functions. It is the main anchor point of the thin filaments that interact with α-actinine, and of two large proteins, namely Nebulin and Titin. In particular, the latter is involved in the regulation of the length of the sarcomere, in the assembly and alignment of the filaments. Moreover, the Z discs are involved in the signalling pathways used to maintain homeostasis [16] and muscle contraction [17].

The thin filaments are bound to the Z disc, cross the I-band, extend towards the center of the sarcomere and at the level of the A-band overlap the thick filaments. They are composed of two helices of F-actin, one right-handed and the other left-handed, which wrap around each other and are associated with two other proteins with a regulatory function, namely Troponin and Tropomyosin. Furthermore, at both ends these filaments bind tropomodulin and CapZ. F-actin, filamentous actin derives from the polymerization of monomers of G-actin, globular, which contains the binding sites for myosin. Tropomyosin is a filamentous protein consisting of two super-volatile α-helix domains that primarily regulate myosin binding sites interacting with troponin. Moreover, by binding with actin, it stabilizes the fine filaments, increasing their rigidity and decreasing their fragmentation. Tropomyosin has a fundamental regulatory function for muscle contraction because of its position on the thin filament it physically hides the actin binding sites for myosin. Troponin is a complex protein consisting of three proteins known as troponin C, troponin I and troponin T. The TnC binds Ca2 + with its N-terminal portion and this alters its interaction with TnI, which has an inhibitory effect on Actomyosin ATPase. In the inactivated state, TnI binds to TnC and TnT, and its C-terminal portion is firmly bound to actin. In the activation state, the TnC binds to Ca2 + with consequent reduction of the affinity of the TnI for the actin. The role of TnT is still uncertain, on one hand it seems to bind the TnC - TnI dimers to tropomyosin [18], however, on the other hand it seems to be involved in the regulation of the sensitivity of actomyosin ATPase to Ca2 + [19]. At the center of the sarcomere is located the A-band which contains the thick filaments, formed by myosin and the proteins associated with it, which are interdigitated with the thin filaments. The globular myosin heads cyclically bind to the actin present in the thin filaments

forming transversal bridges, which are then located in the A band, while in the H zone only the myosin tails are found. The M line is made up of proteins that bind together the thick filaments. Finally, a very important role is played by nebulin, a giant non-elastic protein that not only acts as a mold for fine filaments, but also determines their length [20] (Fig.3).

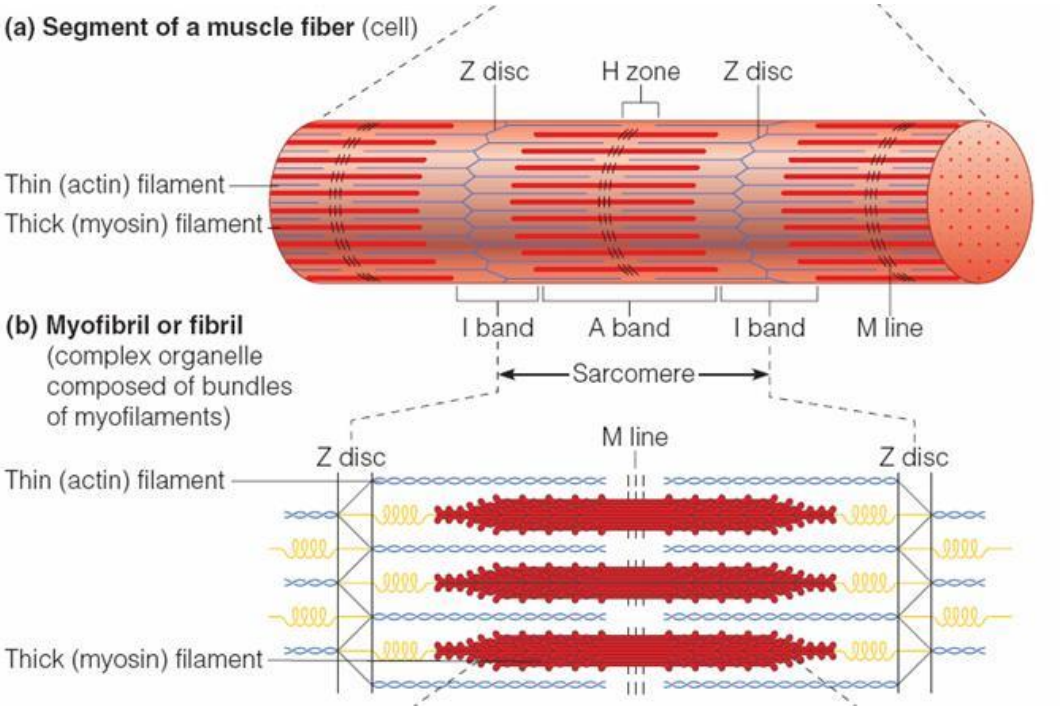


Figure 3: *Structure of a sarcomere.*

The thick filaments consist mainly of myosin II [21], the motor protein of the sarcomere that converts the energy obtained from the hydrolysis of ATP into mechanical work. It consists of two heavy chains (MHC) and four light chains (MLC), two of which are called light regulating chains (RLC) and the other two essential light chains (ELC); two light chains are associated with each heavy chain. From a functional point of view, within myosin, a globular head and an α-helix tail can be distinguished. The N-terminal portion of the heavy chain and the two light chains make up the globular head with catalytic activity that is involved in the formation of cross-bridges or transverse bridges, also known as S-1 fragment [22]. Instead, the C-terminal portion of the two heavy chains constitutes the tail, of which one region contains supervolved domains involved in the polymerization of myosin, while the other, known as the S-2 fragment, connects the myosin heads with the central region of the thick filament. In the myosin head there is both the actin binding site and an ATPasic enzymatic site where the hydrolysis of the ATP energy molecule is linked. In addition to the myosin, inside the filament there is often also the titin, a large protein anchored to

both myosin and Z disc. The titin included in A-band is not extensible and plays a structural role serving as a mold during the assembly of thick filaments. The titin present in the I-band has a PEVK domain, so called because the aminoacids that compose it are proline, glutamic acid, valine and lysine [23] and the N2A segment consisting of four immunoglobulin domains [24]. This portion of titin is attributable to the passive elasticity of the sarcomere, or its ability to maintain the superimposition of the thick and thin filaments when the non-activated myofibrils are elongated or shortened to their resting length [25].

Besides these numerous and important proteins in the skeletal muscle sarcoplasm there are several organelles involved in the muscular contraction mechanism. The transversal T tubules are invocations of the sarcolemma which conduct the nervous action potential within the cell ensuring excitation of the entire fibre [26]. The T tubule forms the so-called *triad* with two cisterns (one on each side) of the sarcoplasmic reticulum and which contain Ca2 + ions. The sarcoplasmic reticulum is in fact the organelle responsible for the accumulation, release and re-uptake of calcium. The action potential that reaches the muscle cell through the alpha motoneurons spreads through the T tubules at the triad level. Here, it stimulates the release of calcium ions by the sarcoplasmic reticulum tanks.

The calcium ions bind to the troponin portion C resulting in a modification of the relationship between the 3 troponin subunits and the tropomyosin molecule displacement unmasking the actin binding sites for myosin and thus making the interaction between the two proteins possible. That is, the process of muscle contraction.

Muscle contraction ends when the calcium ions are reequipped inside the sarcoplasmic reticulum tanks. The implementation of these mechanisms is made possible by two proteins present in the reticulum, the calcium-containing calsequestrin inside the sarcoplasmic reticulum and the SERCA ATPasica pump that allows calcium re-uptake [27]. The cycles of contraction and relaxation are related to phases of release and reuptake of Ca2 + from the sarcoplasmic reticulum and both these events require energy in the form of ATP [28]. ATP is generated by mitochondria which, unlike what was thought in the future, are not separate organelles, but are organized in an ordered array capable of producing the energy required in the presence of oxygen [29]. These organelles are subject to structural and functional changes related to age and physical exercise. For example, with aging, the sarcoplasmic reticulum is fragmented while the number and size of mitochondria increase with aerobic training programs [30].

3. Function

Skeletal muscle supports many functions of the organism, both metabolic and mechanical. As far as metabolic functions are concerned, it represents a reserve of carbohydrates and amino acids, contributes to the basal energy metabolism, and consumes the largest share of energy substrates and oxygen during physical activity. From a mechanical point of view, skeletal muscle, through the conversion of chemical energy into mechanical energy, generates the strength and power necessary for the performance of social, physical and occupational activities [31]. The ability of skeletal muscle to perform these numerous functions is due to the presence of fibres with different contractile and metabolic properties. They have been classified on the basis of multiple criteria: the color of the fibres, red or white, in relation to the myoglobin content, the degree of fatigue, speed of shortening, the speed of release of calcium by the sarcoplasmic reticulum, the prevalence of a glycolytic or oxidative metabolism [32], and finally the expression of certain protein isoforms [33]. All the fibres innervated by a motoneuron form a motor unit and have the same histological, biochemical and contractile characteristics. The predominant classification of the fibres takes into account the presence of isoforms of myosin heavy chains (MHCs) and provides the distinction in fibres of type 1, 2A, 2X and 2B, the latter present however only in small mammals [34].

3.1. Skeletal muscle fibre types

Since the beginning of the studies about the skeletal muscle fibre dynamic properties and types from the pioneering work by Close [35], different classifications have been used. These classifications are based upon physical contractile properties or metabolic and biochemical properties. Fibres have been therefore classified based on the speed of shortening (fast versus slow); colour of the muscle fibres which correlates with the myoglobin content (white versus red), degree of fatigue ability during sustained activation (fatigable versus fatigue-resistant); contractile properties of the motor units in response to electrical stimulation; predominance of specific metabolic pathways or enzymes (oxidative versus glycolytic); calcium handling by the sarcoplasmic reticulum (slow versus fast), protein isoform expression and enzyme-histochemical stain reaction based on ATPase staining techniques or on oxidative enzymes [36] [37]. Understanding the physiological diversities of the different muscle fibres has been recognized as nonetheless fundamental towards the understanding of the pathological changes induced through different disease mechanisms. To this regard and for a better understanding of the history and the current standpoint a comprehensive review has been published by Schiaffino and Reggiani (2011) [38]. For the purpose of our analysis, we will limit to report only the findings relevant to this thesis. One of the first evidences of the heterogeneity of the muscle fibres, through the development of enzyme

histochemical procedures for myosin ATPase led to the discovery of type 2A and type 2B fibre populations, which are abundant in fast-twitch muscles [39] [40]. Further analysis has been able to better determine the biochemical differences of the known muscle fibres and better characterize them. This has been performed analysing muscles composed predominantly by one or another fibre type and it has been found that both 2A and 2B fibres have high levels of glycolytic enzymes, in spite of the different oxidative enzyme complement. This led also to the classification of slow oxidative (type 1), fast-twitch oxidative glycolytic (type 2A) and fast-twitch glycolytic (type 2B) fibres [41]. The next step was the discovery of the so-called type 2X muscle fibres. This occurred thanks to the development of monoclonal antibodies against a third type of Myosin heavy chain (MyHC) [42] [43] [44]. A further step has been made through independent studies with improved electrophoretic procedures that lead to the discovery of a distinct type 2D MyHC band [45]. These two "new" heavy chain isoforms have been confirmed later on thanks to Western Blotting techniques [46]. Motor units composed of type 2X fibres have twitch properties (contraction and halfrelaxation time) similar to those of 2A and 2B units, and their resistance to fatigue is intermediate between that of 2A and 2B units [47]. Most studies regarding shortening velocity have been conducted in murine models. In rats, 2X fibres have an intermediate velocity, placing itself between the 2A and 2B fibres [48] [49]. A spectrum of shortening velocity has been therefore compiled thanks to biochemical and physiological studies of single fibres 1 ↔ 1/2A ↔ 2A ↔ 2A/2X ↔ 2X ↔ 2X/2B ↔ 2B. In human muscles, MyHC-2B is not detectable, although the corresponding MYH4 gene is present in the genome, and fibres typed as 2B based on ATPase staining are in fact 2X fibres based on MyHC composition [50]. It is worth noting that fibre distribution changes significantly according to the body district and individual muscles and compartments. In leg muscles, the most studied muscles of the body, slow type 1 fibres are the most abundant in the posterior compartment (Soleus muscle) and type 2 fibres are more abundant in the forelimbs (or upper limbs in humans) [51]. A comprehensive background of the differences between different mammal's muscles is shown in Fig.4.

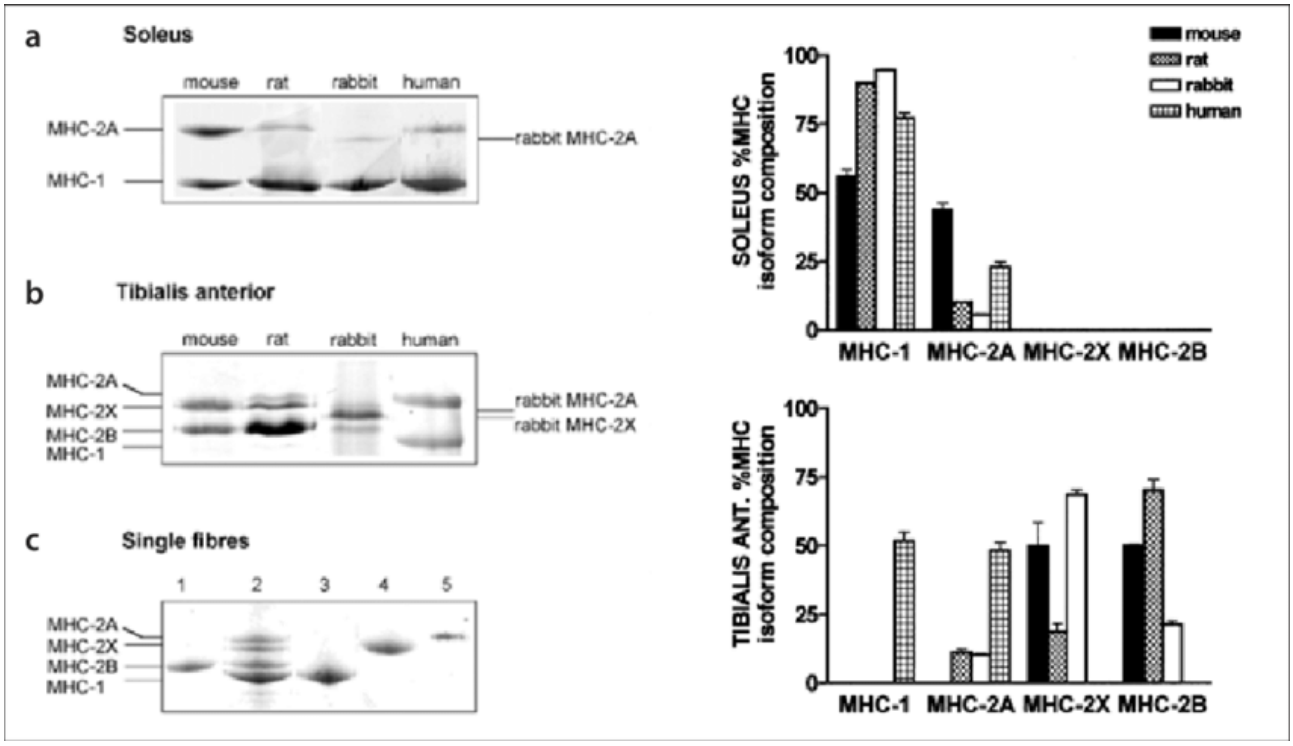


Figure 4. Mammalian myosin heavy chain (MHCs) isoforms separated by polyacrylamide gel electrophoresis (SDS-PAGE). *MHC isoforms content in soleus (a), tibialis anterior (b), and single muscle fibres (c) of rat mouse rabbit and human. (c): lane 1, rat pure fast 2B fibre, lane 3 rabbit pure slow fibre, lane 4 mouse pure fast 2X fibre, lane 5 mouse pure type 2A fibre. Lane 2 shows a mixed rat muscle sample. The histograms on the right report the relative percentage (mean ± S.E.M.) of MHC isoforms of soleus and tibialis anterior muscle of four mammalian species. The figure indicates that: MHC isoforms can be separated by SDS-PAGE; skeletal muscles have variable MHC isoforms distribution. Reproduced from [52].*

3.2. Mechanism of muscle contraction

One of the peculiarities of skeletal muscle is its ability to generate force and movement through excitation-contraction coupling. The initial event that leads to the excitation of a muscle fibre is the transmission of the action potential through the neuromuscular junction, ie the synapse between the motoneuron and the motor unit. The NMJ includes pre and postsynaptic elements, the vesicles at the presynaptic terminal of the motoneuron accumulate in the active zone and contain acetylcholine, which activates the cholinergic receptors located on the postsynaptic muscle membrane [53]. At this point, the action potential is propagated from the sarcolemma to the T tubules, whose membrane contains dependent Na + voltage channels, up to the triad where each tubule T is in contact with the two terminal cisterns of the sarcoplasmic reticulum representing a deposit of calcium.

The depolarization activates the dihydropyridine receptors, DHPR, present on the T tubules, which interact with the ryanidine receptors, RyR, located on the sarcoplasmic reticulum tanks. Activated DHPRs undergo a conformational change that allows the opening of the RyR and the subsequent release of Ca2 + from the sarcoplasmic reticulum to sarcoplasm [54]. In resting conditions, the

concentration of calcium ions in the sarcoplasm is low and tropomyosin sterically blocks the myosin-binding site. When the concentration of Ca2 + increases the ion binds to the TnC, causing the displacement of both the TM, connected to the TnC through the TnT, and the TnI. Both of these events cause the actin-binding site to be exposed and sets the stage for the formation of acto-myosin bridges (Fig.5).

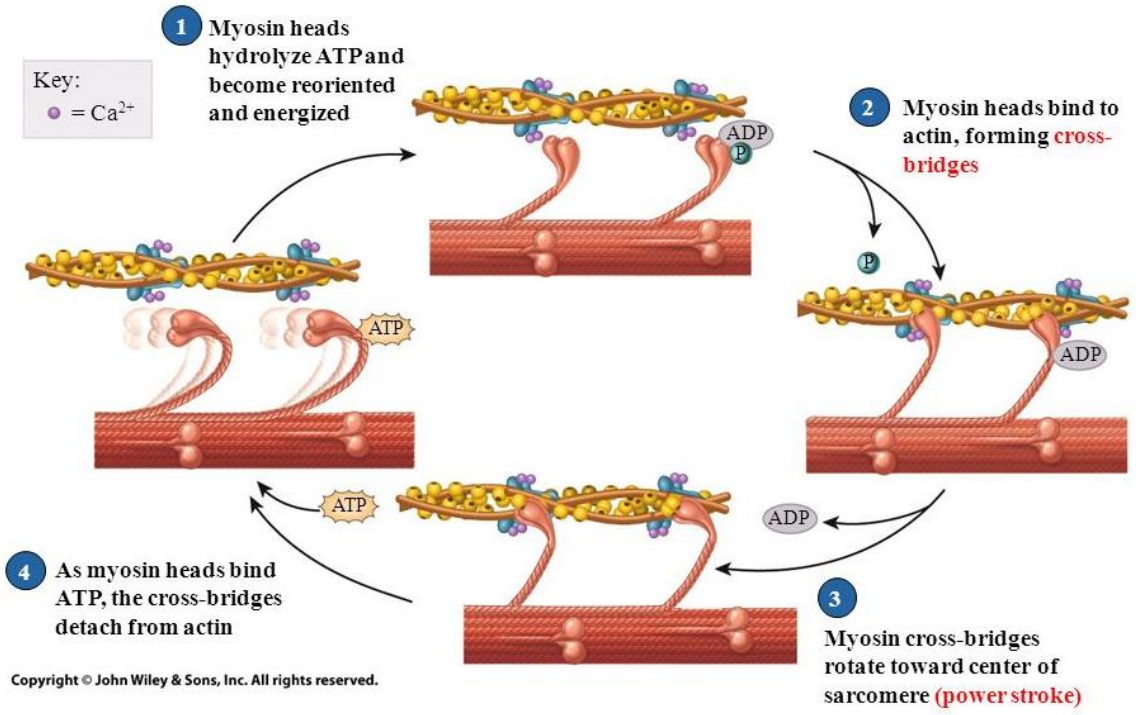


Figure 5: *The Contraction cycle.*

These *cross-bridges* are formed when the myosin head binds to actin causing the thin filament to move toward the midline of the sarcomere and generating force [55]. This mechanism is explained in detail by *Huxley's Theory of Flowing Filaments* proposed in 1954, so called because in this model the filaments of actin and myosin slip one on the other [56]. The molecular events of a contractile cycle are exemplary in six stages. Initially in the state of rigor myosin is linked to actin until an ATP molecule binds to its binding site on the myosin head causing the transverse bridge to break. ATPase in the myosin head catalyses the hydrolysis of ATP to ADP and Pi. At this point, if the binding sites for actin are exposed, myosin can bind to actin. Myosin binds a new actin molecule through a weak link and subsequently release Pi from the ATP site by activating the *working stroke*, which strengthens the binding with the actin and slides the thin filaments onto the thick ones. As a final step, ADP is released and myosin is closely linked to actin in the state of rigor, waiting for a new ATP molecule to be able to break the acto-myosin bond and restart the cycle [57].

The force generated by each transverse bridge is transmitted to the entire fibre and when it reaches the tendons, it generates movement.

3.3. Skeletal muscle energy production and metabolism

The skeletal muscle is a tissue with a complex mechanism of energy production and usage.
Muscle-promoted actions require ATP as a source of energy, but the intracellular reserve of ATP is rather limited and in the case of muscle activation, it is bound to run out in seconds [58]. To compensate for the lack of ATP the cell is able to activate different pathways that depending on the intensity and duration of the activity can be anaerobic or aerobic. Anaerobic metabolism is activated during short duration and high intensity activities, and the two main pathways leading to the fast production of ATP are the degradation of the creatine phosphate and the glycolysis.
There are three main pathways that lead to ATP hydrolysis (Fig.6): Creatine kinase (CK) activity, glycolysis and oxidative phosphorylation. These mechanisms are represented proportionally different between different muscles thanks to the different composition of fast and slow-twitch muscle fibres.

3.3.1. Creatine Kinase Pathway

The creatine kinase pathway uses as a substrate metabolic creatine phosphate (PCr), a high-energy molecule presents mainly in fast fibres. Phosphorylation of ADP by creatine phosphate provides a large amount of ATP at the onset of contractile activity. When the chemical bond between creatine and phosphate is broken, the amount of energy released is the same as when the terminal phosphate is cleaved from ATP. The phosphate and the energy that are in this way released thanks to the enzymatic reaction of the creatine kinase can be transferred to previously formed ADP to form new ATP. The amount of ATP formation is however limited by the initial concentration of creatine phosphate present in the cell. This finding has led to further research. In literature, there are both the evidences that creatine monohydrate is one of the most useful supplements for performance enhancing, especially in short-lived, bursting muscle activity [59] and the evidences suggesting no effect of creatine monohydrate administration on skeletal muscle activity [60]. In human muscles at rest, PCr content is slightly higher in fast than slow fibres [61] [62]; moreover, the total activity of CK is equal in fast and slow fibres [63] or slightly higher in fast fibres [64] but the greater PCr content and the higher activity of CKMM (muscle muscle) suggest that ATP regeneration from PCr is likely more effective in fast than in slow fibres.

3.3.2. Glycolitic Pathway

Similarly, to what happens in the case of phosphocreatine, also in the case of anaerobic glycolysis, the fibres, which are mainly involved, are the fast fibres [65]. The confirmation of these early studies came with the PCR and microarray studies in both human and mouse models [66] [67]. From 1 mol of glucose, 2-3 mol ATP are produced. This anaerobic pathway leads to production of pyruvate, which is converted to lactate by the enzyme lactate dehydrogenase and decarboxylated to acetyl-CoA, which later enters the tricarboxylic acid cycle.

3.3.3. Oxidative Pathway

The greatest energy production takes place in the mitochondria. The two main precursors for ATP resynthesize are pyruvate by pyruvate dehydrogenase enzyme and fatty acids by β-oxidation. Both these pathways lead to production of acetyl-CoA. Contrary to the previous two pathways nominated in this chapter, the oxidative pathway is mainly represented in the slow/oxidative fibres. The first reason for this difference is the substrate availability, namely acetyl-CoA, which is produced in larger quantities in slow fibres [68]. The second reason is the density of mitochondria, which in humans, is highest in slowest fibres and lowest in fastest fibres [69]. Moreover, the ultrastructure of mitochondria is also different in these fibres, presenting more densely packed cristae in the slower fibre types [70]. The last factor marking the difference is the concentration of enzymes and their activity. Citric acid cycle enzymes and the electron transport chain have double capacity in slow twitch muscle fibres compared to fast twitch ones [70]. In slow muscle fibres a particular condition of balance is created between consumption and regeneration. This balance is never achieved in fast twitch.

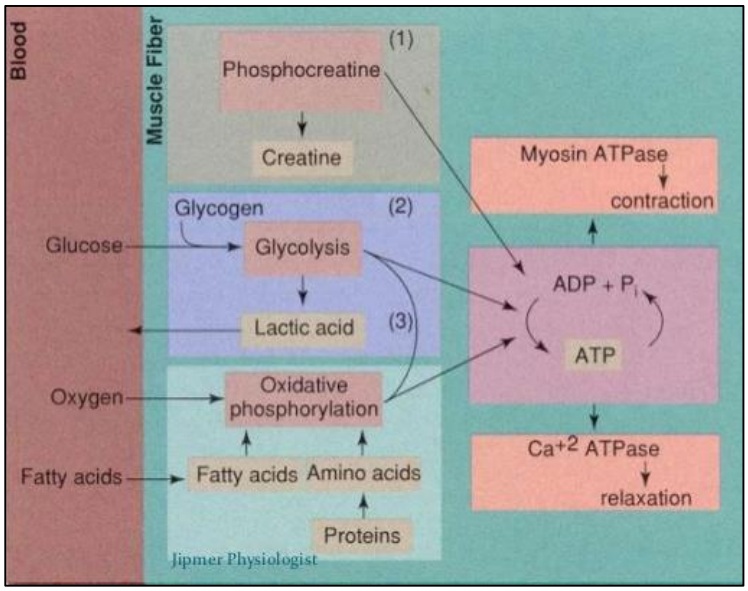


Figure 6: *Sources of ATP for Muscle Contraction.*

CHAPTER 2

MUSCLE PLASTICITY

An important characteristic of skeletal muscle is plasticity, which is the ability to modulate its morpho-functional properties in accordance with the environmental stimuli to which it is subjected [71]. The stimuli able to determine these changes are many, for example disuse, hypoxia, changes in the diet and above all physical exercise [72].

1. Adaptations to exercise

The most studied mechanism capable of stimulating muscle plasticity is physical exercise. The changes, induced by physical activity may be different and vary according to the type of exercise: resistance training or strength training. Resistance training consists of muscle activation at an intensity that increases the ability to sustain a prolonged effort without muscle straining and is typical of running, walking, swimming and cycling. The first changes that occur are the increase in angiogenesis, which allows to increase the oxygen supply and the expansion of the mitochondrial compartment, an increase in the enzymes of the Krebs cycle and the reserves of fatty acids and glycogen, to support therefore of an oxidative type of metabolism both in the slow type fibres (fibres 1) and in those of the fast type (fibres 2) [73]. Strength training, on the other hand, is aimed at increasing the ability of the muscle to develop strength. This result is obtained through the induction of a hypertrophy mechanism related to an increase in protein synthesis. Protein synthesis appears to be associated with the activation and fusion of satellite cells leading to the formation of new myonuclei [74]. Two signalling pathways that regulate muscle mass and hypertrophy have been identified [75]. The first pathway is the insulin-like growth factor IGF-1, which binds to its tyrosine-kinase receptor IGF1R found on the sarcolemma, which leads to the activation of the *Insuline Receptor Substrate*, IRS1. IRS1 activates the phosphoinositide-3-kinase that activates the Akt protein, which is responsible for the activation of mTOR, which regulates factors involved in the control of protein synthesis [76]. The second crucial pathway is that of *Myostatin* which acts as a negative regulator of muscle growth [77]. At the skeletal muscle level, *Myostatin* negatively regulates the Akt pathway and interferes with myoblast differentiation [78]. Furthermore, mutations in the *Myostatin* gene or blockade of this signalling pathway lead to muscular hypertrophy. To stimulate muscle hypertrophy, it is important to implement exercise with an adequate diet, and a recent meta-analysis has shown that a protein diet can lead to an increase in muscle mass [79]. Another important consideration concerns the integration with branched chain amino acids, such as leucine, isoleucine and valine, which are able to activate the mTOR signalling pathway and protein synthesis [80]. Despite the innumerable researches carried out, not all the factors involved in the regulation of muscle mass have been discovered, nor the most effective strategies to counteract the effects related to the loss of muscle mass, have been identified. These are critical issues because the

reduction of muscle mass is a negative prognostic factor in terms of survival as it is inevitably associated with a reduced functionality that limits daily activities [81]. It is also crucial to understand if the same signalling pathways are involved in different pathologies and if the same strategies can be applied to avoid mass loss and improve the quality of life. Despite the fact that there is still a lot to discover, routes have been identified of signalling involved in both atrophy and muscular hypertrophy. The aforementioned *IGF1* regulates muscle growth by increasing protein synthesis and by binding to its receptor, IGFR, and the active IRS1 Akt. Akt activates the mTOR 1 complex (mTORC1) that controls protein synthesis by phosphorylation of the S6 kinase (S6K) and the binding protein of eIF4E (4E-BP). The mTOR complex 2 (mTORC2), on the other hand, contributes to the activation of Akt. Akt inhibits glycogeno syntasinase 3b (GSK3b) and removes inhibition on eIF2B, promoting protein synthesis. In addition, Akt inhibits Forkhead box O (FoXO) and decreases the expression of MAFbx and *MuRF1* whose targets are eIF3-f and myosin chains, respectively. Recently it has been discovered Fbxo40 able to ubiquitinate IRS1 following stimulation of IGF1. *Myostatin* instead acts negatively on muscle mass, through the activation of Smad2 and Smad3, which inhibit Akt [76] (Fig. 7).

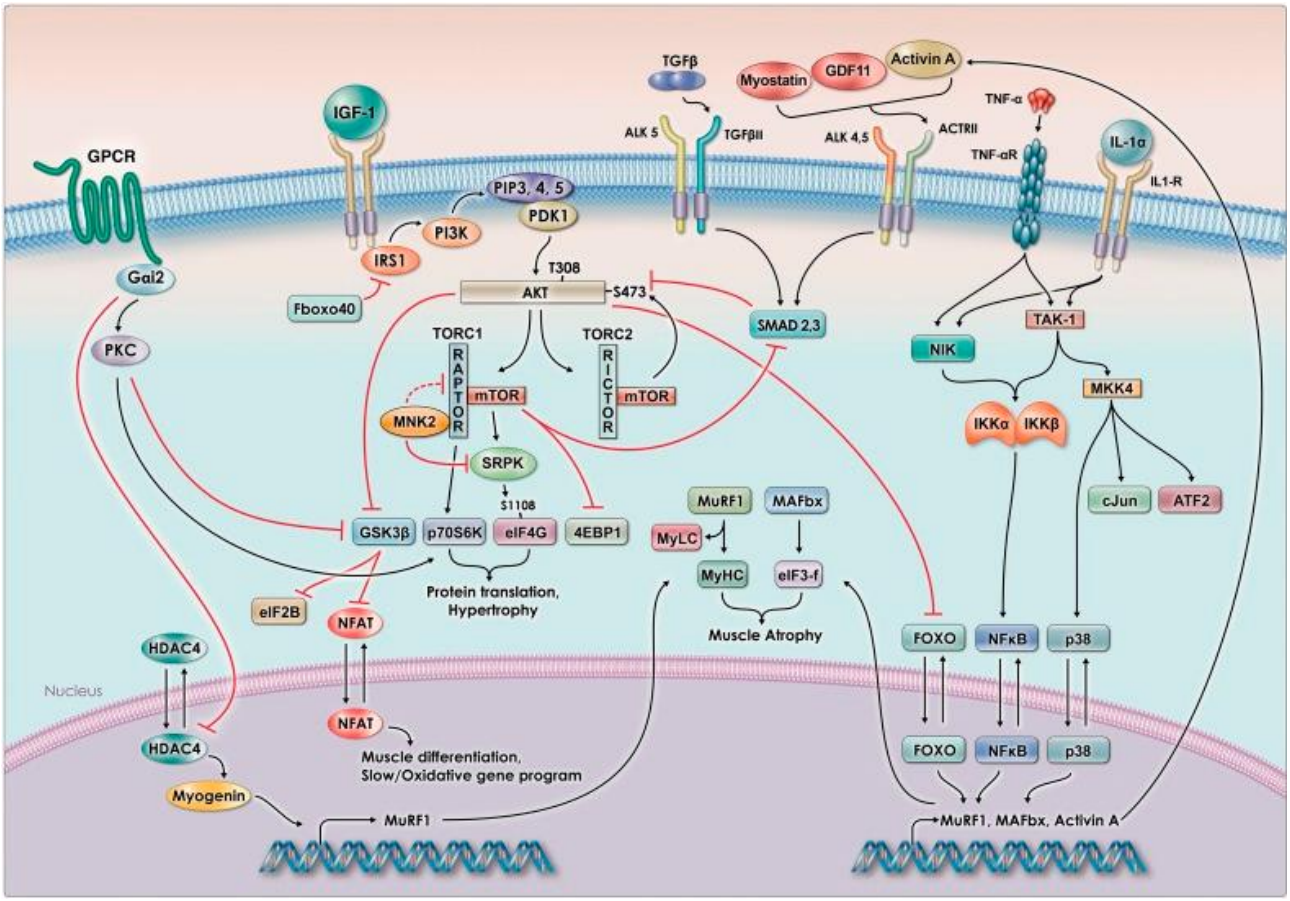


Figure 7: Signalling pathways involved in atrophy and hypertrophy [75]. *Signalling activated by insulin-like growth factor1 (IGF1) positively regulates muscle mass, primarily via induction of protein synthesis, downstream of Akt and mTOR. The myostatin/GDF11/activing pathway negatively regulates muscle size, as a result of the phosphorylation of SMAD2/3 – primarily by inhibiting Akt. IGF1 acts via the IGF receptor (IGFR), and the insulin receptor substrate 1 (IRS1), – activating Akt. Akt activates mTOR complex 1 (mTORC1). mTORC1 is a multiprotein complex that requires the protein raptor for its function and is acutely inhibited by FKBP/rapamycin. mTORC1 controls protein synthesis by phosphorylating S6 kinase 1 (S6K) and eIF4E-binding protein (4E-BP). The multiprotein complex mTORC2 includes the protein rictor and contributes to the activation of Akt. Downstream targets of Akt include glycogen synthase kinase 3b (GSK3b) and Forkhead box O (FOXO) transcription factors. Inhibition of GSK3b by Akt relieves inhibition onto the initiation factor eIF2B, and thereby increases protein synthesis. Activation of Akt also inhibits FOXO and decreases expression of the E3 ubiquitin ligases Muscle Atrophy Fbox (MAFbx) and Muscle Ring Finger1 (MuRF1). Substrates of MAFbx and MuRF1 are the initiation factor eIF3-f and myosin chains, respectively. Another more recently discovered E3 ligase is Fbxo40, which can ubiquitinate IRS1 upon IGF1 stimulation, short-circuiting this pathway unless the muscle is capable of synthesizing new IGF1, via maintenance of TORC1/protein synthesis signalling. To induce hypertrophy, in addition to the classical IGF-1/Akt pathway, more recently the Galpha-i2 pathway has been shown to induce hypertrophy via PKC, bypassing Akt. In addition to the PKC pathway downstream of Galpha-i2, there is a PKC-independent pathway which involves inhibition of HDAC4.The myostatin/TGFb pathway acts via several receptors and results in the activation of Smad 2,3. Activation of Smad proteins inhibits the function of Akt and the expression of MAFbx and MuRF1 by FOXO transcription factors. The function of Smad 2,3 is also inhibited by mTORC1.*

1.1. Protein Synthesis signalling pathway

Several stimuli, such as insulin, nutrients and various growth and survival factors trigger a signalling cascade dependent on sequential activation of PI3K, Akt/PKB and mTOR/FRAP kinases (Fig. 8) and culminate in skeletal muscle fibre enlargement [82] [83].

Akt is activated by phosphorylation within the C-terminus at Ser473 and within the activation loop at Thr308 by phospholipid-dependent kinases. Direct phosphorylation of mTOR/FRAP at Ser2448 by Akt is a key regulatory event controlling its kinase activity, leading to inactivation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1), an inhibitor of translation initiation, and to activation of p70 S6 kinases which in turn phosphorylates the 40S ribosomal subunit protein S6 and stimulates the translation of 5' oligopyrimidine tract containing mRNAs.

The inhibition of mTOR signalling results in a reduction in the initiation phase of mRNA translation with downregulation of protein synthesis. mTOR consists of two complexes: mTORC1 and mTORC2. mTORC1 and mTORC2 trigger distinct pathways that lead, respectively, to increased protein synthesis and to inhibited protein degradation.

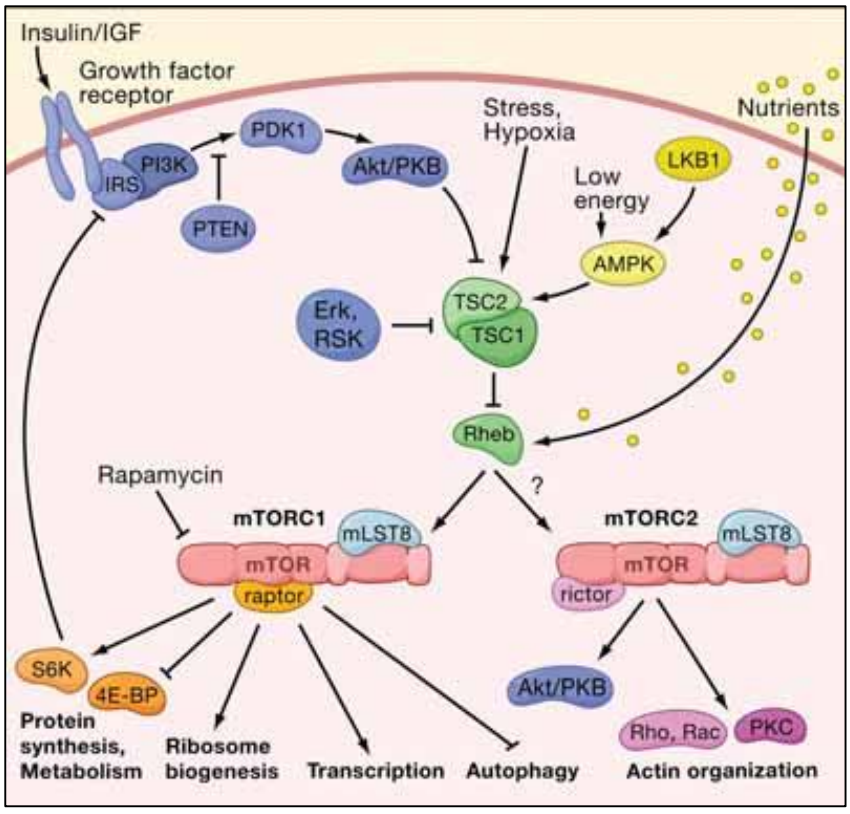


Figure 8: PI3K/Akt pathways. *A schematic representation of mammalian target of rapamycin (mTOR) signaling pathway for the regulation of skeletal muscle protein synthesis in response to growth factors, nutrition and stress.*

2. Atrophy

Muscle atrophy, conversely to muscle hypertrophy, is a decrease in muscle fibre size. The reason for this is an imbalance between the processes that lead to protein synthesis and the processes that lead to muscle breakdown [84]. Muscles are large reservoirs of amino acids that can be used for the body's metabolism and the maintenance of body functions during catabolic periods such as sepsis, burns, heart failure, cancer, disuse, aging, glucocorticoid use etc. [85]. Therefore, it has been well recognised that maintenance of skeletal muscle mass, through different strategies but mainly strength training, plays an important role in health promotion and disease prevention [86]. Many pathological conditions characterised by muscle atrophy (sepsis, cachexia, starvation, metabolic acidosis, severe insulinopenia, etc.) are associated with an increase in glucocorticoids levels [87], suggesting that these hormones could trigger the muscle atrophy observed in these situations. In contrast, glucocorticoids do not appear to be required for disuse atrophy [88], but may clearly exacerbate the deleterious effects of disuse on skeletal muscle mass [89].

For all the previously mentioned evidence, further research is needed in order to understand the cellular and molecular pathways involved with atrophy, and much indeed has been done [90] [91].

2.1. Atrophy-inducing pathways

The main mechanisms leading to cellular protein degradation are: the ubiquitin proteasome system [90], autophagy [85] and oxidative stress [92].

2.2. Ubiquitin-Proteasome Degradation Pathway

The Ubiquitin Proteasome system is the main mechanism responsible for protein degradation and seems reasonably correlated with the onset of muscle atrophy. Initially ubiquitin (Ub) forms a covalent bond with the target protein to be degraded and in this way, it is recognized by the proteasome 26S, a multicatalytic complex that degrades ubiquitinated proteins into small peptides [93]. A fundamental role is played by the enzymes E1, which activates the Ub, E2, the protein carrying the Ub, and above all E3, the protein ligase. While E1 and E2 prepare the Ub for conjugation, E3 recognizes the protein to be degraded and catalyses the transfer of the activated Ub from E2 to the target protein [94] (Fig.9).

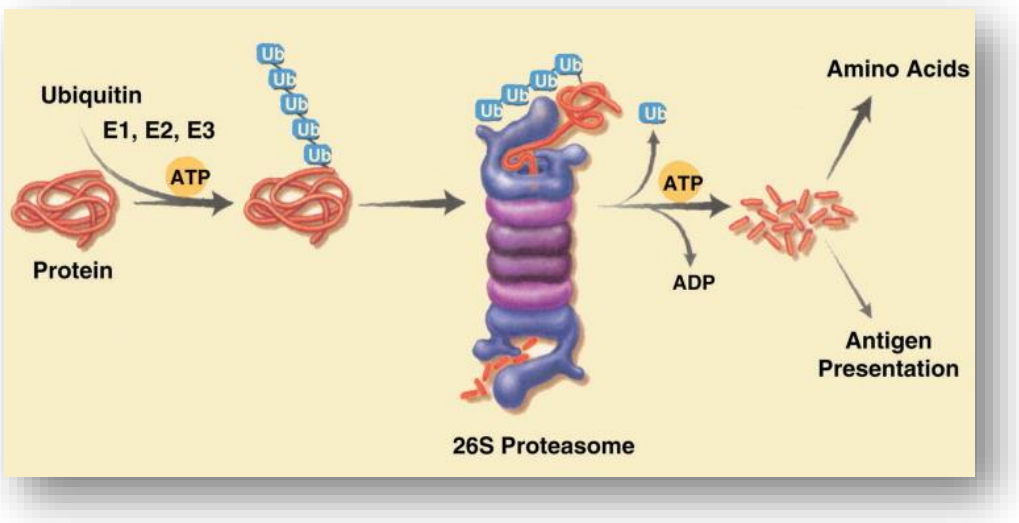


Figure 9: The Ubiquitin Proteasome System. *Ubiquitin is activated by the ubiquitin-activating enzyme (E1) and then transferred to an ubiquitin-conjugating enzyme (E2). E2 transfers the activated ubiquitin moieties to the protein substrate that is bound specifically to a particular ubiquitin ligase (E3). The transfer of ubiquitin takes place either directly (in the case of RING finger ligases) or via an additional thiolester intermediate on the ligase (in the case of HECT domain ligases). Repeated conjugation of ubiquitin moieties to each other generates a polyubiquitin chain that serves as the binding and degradation signal for the 26S proteasome. The protein substrate is degraded, generating short peptides and free ubiquitin that can be further reused. Ub, ubiquitin. From Nader Rahimi – Molecular Cancer Therapeutics [95].*

The human genome encodes more than 650 ubiquitin ligases that are involved in the regulation of numerous cellular processes, such as cell cycle, metabolism, oncogenesis and muscle mass [96]. Only some of these ligases are muscle specific and are upregulated in muscle loss [97]. Their identification dates back to 2001 when two independent research groups, led by Alfred L. Goldberg and David J. Glass, through the comparison of gene expression profiles in different experimental models of muscle atrophy (diabetes, cancer, denervation ...), identified a group of genes that were dysregulated in each of the models used. The genes that were mainly overexpressed are those that code for two specific muscle ubiquitin ligases, ie MAFbx or *Atrogin1* and *MuRF1* [98] [99]. *Atrogin1* is responsible for the degradation of MyoD, a key transcription factor for myogenesis [100], and of eIF3f, activator of protein synthesis [101]. *MuRF1* controls the half-life of important muscle structural proteins, such as myosin light and heavy chains, and troponin I [102] [103] [104].

2.3. Transcriptional regulators of MAFbx and MuRF1

Transcriptional regulation of MAFbx and *MuRF1* in mice occurs via a large family of conserved DNA binding transcription factors that regulate many processes among which: metabolism, cellular proliferation, differentiation, apoptosis, and longevity and stress tolerance [105]. These regulators are part of the Forkhead box containing protein, O-subclass (FoXO) family of proteins. [106]. The transcription activity of these proteins is largely controlled by shuttling between the cytoplasm and the nucleus where FoXOs can bind specific DNA sequences. [107] Upregulation and therefore increased expression at the nuclear level of *FoXO1* member of this family is associated with skeletal muscle mass loss [108]. Moreover another member of this family, namely *FoXO3*, seems to be sufficient for a significant reduction in muscle mass [109] [106] and overexpressed *FoXO3* is linked to upregulation of MAFbx and *MuRF1* promotor activities and mRNA expression. It is interesting to note that the negative regulation of the positive regulation for atrophic processes seems to be performed by a positive regulator of protein synthesis because FoXO transcription factors seem to be rendered inactive following phosphorylation thanks to Akt (an important initiator of protein synthesis) which results in the removal of the transcription factors from the nucleus where they are sequestered in the cytoplasm [110].

MuRF1 and MAFbx transcriptional regulation is dependent also on H2O2 and local inflammation mediators, in particular TNF-alpha, but also linked to the NF-kB pathway of inflammation itself [111] [112]. Therefore, numerous pathways can influence the activity of the two main atrogins. Consequently, they also act on different pathways with MAFbx being involved more with blocking protein synthesis and *MuRF1* with enhancing protein degradation (Fig 10) [113]. Among the most notable effects, we can cite the binding and degradation of myosin heavy chain (MHC) following treatment with Dexamethasone, degradation of myosin-binding protein C and myosin light chain-1 and 2 during denervation and fasting conditions [114].

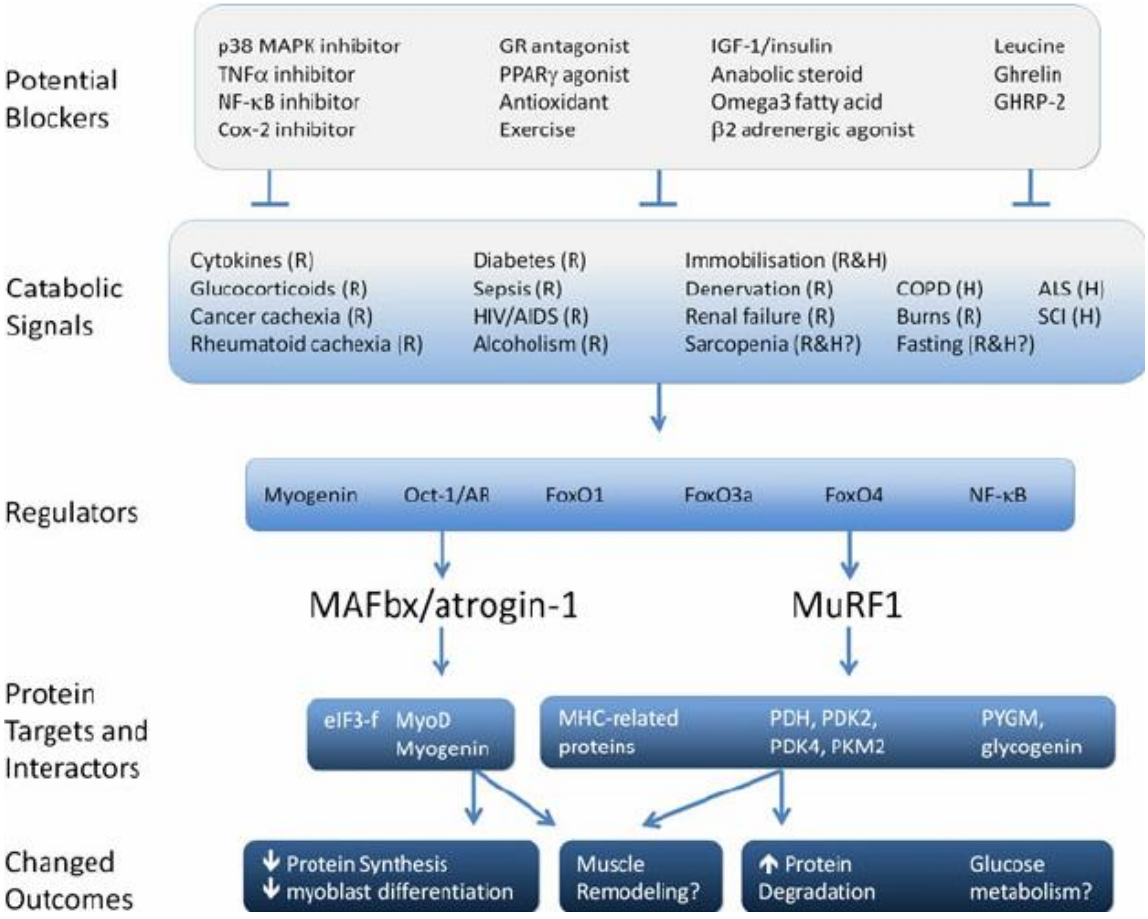


Figure 10: *Catabolic signalling pathways involved in MAFbx/Atrogin1 and MuRF1 regulation and proposed physiological outcomes in skeletal muscle (rodent, R; human, H). A range of diseases, conditions and injuries function as catabolic signals to regulate MAFbx/Atrogin1 and/or MuRF1 gene expression potentially via several FoxO, myogenin, NF-κB and Oct1/androgen receptor (AR) transcription factors. Numerous inhibitors have had varied success in their ability to block MAFbx/Atrogin1 and/or MuRF1 expression and skeletal muscle atrophy. Protein substrates of MAFbx/Atrogin1 include eIF3-f, MyoD and myogenin. MuRF1 may target MHC related proteins to affect overall protein degradation and atrophy while it is not certain as yet whether other interactors of MuRF1 are degraded to affect glucose metabolism. Together, MAFbx/Atrogin1 and MuRF1 are increasingly being implicated in muscle remodelling [image from (Foletta et al. 2011)] [113].*

2.4. Role of autophagy in skeletal muscle atrophy

Autophagy plays a fundamental role in the replacement of cellular components both in physiological conditions and in response to various damaging stimuli [115]. Three different autophagy processes, known as macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), have been described, and most autophagy processes in the muscle are macroautophagic processes [116]. The two final steps of the autophagy process, the destruction and recycling of degradation products, take place in lysosomes whatever the type of autophagy is in progress. What differentiates them is both the type of degraded product and the way in which it is

transported in the lysosomes [117]. Macro-opa-phage degraded soluble receptor and adaptive proteins, sequestered within autophagosomes, ie vesicles with a double membrane, are degraded, that blend directly with lysosomes [118]. With microautophagism, the cytosolic material is incorporated through the invaginations that form directly on the lysosome membrane [119]. CMA does not involve the formation of vesicles, but the material to be degraded, linked to a molecular chaperone, crosses the lysosomal membrane by means of a translocation complex [120]. Because of this particular mechanism, only the proteins can be transported through the CMA, while through the process of macroautophagia and microautophagia it is possible to ferry proteins, lipids, glycogen, organelles and pathogens [121]. More than 31 genes associated with autophagy (ATG) have been identified that encode the Atg proteins involved in the different phases of the autophagy process. The formation of the autophagosome (Fig. 11), the vesicle consisting of a double membrane and responsible for transporting the material towards the lysosome, is regulated by a protein complex containing the phosphatidylinositol-3-kinase (PI3K) of type III and Atg6 also known as Beclin-1 [122]. The Atg8 protein, known as LC3, plays a crucial role as regards the interaction between the autophagic and the ubiquitin system and the removal of protein aggregates is due to LC3, which on one hand interacts with *p62* and NBR1 receptors and other alloy of specific ubiquitin proteins [123]. It has been shown that the selectivity of degradation processes depends on the number of ubiquitin molecules linked to the target protein; in particular, the polyubiquitination is associated to the UPS pathway, while the monoubiquitination is associated to the autophagy one. In addition, the *p62* receptor plays a primary role by binding to the ubiquitinated protein complexes and facilitates degradation by autophagy [124]. The involvement of autophagy in the atrophic process was discovered in the 1970s when some experimental evidence suggested that lysosomal degradation contributed to protein degradation in denervated muscle [125]. The autophagic process (Fig. 11) is activated in different catabolic conditions, such as fasting, denervation, aging, disuse and critical pathologies, and thus leads to muscular atrophy. For example, during fasting periods, autophagy occurs in particular against fast fibres with a glycolytic metabolism [126]. It would be a mistake, however, to consider autophagy as an exclusively damaging process for skeletal muscle. In fact, besides regulating cellular homeostasis, it has been shown that the deletion of some ATG genes contributes to fibre degeneration and loss of muscle mass characterized by the accumulation of abnormal inclusions and mitochondria [127].

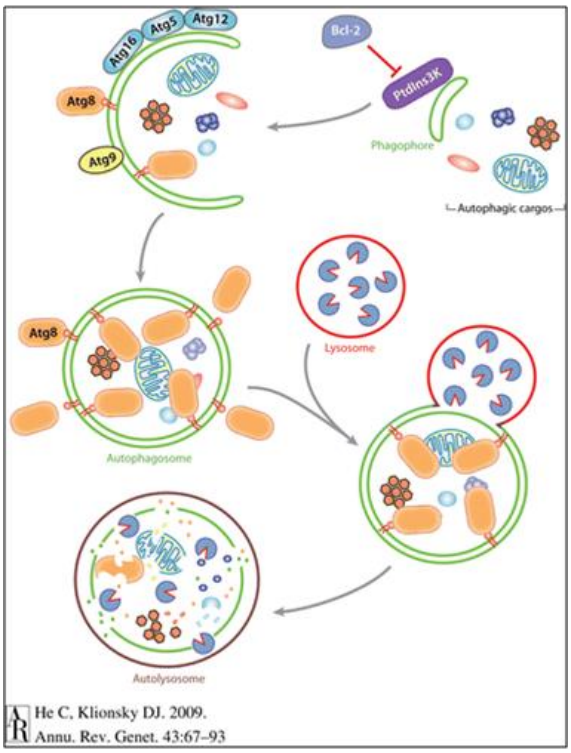


Figure 11: Schematic model of autophagy. *The class III PtdIns3K complex mediates nucleation of the phagophore membrane, enwrapping cytosolic proteins, protein aggregates, and organelles (such as mitochondria). Bcl-2 blocks this step by binding and inhibiting Beclin 1, a component in the PtdIns3K complex. Atg12–Atg5- Atg16 and Atg8–PE conjugates (LC3-II in mammalians cells) are recruited to the phagophore, together with the transmembrane protein Atg9, facilitating the phagophore expansion step. Upon vesicle completion, most of the Atg proteins are dissociated from the autophagosome, allowing autophagosome-lysosome fusion and cargo degradation by lysosomal proteases [image from (He & Klionsky, 2009)] [128].*

2.5. Role of mitochondria in muscle mass maintenance

Alterations in the number and morphology of the mitochondria can have deleterious consequences in the maintenance of muscle mass, also because these organelles communicate with the myonuclei to help the adaptation of the muscle to the physiological and pathological conditions [129]. Mitochondrial DNA (mtDNA) encodes only 1% of the proteins of these organelles, while the remaining 99% derives from nuclear DNA, so most of the proteins must be transported within the mitochondria [130]. This step is regulated by mitoproteases, or specific proteases that rapidly degrade incorrectly bent or oxidized proteins, playing a preventive role against mitochondrial damage [131]. The mitochondria are endowed with remarkable plasticity; in fact, they are able to modify shape, size and distribution alternating melting events, in which these organelles are elongated and interconnected, and fission, which foresees the rupture of the mitochondrial network [132]. Fusion facilitates the distribution of metabolites, proteins and mtDNA, allows optimal energy management and greater resistance to oxidative stress. Moreover, the fusion between healthy and

damaged mitochondria allows maintaining the global functionality of these organelles and avoids the accumulation of non-functioning material [133]. This process begins with the link between the mitochondria, continues with the fusion of the outer membranes, and ends with the fusion of the internal membranes. Mitofusin 1 (*MFN1*) and Mitofusin 2 (*MFN2*) are localised in the outer membrane and promote both the binding of adjacent mitochondria and fusion of external membranes [134]. When *MFN2* is phosphorylated by PTEN-induced kinase (PINK1), a protein that activates mitochondria autophagy (mitophagia), is bound by the ubiquitin ligase Parkin and this causes a blockage of the mitochondrial fusion process [135]. Another protein involved in fusion is the optical atrophy 1 protein (*OPA1*) that regulates the fusion of internal membranes by controlling ridge remodelling and assembly of electron transport chains in supercomplexes that increase mitochondrial respiration [136].

Fission, on the other hand, is a process that allows separating the damaged components of the mitochondrial network, making it possible to remove them through mitophagy [137]. However, excessive fission leads to the formation of isolated mitochondria that are less efficient in the production of ATP and are dysfunctional because they consume ATP produced only to maintain their membrane potential [138]. This mechanism is driven by Fis-1 and related protein dinamine 1 (*DRP1*), that interacts with external membrane components that function as receptors, since *DRP1* does not have hydrophobic binding domains [139]. It is interesting to note that, like the fusion promoting proteins, *DRP1* is also a substrate for the Parkin protein, which promotes its degradation through the UPS [140] (Fig.12).

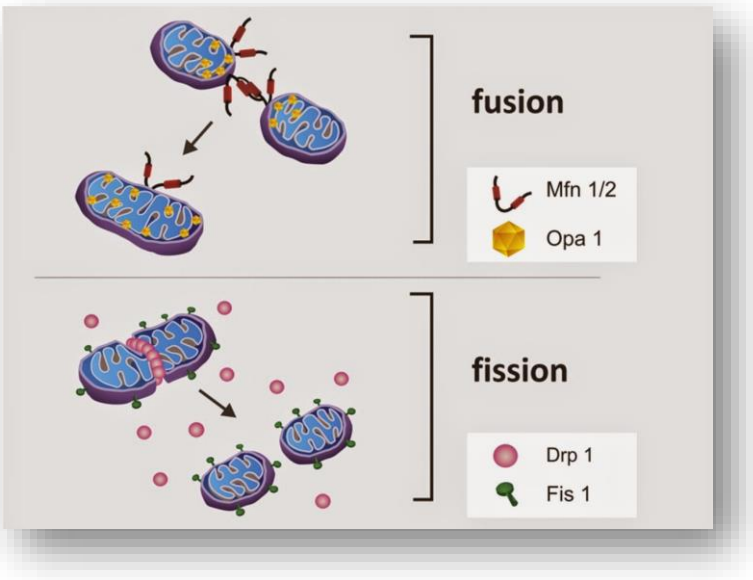


Figure 12: *Schematic representation of the mitochondrial fusion and fission with the respective proteins acting in the two processes.*

When the mitochondria suffer irreversible damage they undergo fission and are subsequently degraded by a specific autophagic pathway called mitophagia, which eliminates the damaged organelles, is also essential for the maintenance of mitochondrial turnover [141]. The mitofagia is controlled by the Bnip3L / Nix, Bnip3, Parkin and PINK1 proteins, and loss-of-function mutations of the latter proteins have been found in some inherited recessive forms of Parkinson's disease as evidence of how important this mechanism is. In physiological conditions PINK1 enters the inner mitochondrial membrane via a dependent voltage mechanism, is cleaved by the PARL, a protein located on the mitochondrial membrane, which plays a crucial role in the control of the integrity and mitochondrial function [142]. The resulting peptides are degraded through the ubiquitin-proteasome system [143] and then in the functioning mitochondria the PINK1 levels are very low. If, however, the mitochondrial membrane potential is lost, PINK1 accumulates in the outer membrane and phosphorylates both the ubiquitinate proteins of the mitochondrial outer membrane (OMM) and Parkin [144]. Parkin in turn promotes the addition of new ubiquitin chains to the OMM proteins that will be phosphorylated by PINK1. At this point, the phosphorylated ubiquitins are linked by the optineurin, NDP52 and *p62* adaptive proteins that in turn label the OMM proteins with LC3 to activate the mitophagic process [145]. Bnip3 and Bnip3L contain highly conserved domains that bind LC3 and GABARAP, a protein involved in the maturation of autophagosome, therefore they act as receptors for autophagy that bring mitochondria closer to the autophagosome. The plasticity that distinguishes the muscle fibres depends largely on the dynamics of the mitochondria [146] and in particular, the breakdown of the balance between fusion and fission is related to the altered mitochondrial degradation typical of the atrophic muscle [147]. Fission is referred to as one of the processes that causes atrophy in aging or other systemic diseases, while fusion represents a defence mechanism against DNA mutations [148]. Experimental evidence shows that the genes of autophagy and mitochondrial fragmentation may depend on the activation of AMPK (AMP-activated kinase) [149]. AMPK is activated when the AMP / ATP ratio increases and causes an increase in protein degradation helping the expression of the *Atrogin1* and *MuRF1* genes [150]. Critical for mitochondrial alteration is the binomial AMPK / FoxO3, where FoxO3 binds *Atrogin1* and *MuRF1*. The coactivator 1 of the peroxisome gamma proliferator (*PGC-1α*), a protein that binds to transcription factors and alters chromatin to promote gene expression, balances the action of FoxO3. *PGC-1α* promotes the expression of genes involved in mitochondrial biogenesis and oxidative metabolism such as gluconeogenesis and β-oxidation of fatty acids [151]. This cofactor has been shown to play a role in maintaining muscle mass as the values of *PGC-1α* decrease in conditions associated with muscular atrophy [152] [153]. Although *PGC-1α* is a

transcription activator, it is actually able to block protein degradation by inhibiting FoxO3 and decreasing the expression of *Atrogin1* and *MuRF1* [154]. *PGC-1α* has been described as a master regulator of oxidative metabolism that controls metabolic and mitochondrial gene expression [155] with consequent improvement in the oxidative capacity, which also implies a greater ROS production. In addition, *PGC-1α* regulates the expression of several antioxidant enzymes such as superoxide dismutase 1 and 2 (SOD1 and SOD2) and glutathione peroxidase [156] [157].

2.6. Role of oxidative stress: reactive oxygen species' influence on muscle mass

The term oxidative stress refers to the damaging effect induced by reactive oxygen species (ROS). Oxidative stress is a type of free radicals, ie chemical species characterized by the presence of one or more electrons unpaired in the orbitals and therefore they are highly reactive [158]. The most common ROS are hydrogen peroxide (H2O2), superoxide anion (O2-), hydroxyl radical (• OH), and are physiological products of oxidative metabolism that occurs in the mitochondria, during which some electrons escape from the chain of transport [159]. Mitochondria are a production site for lipid precursors, nucleic acids and amino acids, and ROS can oxidize them causing mitochondrial dysfunction [160]. Moreover, mitochondria have positive effects, for example, they are responsible for the defense against infectious agents and for the regulation of cellular signalling pathways [161]. Some experimental evidence has shown that the production of ROS influences the expression of PGC1α in some models of atrophy. In fact, *PGC-1α* is decreased in disuse atrophy, denervation, aging and diabetes, however it remains unclear whether there is a correlation between glucocorticoid-induced atrophy and *PGC-1α*-mediated oxidative stress [162] [163].

2.7. Antioxidant enzyme

Antioxidant enzymes are key elements that help maintain the redox homeostasis, thus preventing excessive harmful effects at cellular level. There are three main antioxidant enzymes: superoxide dismutase, catalase and glutathione peroxidase. There are some minor enzymes involved with maintenance of redox balance; however they are beyond our analytical interest, and therefore will not be discussed further here. Superoxide dismutase (SOD) transforms superoxide to hydrogen peroxide and oxygen. There are three isoforms of this enzyme with different locations. Of particular interest for antioxidant enzyme, seems to be SOD1, which is mainly found in the cytosol, which is the site of skeletal muscle where antioxidation capacity mostly occurs [164]. Catalase is an enzyme that breaks down hydrogen peroxide to water and oxygen and this enzyme is found mainly in muscle fibres with high oxidative capacity [165]. The other main enzyme, glutathione peroxidase is

actually made up by a family of enzymes with peroxidase activity, whose substrate is mainly hydrogen peroxide.

2.8. Role of NRF2 in oxidative stress

The nuclear factor erythroid 2 – related factor (*NRF2*) is a transcription factor related to the transcriptional activation of antioxidant genes. When *NRF2* is activated (Fig 13), it mediates and induces the expression of an array of enzymes and signalling proteins to regulate drug metabolism, antioxidant defence, and oxidant signalling, thereby influencing oxidant physiology and pathology. By regulating oxidant levels and oxidant signalling, *NRF2* participates in the control of several programmed functions, such as autophagy, inflammasome signalling, UPR (unfolded protein response), apoptosis, mitochondrial biogenesis, and stem cell regulation [166].

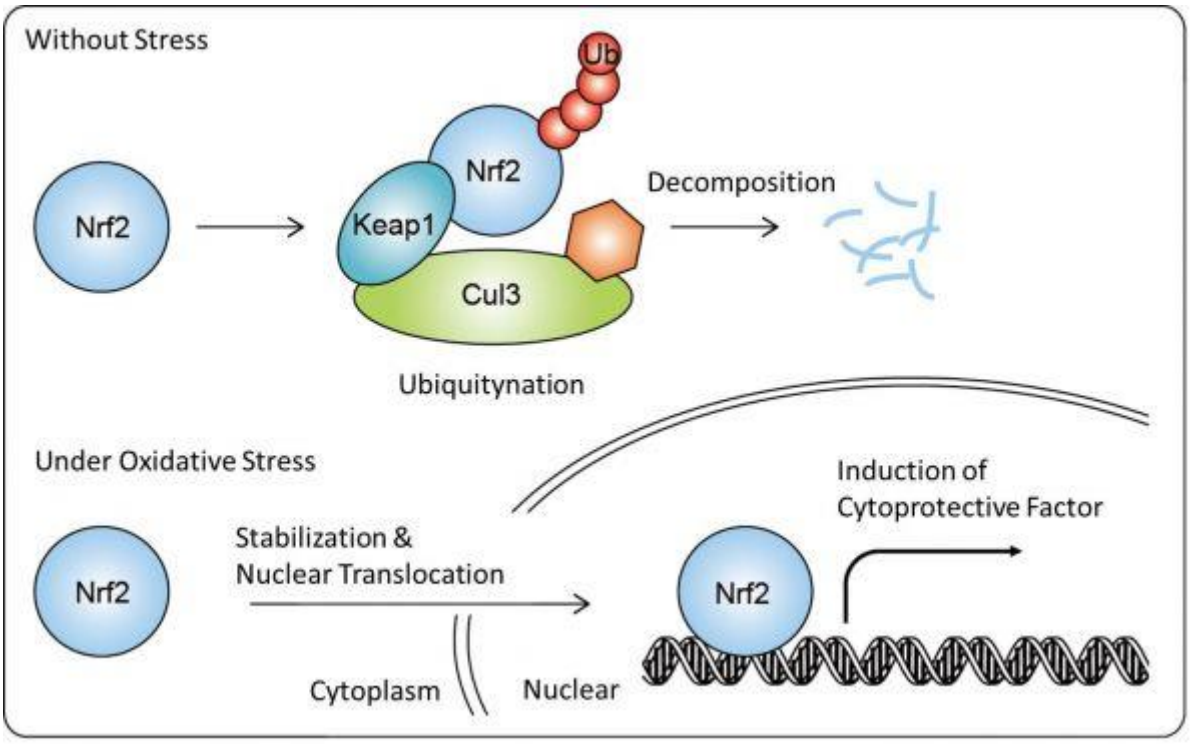


Figure 13: A diagrammatic representation of Nrf2 activation by oxidative stress. *The acrivation of Nrf2 can lead to proteasomal degradation. However, reactive oxygen species cause a change the dissociation of Nrf2. The free form of Nrf2 translocates to the nucleus where it interacts with the antioxidant response element (ARE) to increase the expression of many antioxidant and detoxifying enzymes. Ub: ubiquitin; Keap1: Kelch-like ECH-associated protein 1; Cul3: Cullin3.*

CHAPTER 3

GLUCOCORTICOIDS AND STEROID MYOPATHY

1. Structure of adrenal steroids

One of the major roles of the adrenal gland is the synthesis and secretion of steroids. All steroids are synthesized from the precursor cholesterol, and share a common basic structure; three cyclohexane rings fused to a cyclopentane ring (Fig. 14). The chemical properties of these molecules are dependent upon the number of carbons atoms and side groups bonded to the basic four rings structure. There are 5 main groups of steroid produced by the adrenal gland, identified by the number of carbon atoms they contain. For example, androgens and progestogens both have 19 carbons; estrogens have 18 carbons; mineralocorticoids and glucocorticoids (GCs) have 21 carbons [167].

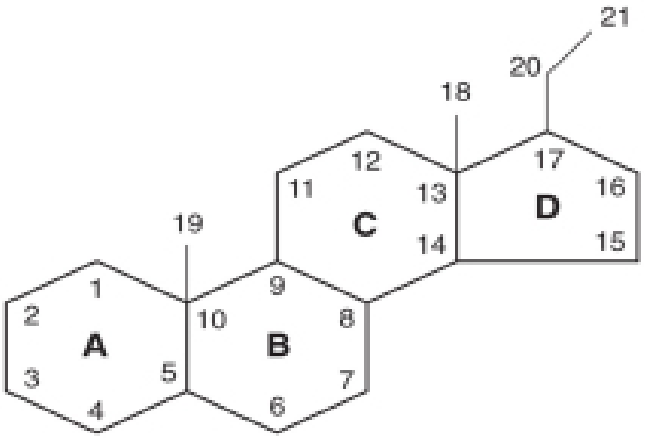


Figure 14: *Standard structure and nomenclature of adrenal steroids. The numbers designate the carbon atoms and letters designate the rings.*

2. Steroidogenesis

All adrenal steroids are synthetized from the precursor cholesterol in the cortex. The predominant source of cholesterol is through the uptake of low-density lipoproteins (LDL) from the circulation. Adrenal tissue expresses specific cell surface receptors that bind and internalise circulating LDL, by receptor-mediated endocytosis. Once in the cytosol, the LDL is hydrolysed, liberating free cholesterol. Cholesterol can also be synthesized de novo within the cortex by the enzyme acetyl coenzyme A (acetyl-CoA) [167].

The three zones of the cortex have distinct enzymic profiles, allowing them to specialise to the synthesis of specific steroids. The first step of steroidogenesis takes place in all zones, and involves the transport of cholesterol, from the cytosol, to the inner mitochondrial membrane, where it is subsequently converted to pregnenolone by cytochrome P450scc. The zona glomerulosa is specialized for synthesising mineralocorticoids, due to the high expression of aldosterone synthase

(P450c18) in this zone. This enzyme is not expressed in either the zona fasciculata or the zona reticularis; consequently, these zones are unable to synthesize aldosterone [167]. By contrast, the zona fasciculata and zona reticularis express P450c17, which is absent from the zona glomerulosa. This enzyme has both 17α-hydrolylase and 17-20-lyase activity, the latter being dependent upon the availability of the flavoprotein cytochrome b5. In the zona fasciculata, the 17α-hydrolylase activity of P450c17 predominates, generating 17-OH-pregnenolone, a prerequisite for GC synthesis in this zone. In the zona reticularis, the comparatively high expression of cytochrome b5 allows P450c17 to carry out 17, 20-lyase activity, which is necessary for the generation of the adrenal androgen precursors, dehydroepiandrosterone (DHEA) and androstenedione in this zone (Fig. 15) [167].

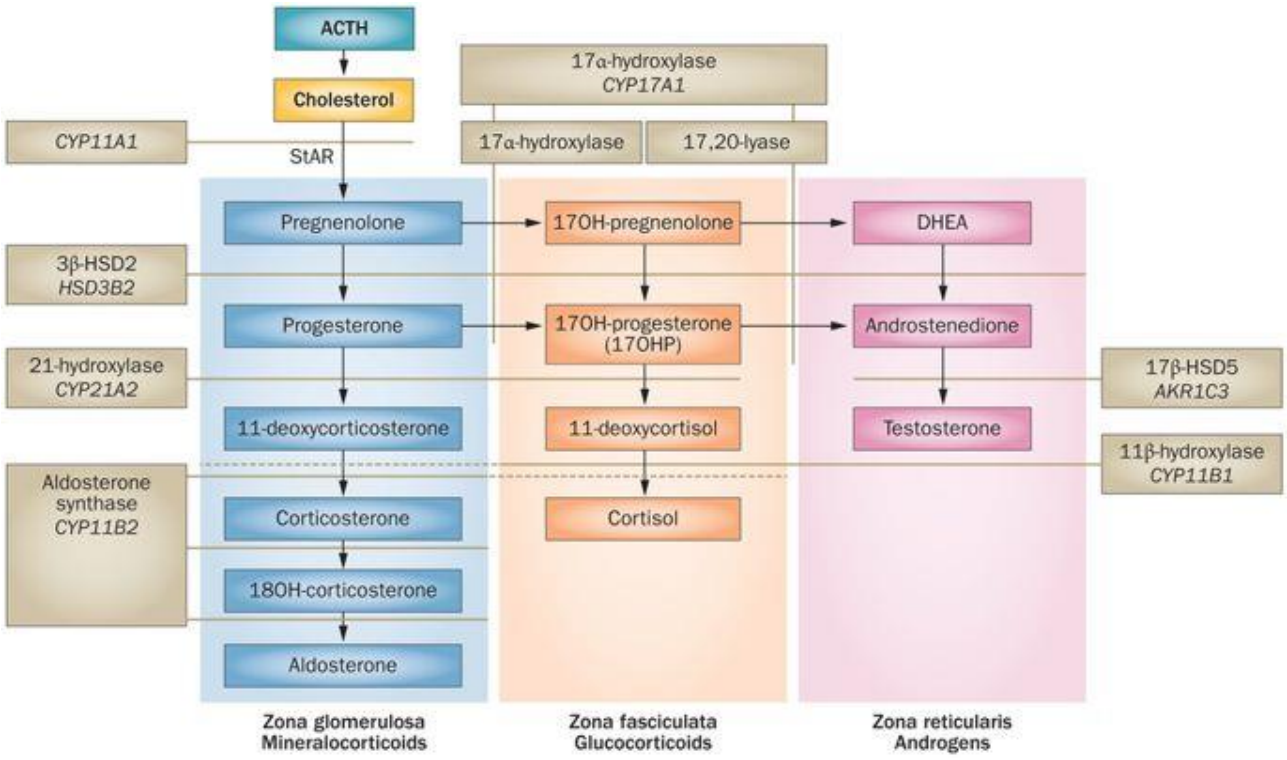


Figure 15: *The 3 zones of the adrenal cortex have distinct enzymic profiles allowing for the synthesis of specific steroids in each zone.*

3. Glucocorticoids synthesis

GCs are synthetized in the fasciculata area of the adrenal cortex. The first step of cortisol synthesis is the conversion of cholesterol to pregnenolone within the mitochondria, and involves hydroxylation and side chain cleavage at C20 by P450scc. Pregnenolone is released from the mitochondria, and subsequently converted to 17-OH-progesterone by one of two possible pathways. In the predominant pathway, pregnenolone is firstly converted to progesterone in the cytosol by 3β-HSD, by a reaction involving isomerisation of the double bond at C5 and dehydrogenation of the 3-OH group. P450c17 then converts progesterone to 17-OH-progesterone by hydroxylation of C17 using its 17α-hydroxylase activity, and cleavage of the 2 carbon side chain at C17 using its 17-20-lyase activity. The alternative pathway for 17-OH-progesterone synthesis utilises the same enzymes, however, P450c17 first converts pregnenolone to 17-OH- pregnenolone, which in turn is converted to 17-OH-progesterone by the actions of 3β-HSD. The next step in cortisol biosynthesis is the conversion of 17-OH-progesterone to 11-deoxycortisol by 21-hydroxylase (P450c21), in a reaction that involves hydroxylation of C21. The last step takes place in the mitochondria, and involves the conversion of 11-deoxycortisol to cortisol by the enzyme 11β-hydroxylase (P450c11) [167].

4. The hypothalamic-pituitary-adrenal axis

GC secretion is controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 16). Neural stimuli from the brain drive the hypothalamus to secrete corticotrophin-releasing hormone (CRH) into the hypophyseal portal vein, where it travels to the anterior pituitary and binds to the type I CRH receptors. This in turn stimulates the release of adrenocorticorticotrophic hormone (ACTH) from the anterior pituitary into the circulation, where it acts on the adrenal gland increasing cortisol secretion. A negative feedback system is in place whereby cortisol can inhibit the release and synthesis of CRH and ACTH.

ACTH secretion varies on a pulsatile basis, with peaks at approximately 30 minutes intervals. Furthermore, ACTH levels vary throughout a 24 hours cycle, in a pattern known as the circadian rhythm. Consequently, cortisol secretion is also pulsatile, and follows the circadian rhythm with levels peaking.

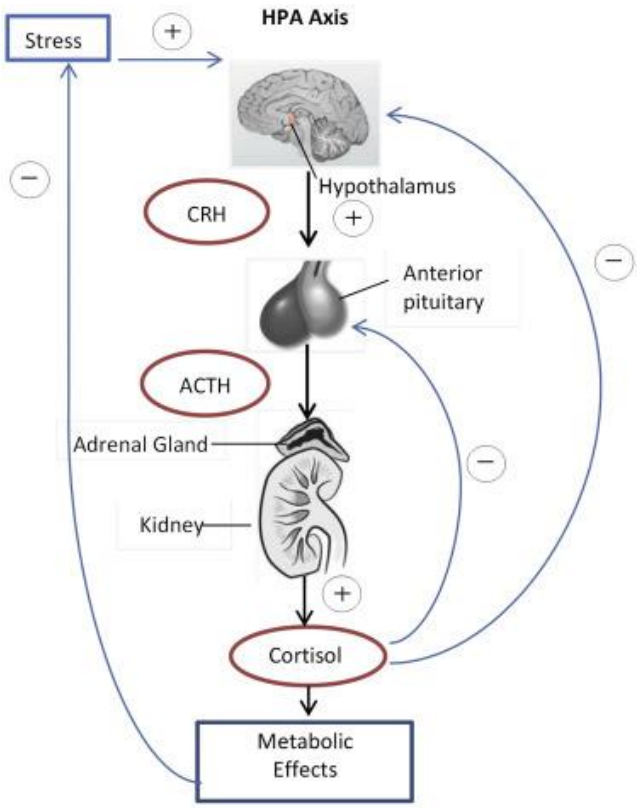


Figure 16: The hypothalamic-pituitary-adrenal (HPA) axis. *A negative feed back mechanism is in place whereby cortisol inhibits its own release.*

5. Glucocorticoids action

In the cytosol, the predominant actions of glucocorticoids are through the glucocorticoid receptor (GR), which regulates the transcription of specific genes. The GR is a member of steroid hormone receptor family, which are ligand-activated nuclear receptors. All members of this family share a common structure, consisting of a C-terminal ligand binding domain, a DNA binding domain and an N-terminal transactivation domain [168]. The GR shuttles between the cytoplasm and nucleus upon ligand binding. In its unbound form, the GR is localized to the cytosol where it forms a heterocomplex with 2 molecules of heat shock protein-90 (hsp90), stoichiometric amounts of heat shock protein-70 (hsp70), p23 and immunophilin [169] [170]. The association between GR and hsp90 opens the hydrophobic steroid-binding cleft within the GR, allowing access by the steroid ligand [171]. Upon steroid binding, the hsp-immunophilin complex dissociates and the liganded GR rapidly translocates into the nucleus where it interacts with positive / negative GC responsive elements (GREs) within the DNA of gene targets - activating / repressing gene transcription (Fig. 17) [172] [173].

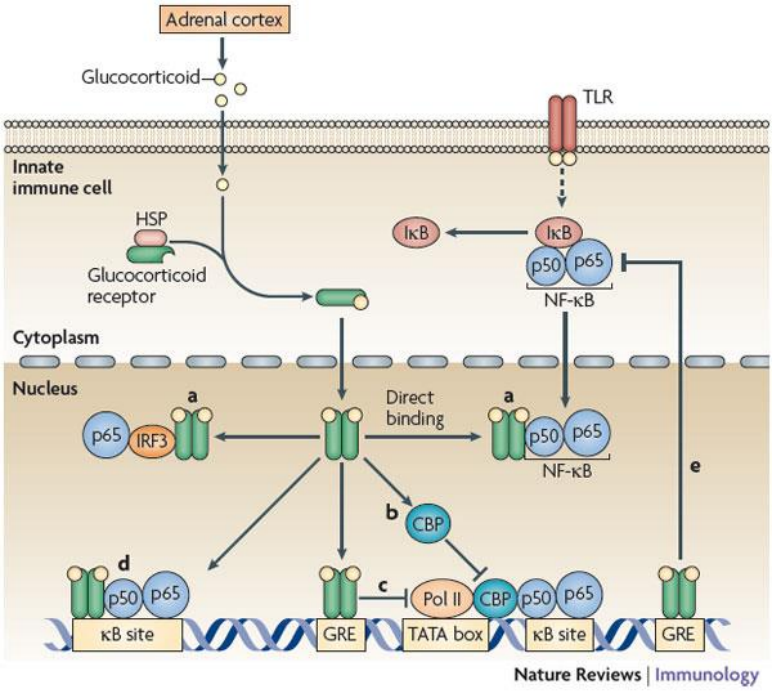


Figure 17: The mechanism of GC action. *Upon steroid binding, the GR dissociates from its protein complex, translocates into the nucleus and modulates gene transcription.*

Structurally, the GR can be organized into 3 functional domains: an N-terminal domain, a DNA-binding domain and a ligand-binding domain (Fig. 18) [174]. The DNA-binding domain is composed of two highly conserved zinc fingers, located centrally within the amino acid sequence. The first zinc finger is primarily responsible for site specific GR-DNA binding, since amino acid residues located in this region make specific interactions with bases located within the GRE [175] [176]. The second zinc finger functions to stabilize GR-DNA interactions, and plays a role in homodimerisation at the GRE [175] [176]. Much of the GRs transcriptional activity is dependant upon the AF1 activation region, located within the N-terminal domain. AF1 has been shown to associate with a number of transcriptional co-activators and co-repressors including TFIIB, CBP and SRC1 [177] [174]. In addition to its role in binding steroid-ligands and chaperones, the ligand-binding domain also has an activation subdomain (AF2) which, like AF1, binds co-activators and co-repressors including SRC1 [168] [178].

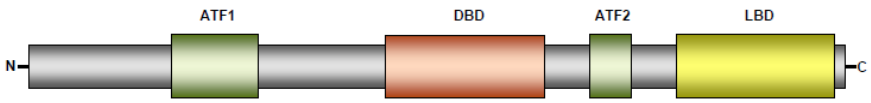


Figure 18: The GC receptor domain structure. *(DBD= DNA binding domain, LBD= ligand binding domain).*

In addition to regulating the transcription of genes, which have GREs, the GR can also regulate transcription of genes that do not possess these elements: by interact with, and regulating the activity

of transcription factors bound to their own response element [179]. The primary changes in gene transcription meditated by the GR can be either positive or negative, and can take place in as little as 15 minutes following GCs exposure [180]. In addition, the GR can elicit secondary events whereby the expression of GC responsive genes can modulate the expression or activity of other proteins.

The GR gene is subject to alternative splicing at the first and last exons. The most abundant spliced variants are GRα and GRβ. GRα has a high affinity for GCs, and is expressed ubiquitously, whereas GRβ has a very low affinity for GCs, limited tissue distribution, and may act as a dominant negative regulator of GRα activation [168]. Some GC effects have been reported to occur within minutes, and are insensitive to transcriptional inhibition [181] [182] [183] [184]. There is evidence that these non-genomic GC effects are, at least partly, mediated by a membrane bound GR [185] [186]. Reported non-genomic effects include activation of the insulin signaling components PI3K and PKB/akt [187]. The precise signaling events leading to these effects has not been fully elucidated, but are thought involve caveolin-1 [188]. Recently rapid non-genomic effects of CG were showed also in mammalian skeletal muscle [189].

6. Steroid Myopathy

Steroid myopathy is a condition of skeletal muscle associated with an excess of glucocorticoids (GC). In many pathological conditions characterized by muscular atrophy, there is an increase in the serum concentration of these hormones, suggesting a potential role in the onset of the atrophic process [190]. Agonists of these hormones have been introduced over the years, synthetic glucocorticoids, which are used in the treatment of numerous inflammatory, autoimmune and allergic diseases. However they are associated with the important side effects that arise mainly in long-term treatments that lead to development of the iatrogenic form of Cushing Syndrome, whose signs and symptoms are: buffalo hump, round face, obesity, stretch marks, high blood pressure, thin fragile skin etc. These side effects include the increase in the concentration of sugars in the blood with the possibility of developing diabetes, increased levels of triglycerides and fatty acids, decreased calcium absorption that can cause osteoporosis, increased risk of ulcers and gastritis, and the establishment of a clinical picture similar to Cushing's Syndrome with abdominal obesity and hypertension. The potential effect that is at the heart of this study is however, the myopathic condition the use of these hormones can cause. People with steroid myopathy have muscular weakness located mainly in the lower limbs with difficulty performing simple daily activities such as climbing stairs. The main characteristic of this condition is an atrophy that explains the weakness of the muscles. Approximately 50-80% of patients with non-iatrogenic Cushing Syndrome have some degree of muscle weakness that affects men more, but the incidence of muscle weakness

caused by glucocorticoid administration is not known [191]. Furthermore, the relationship between the time of administration and the appearance of the effects has not been outlined, since both acute cases after a single administration are recorded, and chronic forms due to a treatment of several weeks are recorded.

Dexamethasone (DEX) is the synthetic steroid whose administration seems to be more related to the onset of steroid myopathy [192] (Fig.19). Numerous mechanisms have been described in the literature through which corticosteroids would be able to induce atrophy [193], however the primary cause of steroid myopathy is not yet known. So far, the skeletal muscle investigations have been focused on the established phase of steroid myopathy, a phase that occurs after repeated administration of glucocorticoids. In this condition is difficult to identify the phenomenon responsible for triggering the myopathic process, ie the one with a primary pathogenic role, and differentiating it from those phenomena that could be a consequence of an already stabilized atrophic and myopathic process. To understand the pathogenesis of this disease it is necessary to analyze the pathways involved with the action of steroids.

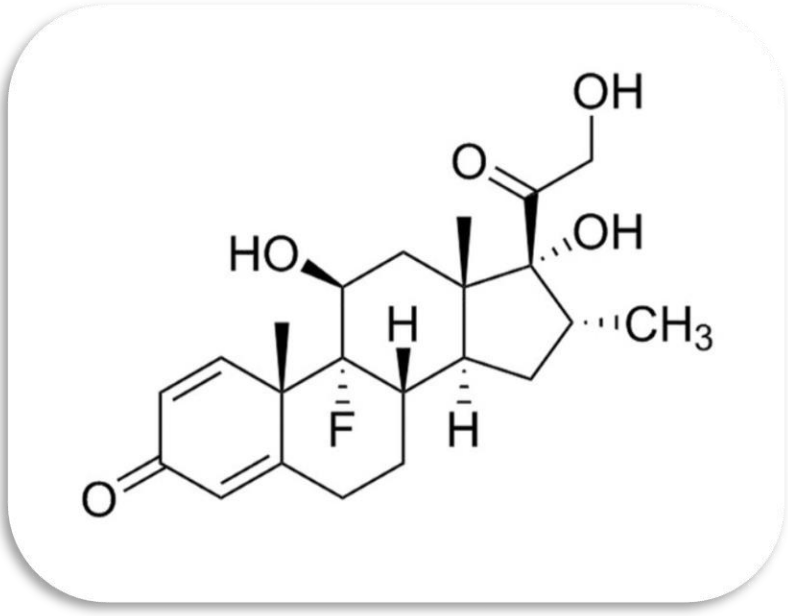


Figure 19: *Chemical structure of Dexamethasone.*

7. How do steroids exert their atrophic action?

Although many mechanisms for steroid-induced muscle atrophy have been described, it is not clear which of them plays the major role and which is the underlying kinetics. We will now list and briefly analyse the main pathways through which steroids exert their actions.

7.1. Anti-anabolic activity

Glucocorticoids are able to inhibit the transport of amino acids into muscle cells, limiting protein synthesis [194]. The inhibition of protein synthesis by GC mainly results from the inhibition of mTORC1, the kinase responsible for the phosphorylation of 4E-BP1 and S6K1 [195]. Repression of mTORC1 signaling results in a reduction in the initiation phase of mRNA translation with down-regulation of protein synthesis. Recent studies indicate that the repression of mTORC1 signaling in response to GC is the result of enhanced transcription of REDD1 and *Klf15*, two repressors of mTORC1 signaling [196]. These two genes are direct targets of the GR in the skeletal muscle. By inhibiting Rheb, a positive effector of mTORC1, REDD1 represses mTORC1 function, leading to decreased phosphorylation of both 4E-BP1 and S6K1. The action of the transcription factor *Klf15* is more complex. By stimulating the expression of BCAT (branched-chain aminoacid BCAA aminotransferase), an enzyme which degrades BCAA, *Klf15* accelerates the intracellular catabolism of BCAA, which are believed to activate mTOR and therefore inhibits mTOR activity [197]. Furthermore, *Klf15* cooperates with *FoXO1* to upregulate the promoter activity of E3 ubiquitin ligases *Atrogin1* and MuRF-1. Therefore, overexpression of *Klf15* causes myotube atrophy in an Akt-independent way. Recent evidence suggests that mTOR signaling could be also inhibited directly by FoXO [198].

In addition, they negatively regulate insulin and IGF-1 stimulation of 4E-BP1 phosphorylation and ribosomal kinase S6 (S6K1), which in turn regulate mRNA translation [199]. Finally, dexamethasone positively regulates *Myostatin*, which has an inhibitory action on muscle growth and differentiation [200].

7.1.1. IGF-1

GC can also cause muscle atrophy by altering the production of growth factors, which control locally the muscle mass development. GC inhibit the production by the muscle of *IGF-1* [201] a growth factor which stimulates the development of muscle mass by increasing protein synthesis and myogenesis while decreasing proteolysis and apoptosis. The action of *IGF-1* towards muscle growth is probably mediated through the IGF-1 receptor (IGF-1R) as mice lacking IGF-1R in skeletal muscle exhibit impaired muscle development characterized by reduction in fibre number and area

[202]. For these reasons, decreased muscle *IGF-1* has been thought to play a key role in GC-induced muscle atrophy. This hypothesis has been recently confirmed both in vitro and in vivo. First, by activating the PI3K/Akt/mTOR pathway and blocking nuclear translocation of the transcription factor *FoXO, IGF-1* down-regulates the different proteolytic systems (lysosomal, proteasomal and calpain-dependent) and the expression of Atrogenes such as *Atrogin1*, *MuRF1*, *Cathepsin-L* induced by GC in myotubes [203]. Second, *IGF-1* suppress the muscle cell atrophy caused by GC in vitro [204]. Third, systemic administration [205] or local overexpression of *IGF-1* into skeletal muscle prevents GC-induced muscle atrophy [206]. Taken together, these results indicate that *IGF-1* has a dominant effect, overriding GC to turn off catabolism. In addition, they support the key role of decreased muscle *IGF-1* in the atrophy caused by GC. Therefore, restoration of *IGF-1* may provide a strategy to reverse the catabolic effects of GC excess.

7.1.2. Myostatin

Myostatin, formerly known as growth and differentiation factor 8, a member of the transforming growth factor-β superfamily, is an important negative regulator of skeletal muscle mass. Disrupted *Myostatin* gene expression, either by gene targeting in mice or because of naturally occurring mutations in cattle, is associated with increased skeletal muscle mass resulting from muscle fibre hyperplasia as well as hypertrophy [207]. To investigate the regulation of *Myostatin* gene expression, Ma and collegues [208] cloned and characterized the 5′-upstream regulatory region of the human *Myostatin* gene and found that the promoter contains a number of response elements important for muscle growth, including seven putative glucocorticoid response elements (GREs). They also demonstrated that dexamethasone dose-dependently increases endogenous *myostatin* transcription in C2C12 myocytes through a glucocorticoid receptor-mediated mechanism. These findings led us to postulate that the increase in *Myostatin* gene expression by glucocorticoids might contribute to the pathogenesis of glucocorticoid-induced skeletal muscle atrophy.

GC stimulate the production by the muscle of *Myostatin* (Mstn) [208], a growth factor which inhibits the muscle mass development by down-regulating the proliferation and differentiation of satellite cells [209] as the protein synthesis [210]. The stimulation of Mstn expression is thought to result from increased Mstn gene transcription, but also from posttranscriptional mechanisms. Indeed, although the mouse Mstn gene promoter contains a GRE motif, the stimulation of Mstn gene transcription in response to GC is modest [208]. Furthermore, recent evidence indicates that the down regulation of miR-27a by GC may contribute to stabilize the Mstn mRNA [211] [212]. The action of Mstn towards skeletal muscle is illustrated *in vitro* and *in vivo* [213] where Mstn causes muscle atrophy, albeit modest, by reversing the IGF-I/PI3K/Akt hypertrophy pathway. Through

inhibition of Akt phosphorylation, Mstn increases the levels of active *FoXO*, allowing increased expression of Atrogenes [214]. Furthermore, transgenic mice, which express Mstn selectively in skeletal muscle, have also muscle atrophy [215]. But the most convincing demonstration of the role of Mstn on muscle mass is the fact that targeted disruption of Mstn gene expression in mice leads to dramatic increase in skeletal muscle mass due to fibre hyperplasia and/or hypertrophy [216]. Similarly, Mstn knock-out myotubes are larger than control ones together with an increased activation of the Akt/mTOR signaling pathway and protein synthesis [217]. For these reasons, increased muscle Mstn has been thought to play a key role in GC-induced muscle atrophy [208], [218]. This hypothesis has been recently confirmed *in vivo* [219] using a model of Mstn knock-out (KO) mice. In contrast to wild type mice, Mstn KO mice did not develop a reduction of muscle mass nor fibre cross-sectional area after GC treatment. This observation indicates that Mstn is mandatory for the atrophic effects of GC on muscle. The mechanism by which Mstn deletion prevents muscle atrophy caused by GC is not known. However, the observation that prevention of muscle atrophy by Mstn deletion is associated with the blockade of the upregulation of Atrogenes expression and proteosomal activity caused by GC suggests this protection of muscle mass results at least in part from the inhibition of the muscle proteolysis [219]. Taken together, these results suggest that increased Mstn contributes to the atrophic effects of GC on skeletal muscle. Therefore, besides stimulating *IGF-1*, inhibition of Mstn may provide another strategy to reverse the catabolic effects of GC excess. The upregulation of *Myostatin* gene expression in DEX-treated mice could be mediated by the glucocorticoid receptor to glucocorticoid responsive elements in *Myostatin* promoter [220]. The role of Mstn in other models of muscle atrophy is still disputed.

7.2. Catabolic activity

Glucocorticoids stimulate proteolysis by activating the autophagy system and the ubiquitin-proteasome system and degradation occurs in both myofibrillar and extracellular matrix [221]. In particular, they promote the expression of *Atrogin1* and *MuRF1*, ubiquitin ligases specific muscle, or directly increase the ubiquitination rate. Furthermore, *MuRF1* is activated by the glucocorticoid receptor and *FoXO1*, while *FoXO3* is involved in *crosstalking* with GCs and *Atrogin1* [222] [223].
Transcription factors *FoXO* play a major role in the muscle cell catabolism caused by GC. The role of these transcription factors in the GC-induced muscle cell atrophy has been established by different observations. First, exposure of myotubes or skeletal muscle to GC increases the *FoXO* gene expression, particularly −1 and −3a [224]. Second, in vitro as well in vivo, *FoXO* overexpression causes muscle cell atrophy, together with activation of several Atrogenes such as *Atrogin1*, *MuRF1* and *Cathepsin-L* [106]. Finally, overexpression of a dominant negative form of

FoXO3a prevents muscle cell atrophy together with *Atrogin1* induction caused by GC in vitro [106]. Among the genes most strongly induced in microarray analyses of muscle atrophy due to a variety of wasting diseases are several genes (*Atrogin1*, *MuRF1*, *Cathepsin-L*, *PDK4*, *p21*, *Gadd45*, *4E-BP1*) controlled by the *FoXO* transcription factors [222]. Taken together, these data indicate that increased expression of *FoXO* by GC activates a gene transcriptional program responsible for triggering muscle atrophy. The establishment of an active transcriptional program necessary for the induction of muscle atrophy has thus challenged the view that atrophy is a passive adaptation of the muscle to a lack of anabolic stimuli. All these observations support the role of *FoXO* in muscle atrophy induced by GC but there is not yet direct in vivo evidence for the requirement of *FoXO* in this muscle atrophy model.

A very recent paper [225] suggests a primary involvement of *MuRF1* activation in steroid myopathy.

The role of autophagy system in the atrophic effect of glucocorticoids, is also suggested by the increase in *Cathepsin-L* muscle expression [226] and by the increased conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II, an indicator of, in glucocorticoid-treated animals. In this step the cytosolic form LC3-I is conjugated to the phosphatidylethanolamine to form LC3-II. LC3-II binds to the autophagosome membrane, which in turn merges with the lysosomes forming the autolisosomes in which the components destined for degradation are destroyed by lysosomal hydrolases. At the same time, LC3-II is degraded, for this reason the LC3-II turnover is considered a marker of autophagy [227].

Finally, some in vivo data also suggest that caspase-3 can be implicated in the myofibrillar proteins breakdown induced by glucocorticoids. Indeed, in glucocorticoid-dependent muscle wasting models, such as diabetes mellitus and chronic renal failure, caspase-3 activity in muscle is increased and inhibition of caspase-3 by Ac-DEVD-CHO, a peptide inhibitor, suppresses the accelerated muscle proteolysis [218]. However, the role of glucocorticoids in the induction of caspase-3 activity in these models has not yet been explored.

7.2.1. Glucocorticoid-induced mitochondrial dysfunction

GCs undoubtedly act on mitochondria and oxidative metabolism, but neither the mechanism involved nor the degree of mitochondrial dysfunction has yet been elucidated. These steroids increase the permeability of the mitochondrial membrane and modulate the expression of mitochondrial genes [228]. Furthermore, it has been shown that GCs can have a double effect on these organelles; it seems that short-term exposure causes an increase in mitochondrial function, whereas chronic exposure leads to a decrease in functionality [229]. It was demonstrated that

mitochondrial gene expression could be regulated by GCs via a direct action on mitochondrial DNA and oxidative phosphorilation gene or by an indirect effect through interaction with nuclear genes [230].

Weber et al. [231] treated rats for 3 days with dexamethasone and observed an increased mitochondrial biogenesis suggesting a possible involvement of a mithocondrial glucocorticoids receptor. On the contrary, other groups as Orzechowski [232] suggest that chronic corticosteroid administration reduces mitochondrial oxidative capacity in skeletal muscle. Dumas et al. [233] suggest that the dexamethasone induced hypercatabolic state has no effect on mithocondrial energy metabolism at least in fast muscle.

In particular as regards as the action of GCs on *PGC-1α* Qin at al. [220], [234] found that 7 days of DEX treatment leads, in gastrocnemius muscle of rat, to a reduction of *PGC-1α* protein levels at cellular and nuclear level. The same result was obtained in mice [235]. On the other hand, Menconi and collegues [236] showed that *PGC-1α* mRNA levels were not affected by DEX treatment in mice. Orzechowski et al. [232] observed that rats with a glucocorticoid-induced catabolic state show synthoms of oxidative stress in soleus. Moreover, Oshima and colleagues [237] aimed to clarify this point by using cultured human cell lines and found that corticosteroid may have induced overproduction of reactive oxygen species (ROS), resulting in mitochondrial dysfunction and cellular apoptosis in differentiated myogenic cells.

8. Other effects of GCs on skeletal muscle fibres

There are other effects caused by the administration of GCs, even if they appear to have a lower role. For example, the excitability of sarcolemma is reduced due to the decrease in the concentration of proteins that regulate the transmembrane electrolyte balance [238]. Furthermore, GCs hinder the differentiation and recruitment of satellite cells, the stem cells of muscle, through regulation positive *Myostatin*, which results in the impossibility of responding to muscle injuries [239]. Moreover, another effect can be seen in the form of myosin loss and rhabdomyolysis, although the exact processes that lead to this are not entirely clear [240] [241]. Glucocorticoids have been shown to cause atrophy of fast-twitch or type 2 muscle fibers (particularly 2X and 2B) with less or no impact observed in type 1 fibers [242]. Therefore, fast-twitch glycolytic muscles (i.e., tibialis anterior) are more susceptible than oxidative muscles (i.e., soleus) to glucocorticoid-induced muscle atrophy. The mechanism of such fiber specificity could be related to the higher GC receptor expression in tibialis anterior than soleus muscles [243].

9. Glucocorticoids and Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is the most prevalent muscular dystrophy occurring in 1 out of 3500 live male births, and caused by mutations in the dystrophin gene [244]. DMD is a severe progressive neuromuscular disease that presents with gait disturbances between the ages of 3 and 5 years and loss of ambulation by 12. There are no cures for DMD, but the use of glucocorticoid therapy (prednisone, prednisolone and deflazacort) has been shown to improve muscle strength during the first 6 months of usage followed by stabilization of the course of the disease for up to 2 years, after which there is a decline in function. There are many potential adverse effects of glucocorticoid therapy including osteoporosis, excessive weight gain, and behavioral abnormalities [245]. While there appear to be short-term benefits of glucocorticoid therapy in DMD patients, the long-term functional benefits are unclear [244]. A study performed on mdx mice showed that daily administration of prednisolone resulted in early (initial 50 days) improvements in muscle strength and motor coordination, but these benefits were lost after 100 days of continuous treatment [246]. Further, there was a deterioration of cardiac function and increased fibrosis of the heart with prolonged glucocorticoid treatment [247]. The mechanism by which glucocorticoids improve muscle strength in DMD patients is unknown, and thought to occur through a suppression of inflammation. DMD patients often show pseudohypertrophy of muscles, especially in the calf, which is thought to be due to inflammation. However, other immunosuppressive drugs, that reduce inflammatory infiltrates in the muscles of DMD patients do not improve strength, as seen with prednisone. In mdx mice, treatment with prednisolone increases specific force in muscle, while having no affect on muscle fibre size [248] [249]. The increase in specific force output is similar to what is seen in normal mice and rats given glucocorticoids. The mechanism by which glucocorticoids improve force output in DMD and mdx is still unclear and may be distinct from the anti-inflammatory actions of the glucocorticoids.

10. Diagnosis and possible treatment

The diagnosis of steroid myopathy is not simple. Firstly, because there are both endogenous and exogenous forms and the latter may be due to treatment with compounds with different characteristics and potentials. Furthermore, the affected population is heterogeneous in terms of age, previous diseases and pharmacological treatments in progress.

However, some fixed parameters can be used for diagnosis. Direct methods for measuring muscle mass are the kinetics of amino acids and possibly muscle biopsy, even if, from an ethical point of view, the latter is difficult to apply [250]. A definitely less invasive method is the use of some specific biomarkers, such as the evaluation of serum creatine kinase (CK) levels and urinary

creatinine excretion [251]. CK, along with the detection of a decrease in fibre conduction velocity, is an early marker of steroid myopathy [252].

The treatment of steroid myopathy is different depending on the etiology of the disease. The main cause of Cushing's syndrome is the excessive production of cortisol by the adrenal glands due to neoplasia, so the approach in this case will be of the surgical, chemo and radiotherapy type. If hypercortisolism is exogenous, the dosage and type of steroid used can be changed, as well as the administration of creatinine to preserve muscle mass [253].

One substance that has been proposed for the treatment of steroid myopathy is ghrelin deacilata (UAG), a product of ghrelin catabolism. Ghrelin is a circulating peptide hormone produced mainly by the stomach, which acts on the hypothalamus-pituitary axis induces the secretion of growth hormone (GH) by stimulating food intake and adiposity by binding to its GHSR-1 receptor. The UAG, which is more abundant in plasma than AG, does not bind to GHSR-1a, and has no GH-release activity [254]; for this reason, for many years, it has been considered the inactive product of ghrelin catabolism. Recently it has been shown that UAG exercises a protective activity against muscular atrophy [255]. It has also been shown that both ghrelin and UAG are able to counteract the effects of dexamethasone on both cultured myoblasts [256] and on muscle in vivo [225].

Since the benefits of this molecule appear to be limited by its short half-life, it is necessary to investigate other potential drugs able to act effectively on the mechanisms underlying the myopathic condition established by treatment with dexamethasone.

11. GCs and BCAA

In order to define an effective therapeutic approach for steroid myopathy, a valid alternative could be represented by branched chain amino acids (BCAA: leucine, isoleucine and valine) (Fig. 20), essential for protein synthesis in humans. Many athletes use BCAA supplements to improve their physical performance and increase muscle mass. In addition, amino acids, particularly BCAAs, can be used clinically to alleviate the induced muscular atrophy of low-calories diets [257], and prevent sarcopenia [258]. It has also been reported that the activity of mitochondrial enzymes and the abundance of mRNA gene transcripts encoding mitochondrial proteins are stimulated by a mixture of insulin and essential amino acids in young healthy subjects [259]. The above study cites a unique role for amino acids in regulating both the mitochondrial function of muscle and protein synthesis. BCAAs improve the cellular signaling pathways regulating protein synthesis of skeletal muscle, which in turn can also facilitate an improvement in the production of mitochondrial ATP. It has been demonstrated that the integration of a mixture of amino acids high in BCAA (~ 60%) promotes mitochondrial biogenesis in the heart and musculoskeletal of middle-aged mice, through

increases in the gene expression of mitochondrial transcriptional regulators including peroxisome activated by the proliferator receptor-Î³ coactivator-1α (PGC1α) [260]. It was also observed that chronic BCAA supplementation could increase the activity of mitochondrial function markers (citrate synthase and cytochrome c oxidase) in the skeletal muscle of sedentary mice [261].

In this study object, the effectiveness of BCAA in counteracting the effects induced by dexamethasone will be evaluated.

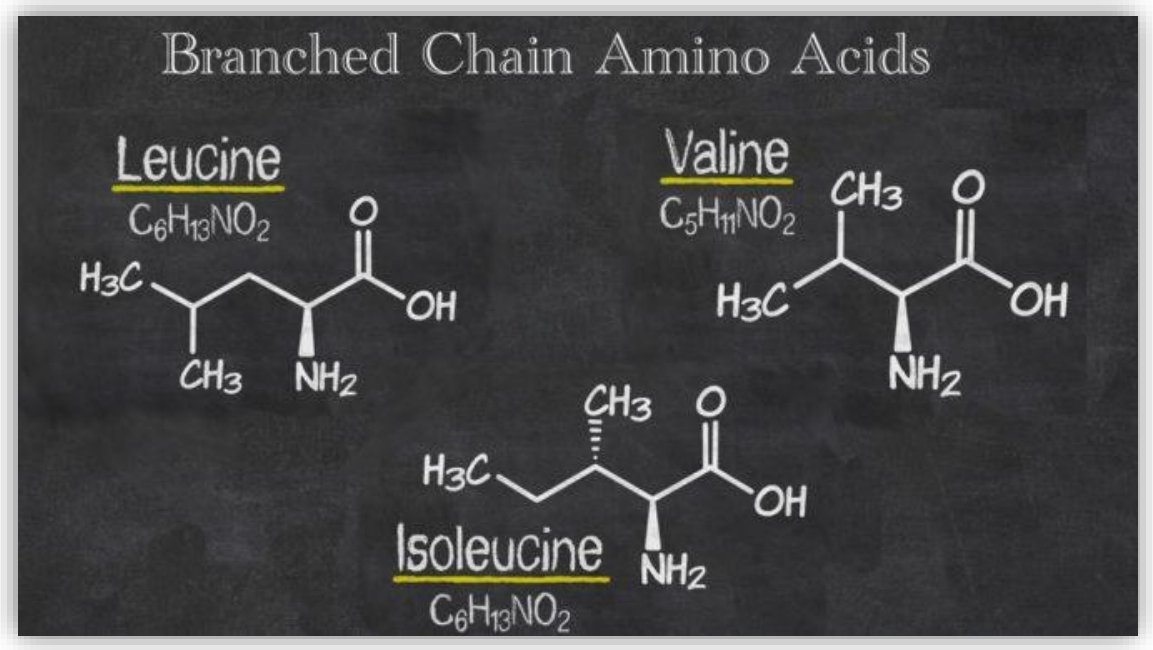


Figure 20: *Chemical structure of Essential Branched Chain Amino Acids.*

CHAPTER 4

STUDY DESIGN

AND

GENERAL OBJECTIVES

The general aim of the study is to identify the pathogenetic mechanisms triggering steroid-induced atrophy. In particular, the objective is to identify the intracellular pathways that are activated following a single administration of Dexamethasone (DEX) in healthy volunteers. Subsequently, the ability to counteract the activation of these pathways by a food supplement based on branched chain essential amino acids will be evaluated. The project encompasses a series of studies that have used both human and animal models. Two studies in particular have assumed an important position in the determination of the experimental protocol of the present study, and therefore will be briefly illustrated below.

The first of these was conducted in 2015 by ***Professor Minetto and co-workers*** [262], at the Department of Human Physiology of the University of Pavia. This work was the first in vivo study to describe the effects of one week's administration of dexamethasone in humans by investigating the mechanics of individual muscle cells and intracellular molecular aspects. The results of this study show that after a week of DEX administration, muscle fibers underwent atrophy, decreased their specific force, and decreased their myosin concentration. Although muscle atrophy is a well-known side effect of a longterm glucocorticoid excess, this study is the first showing that the short-term administration of glucocorticoids, in doses well within the range used clinically, reduced muscle fibre CSA and myosin concentration.
A reduction in circulating muscle proteins points toward the anti-anabolic effect of glucocorticoids as the most plausible explanation for the decrease in CSA and myosin loss. Myosin loss and a decrease in acto-myosin interaction could also explain the observed reduction in specific force of muscle fibers.
The obtained results suggest that the glucocorticoid-induced quantitative and qualitative adaptations of muscle fibers develops after only a few days of glucocorticoids adminiostration.
This suggested the possibility of identifying the first pathogenic mechanisms focusing on the early stages of the myopathic process.

The second study was conducted in 2018 by ***Professor Canepari and co-workers*** [225], at the Department of Human Physiology of the University of Pavia. This study on mice model allowed to perform a time course to assess the adaptations of the intracellular molecular pathways involved with steroid myopathy. In the study, the activation of the catabolic and anti-anabolic pathways and the effect of the co-administration of DEX and unacylated ghrelin (UAG) on muscle atrophy and gene expression was assessed.

To this end, the animals were sacrificed after 1, 3 or 10 hours from a single intraperitoneal injection of dexamethasone (DEX) or DEX+UAG, and the main pathways that control protein degradation, protein synthesis, oxidative metabolism and redox system homeostasis were studied.
The results of this study show that several responses of intracellular signalling pathways occur very early (1h) and simultaneously following single DEX administration and fade away in few hours (10h). The study also demonstrates that UAG is able to selectively inhibit the early-enhanced expression of *Murf*-1 and counteract muscle atrophy suggesting that the primary phenomenon causing steroid myopathy is an early and transient reprogramming of the activity of ubiquitin proteasome system (UPS).
The results also suggest that steroid atrophy could be the result of a cumulative effect of transient gene expression activation by daily injections.

On the basis of the informations obtained from the aformentioned works this project was focused on the study of the intracellular signaling response in skeletal muscle of healthy human subjects following a single glucocorticoids administration order to identify the biomarkers that could be modulated in the clinical practice to counteract steroid induced muscle wasting.

In order to evaluate both the genomic and the proteomic effect of the GCs administration the analysis on muscle samples taken at 4h post DEX or placebo administration were inially performed. The analysis of gene expression in samples taken at 1h post administration allowed to complete the picture about the GCs action. Finally, the analysis of the samples taken at 8h post administration allowed to study the duration of the GCs action.

CHAPTER 5

MATERIALS AND METHODS

1. Subjects

This study is in compliance with international ethical standards and has been approved by the Ethical Commission of the University of Pavia. Each subject participating in this has been provided with an informed consent. The study was conducted on 42 volunteers (29 male and 13 female), with ages comprised between 20 and 30 years, free from neuromuscular diseases and not involved in high performance athletic activities. Enrolled subjects were forbidden to do gym or training sessions the day before the biopsy. The day of biopsy, each volunteer was asked to have a light breakfast consisting only of a cup of hot tea and three biscuits.

For each subject, one baseline pre-dexamethasone administration muscle biopsy (PRE) has been obtained and used as reference. Seven days after PRE, subjects have been administered randomly (in double blind) with a single 8mg dose i.v. of DEX or placebo and muscle biopsy has been obtained in a group of subjects (n=37) at 4h post- administration (POST 4h), in another group at 1h post administration (n=16) (POST 1h) and in the last group at 8h post- administration (n=20) (POST 8h), in separate sessions, at least 1 month apart. The Dexamethasone or placebo administration has been performed in facilities provided by Fondazione Salvatore Maugeri IRCCS under medical surveillance.

Eight volunteers have been also administrated with Big One supplement (Professional Dietetics) based on essential branched amino acids (L- Leucina, L- Lisina, L- Isoleucina, L- Valina, L- Treonina, L- Cistina, L- Istidina, L- Fenilalalina, L- Metionina, L- Tirosina e L- Triptofano) (5.5 g twice a day), which lasted for 14 days. On the morning of the 15th day each subject, after the usual intravenous administration of DEX, was given another dose of supplement and after 4 hours the subject was subjected to a biopsy (n=8) (POST_AA).

In table A all biopsies performed for each volunteer are summarized.

Subjects	PRE	POST_Placebo 1h	POST_DEX 1h	POST_Placebo 4h	POST_DEX 4h	POST_Placebo 8h	POST_DEX 8h	POST_AA	Gender
N° 1	√		√		√			√	M
N° 2	√				√				M
N° 3	√			√					M
N° 4	√	√							F
N° 5	√	√							F
N° 6	√		√		√				F
N° 7	√	√							M
N° 8	√			√					F
N° 9	√			√					M
N° 10	√		√		√			√	M
N° 11	√	√							M
N° 12	√				√				M
N° 13	√		√		√				M
N° 14	√	√							M
N° 15	√		√		√				M
N° 16	√		√		√			√	F
N° 17	√		√		√			√	M
N° 18	√		√		√			√	M
N° 19	√		√		√			√	M
N° 20	√		√		√			√	M
N° 21	√		√		√			√	F
N° 22	√			√		√			M
N° 23	√			√		√			M
N° 24	√				√		√		M
N° 25	√				√		√		M
N° 26	√				√		√		M
N° 27	√				√		√		F
N° 28	√			√		√			F
N° 29	√				√		√		M
N° 30	√			√		√			M
N° 31	√				√		√		M
N° 32	√				√		√		M
N° 33	√			√		√			F
N° 34	√			√		√			F
N° 35	√			√		√			M
N° 36	√			√					F
N° 37	√				√		√		F
N° 38	√			√		√			M
N° 39	√				√		√		M
N° 40	√				√		√		F
N° 41	√			√		√			M
N° 42	√			√		√			M
Tot.	42	5	11	14	23	10	10	8	

Table A: Summary of all biopsies performed for each volunteer.

2. Muscle Biopsy

The bioptic procedure utilizes the Bergstrom method (4 mm external diameter needle biopsy), that represents the routine method for the diagnosis of muscular pathologies, providing adequate sample size for histologic, ultrastructural, DNA and proteomic analysis [263]. In order to obtain the muscle samples, the following steps have been performed:

- Identification and evaluation of the muscle has been performed with ultrasound
- Skin disinfection has been performed with iodopovidone or benzalkonium chloride
- Cutaneous and subcutaneous local anesthesia with 2ml lidocaine has performed in an oval area surrounding the site of incision
- 5 mm long axis skin incision
- Insertion of the Bergstrom needle (4 mm external diameter) until muscle tissue is met
- Obtainment of a small muscle tissue quantity (around 50 mg)
- Wound disinfection and closure with 3M Steri-Strip wound closure strips

Muscle samples (Fig. 21) have been obtained from the Vastus Lateralis muscle, halfway along the line from ASIS to superolateral border of the patella of the dominant lower limb, frozen at -80°C and stored for further analysis.

Figure 21: *Freshly obtained muscle sample from Vastus Lateralis muscle biopsy.*

A single dexamethasone administration does not determine any damage at the muscular level. The unpleasant feeling following the biopsy will be a pain similar to an intramuscular injection. The pain in the biopsy area can persist for around 23 days, but normal daily activities such as walking will not be hindered. Intense physical activity is not advisable for 7 days following the biopsy.

3. Protein analysis

3.1. Sample Preparation: muscle lysis and protein extraction

Frozen muscles in liquid nitrogen are reduced into powder using a ceramic pestle. The powder thus obtained was homogenized with a lysis buffer containing 20mM TRIS-HCl, 1% triton x100, 10%Glycerol, 150mM NaCl, 5 mM EDTA, 100mM NaF and 2mM NaPPi supplemented with 1X inhibitors protease phosphatase (Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis MO) and 1mM PMSF. The lysis of tissue was performed on ice for 40 minutes and successively centrifuged at 13500 rpm for 20 min in a refrigerated centrifuge at 4°C. The supernatant obtained, has been transferred to a clean eppendorf tube and stored at -80°C until ready to use.

3.2. Protein concetration

The protein concentration of the lysates was determined using the RC DC™ (reducing agent and detergent compatible) protein assay (Bio-Rad).

RC-DC TM is a colorimetric assay for protein determination in the presence of reducing agents and detergents. The RC DC protein assay is based on the Lowry protocol [264], one of the most used methods to evaluate protein amount; proteins in the samples are treated with copper and other solution to have a final blue colored product which absorbance it is read at 750 nm and it is directly proportional to protein concentration according to the law of Lambert-Beer. The absorbance value of each sample is read in a spectrophotometer and the concentration protein is calculated by interpolating the values on a calibration curve whose points are scalar concentrations of a solution of known concentration (1.45 mg/ml) of Bovine serum albumin (BSA).

4. Western Blot (WB)

Western Blot (WB), or immunoblot, is a widely used technique in molecular biology, invented in 1979 by Towbin [265], which has the main objective of detecting specific proteins in a tissue sample. The total and/or the phosphorylated forms of our proteins of interest (table A) have been detected using a protocol already described in the literature. The Western Blot technique was and is a simple and rapid allows identifying a particular protein in a mixture of proteins, using the recognition by specific antibodies. The mixture of proteins (protein lysate) is first separated according to their molecular weight by electrophoresis on a polyacrylamide gel and subsequently transferred on a nitrocellulose or Polyvinylidene Fluoride (PVDF) membrane. After it is possible to

proceed to the recognition of target protein through the use of a specific antibody; the binding protein-antibody can be displayed using different techniques, including colored products or chemiluminescence autoradiography.

4.1. Sample preparation for WB

Once the protein concentration has been determined, samples are prepared through dilution with PBS and Loading solutions. Loading solution consists in Laemmli BUFFER 4X, (8% SDS, 20% 2-mercaptoehtanol, 40% glycerol, 0.25 M Tris HCl, pH 6.8), previously prepared [266], and bromophenol blue traces. The sodium dodecylsulfate (SDS, anionic detergent) present in the buffer has a dual function (i) being a detergent, favors the denaturation of proteins in combination with other reducing agents (beta-mercaptoethanol (ii) intercalates every two amino acids, giving the denatured protein a negative electric charge; proteins can be well resolved in accordance with their mass in an electrophoretic run. Glycerol is added to the loading buffer to increase the density of the sample to be loaded and hence maintain the sample at the bottom of the well, restricting overflow and uneven gel loading. To enable visualization of the migration of proteins it is common to include in the loading buffer a small anionic dye molecule (e.g., bromophenol blue). Since the dye is anionic and small, it will migrate the fastest of any component in the mixture to be separated and provide a migration front to monitor the separation progress. Leamlly buffer 4X was add to volume of sample that contain the total amount of proteins to load on gels at the final concentration of 1X.

The total amount of protein that was load onto gels depend on the expression level of target protein that we studied.

In order to allow complete denaturation of proteins, the samples have been heated in water at 95°C for 5min and successively kept 5 minutes at environmental temperature. For specific protein determination, the previously obtained mixture of denatured proteins must be separated into single constiuents. In order to do so, the gel electrophoresis method has been utilized, with the help of minigel precast (BioRad).

4.2. SDS-PAGE

For all the experiment performed in this study a gradient precast gels purchase from BIORAD (any kD) were used. In these gels the percentage of two polymers varies uniformly from 12% (the upper part of the gel) to 20% (at the bottom of the same); and they are designed to provide a complete and well-resolved molecular weight protein separation pattern (300kD-5kD).

Protein samples were loaded into wells on the gel cassette, leaving one lane reserved for the marker (mixture of proteins with known molecular weight: Preistained Protein Ladder Marker by BIORAD) and subjected to electrophoresis (Fig. 22). Electrophoretic run was carried out at constant current (100V and max 400 mA), and proteins migrate through the electrophoretic gel for around 90 minutes,separating themselves into band within each lane, in a running buffer at pH 8.8 (Tris 25mM, Glycine 192mM, 1% SDS). At the end of the gel run, the electrophoretic apparatus was disassembled and the gel recovered for the next step.

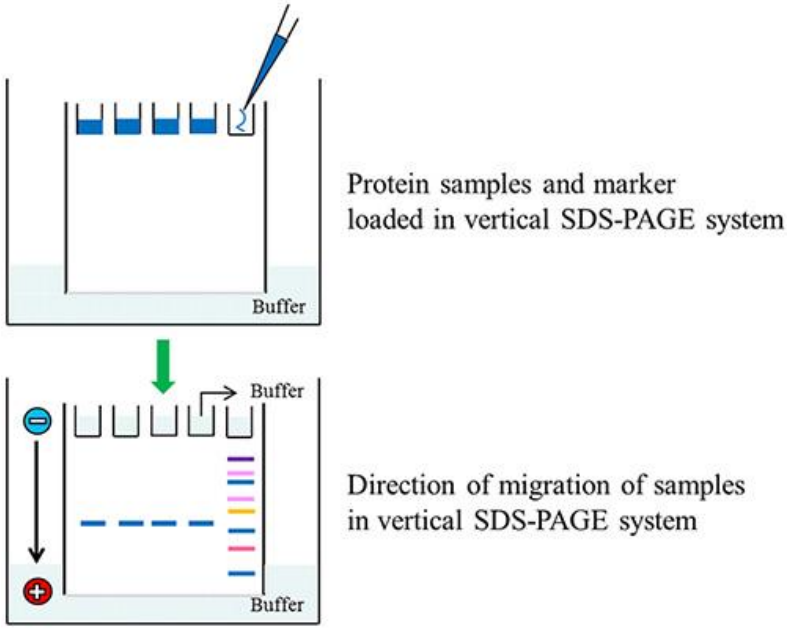


Figure 22: *SDS-PAGE of protein samples and color burst protein marker.*

4.3. Electroblotting

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The primary method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane maintaining their organization by applying an electric field in which the proteins still negatively charged migrate from the negative (gel) to the positive pole (membrane). Because of "blotting" process, the proteins are exposed on a thin surface layer for detection (see

below). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.

In this study the proteins resolved by electrophoresis, are transferred (blotting) to a PVDF membrane (for poly-ubiquitinated proteins). The transfer was carried out at constant voltage at 100V and max 400 mA for 120 minutes at 4°C or 35mA overnight (O/N) in a transfer buffer containing 25mM Tris, Glycine 192mm, and 20% methanol.

The effective proteins transfer to PVDF was verified by staining with Ponceau Red (Sigma) in acetic acid (0.2% Ponceau Red in 3% acetic acid) for 5 minutes under stirring at room temperature; for polyubiquitinated proteins ponceau stained membrane were scanned and images were used for next analysis.

4.4. Target protein detection

To minimize the background, nonspecific binding sites present on the PVDF membrane are saturated with a blocking solution consisting of 5% fat-free milk in TBST 1X (Tris 0.02M, NaCl 0.05M and 0.1% Tween-20) or 2% BSA depending on the protein studied (Table B) for two hours at room temperature with constant shaking. After the blocking phase, the PVDF membrane has been washed with TBST for three times 10’ each and has been probed with a primary antibody (mouse or rabbit-derived), diluted in a solution of TBST 1X containing 5% BSA or 5% MILK (Table A), for the protein of interest and incubated overnight at 4°C on an orbital shaker. After washing and rinsing the membrane to remove any unbound primary antibody, a secondary antibody that binds to the primary antibody has been added and left to react for 1h at ambient temperature. Subsequently, the membrane was washed three times in TBST 1X and then incubated for 60 minutes at room temperature in constant agitation, with a secondary antibody diluited suitably goat anti-mouse or anti rabbit conjugated with the enzyme HRP (Horseradish Peroxidase).

After removing the excess of antibody with two washes of 10 minutes each, in TBST 1X and the last one in TBS 1X (Tris 0.02M and NaCl 0.05M), the protein of interest has been in this way evaluated by enhanced chemiluminescence and the protein content were investigated by the brightness-area product (BAP). Proteins detection was made using ECL advance detection system (Amersham) which highlights the HPRT substrate with a chemiluminescent reaction. The membranes were impressed on photographic films (Kodak) in a dark room, and the exposure time is adjusted in relation to the intensity of the emitted signal.

The PVDF membrane have been successively stripped of all antibodies and unspecific bindings and stored for further analysis of proteins in the same MW range.

	Ab I	MW (kDa)	Ab II	Function
pAKT*	1:2000 – BSA 5% O/N	60	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
AKT*	1:2000 – BSA 5% O/N	60	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
pS6Rp*	1:2000 – BSA 5% O/N	32	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
S6Rp*	1:2000 – BSA 5% O/N	32	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
p4E-BP1*	1:2000 – BSA 5% O/N	15 - 20	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
4E-BP1*	1:2000 – BSA 5% O/N	15 - 20	*Anti-Rabbit 1:10000 MILK 5%	Protein Synthesis
CS**	1:2000 – MILK 5% O/N	52	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
PGC-1α**	1:1000 – MILK 5% O/N	92	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
pAMPK*	1:2000 – BSA 5% O/N	62	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
AMPK*	1:2000 – BSA 5% O/N	62	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
pACC*	1:2000 – BSA 5% O/N	280	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
ACC*	1:2000 – BSA 5% O/N	280	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
Phospho – p38 MAPK**	1:500 – BSA 5% O/N	41	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism

p38 MAPK**	1:500 – BSA 5% O/N	41	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Metabolism
SOD1**	1:1000 – MILK 5% O/N	17	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Stress
Catalase **	1:2000 – MILK 5% O/N	60	*Anti-Rabbit 1:10000 MILK 5%	Oxidative Stress
OPA1**	1:2000 – MILK 5% O/N	112	***Anti-Mouse 1:15000 MILK 5%	Mitochondrial Fusion
MFN1**	1:1000 – MILK 5% O/N	84	***Anti-Mouse 1:15000 MILK 5%	Mitochondrial Fusion
MFN2**	1:2000 – MILK 5% O/N	82	*Anti-Rabbit 1:10000 MILK 5%	Mitochondrial Fusion
Fis1**	1:2000 – MILK 5% O/N	17	*Anti-Rabbit 1:10000 MILK 5%	Mitochondrial Fission
DRP1*	1:1000 – BSA 5% O/N	78	*Anti-Rabbit 1:10000 MILK 5%	Mitochondrial Fission
LC3B*	1:1000 – MILK 5% O/N	16 - 18	*Anti-Rabbit 1:10000 MILK 5%	Autophagy
p62*	1:2000 – MILK 5% O/N	62	*Anti-Rabbit 1:10000 MILK 5%	Autophagy
Polyubiquitinated proteins****	1:2500 – BSA 2% O/N	------	***Anti-Mouse 1:15000 MILK 5%	Autophagy

Table B: Antibodies list used.

***Antibody's company: Cell Signalling**

****Antibody's company: Abcam**

*****Antibody's company: Dako**

******Antibody's company: Enzo Life**

4.5. Data analysis

The bands present on the photographic film were quantified.

The data were expressed as an integrated density (units of optical density per volume of the band). The target protein levels were then normalized with respect to the amount of the housekeeping protein, the actin by Ponceau staining. The data are expressed as the ratio between the target protein and the housekeeping.

5. Gene expression analysis

5.1. RNA extraction from muscle tissue

The muscle tissues still frozen, were pulverized using a sterile pestle and mortar previously treated with RNase Zap to remove RNAse presence. Approximately 20 mg of powder of each sample was used to RNA extraction with SV Total RNA Isolation System (Promega, Italia).

The successful isolation of intact RNA requires four essential steps:

- effective disruption of cells or tissue,
- denaturation of nucleoprotein complexes,
- inactivation of endogenous ribonuclease (RNase) activity,
- removal of contaminating DNA and proteins.

The most important step is the immediate inactivation of endogenous RNases that are released from membrane-bound organelles upon cell disruption. The SV Total RNA Isolation System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β-mercaptoethanol to inactivate the ribonucleases present in cell extracts (2). GTC, in association with SDS, acts to disrupt nucleoprotein complexes, allowing the RNA to be released into solution and isolated free of protein.

Dilution of cell extracts in the presence of high concentrations of GTC causes selective precipitation of cellular proteins to occur, while the RNA remains in solution. After centrifugation to clear the lysate of precipitated proteins and cellular debris, the RNA is selectively precipitated with ethanol and bound to the silica surface of the glass fibres found in the Spin Basket. By effectively clearing the lysate of precipitated proteins and cellular debris, these cleared lysates may be bound to the Spin Baskets by a centrifugation filtration method. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the silica.

RNase-Free DNase I is applied directly to the silica membrane to digest contaminating genomic DNA. The bound total RNA is further purified from contaminating salts, proteins and cellular impurities by simple washing steps. Finally, the total RNA is eluted from the membrane by the

addition of Nuclease-Free Water. This procedure yields an essentially pure fraction of total RNA after only a single round of purification without organic extractions or precipitations.

5.2. RNA quantification

Nucleic acids absorb ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. Using the Beer Lambert Law the amount of light absorbed was related to the concentration of RNA in samples. It has ben evaluated also RNA from proteins contaminants by checking the ratio of the absorbance at 260 and 280nm (A 260/280) that for pure RNA A260/280 is ~2.

5.3. cDNA synthesis

In order to measure messenger RNA (mRNA), it is necessary using a Reverse transcriptase enzyme to convert mRNA into complementary DNA (cDNA) which will be then amplified by Real-Time PCR .

In this study 300 ng of RNA for each sample were reverse transcribed using the Superscript III enzyme (Invitrogen) with this protocol: at 300 ng of RNA of each sample, were addicted 1µl of random primers, 1µl Deoxyribonucleotides (10mM each dATP, dGTP, dCTP and dTTP at neutral pH), and RNAse free water to reach the final volume of 13.5 µl. The mix obtained was heat at 65°C for 5 minutes and incubate on ice for at least 1 minute.

Then 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl) and 0.5 µl of SuperScript™ III RT (200 units/µl) were addicted. The mix was incubated at 25°C for 5 minutes, at 50°C for 60 minutes. Increase the reaction temperature to 70°C for 15 min to inactivate reaction.

5.4. Primer design

Primers were designed using Primer 3 software (Table C). Each primer sequence was test to not have tendency to form secondary structure using Oligonucleotides proprieties calculator (on-line free) that provides a user web interface for calculating the physical properties of oligonucelotides.

Primers were purchase from SIGMA ALDRICH company, resuspended in sterile water at the final concentration of 100µM and stored at -20°C.

	Forward Primer	***Reverse Primer***
MuRF1	CTTCCCTTCTGTGGACTCTTCCT	CCTGAGAGCCATTGACTTTGG
MuRF2*	----------	----------
MuRF3	CAACCTGGAGAAGCAGCTCA	TGCCATAGAGGATTCGAGGC
Atrogin1	GCAGCTGAACAACATTCAGATCAC	CAGCCTCTGCATGATGTTCAGT
FoXO1	AAGAGCGTGCCCTACTTCAA	CTGTTGTTGTCCATGGATGC
FoXO3a	TGTTGGTTTGAACGTGGGGA	GTTTGAGGGTCTGCTTTGCC
Myostatin	TGGCTCAAACAACCTGAATCC	TTCAGTTATCACTTACCAGCCCA
Klf15	GGGAGAGAGGTGAAAAGCGT	TTGTCTGGGAAACCGGAGGA
CS	TCTGGCCTGCTCCTTAGGTA	TGACACACCTACTTTGCAGGAA
PGC-1α	CAGGATTTCATCTGAGTGTGGA	GCGAGAGAGAAAGGAAAAGAACAA
NRF2	CACAGAAGACCCCAACCAGT	CTGTGCTTTCAGGGTGGTTT
OPA1	AGCCTCGCAATTTTTGG	AGCCGATCCTAGTATGAGATAGC
MFN1	ATGACCTGGTGTTAGTAGACAGT	AGACATCAGCATCTAGGCAAAAC
MFN2	CACATGGAGCGTTGTACCAG	TTGAGCACCTCCTTAGCAGAC
Fis1	GTCCAAGAGCACGCAGTTTG	ATGCCTTTACGGATGTCATCATT
DRP1	AAGAACCAACCACAGGCAAC	GTTCACGGCATGACCTTTTT
p62	GCTTCCAGGCGCACTACC	CATCCTCACGTAGGACATGG
Beclin1	TGGAAGGGTCTAAGACGT	GGCTGTGGTAAGTAATGGA
β 2-microglobulin	GCTGTGCTCGCGCTACTCTCTCT	TCTGCTGGATGACGTGAGTAAACCT

Table C: primers used for gene expression experiments.

***MuRF2 Taqman gene expression assay (Applied Biosystems).**

5.5. Primer efficiency validation

In principle, amplicons double at each cycle. The actual production, however, depends of the efficacy of each amplification cycle.

The traditional method for determining amplification efficiency requires a calibration curve, where a sample is serially dilute at known concentration.

For each primer basis a calibration curves with serial diluition (10-1) was performed; Ct values obtained were blotted versus the initial amounts of input material on a semi-log10 plot, and the data were fit to a straight line. It is possible to determine the efficiency for each reaction, by calculating the slopes of the standard curves generated by using the equations:

Exponential Amplification:

$$10^{(-1/\text{slope})}$$

or, Reaction Efficiency:

$$[10^{(-1/\text{slope})}] - 1.$$

Optimal values for slope and efficiency are -3.33 and 1, respectively. These calculations are usually automatically determined by the software and provided with the results. In this project only primers that have an efficiency higher that 0.9 were accepted.

Specificity of each reaction as well as primers dimers possibly formation should be ascertained after completion of the amplification protocol, by performing the Melting procedure (58-99°C; 1°C/5 sec). When most of the fluorescent signal originates from the product of interest during the amplification procedure, a single melting peak is obtained. In contrast, should there be amplification of secondary products, of primer dimers or of non-specific amplicons, several melting peaks are generated at temperatures lower than the melting point expected for the product of interest, precluding any quantitative assessment. In this study all primers pairs that showed a good efficiency, but an atypical melting curve were not used for real time experiment.

6. Real Time PCR

Real Time PCR allows reaction to be characterized by the point in time during cycling when amplification of a PCR products achieves a fixed level of fluorescence, rather than the amount of PCR product accumulated after a fixed number of cycle (PCR end-point).

An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed.

As shown in Fig. 23, the initial cycle of PCR, there is no significant change in fluorescence signal. This predefined range of PCR cycles is called baseline.

The software generate a baseline subtracted amplification plot by calculating a mathematical trend using Rn values (the fluorescence emission intensity of the reporter dye) corresponding to the baseline cycles. Then an algorithm searches for the point on the amplification plot at which the delta Rn (Rn-baseline) crosses the treashold. The fractional cycle at which this occurs is defined as the Ct.

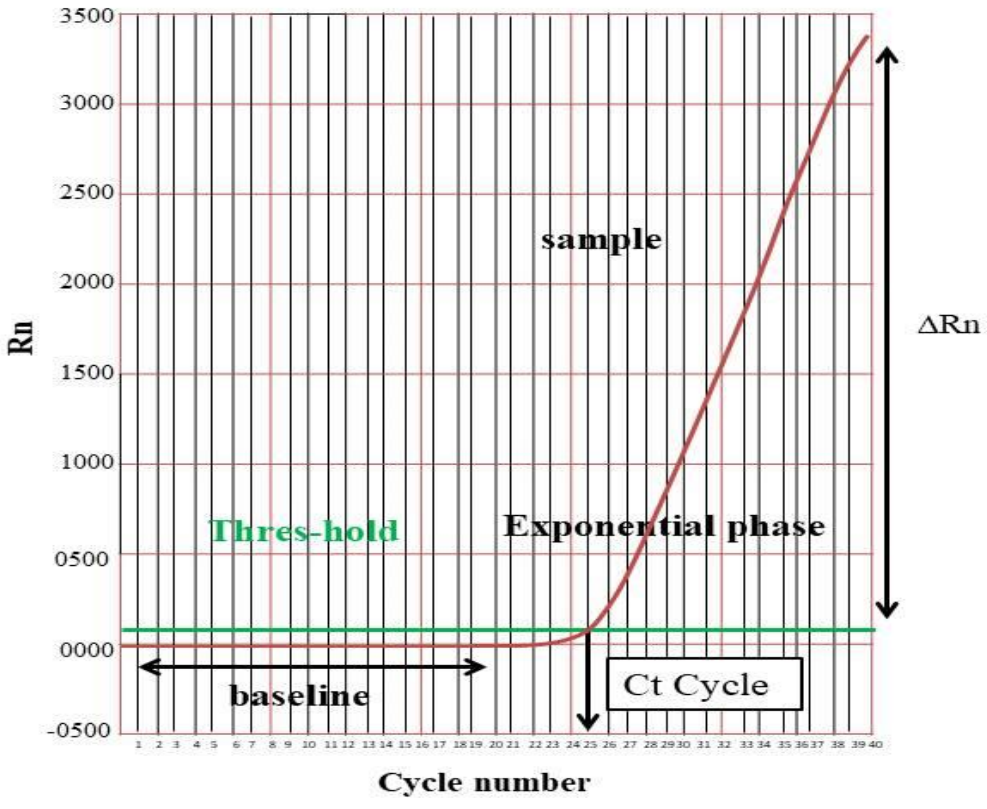


Figure 23: Model of a single sample amplification plot. *In the baseline no significant change in fluorescence signal (Rn) occurs, in the exponential phase fluoresce signal increase in proportional to amplification products increase formation. The threshold line is the level of fluorescence signal automatically determine by the sequence detection system software and it is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The Ct cycle is the number of amplification Cycle when the fluorescence signal cross the threashold.*

In this project Real-time PCR experiments were performed using AB 7500 instrument (Applied Byosistems); and PCR reactions were performed using SYBR Green chemistry (Power Syber Green, Applied Byosistems). Real time PCR reaction was composed by:

- 3µl cDNA
- 12µl sterile water
- 15µl Power Syber Green
- 0,6µl Primers (mix of Forword and Reverse)

Each sample was run in duplicate. PCR thermal cycler parameters were set to standard mode of 10 minute incubation at 95°C before repeat cycling at 95°C for 15 seconds, followed by a 1 minute incubation at 60°C for 40 cycles; were fluorescence signal was detected.

To compensate for variations in input RNA amounts and efficiency of reverse transcription, 18s ribosomal rRNA was quantified and all results were normalized to these values.

6.1. RT-PCR analysis

The ΔCT was calculated by subtracting the CT of baseline biopsy (PRE) to CT of post DEX or placebo administration (POST) for both target gens and housekeeping gene; after this the value obtained from target genes were normalized on the value obtained from housekeeping gene.

7. Statistical analysis

Quantitive variables were expressed as means ± SD. Differences across groups were assessd by applying a Paired t-test. The threshold for statistical significance (alfa) was set at 5% ($p<0,05$).

CHAPTER 6

RESULTS AND DISCUSSION

1. Gene expression at 4h post-administration

1.1. Anti-anabolic pathway

*4h Post – **DEX** administration*

*4h Post – **Placebo** administration*

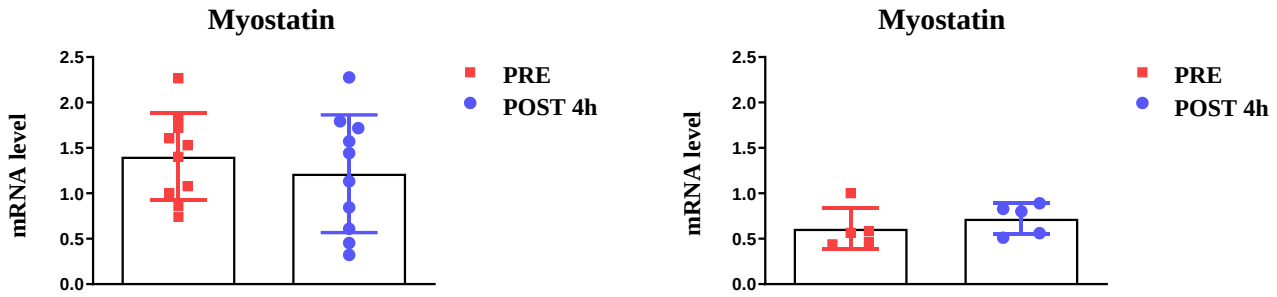


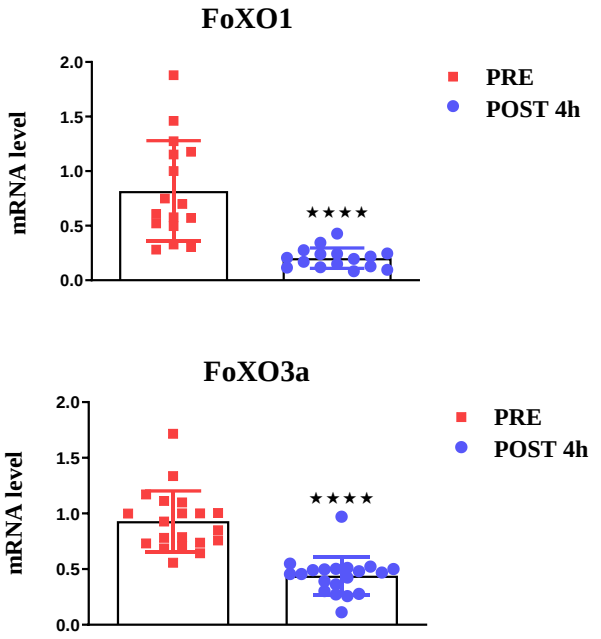
Figure 24: *Analysis of the anti-anabolic marker Myostatin at 4h Post – DEX (on the left) and Placebo (on the right) administration.*

Myostatin is utilized as a putative marker for the inhibition of muscle synthesis.

It has been suggested that DEX-induced muscle atrophy might be associated with the up-regulation of mRNA and protein expression of *Myostatin* both in rats [267] and in mice [268], However, no significant changes have been observed concerning mRNA levels in either DEX or Placebo (Fig. 24). Results are in accordance with those obtained in mice by Canepari and co-workers [225] and suggest that *Myostatin* does not plays a major role in the triggering steroid myopathy.

1.2. Catabolic pathways

*4h Post – **DEX** administration*



*4h Post – **Placebo** administration*

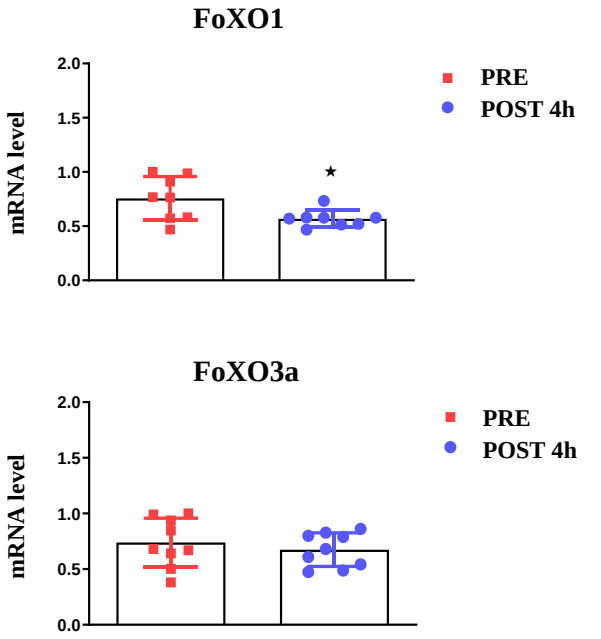
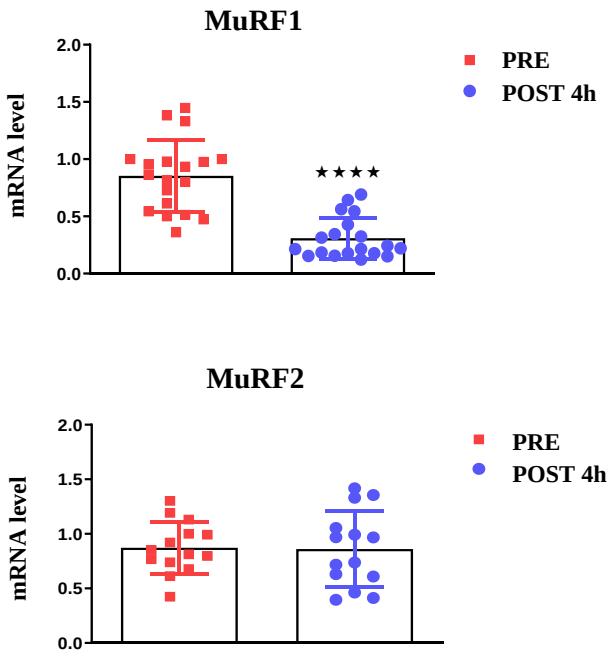
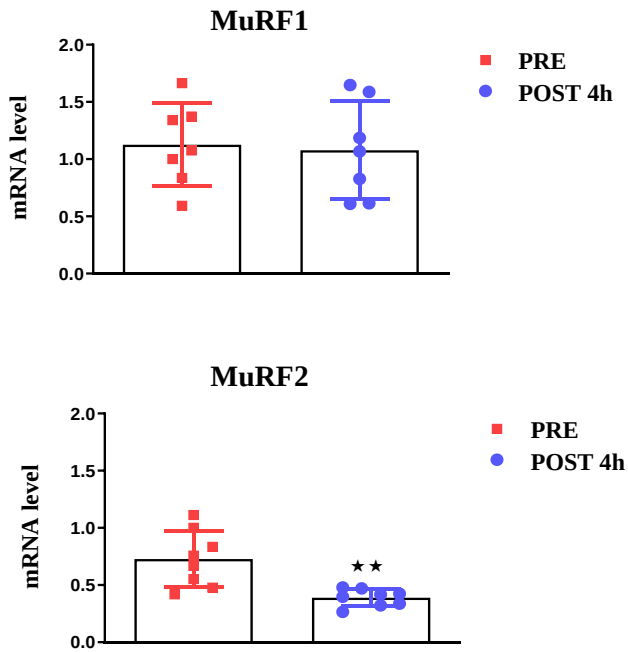


Figure 25: *Analysis of markers involved in protein degradation (FoXO1 – FoXO3a) at 4h Post – DEX (on the left) and Placebo (on the right) administration.★ p ≤ 0,05 vs PRE.*

*4h Post – **DEX** administration*



*4h Post – **Placebo** administration*



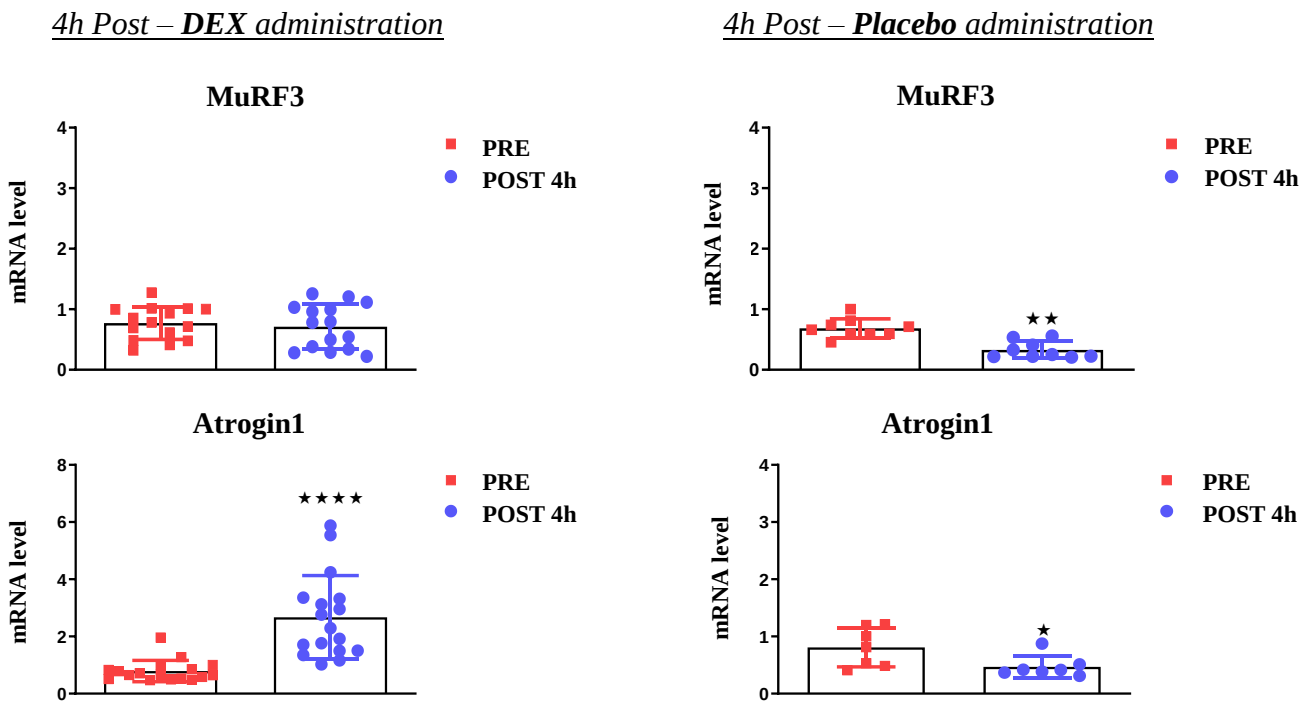


Figure 26: *Analysis of markers involved in protein degradation (MuRF1 – MuRF2 – MuRF3 – Atrogin1) at 4h Post – DEX (on the left) and Placebo (on the right) administration.★ $p \leq 0,05$ vs PRE.*

The major intracellular signalling pathways controlling protein degradation through the ubiquitin proteasome system (*FoXO1, FoXO3a, MuRF1, MuRF2, MuRF3* and *Atrogin1*) were analysed.

A significant decrease in the mRNA expression of FOXO factors and *MuRF1* atrogene is shown after DEX and placebo administration (Fig. 25 and 26). Moreover, no increase in polyubiquitinated proteins was found (Fig. 34). It should indicate the absence of UPS system activation. On the other hand, a significant increase in *Atrogin1* expression due to DEX administration was evident. As *FoXO3a* is generally believed to control both *Atrogin1* and *MuRF-1* and *FoXO3a* is downregulated, a down-regulation of both atrogens would be expected [106], but different regulation mechanisms could also be [269] [270].

However, while FoXO regulate *Atrogin1* and *MuRF1* in rodents, observations in several clinical models of human muscle atrophy including ALS [271], COPD [272], ageing [273] and spinal cord injury [203] suggest that *Atrogin1* regulation by *FoXO3a* is not a major mechanism.

Others have also demonstrated a similar discordance in the regulation of FoXO of *Atrogin1* and *MuRF1* in human skeletal muscle following running [274] [275] and after short-term limb immobilisation [276]. Differential regulation by FoXOs in animals versus humans may help explaining other inconsistencies found between model systems. For example, fasting increases MAFbx in mice [99] whereas this condition has no apparent effect in humans [277].

Moreover, there are some evidences that *Atrogin1* than by *MuRF1* influences human skeletal muscle atrophy more [278] [271].

Collectively, the analysis of catabolic pathways suggests that DEX administration cause the activation of the ubiquitin-proteasome system and in turn muscle rotein breakdown.

1.3. Transcription Factor Krüppel

*4h Post – **DEX** administration* *4h Post – **Placebo** administration*

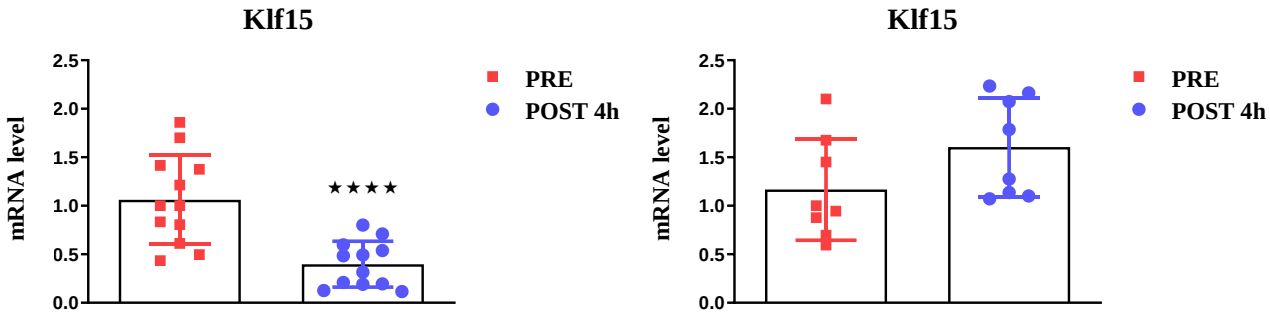


Figure 27: *Analysis of Transcription Factor Krüppel (Klf15) at 4h Post – DEX (on the left) and Placebo (on the right) administration.★ $p \leq 0,05$ vs PRE.*

Recently, it has been shown that steroids rely on Krüppellike factor 15 (*Klf15*), a Krüppel like transcription factor, mediating ergogenic muscle performance effects [279]. As shown in Fig.27 a significant decrease in *Klf15* expression was found. *Klf15* is a direct target of the GCs [280] [281]. It was found that different steroid dosing are able to induce divergent *Klf15* gene expression and genetic program. There is a dose-dependent difference in occupancy of the *Klf15* promoter by GCs, eliciting differential effects. In skeletal muscle, molecular pathways of atrophy were triggered by daily dosing of DEX and this was accompanied by decreased expression of *Klf15*, elevated expression of *Atrogin1* and reduced performance of voluntary hind limb and respiratory skeletal muscles. In contrast, intermittent dosing of DEX induced an increase in *Klf15* expression and did not trigger atrophy but, on the contrary, improved muscle performance [282]. The GC induced decrease in Klfl5 expression 4h after a single injection indicate that GS elicetd their action by 4h and suggest that the catabolic adaptations observed could depend on GC through Klfl5.

1.4. Oxidative metabolism

*4h Post – **DEX** administration*

*4h Post – **Placebo** administration*

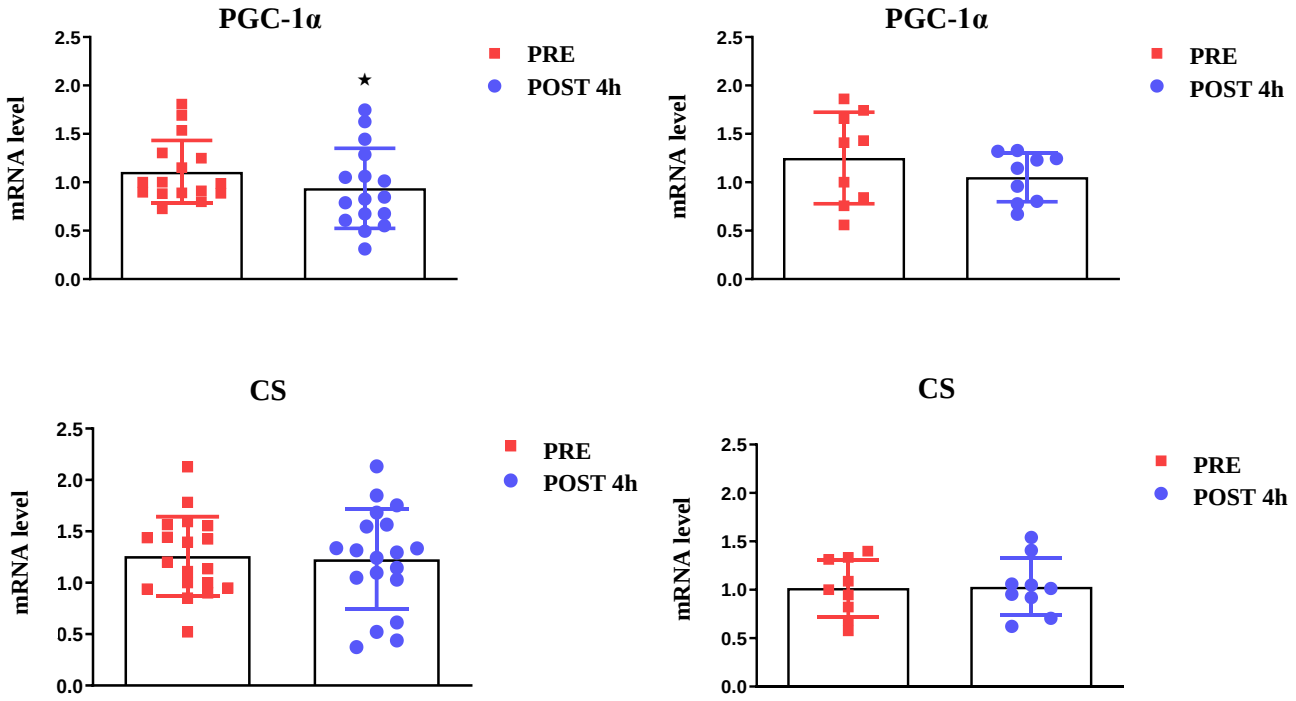


Figure 28: *Analysis of markers involved in the oxidative metabolism (PGC-1α – CS) at 4h Post – DEX (on the left) and Placebo (on the right) administration.* ★ *p ≤ 0,05 vs PRE.*

PGC-1α and *CS*, markers involved in oxidative metabolism, were studied. Gene expression of *PGC-1α* appears to be significantly decreased 4 hours after the administration of DEX (Fig. 28). These data suggest a metabolic alteration linked to the administration of DEX. DEX could reduce *PGC-1α* expression in muscle cells by several mechanisms [283]. DEX can act directly on *PGC-1α* gene expression by binding to the GRE present on its promoter [284]. DEX can also change the activity of other trascriptional factors that partecipate in *PGC-1α* trascription (e.g. MEF2, NFAT). Morover DEX could also increase the level of an unidentified microRNA(s) that targets *PGC-1α* in skeletal muscle. Finally, DEX could induce a decreases in activity of p38 MAPK [234] (Fig. 37). The action of p38 MAPK on *PGC-1α* induced by DEX may represent a novel mechanism by which this agent induce muscle atrophy [234].

Interestingly, a decrease in *PGC-1α* gene expression and a consequent oxidative metabolism pathway dysfunction may result in reduced ATP production leading to an increase in the AMP / ATP ratio. Such imbalance could lead to the activation of AMPK, the kinase activated by the AMP and the Acetyl-CoA carboxylase, as it will be shown later in the section of protein expression later (Fig. 36).

1.5. Oxidative stress

*4h Post – **DEX** administration*

*4h Post – **Placebo** administration*

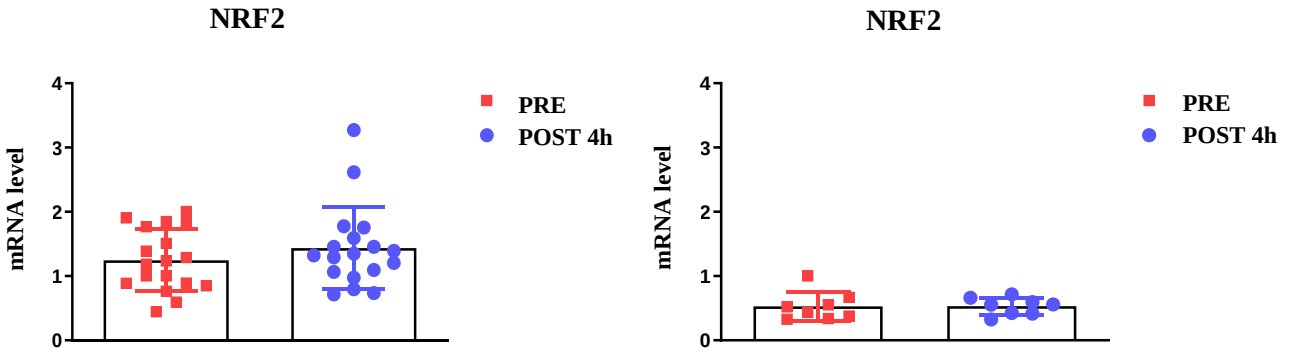


Figure 29: *Analysis of oxidative stress marker NRF2 at 4h Post – DEX (on the left) and Placebo (on the right) administration.*

Fig. 29 shows that the expression levels of the transcription factor *NRF2* do not change 4 hours after DEX administration. *NRF2* is redox sensor, which is upregulated by increased ROS in the cell and in turn activate the transcription of redox buffers (e.g. catalase, SOD). Given that, no change in expression occurs and that previous works from our laboratory, suggest that *NRF2* is likely sentive to very small variaitons of redox balance, it can be suggested that 4h after DEX administration no redox imbalance was present. The result is interesting as oxidative stress is considered among the potential triggers of steroid myopathy.

1.6. Mitochondrial dynamics: Fusion

*4h Post – **DEX** administration* *4h Post – **Placebo** administration*

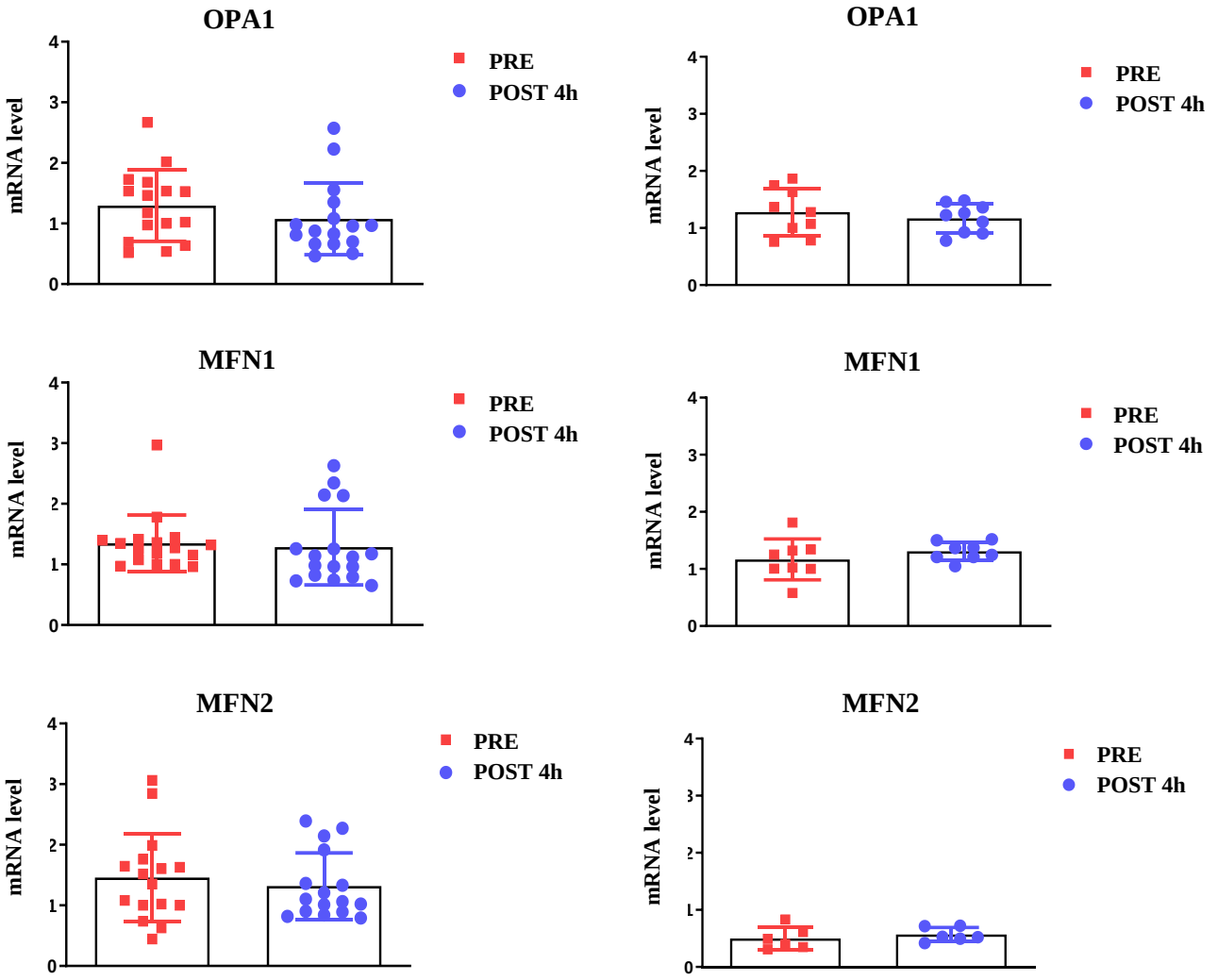


Figure 30: *Analysis of mitochondrial fusion markers (OPA1 – MFN1 – MFN2) at 4h Post – DEX (on the left) and Placebo (on the right) administration.*

Mitochondrial dynamics has been shown to be one of the most relevant factors affecting muscle integrity, i.e. a metabolic program could modulate the balance between muscle protein synthesis (MPS) and degradation (MPB). Mitochondrial dynamics is controlled by an interplay between fusion and fission. When fission prevailes over fusion, mitochondrial dysfuntion can occur triggering muscle wasting. The gene expression of mitochondrial remodeling factors, *OPA1*, *MFN1* and *MFN2*, involved in the process of fusion of the internal mitochondrial membrane was analyzed. They were not significantly changed 4 hours post administration of DEX (Fig. 30).

Mitochondrial fusion process seems not to be alterated by DEX.

1.7. Mitochondrial dynamics: Fission

*4h Post – **DEX** administration*

*4h Post – **Placebo** administration*

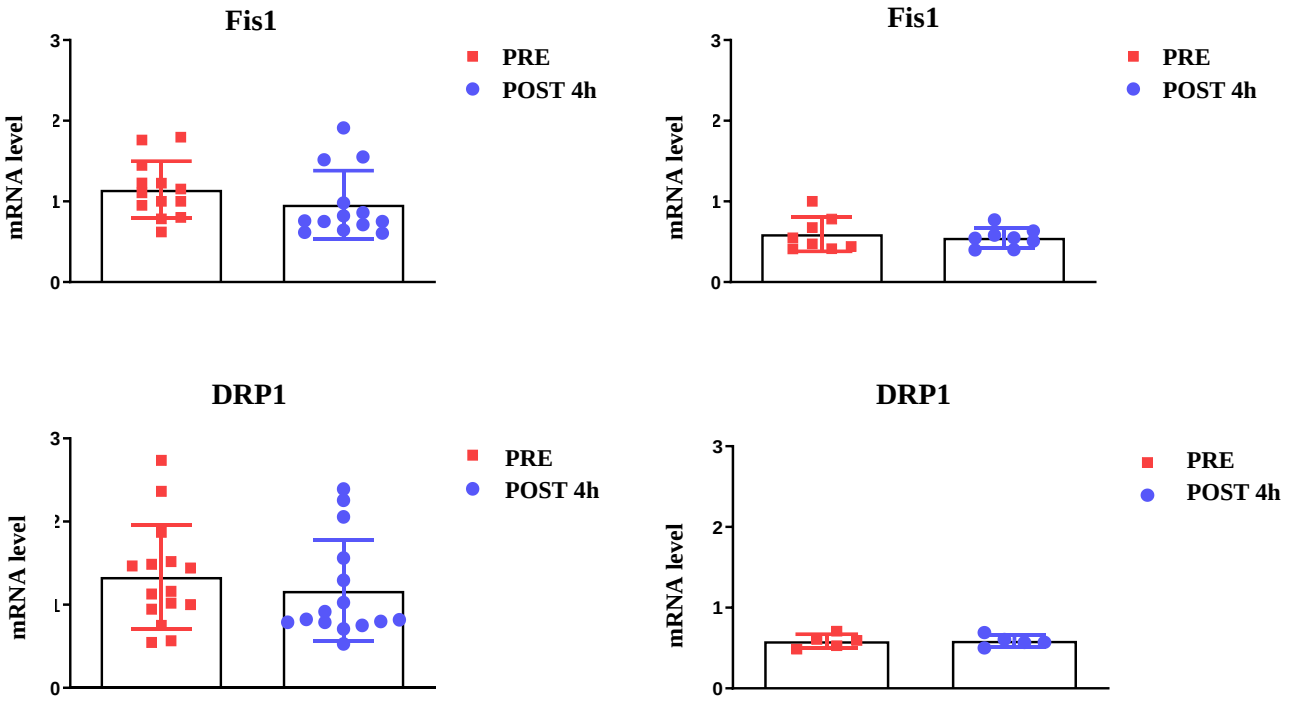


Figure 31: *Analysis of mitochondrial fission markers (Fis1 – DRP1) at 4h Post – DEX (on the left) and Placebo (on the right) administration.*

The gene expression of mitochondrial remodeling factors, *Fis1* and *DRP1*, involved in the process of mitochondrial fission was analyzed. Both the factors were not significantly changed after 4 hours post administration of DEX (Fig. 31).

Mitochondrial fission process appears not to be alterated.

1.8. Autophagy

*4h Post – **DEX** administration*

*4h Post – **Placebo** administration*

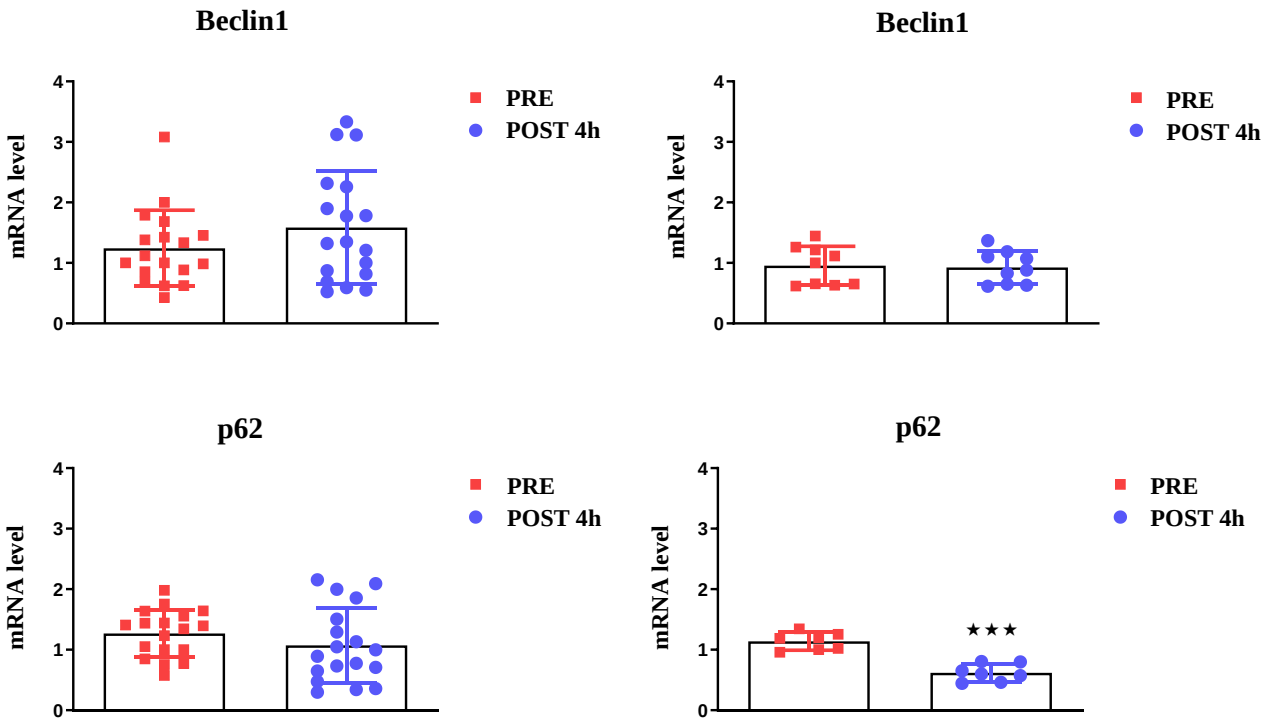


Figure 32: *Analysis of autophagy markers (Beclin1 – p62) at 4h Post – DEX (on the left) and Placebo (on the right) administration.* ★ *p ≤ 0,05 vs PRE.*

The gene expression of *Beclin1* and *p62*, two proteins which are widley used as markers of the activity of autophagy, were analyzed. Both the factors were not significantly changed after 4 hours post administration of DEX (Fig. 32).

The autophagy process, which together with the ubiquitine proteasome system controls MPB, is not activated by DEX.

2. Protein expression at 4h post-administration

2.1. Protein synthesis pathways

*4h Post – **DEX** administration*

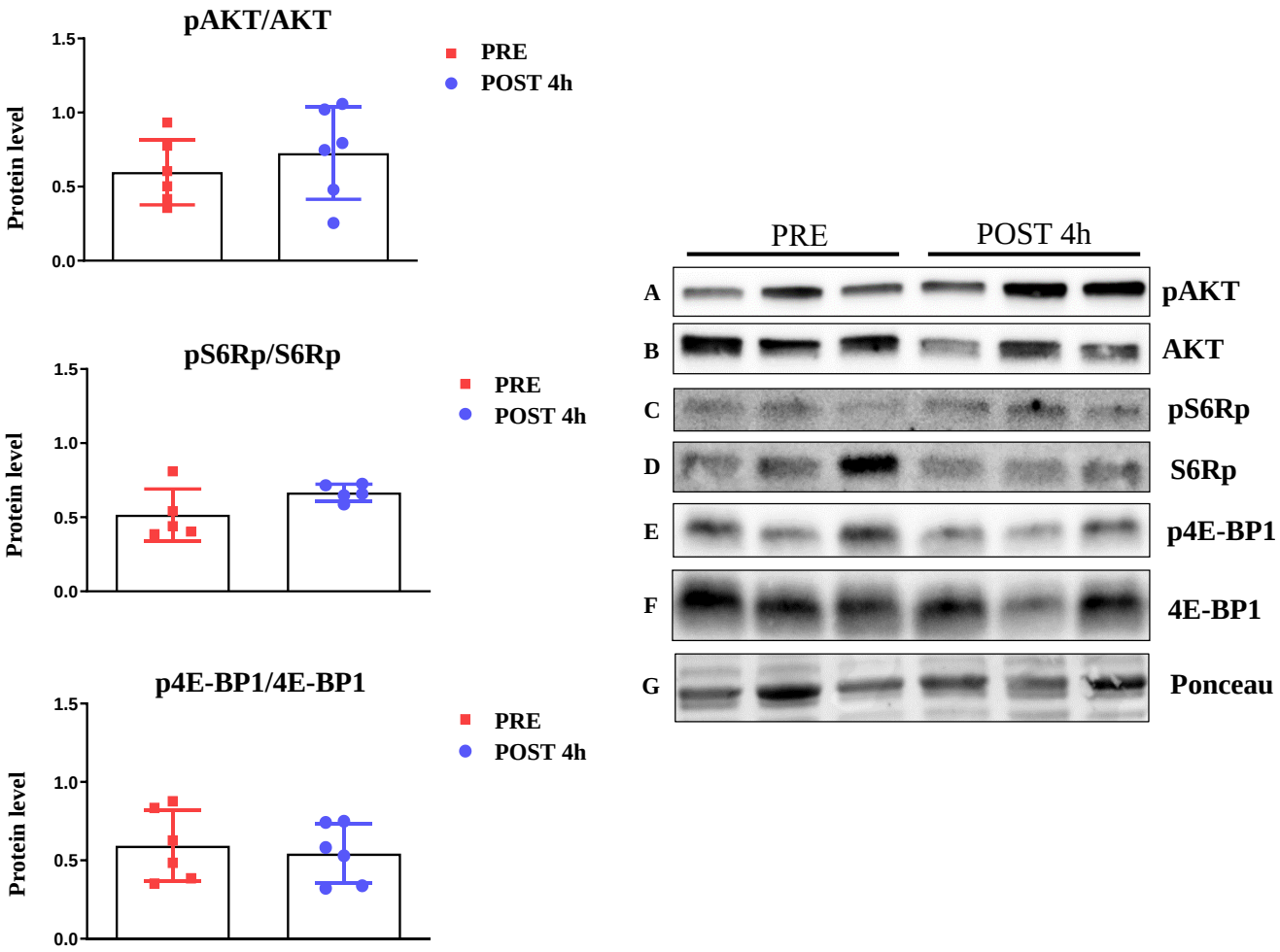


Figure 33: *Analysis of protein synthesis markers (pAKT/AKT – pS6Rp/S6Rp – p4EBP1/4EBP1) at 4h Post – DEX administration and the respective western blot images (from A to F). Actin image by Ponceau staining (G).*

The response of the IGF-1/AKT/mTOR pathway controlling protein synthesis was investigated by measuring the ratio between phosphorylated and total form of AKT, 4E-BP1 and S6Rp. Fig. 33 shows that the synthesis pathway is not significantly affected at 4h post DEX administration. These results suggest a limited or nearly absent influence of dexamethasone on this pathway. The decrease in protein synthesis seems not play a major role in the initial development of steroid atrophy. Data are in accordance with data on mice by Canepari and co-workers [225].

2.2. Catabolic pathway

*4h Post – **DEX** administration*

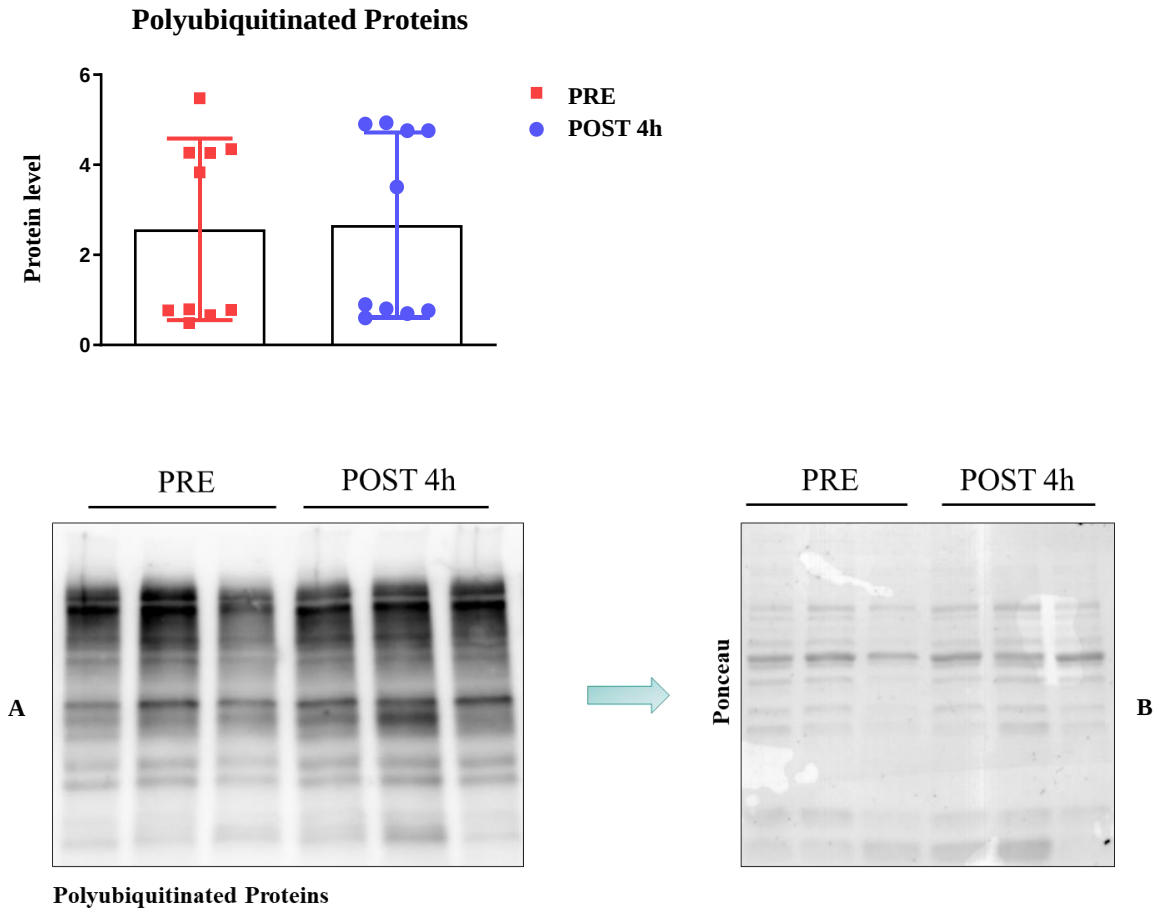


Figure 34: *Analysis of Polyubiquitinated proteins at 4h Post – DEX administration and the respective western blot image (A). Actin image by Ponceau staining (B).*

Levels of poly-ubiquitinated proteins, which are targeted by the UPS system, did not significantly increase at 4h from DEX administration (Fig. 34).

Data with placebo are not shown as identical to DEX.

2.3. Oxidative metabolism

4h Post – ***DEX*** administration

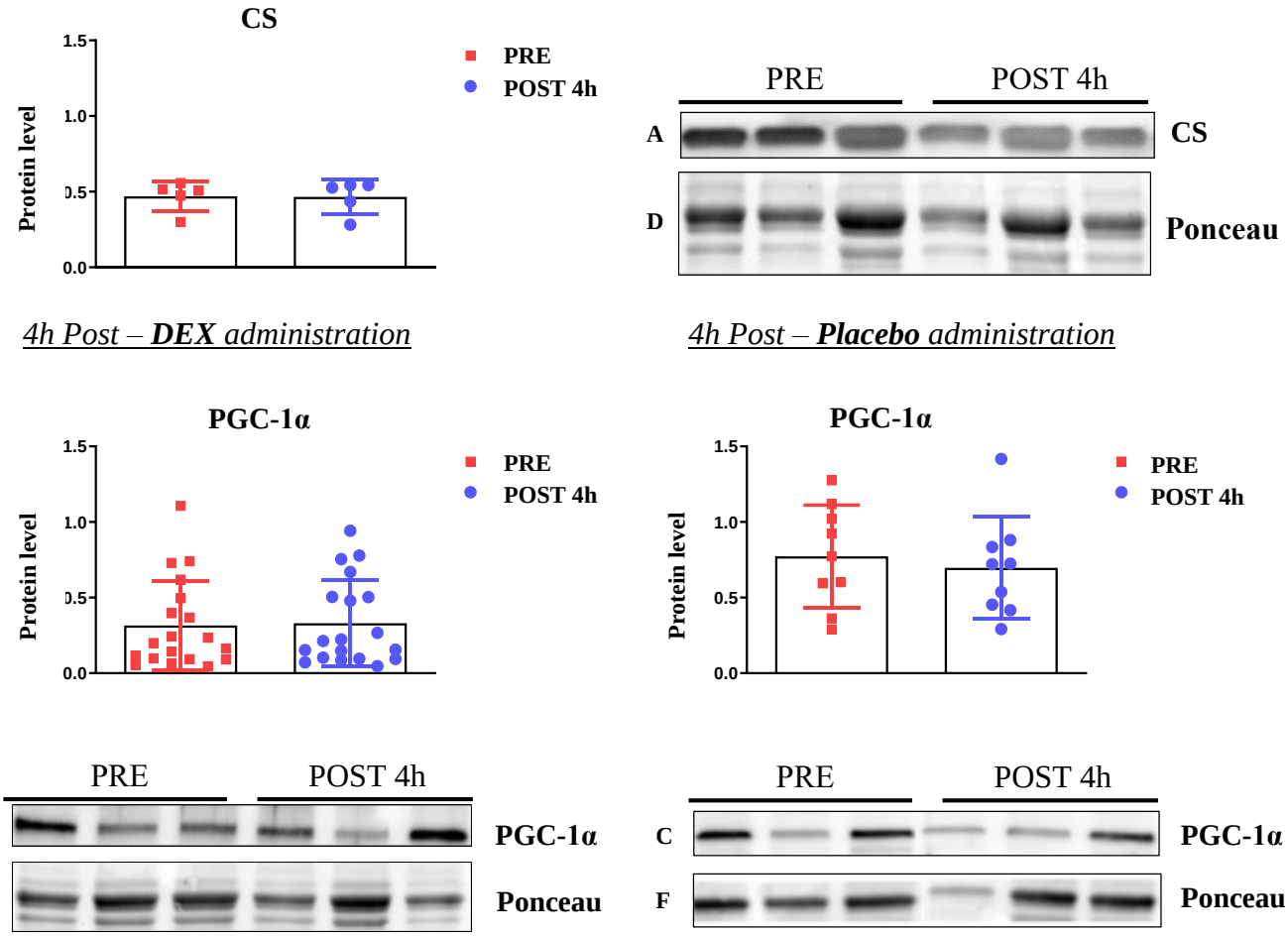


Figure 35: *Analysis of oxidative metabolism markers (PGC-1α – CS) at 4h Post – DEX (on the left) and Placebo (on the right: only for PGC-1α) administration and the respective western blot images (DEX: A and B; Placebo: C). Actin images by Ponceau staining (D, E and F).*

The results in Fig. 35 show that protein contents of both *PGC-1α* and *CS* do not change at 4 hours after the administration of DEX.

2.4. Energy imbalance

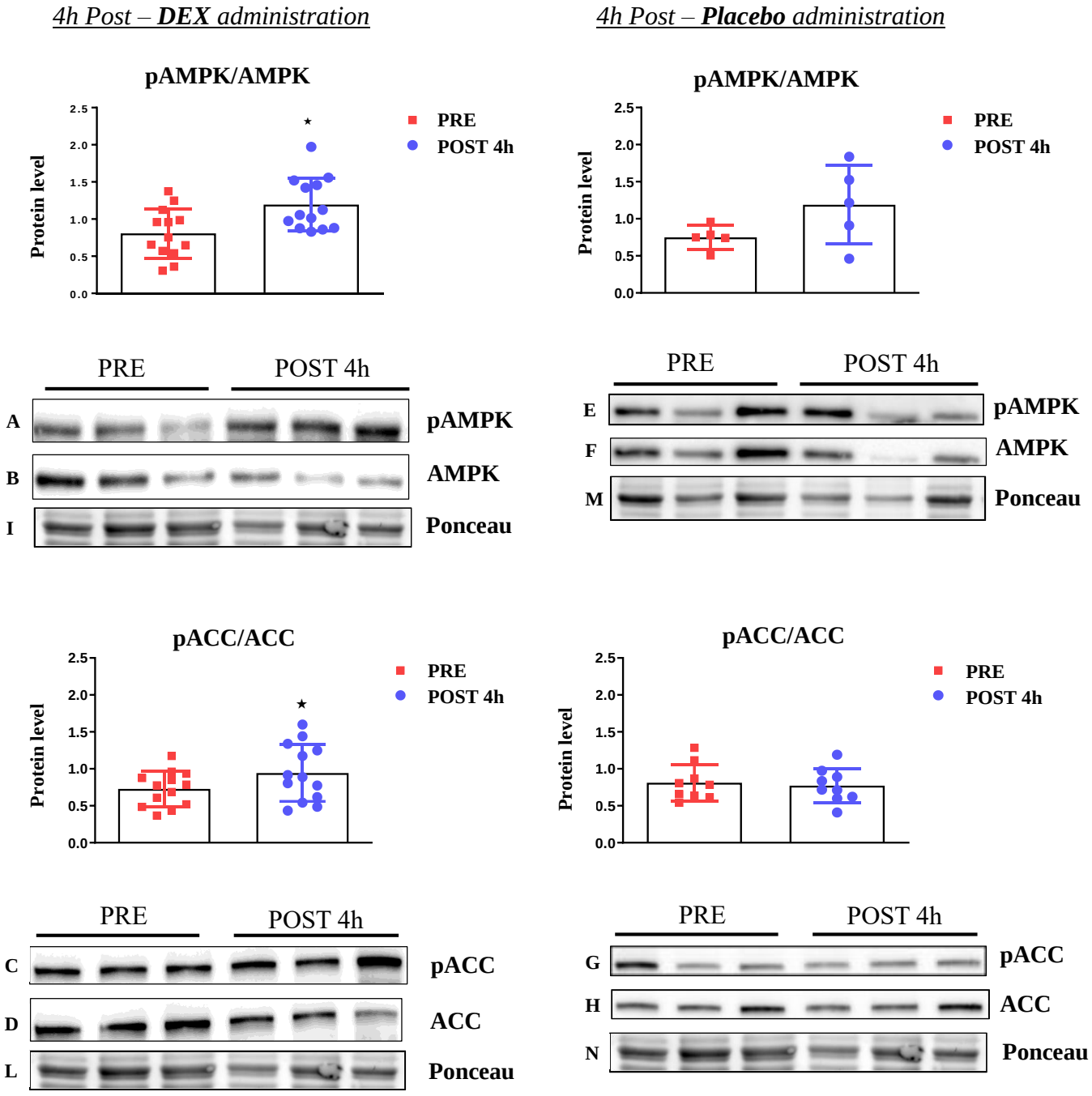


Figure 36: *Analysis of the cellular kinase AMPK and ACC, a kinase downstrem of AMPK, markers of energy imbalance at 4h Post – DEX (on the left) and Placebo (on the right) administration and the respective western blot images (DEX: A, B, C and D; Placebo: E, F, G and H). Actin images by Ponceau staining (I, L, M and N).★ $p \leq 0,05$ vs PRE.*

The energy imbalance of the muscular cells was assessed by measuring the ratio between phosphorylated and total form of AMPK (kinase activated by the AMP and the Acetyl-CoA carboxylase) and ACC, a kinase downstrem of AMPK. The AMPK system is activated when cells need energy and triggers several downstream phenomena among which protein degradation to

provide aminoacids for energy production and for the synthesis of essential proteins. A significant increase in both pAMK and pACC was found (Fig. 36) suggesting an activaton of the AMPK system. The decrease in *PGC-1α* mRNA expression (Fig. 28) could cause a reduced ATP production due to impairment of the oxidative metabolic pathway, generating energy imbalance and leading to the activation of AMPK and ACC in order to revert the imbalance.

2.5. p38 MAPK

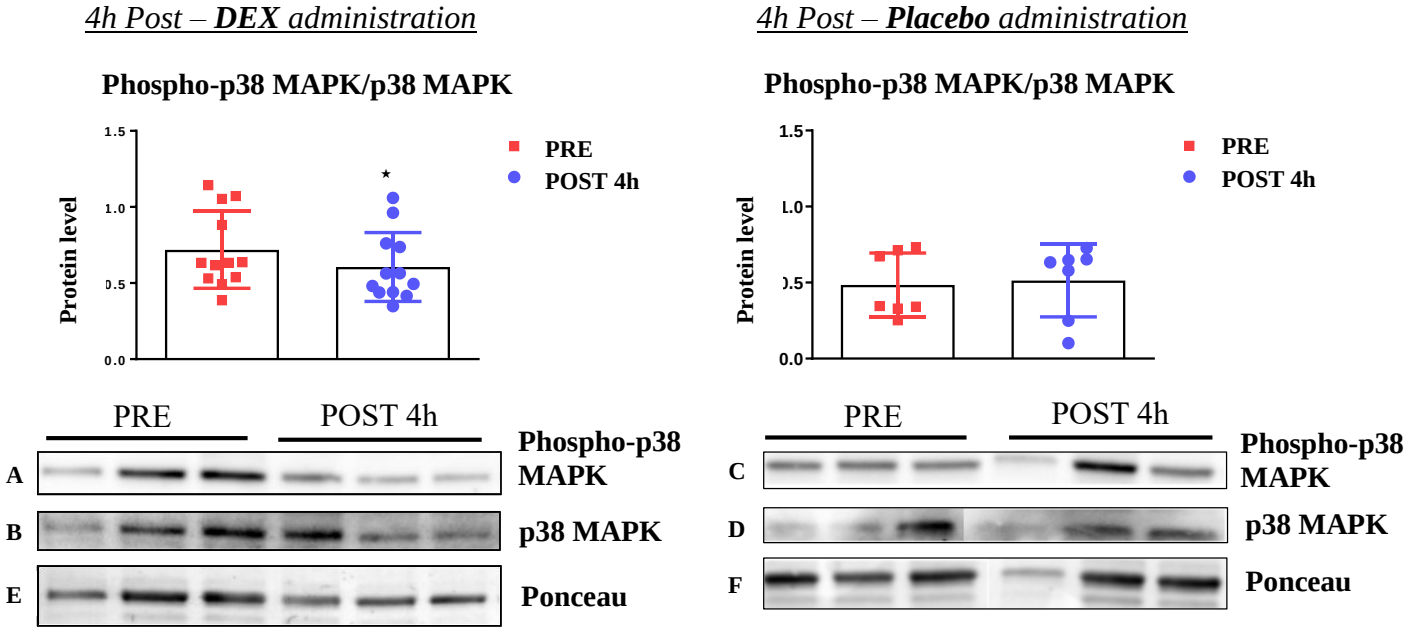


Figure 37: *Analysis of Mammalian p38 mitogen-activated protein kinase (p38 MAPK) at 4h Post – DEX (on the left) and Placebo (on the right) administration and the respective western blot images (DEX: A and B; Placebo: C and D). Actin images by Ponceau staining (E and F).★p ≤ 0,05 vs PRE.*

A significant decrease in p38 MAPK phosphorylation was found (Fig. 37). It has been shown that p38 MAPK could phosphorylates and activate *PGC-1α* [285] [286] [234]. Activity of p38 MAPK has been linked to increased transactivating activity of *PGC-1α* and to its nuclear migration [287]. A plausible explanation for the decreased gene expression of *PGC-1α* caused by DEX could be the associated reduction in p38 MAPK activity [234]. A reduction in p38 MAPK phosphorylation caused by DEX in rat skeletal muscle was also consistent with prior reports from cell culture systems [288].

2.6. Oxidative stress

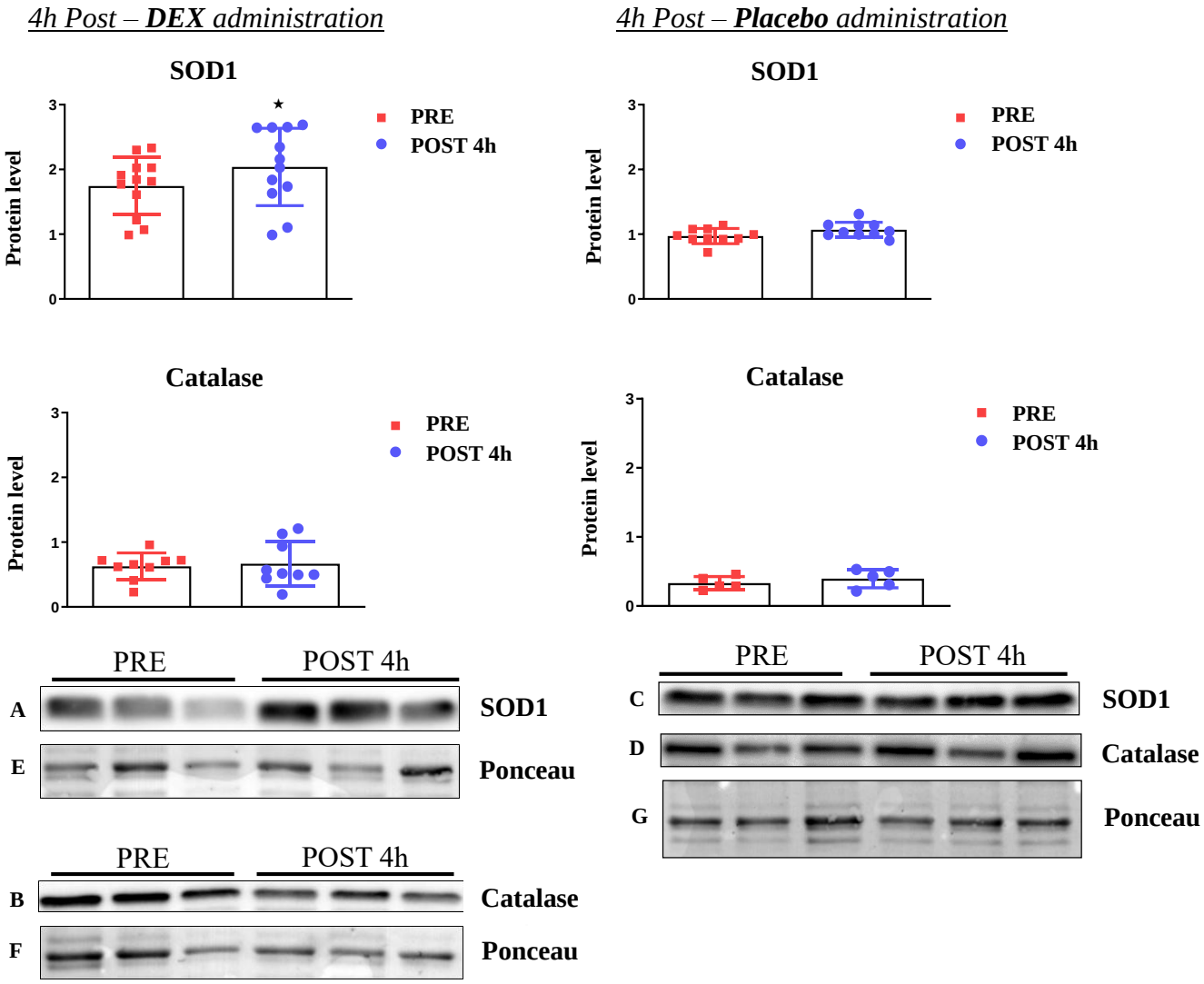


Figure 38: *Analysis of oxidative stress markers (SOD1 – Catalase) at 4h Post – DEX (on the left) and Placebo (on the right) administration and the respective western blot images (DEX: A and B; Placebo: C and D). Actin images by Ponceau staining (E, F and G).★ $p \leq 0{,}05$ vs PRE.*

Antioxidant enzymes are essential for the maintenance of redox homeostasis and prevent any harmful effects caused by the accumulation of free radicals that can be formed following mitochondrial dysfunction.

Fig. 38 shows that there was a significant increase of SOD1 protein level at 4 hours after DEX administration, while there were no changes in the protein level of catalase. The increase in SOD1 protein expression suggest the presence of a mild redox imbalance at 4h after the administration of DEX. As expression of *NRF2* was not upregulated at the same time, up-regulation of SOD likely compensate for an earlier, small and transient increase in ROS.

2.7. Mitochondrial dynamics: Fusion

*4h Post – **DEX** administration*

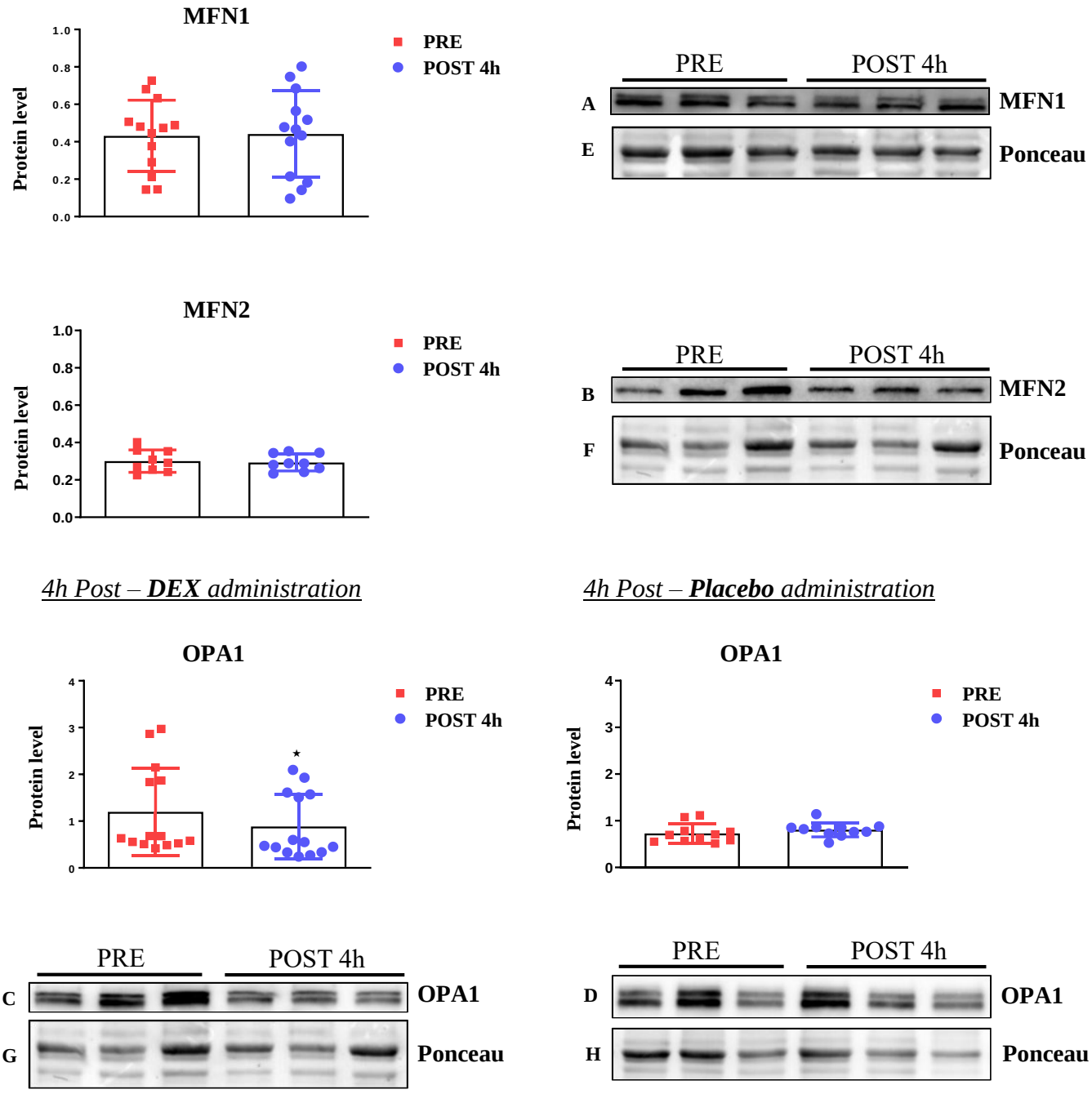


Figure 39: *Analysis of mitochondrial fusion markers (MFN1 – MFN2 – OPA1) at 4h Post – DEX (on the left) and Placebo (on the right) administration and the respective western blot images (DEX: A, B and C; Placebo: D). Actin images by Ponceau staining (E, F, G and H).★ $p \leq 0,05$ vs PRE.*

Protein levels of mitochondrial remodeling factors, *MFN1*, *MFN2* and *OPA1*, involved in the process of fusion of the internal mitochondrial membrane were analyzed. The results showed that only *OPA1* decreased significantly 4 hours after the administration of DEX indicating an imparment of the mitochondrial fusion process (Fig. 39).

2.8. Mitochondrial dynamics: Fission

*4h Post – **DEX** administration*

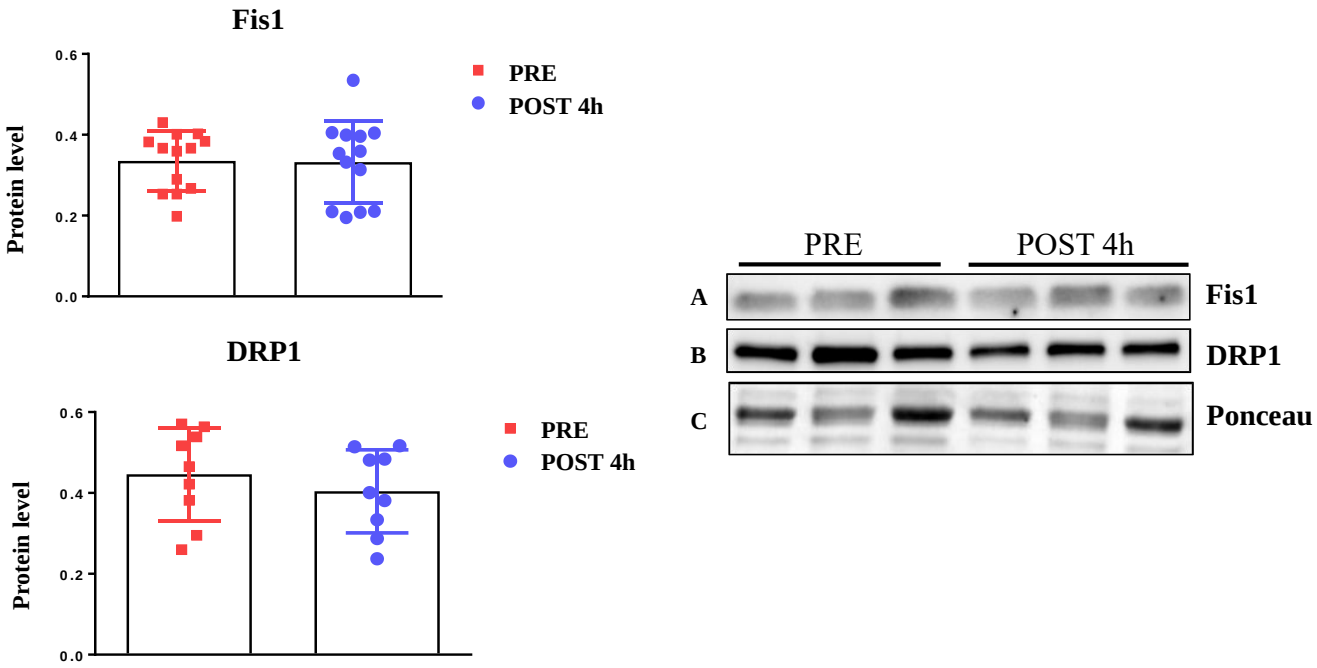


Figure 40: *Analysis of mitochondrial fission markers (Fis1 – DRP1) at 4h Post – DEX administration and the respective western blot images (A and B). Actin image by Ponceau staining (C).*

With regard to mitochondrial fission, the *Fis1* and *DRP1* markers were analyzed and no significant changes were found (Fig. 40). The mitochondrial fission process appears not to be alterated. Data with placebo were not shown as identical to DEX.

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2.9. Autophagy

4h Post – **DEX** administration

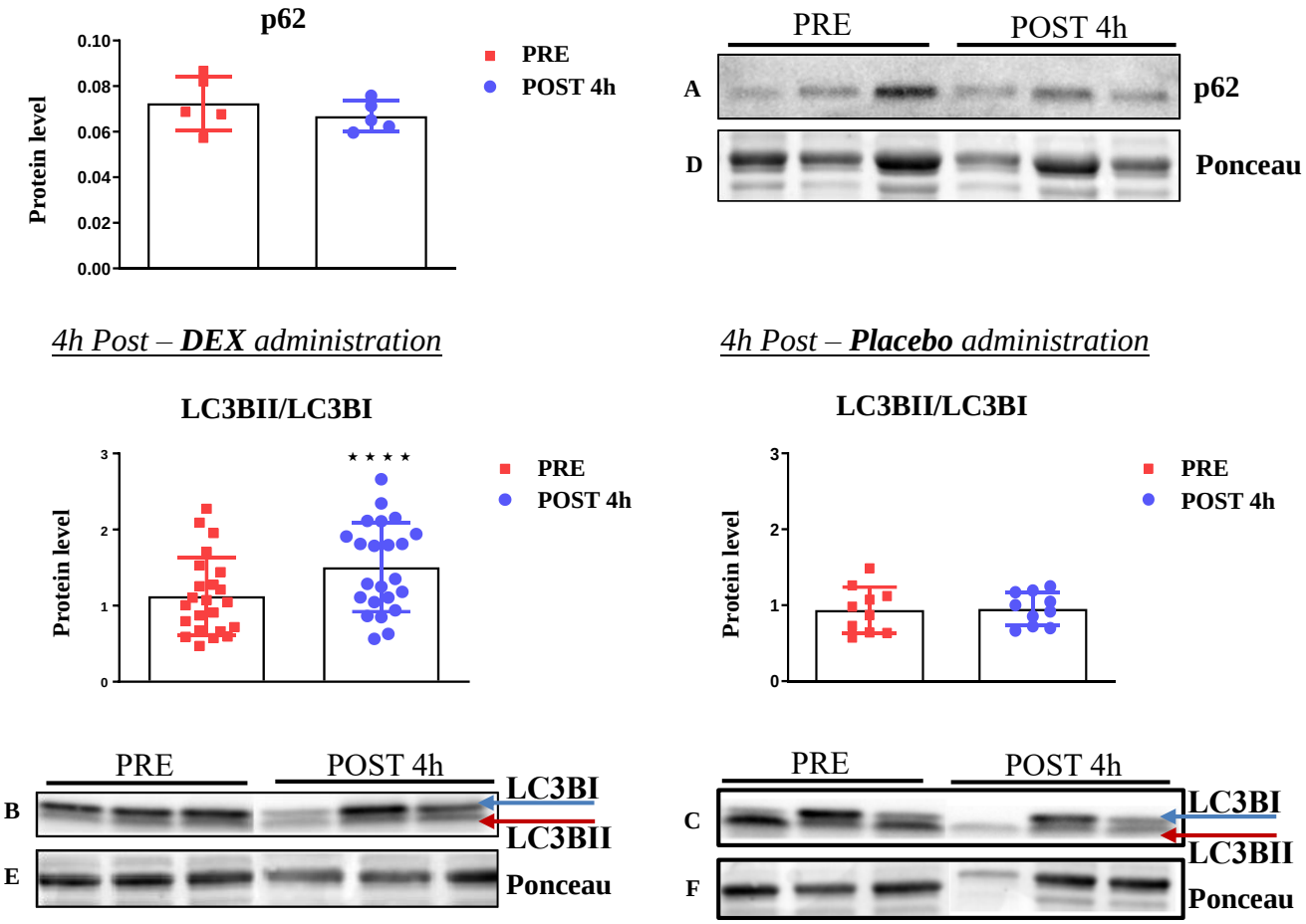


Figure 41: *Analysis of autophagy markers (p62 – LC3BII/LC3BI) at 4h Post – DEX (on the left) and Placebo (on the right) administration and the respective western blot images (DEX: A and B; Placebo: C). Actin images by Ponceau staining (D, E and F).★ $p \leq 0,05$ vs PRE.*

With regard to autophagy, the protein expression of *p62* and the relationship between the active form and the inactive form of LC3B (LC3BII / LC3BI) was analyzed. While the protein expression of *p62*, a protein involved in the process of elimination of protein aggregates, does not undergo changes, the ratio LC3BII / LC3BI is significantly increased at 4 hours after DEX administration (Fig. 41) indicating the activation of autophagy process. The activation of the autophagic process can be useful to the cell both to eliminate damaged and therefore non-functioning intracellular proteins and organelles and to stimulate the production of ATP [149] [289]. Since results demonstrate a decrease in production of ATP (activation of AMPK and ACC in Fig. 36) and a decrease in mitochondrial fusion process, (decreased protein expression of *OPA1* in Fig. 39) is reasonable to hypothesize the activation of the autophagic process as a preventive/compensatory mechanism that the cell enacts to try to counteract the effect of glucocorticoids. The activation of

autophagy could be understood as an attempt by the cell to obtain energy in conditions of ATP deficiency, coherently with what reported in the literature [290]. Since *p62* is involved in the communication between ubiquitinated proteins and autophagy, but its expression does not change this seems to confirm the previous results obtained from the analysis of ubiquitinated proteins (Fig. 34).

3. Gene expression at 1h post-administration

3.1. Anti-anabolic pathway

*1h Post – **DEX** administration*

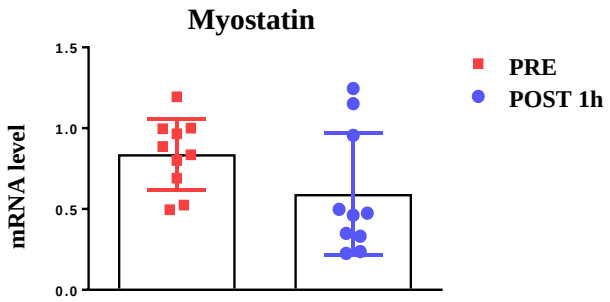


Figure 42: *Analysis of the anti-anabolic marker Myostatin at 1h Post – DEX administration.*

No significant changes have been observed concerning mRNA levels of *Myostatin* 1h post DEX administration (Fig. 42). The result confirms the hypothesis that *Myostatin* does not play a primary role in trigger steroid myopathy.

3.2. Catabolic pathways

*1h Post – **DEX** administration*

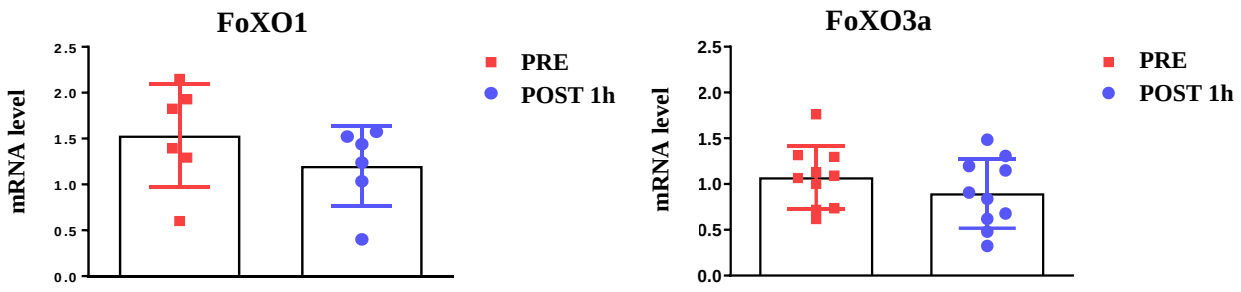


Figure 43: *Analysis of markers involved in protein degradation (FoXO1 – FoXO3a) at 1h Post – DEX administration.*

*1h Post – **DEX** administration*

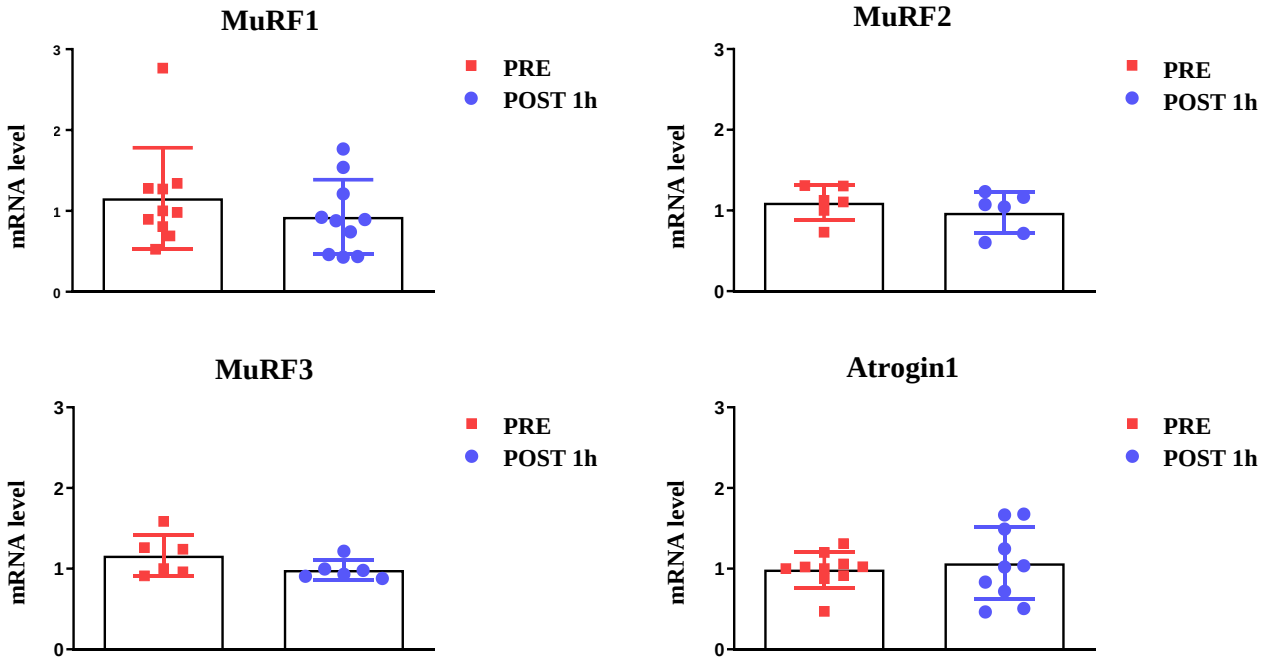


Figure 44: *Analysis of markers involved in protein degradation (MuRF1 – MuRF2 – MuRF3 – Atrogin1) at 1h Post – DEX administration.*

As shown in Fig. 43 and 44, the catabolic pathway UPS did not appear to be activated at 1h after DEX administration.

3.3. Transcription Factor Krüppel

*1h Post – **DEX** administration*

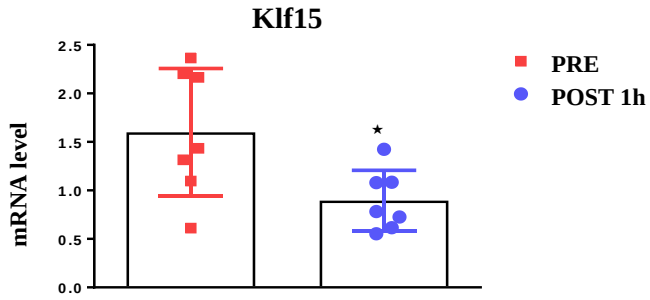


Figure 45: *Analysis of Transcription Factor Krüppel (Klf15) at 1h Post – DEX administration.★ p ≤ 0,05 vs PRE.*

As shown in Fig. 45 a significant decrease in *Klf15* expression was found at 1h after DEX administration. The result confirm the hypotesis that DEX suppresses *Klf15* [282].

3.4. Oxidative metabolism

*1h Post – **DEX** administration*

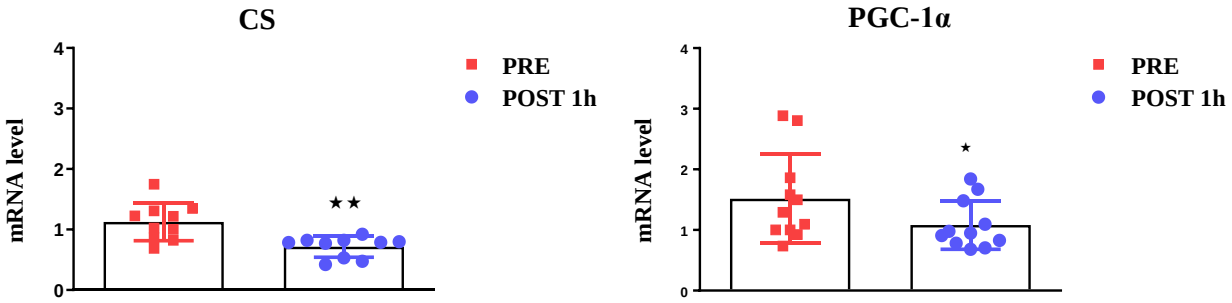


Figure 46: *Analysis of markers involved in the oxidative metabolism (PGC-1α – CS) at 1h Post – DEX administration. ★ p ≤ 0,05 vs PRE.*

Gene expression of *PGC-1α* and *CS* appear to be significantly decreased 1 hours after the administration of DEX (Fig. 46). Data suggest that a metabolic alteration could be the primary mechanism triggering steroid atrophy.

3.5. Oxidative stress

*1h Post – **DEX** administration*

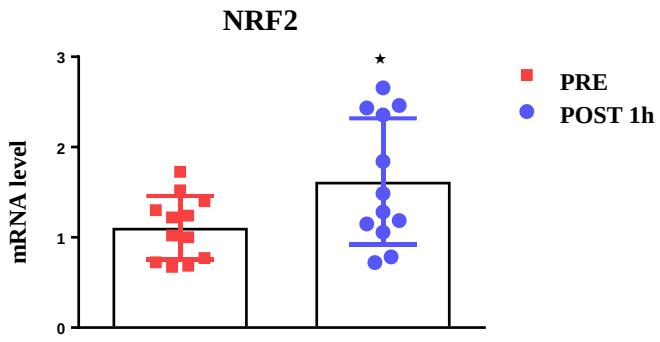


Figure 47: *Analysis of oxidative stress marker NRF2 at 1h Post – DEX administration.*

As shown in Fig. 47 the transcription factor *NRF2* significantly changes 1 hour after DEX administration. The results suggest that the decreased expression of *PGC-1α* and the consequent metabolic imparment generate a condition of redox imbalance. This condition is also confirmed by the increase in protein level of SOD1 at 4h after DEX administration (Fig. 36).

3.6. Mitochondrial dynamics: Fusion

*1h Post – **DEX** administration*

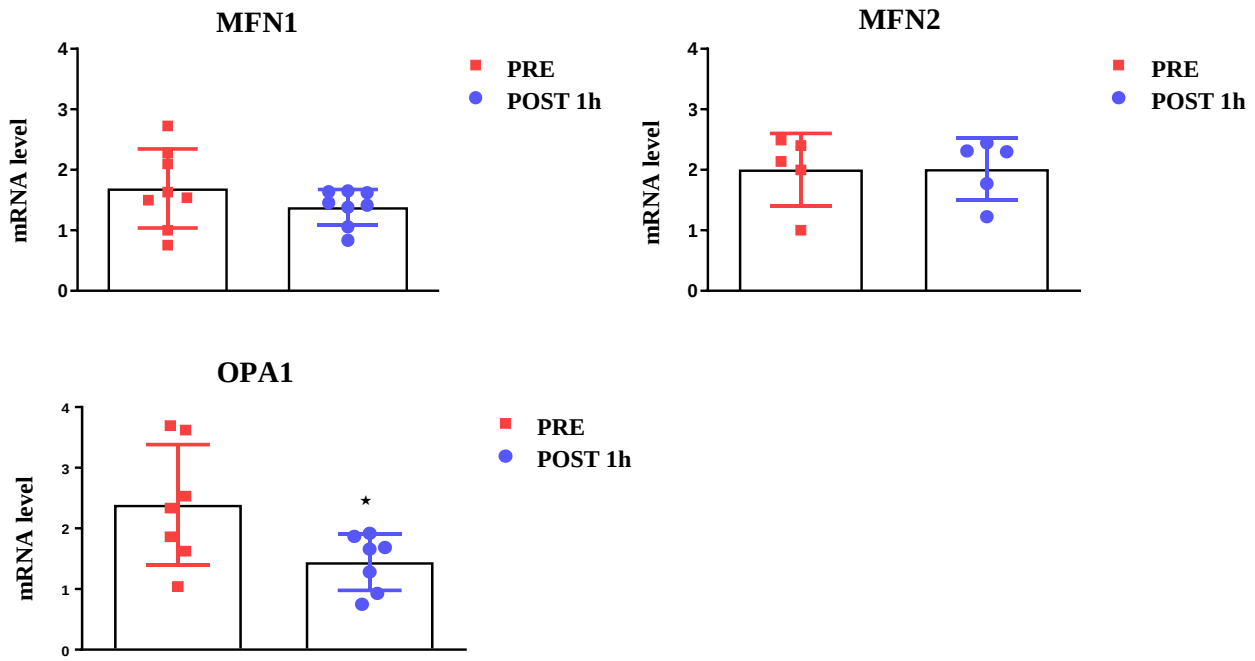


Figure 48: *Analysis of mitochondrial fusion markers (OPA1 – MFN1 – MFN2) at 1h Post – DEX administration.*
★ *p ≤ 0,05 vs PRE.*

As shown in Fig. 48 the mRNA expression of *OPA1* was significantly decreased 1 hour post DEX administration. A reduction in fusion mitochondrial process could be induced by the decreased expression of *PGC-1α* (Fig. 46). Both results suggest mitocondrial dysfunction that in turn could lead to higher production of ROS and to redox imbalance (Fig. 47).

3.7. Mitochondrial dynamics: Fission

*1h Post – **DEX** administration*

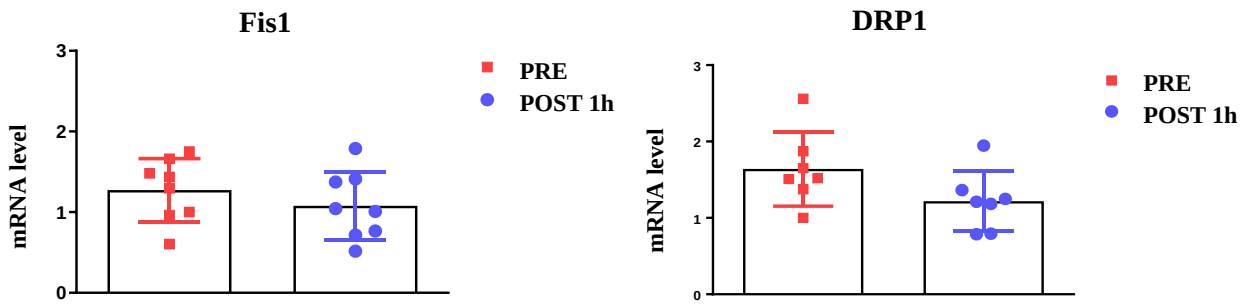


Figure 49: *Analysis of mitochondrial fission markers (Fis1 – DRP1) at 4h Post – DEX (on the left) and Placebo (on the right) administration.*

As shown in Fig. 49 no alteration of fission process accurred at 1h after DEX administration.

3.8. Autophagy

*1h Post – **DEX** administration*

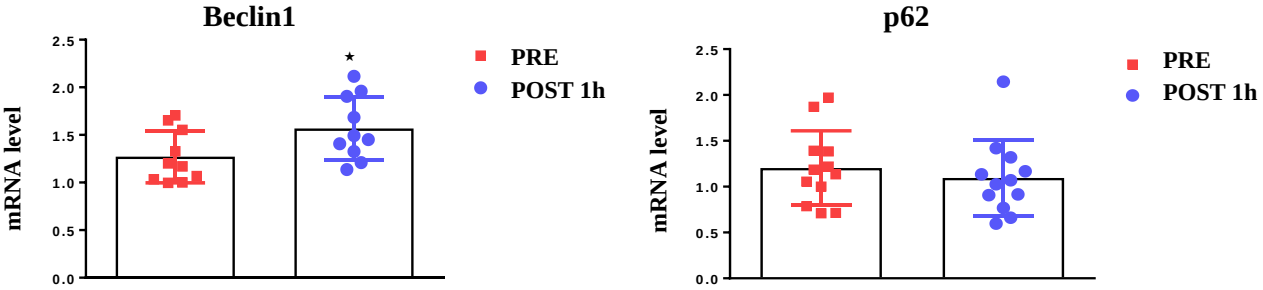


Figure 50: *Analysis of autophagy markers (Beclin1 – p62) at 1h Post – DEX administration.★ $p \leq 0,05$ vs PRE.*

As shown in Fig. 50 the gene expression of *Beclin1* was significantlyincreased 1h post DEX administration. The activation of the autophagic process was also confirmed by the significant increase of the ratio LC3BII/ LC3BI at 4 hours after DEX administration (Fig.41).

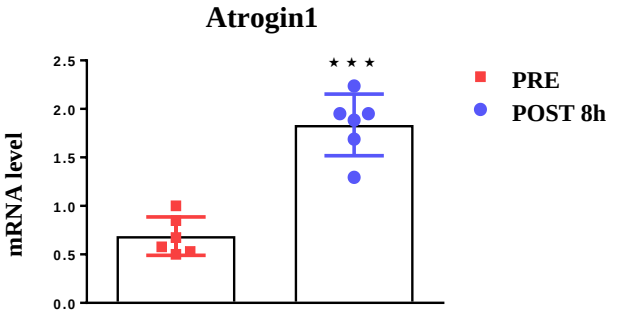
The results obtained show that the autophagy process is activated togheter with the decreased expression of *PGC-1α*, mitocondrial dysfunction and the condition of redox imbalance.

Collectively the latter results suggest that a primary action of DEX could be on *PGC-1α* gene expression, which could cause a consequent impairment of mitochondrial dynamic, which in turn would lead to redox imbalance due to increased ROS production. The activation of the autophagy process could be usefull to eliminate both the not functional mitochondria and oxidated proteins.

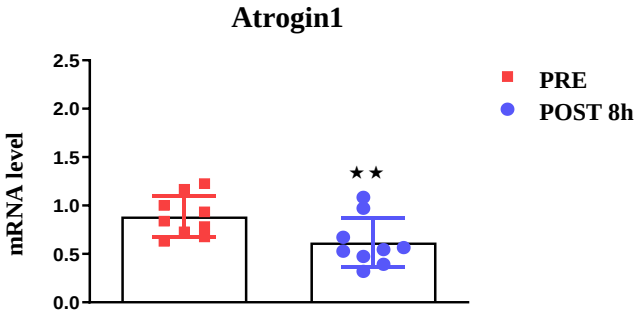
4. Gene expression at 8h post-administration

A. Catabolic pathway

<u>8h Post – **DEX** administration</u>

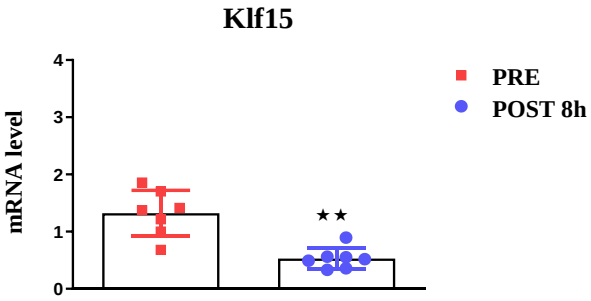


<u>8h Post – **Placebo** administration</u>

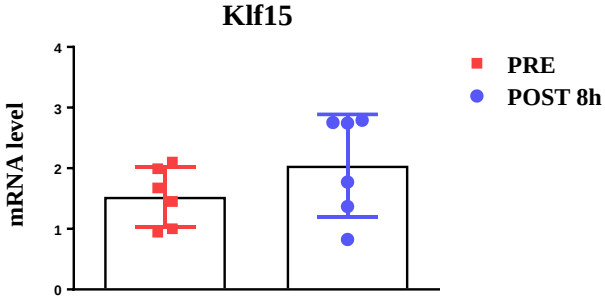


B. Transcription Factor Krüppel

<u>8h Post – **DEX** administration</u>

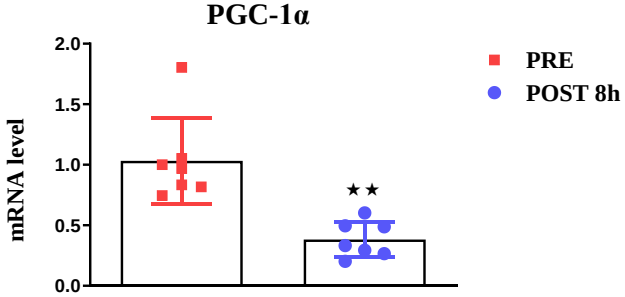


<u>8h Post – **Placebo** administration</u>



C. Oxidative metabolism

<u>8h Post – **DEX** administration</u>



<u>8h Post – **Placebo** administration</u>

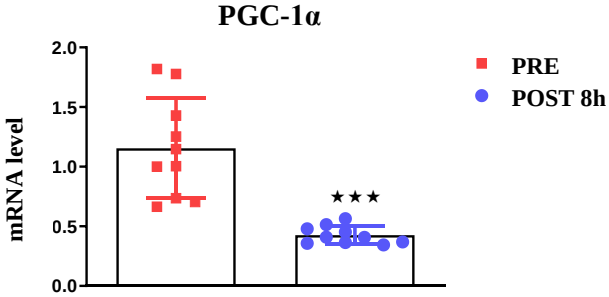


Figure 51: *(A) Analysis of marker involved in catabolic pathway (Atrogin1); (B) analysis of Transcription Factor Krüppel (Klf15); (C) analysis of marker involved in oxidative metabolism (PGC-1α). All markers have been analyzed at 8h Post – DEX (on the left) and Placebo (on the right) administration.★ $p \leq 0,05$ vs PRE.*

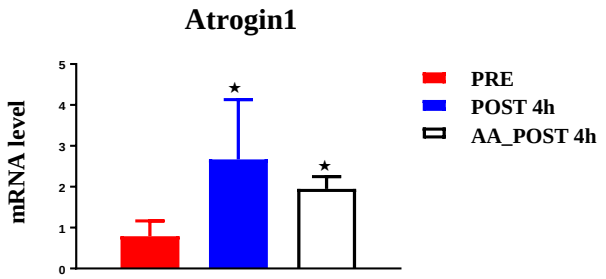
In order to evaluate the duration of DEX effect on intracellular pathways the analysis of the gene expression of *Atrogin1* (Fig. 51.A) and Klf5 (Fig. 51.B) and *PGC-1α* (Fig. 51.C) at 8h after DEX administration was performed. Gene expression of *PGC-1α* resulted significantly decreased after both DEX and placebo administration indicating that the observed effect could not be related to the DEX action. On the contrary, the significant increase in *Atrogin1* and decrease in Klf5 in DEX and not in placebo suggest that DEX action on this factors is still present.

The analysis of gene expression of the factors involved in mitochondrial dynamics, oxidative stess and autophagy at 8h after DEX administration will be performed.

5. Gene expression at 4h post DEX-administration, after 15 days of Branched-chain essential amino acids (AA)

A. Catabolic pathway

*4h Post – **DEX (AA)** administration*



B. Oxidative metabolism

*4h Post – **DEX (AA)** administration*

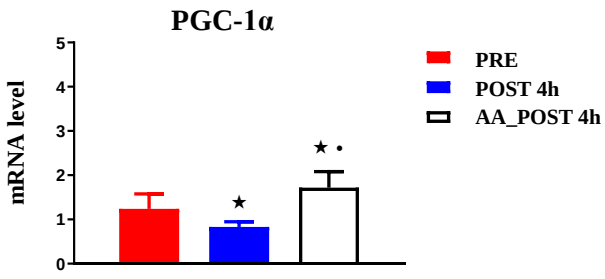


Figure 52: *(A) Analysis of marker involved in catabolic pathway (Atrogin1); (B) analysis of marker involved in oxidative metabolism (PGC-1α). Both markers have been analyzed at 4h Post – DEX administration after 15 days of AA.* ★ *p ≤ 0,05 vs PRE;* • *p ≤ 0,05 vs POST 4h.*

Amino acids, particularly BCAAs, are used to alleviate the induced muscular atrophy of low-calories diets [257], and prevent sarcopenia [258]. It has also been reported that the activity of mitochondrial enzymes and the abundance of mRNA gene transcripts encoding mitochondrial proteins are stimulated by a mixture of essential amino acids in young healthy subjects [259]. It has been demonstrated that the integration of a mixture of amino acids high in BCAA (~ 60%) promotes mitochondrial biogenesis in the heart and musculoskeletal of middle-aged mice, through increases in the gene expression of mitochondrial transcriptional regulators including peroxisome activated by the proliferator receptor-Î³ coactivator-1α (*PGC-1α*) [260]. It was also observed that

chronic BCAA supplementation could increase the activity of mitochondrial function markers (citrate synthase and cytochrome c oxidase) in the skeletal muscle of sedentary mice [261].

The effectiveness of BCAA in counteracting the effects induced by DEX on *Atrogin1* and *PGC-1α* (Fig. 52) was assessed.

Results demostrared that 15 days of BCAA supplementation after DEX administration was able to significantly reduce DEX- induced increase in *Atrogin1* and decrease in *PGC-1α* expression.

CHAPTER 7

CONCLUSIONS

Regarding ***gene expression*** (Table D), the experimental evidences of this study show that:

Marker	1h post DEX-administration	4h post DEX-administration	8h post DEX-administration
PGC-1α	↓ Decrease	↓ Decrease	↓ Decrease
CS	↓ Decrease	≈*	**
NRF2	↑ Increase	≈*	**
OPA1	↓ Decrease	≈*	**
Beclin1	↑ Increase	≈*	**
Klf15	↓ Decrease	↓ Decrease	↓ Decrease
Atrogin1	≈*	↑ Increase	↑ Increase

Table D: Gene expression's results obtained at 1h, 4h and 8h post DEX-administration.

***No changes.**

****Markers not yet tested.**

Regarding ***protein expression*** (Table E), the experimental evidences of this study show that:

Marker	4h post DEX-administration
p38 MAPK phosphorylation	↓ Decrease
AMPK phosphorilation	↑ Increase
ACC phosphorilation	↑ Increase
OPA1	↓ Decrease
LC3BII / LC3BI	↑ Increase

Table E: Protein expression's results obtained at 4h post DEX-administration.

Chronic glucocorticoids administration has a dramatic impact on muscle structure and function. In mice, a variety of responses in the intracellular pathways controlling muscle mass, mitochondrial dynamics and metabolism, and redox balance have been shown to occur [193]. It is still debated which of them plays a primary role triggering muscle atrophy and which is a consequence of muscle deterioration. A previous work from my laboratory has studied the time course of GCs action on intracellaulr pathways and suggested that activation of the ubiquitine proteasome system is the earliest and primary phenomenon underlying muscle wasting [225].

In humans, limited information is available on the earliest responses to DEX administration. Therefore, it is even less understood which event plays a primary role.

To address this issue, the work of my thesis focused on the phenomena occurring at very early time (1. 4, 8 hours) following DEX administration. A large number of markers of the major pathways controlling muscle protein breakdown (MPB), i.e. the ubiquitine proteasome system and authophay, muscle protein syntesis (IGF1/AKT/mTor pathways; miostatin), mitochondrial dynanics (*PGC-1α* and proteins involved in controlling mitochondrial fusion and fission), redox balance (*NRF2* and ROS buffering system), the response to the cell to energy imbalance (AMPK system), were studied.

Moreover, *Klf15* transcription factor and p38MAPK, which are directly controlled by GCs at a transcriptional level, were also studied.
The major findings are summarised in tables D and E. They indicate that the mechanism of GCs action is surely complex and not definetly settle. In fact, the results would be consistent with an upregulation of *FoXO*, which in turn would help to put all the findings in a comprehensive picture. The surprisingly observation that *FoXO* is not upregulated suggests more complex, but interesting and novel triggering mechanisms of steroid myopathy which are described below.

Muscle atrophy in humans would depend on (i) activation of *Atrogin1* (expression higher after 1 and 4 hours) and therefore on enhanced ubiquitine proteasome system activation, and (ii) enhanced autophagy (higher *Beclin1* expression after 1h and higher LC3BII/BI protein content after 4h).
GCs could enhance *Atrogin1* expression through a decrease in the expression of *Klf15*. The latter is a transcription factor known to be directly controlled by GCs at a transcriptional level and in turn to elicit some of the actions of GCs among which a control on *Atrogin1* expression [193] [282].
As regards, GCs activation of autophagy catabolic system, it would depend on the following sequence of events: GCs could directly act on *PGC-1α* expression decreasing it [284]; the latter could cause a decrease in *OPA1* (lower expression after 1h and lower protein content after 4h) which could cause decreased mitochondrial fusion and mitochondrial dysfuntion; Mitochondrial disfunction could cause a transient increase in ROS production [291] and a transient redox imbalance (increased *NRF2* expresion after 1h and increased SOD1 protein content after 4 h); ROS would enhance autophagy [292] [293]. Mitocondrial disfunciton is suggested not only by *OPA1* down-regualtion, but also by activation of the AMPK catabolic system. AMPK senses energy balance in the cell and is phosphorylated when the ratio AMP/ATP increases. Mitocndrial dysfunction can impair oxidative metabolism and limit ATP synthesis. AMPK can also directly promote autophagy under oxidative stress [294]. Though autophagy can avoid the energy crisis by hypoxia or oxidative stress by fully breaking down the damaged organelles and proteins, cells also take advantages of decomposed organelles components and de novo form new proteins or membrane structure to maintain metabolic fitness.

In addition, GCs could decrease *PGC-1α* also through a decrease in p38 MAPK (lower protein content after 4h). In fact, p38 MAPK is known to be directly controlled at a transcriptional level by GCs [229] [293] and to affect *PGC-1α* expression.

It would be noted that DEX could reduce *PGC-1α* expression in muscle cells by several mechanisms [283]. DEX is able to act directly on *PGC-1α* gene expression by binding to the GRE

present on its promoter [284]. DEX can also change the activity of other trascriptional factors that partecipate in *PGC-1α* trascription (e.g. MEF2, NFAT). Moreover, DEX could also increase the level of an unidentified microRNA(s) that targets *PGC-1α* in skeletal muscle. Finally, DEX could induce a decrease in activity of p38 MAPK that, in turn, can play a role in detrmining *PGC-1α* activity [229].

Moreover, the activation of autophagy process, suggested by the increase in *Beclin1* gene expression at 1h after DEX administrationa and by the increased ratio LC3II/LC3I at 4h after DEX administraion, could be usefull not only to eliminate damaged cell components (mitochondria and oxidated proteins), but also to extract energy from them.

Collectively, the present results of gene expression, suggest that DEX acts through different routes in humans compared to mice [225] (Table F), mainly altering oxidative metabolism and stimulatin authophagy through ROS and enhancing ubiquitine proteasome activity through *Klf15* and *Atrogin1* (Fig. 53).

Marker	1h post DEX-administration
FoXO3a	↑ Increase
Atrogin1	↑ Increase
MuRF1	↑ Increase
Myostatin	↑ Increase

Table F: Gene expression's results obtained in mice at 1h post DEX-administration [225].

The atrophic program remains active at least 8h after DEX admistration (decrease in in *Klf15* and increase in *Atrogin1* gene expression) whereas the metabolic imparment seems to be solved or no longer related to DEX administration (decrease in *PGC-1α* gene expression in both DEX and placebo).

An important, although still preliminary, result of this study is the observation that a mixture of essential branched-chain amino acids seem able to act directly both on oxidative metabolism and on the catabolic process, counteracting the effects of dexamethasone on muscle

These supplements have no side effects, making them a valid aid in subjects treated for inflammatory, autoimmune and allergic disease.

This is the first project drawn up with the aim of analyzing the consequences of a single DEX administration on human skeletal muscle. The results shown in this thesis are not conclusive. Further analysis are ongoing, in particular at 8h after DEX and placebo administration in order to investigate more precisely the relationship between metabolic dysfunction and atrophy.

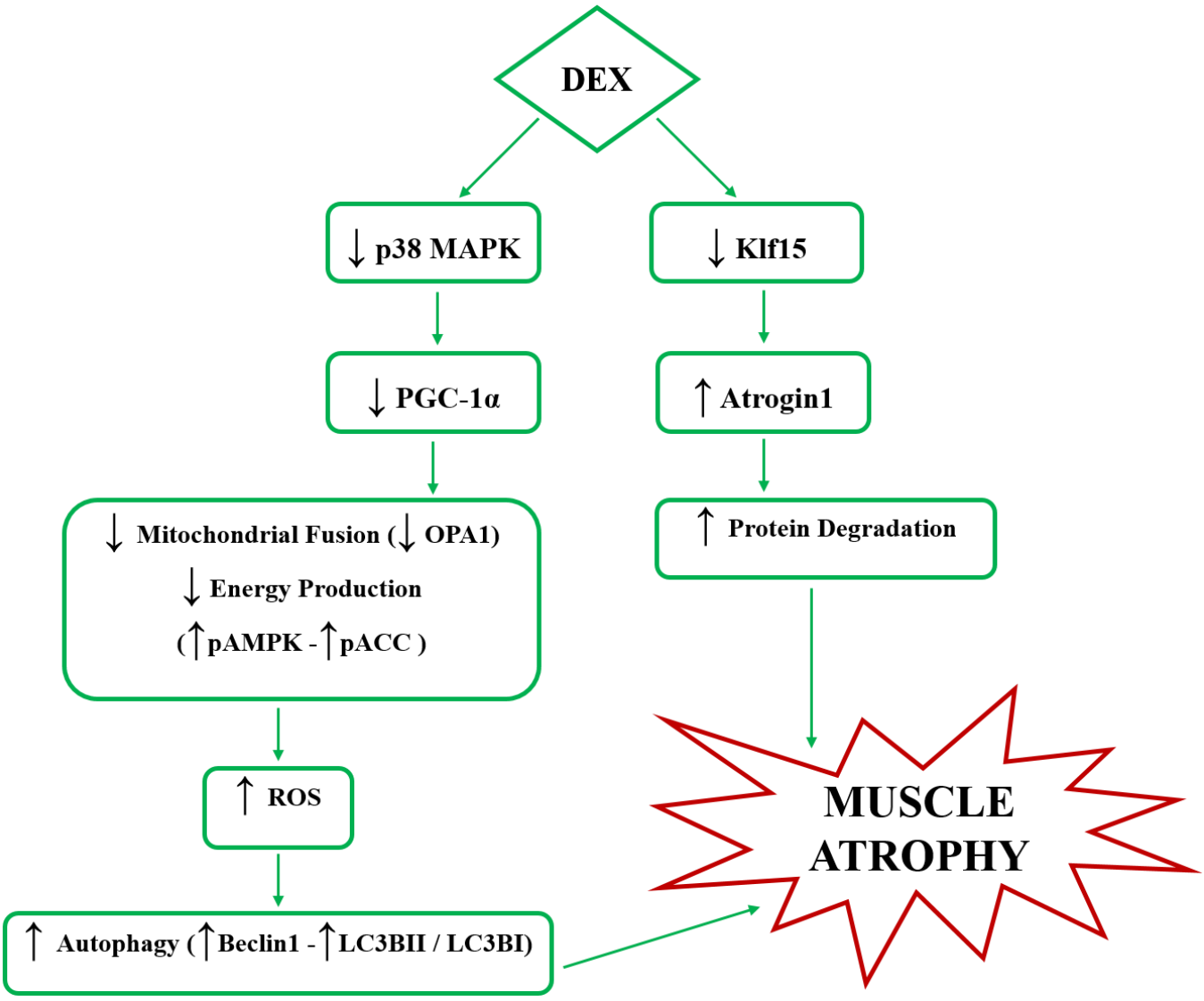


***Figure 53**: Flowchart of the possible mechanism leading to skeletal muscle atrophy after Dexamethasone administration.*

CHAPTER 8

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