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Autophagic modulation in preserving skeletal muscle integrity

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Abstract

Introduction:

Autophagy is a physiological mechanism, responsible for cell homeostasis, aimed to remove damaged organelles and unfolded proteins avoiding their accumulation in the cytoplasm. Autophagic flux impairment seems to be involved in several diseases, including muscular dystrophies and myopathies (Park *et al*., 2009; Sandri *et al*., 2013), cancer (Lu *et al*., 2013), diabetes (Kang *et al*., 2016), Parkinson's, Alzheimer's, Huntington's disease (Cheung *et al*., 2011) as well as Lysosomal Storage Disorders (Settembre *et al*., 2008).

According to few papers in literature, chemotherapeutic drug treatments are able to trigger autophagy (Fanzani *et al*., 2011). Previous works, conducted by our group, demonstrated that C2C12 myotubes treated with etoposide (eto), a chemotherapeutic drug known to induce cell death and oxidative stress, undergo an abnormal autophagic activation, as well as nuclear disorganization and cytoplasmic shrinkage (Salucci *et al*., 2013).

Amino acid supplementation, stimulated by glutamine, has been described as protective from the degradative effect of proinflammatory cytokines (TNF-α) implicated in many degenerative processes (Bonetto *et al*., 2011). Moreover, dietary glutamine intake represents a conditioning therapy before chemotherapy, to counteract drug side effects (Yoshida *et al*., 2001; Mora *et al*., 2002; Gaurav *et al*., 2012; Pesarini *et al*., 2014).

Starvation is a common used strategy to conduct studies on autophagy, since nutrient deprivation is an effective stimuli for autophagic induction (Desgeorges *et al*., 2014).

Aim:

The aim of this work was to find a strategy, based on diet modulation, to prevent antineoplastic treatment induced damage. An *in vitro* model of skeletal muscle has been exposed to etoposide, to mimic chemotherapeutic induced damage. Glutamine supplementation and nutrient deprivation have been chosen as pre-treatment to counteract autophagic flux impairment and myotube atrophy.

Methods:

Differentiated C2C12 cells have been exposed to eto after a previous treatment with glutamine or starvation. Cytofluorimetric, morphological and morpho-functional analyses revealed that eto treatment induces damage to the lysosomal compartment, causing the accumulation of autophagic vacuoles, and a reduction of myotube area. Interestingly, both pre-treatments were able to rescue myotubes from damage. Glutamine supply, in particular, seemed to be a good strategy to preserve cell size and prevent the autophagic impairment, partially restoring the normal lysosomal activity. Starvation, secondly, was able to activate a survival autophagy, avoiding autophagosome and damaged organelle accumulation induced by etoposide.

Conclusions and future purposes:

These findings suggest that a diet modulation could prevent the eto-induced abnormal autophagic activation and hamper the atrophic pathway activation in differentiated C2C12 cells.

Further molecular studies are already ongoing to validate these data.

Once achieved comprehensive results *in vitro*, it could be interesting to go towards an *in vivo* approach, to investigate if a diet modulation could prevent muscle atrophy in tumor-bearing rats undergoing chemotherapy.

Introduction

Skeletal Muscle structure

Skeletal muscles are the body's agent of motion and they represent the 40-50% of the whole human body (Sandri, 2010). This highly structured tissue has the important functions to sustain the mechanical tension, allow movements, produce heat and assist the blood flow return through contraction.

Skeletal muscle is structurally well organized (***Fig.1***). Muscle cells are surrounded by the *endomysium,* the thinner portion of intramuscular connective tissue, rich in vessels and nerves; it is directly in contact with basal membrane and sarcolemma and represents the site of nutrient exchange between cells and blood. Bundle of fibers are then surrounded by another connective tissue layer, called *perimisyum.* Finally the *epimysium* is a lamina which covers muscle bellies and clearly defines muscle volume. The latter is thicker than the other elements and is composed by collagen fibers with a large diameter; moreover, at muscle ends, it merges with tendons of origin and insertion (Turrina *et al*., 2013).

Muscle cells, called *fibers* due to their particular shape, contain from hundreds to thousands myofibrils, characterized by the repetitive presence of sarcomeres, consisting in turn of actin and myosin filaments.

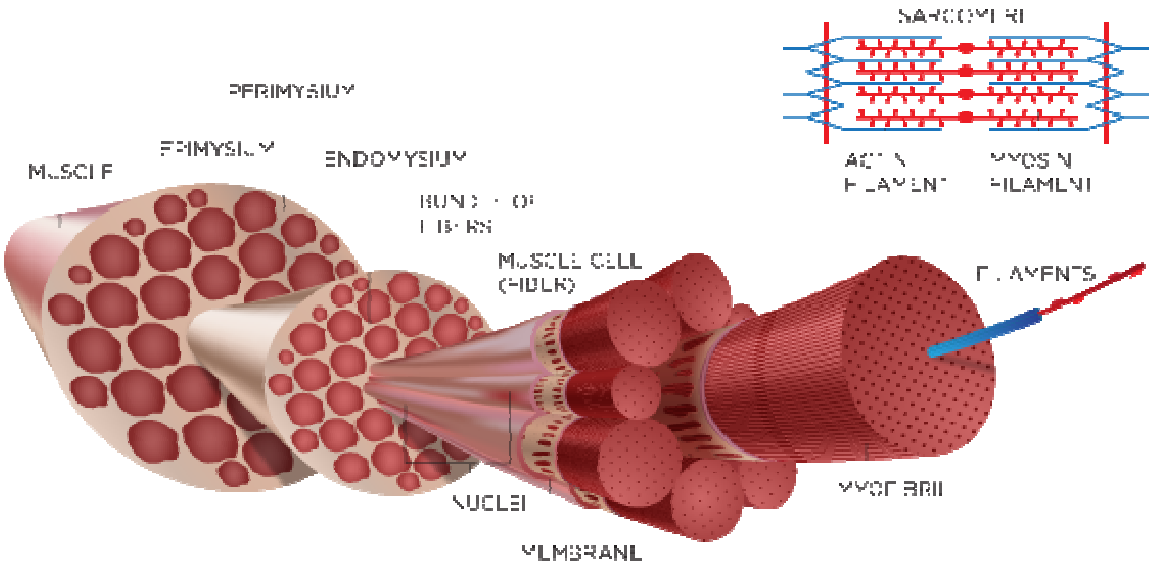

Fig.1

Skeletal muscle structure.

The sarcomere represents the muscle functional unit (Fusi *et al*., 2014), since it contains all the components needed for muscle contraction. Hundreds of sarcomeres are matched in a repetitive way to form contractile organelles, called myofibrils (Sandri, 2010), which are packaged in parallel to compose skeletal muscle fibers. Two *Z disks* delimit the sarcomere (***Fig.2***); from each *Z disk* actin filaments (thin filaments) take attack, intersected with thick myosin filaments, localized in the middle, in order to allow their interaction to produce muscle contractions. Other structures are detectable at this level: myosin filament zone is commonly called *A band* (or dark band), whereas actin zone is called *I band* (or light band). Thick filaments take attack from the *M line*, situated in the medial *H band*.

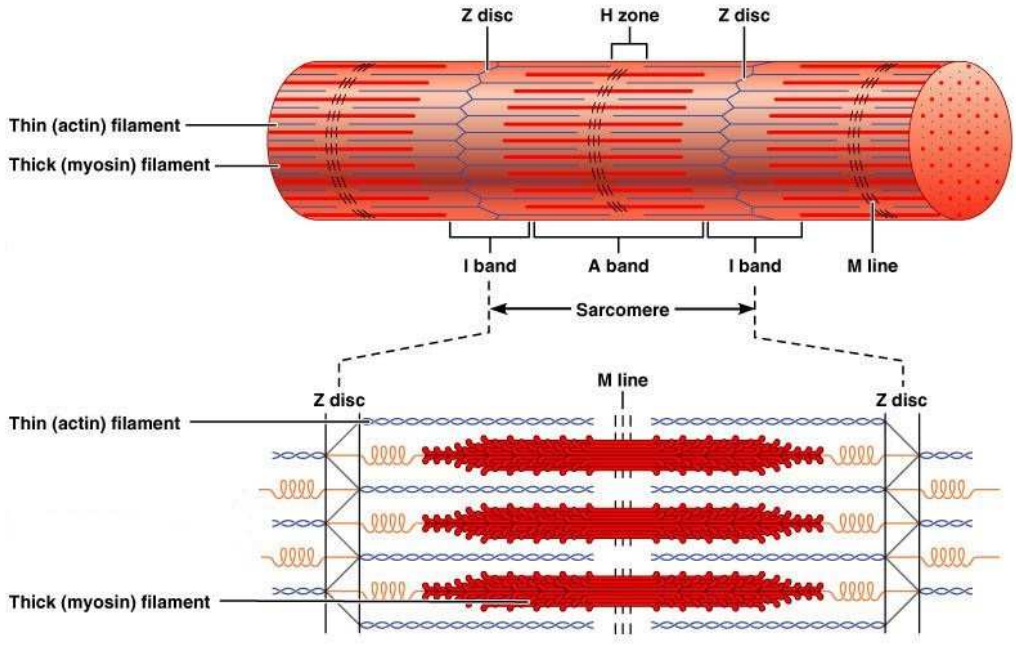

Fig.2
Sarcomere organization.

Other organelles are embedded into myofibers such as mitochondria, important for ATP generation, and sarcoplasmic reticulum, for calcium release, useful for myofiber contraction (Sandri, 2010).

Besides giving support to the body and allowing the movement, muscles are important sites for metabolism control. Indeed, during catabolic conditions, stress and nutrient deprivation, protein are mobilized to sustain gluconeogenesis and provide alternative energy for organs. Two different pathways are responsible for this "survival mechanism": the ubiquitin-proteasome system and the autophagy-lysosome system, which are both proteolytic pathways, highly regulated and involved in muscle atrophy occurrence. Sandri *et al*. (2013) report that whether autophagy-lysosome as ubiquitin-proteasome system are involved in the pathogenesis of muscle wasting in different types of muscle disorders. Autophagy-lysosome seems to be crucial for removing dysfunctional organelles and unfolded proteins, whereas ubiquitin-proteasome is responsible for protein quality control (Sandri *et al*., 2013).

Muscle mass maintenance is the result of the highly regulated balance between synthesis and degradation. Modifications in this balance lead to two different and opposite conditions, known as hypertrophy and atrophy. The first is the consequence of an enhanced protein synthesis, stimulated by mechanical overload or anabolic hormonal intake (Romanello *et al*., 2016); the second occurs when protein degradation exceeds synthesis, leading to muscle weakness and atrophy (Sandri, 2008). Loss of muscle mass occurs in several conditions such as denervation, aging, inactivity, microgravity, fasting or in many systemic diseases including cancer, sepsis, AIDS, diabetes, cardiac and renal failure (Lecker *et al*., 2006); hence, to find novel therapeutic strategies to counteract muscle wasting represents an interesting investigation field for many researchers.

Autophagy

The term “autophagy” derives from Greek and it means “self eating”.

Autophagy is an highly regulated physiological mechanism, aimed at removing misfolded or aggregated proteins, damaged organelles and intracellular pathogens, to avoid their accumulation within cells (Balduini *et al*., 2012).

An autophagic activation occurs also in stress conditions, such as limited nutrient availability, to provide cells with amino acids and fatty acids (Lee *et al*., 2012). Thus, it is generally thought as a survival mechanism (Glick *et al*., 2010). Anyway, autophagy is an essential process for cell homeostasis both in normal and pathological conditions (Lee *et al*., 2012).

In recent years, researchers focused their attention on the relationship between autophagy and disease. An autophagic flux impairment, indeed, seems to be involved in the onset of several pathologies (Levine *et al*., 2008; Mizushima *et al*., 2008) such as muscular dystrophies and myopathies (Park *et al*., 2009; Sandri *et al*., 2013), cancer (Lu *et al*., 2013), diabetes (Kang *et al*., 2016), Parkinson’s, Alzheimer’s, Huntington’s disease (Cheung *et al*., 2011) and LSDs – lysosomal storage disorder’s (Settembre *et al*., 2008). Moreover, the autophagic activation occurs in order to protect cells against genome instability and prevent necrosis, as well as to promote antigen presentation on the cell surface.

Autophagic regulation occurs through the transcription of several genes responsible for different stages of the pathway (Kourtis *et al*., 2009); since these genes are thought to regulate the loss of muscle components, they are called atrophy-related genes or Atrogenes (Atg) (Sandri *et al*., 2004).

Three different types of autophagy are known: macro-autophagy (often reported only as autophagy), micro-autophagy and chaperone-mediated autophagy (CMA) (***Fig.3***; Okamoto, 2014).

In macro-autophagy, cytoplasmic materials are engulfed by double membrane vesicles, called autophagosomes, which fuse with lysosomes forming the

autolysosome; these vesiscles are able to degrade the cargo thanks to the activity of hydrolytic enzymes. In micro-autophagy, on the contrary, cytosolic components are directly taken up by lysosomes, through an invagination of the lysosomal membrane. Finally, in CMA, proteins are translocated in a complex with chaperone proteins, which are recognized by LAMP2, a lysosomal membrane receptor, for the degradation (Saftig *et al*., 2008).

Degradation products can be re-used by the cell to form new macromolecules useful for metabolism, giving to autophagy an important "recycling" role (Mizushima, 2007).

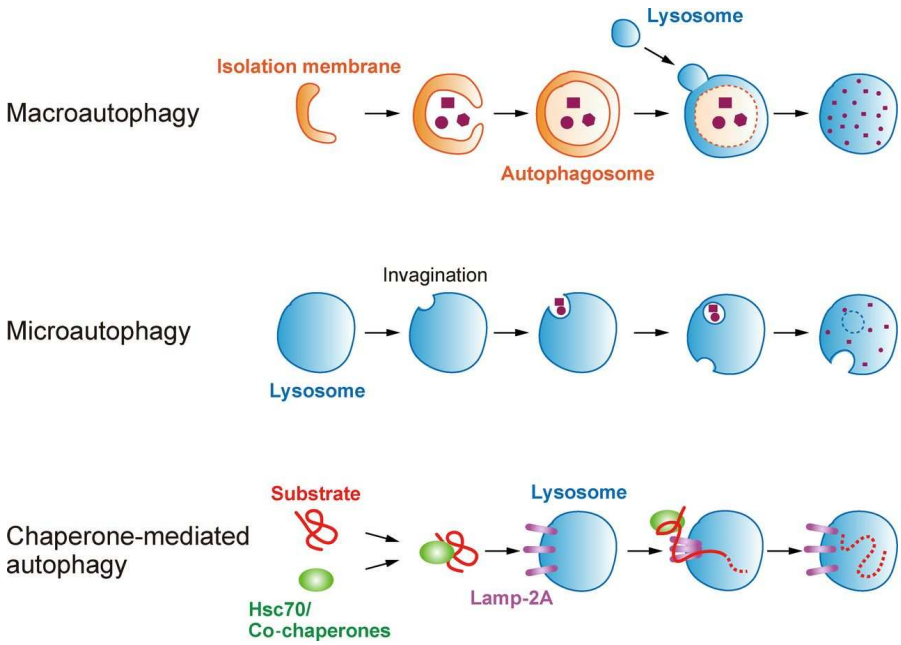

Fig.3

picture from:

Okamoto, 2014. J Cell Biol. 205:435-45.

Due to the possibility to assume macro-autophagy as a target for some therapies, several researchers, in recent years, focused their attention on understanding its activation, impairment, modulation and blockage.

Macro-autophagy (hereafter reported only as autophagy) has five key stages (***Fig.4***; Glick *et al*., 2010):

a) phagophore formation (isolation membrane);
b) Atg5-Atg12-Atg16L complex formation;
c) LC3 activation;
d) cargo selection;
e) autolysosome: fusion autophagosome-lysosome and proteolitic degradation.

a) Phagophore formation

The formation of the phagofore, also called "isolation membrane", is the first step of autophagy (Tooze *et al*., 2010). This membrane first engulfs cytoplasmic material, selectively or randomly, then closes around the cargo and delivers it to the lysosomal degradative system. Autophagosome formation starts from PAS, Phagophore Assembly Sites, and requires the activity of the kinase PI3K (class III phosphoinositide 3-kinase, also known as Vps34). PI3K acts with a large number of macromolecular complex, among which Beclin-1, to form PI 3-phosphate (PI(3)P) (Weidberg *et al*., 2011). Beclin is an early marker of autophagy, precisely responsible for the autophagosome formation (Kang *et al*., 2011).

The elongation of the membrane, to form the autophagosome, is highly regulated by the activity of two ubiquitination-like reactions, described below: the Atg5-Atg12 conjugation (Geng *et al*., 2008) and the LC3 protein processing and activation (Shpilka *et al*., 2011).

b) Atg5-Atg12-Atg16L complex formation

Atg5-Atg12-Atg16L complex formation is thought to be responsible for the curvature of the phagophore, to form the autophagosome vesicle. This complex

can perform its function binding the LC3II protein, localized on the growing phagophore membrane (Glick *et al*., 2010).

Atg7 is the starter for complex formation and it behaves like an enzyme, activating Atg12 which is then transferred to Atg10. This last potentiates the linkage between Atg5 and Atg12, subsequently associated with Atg16L, forming the final complex Atg5-Atg12-Atg16L, whose role is to recruit asymmetrically processed LC3 proteins, to promote phagophore curving.

Although this step is crucial for the autophagic pathway, complex formation is not dependent on autophagy induction. Since the complex dissociates from the membrane once the autophagosome is formed, conjugated Atg5-Atg12 is considered an unsuitable read-out for autophagy (Bath *et al*., 2010).

c) LC3 activation

The second mechanism involved in autophagosome formation is the processing of LC3B (protein light chain 3). LC3 is the mammalian homologue of yeast Atg8 gene; it is a protein normally situated in the cytoplasm and it is important for membrane growth (Nakatogawa *et al*., 2009). When autophagy is activated, Atg4 cleaves LC3 to generate LC3B-I, which is transferred to Atg3 for the final activation in the lipidated form LC3B-II.

The LC3 localization, both on the internal and external vesicle surface, is due to the Atg5-Atg12 complex activity.

Although LC3 synthesis and processing are important steps for the autophagic pathway progression, its real role is still unclear; Schwarten and colleagues (2009) postulated that this protein could have an important role in selecting the cargo.

Since LC3 levels are strongly increased during autophagy, it is a commonly used marker to assess the autophagic trend (Glick *et al*., 2010).

d) Cargo selection

For years autophagy has been described as a random process. Indeed, Transmission Electron Microscopy (TEM) analyses, performed by Glick and colleagues, showed the presence of various cytoplasmic material, including mitochondria, endoplasmic reticulum and Golgi membranes within autophagic vacuoles (Glick *et al*., 2010). However, latest analyses showed that the phagophore can selectively interact with damaged organelles and unfolded proteins, suggesting a targeted activity against the accumulation of dangerous material within the cell.

Some authors suggested that LC3B-II, the activated form of LC3 protein, behaves like a receptor and interacts with specific molecules, situated on the target, to promote their engulfment and degradation. One of the well known molecules, in this sense, is the p62/SQSTM1 (Sequestrosome1), which promotes the turnover of poly-ubiquinated proteins (Kirkin *et al*., 2009).

Moreover, Atg32 has been identified as a marker for the selective removal of mitochondria, process known as mitophagy (Kim *et al*., 2007).

e) Autolysosome: fusion autophagosome-lysosome and proteolitic degradation.

The fusion of the completed phagophore with the lysosome, to form the autolysosome, is the last step of this self-degradative process (Mizushima *et al*., 2007). Once the phagophore completed its closure, the low pH of the autophagic vesicle and the activity of lysosomal acid proteases are the main responsible for cargo degradation (Eskelinen, 2005). Lamp-1 and Lamp-2 are two lysosomal membrane proteins, crucial for lysosome biogenesis and for the end of the autophagic pathway (Eskelinen, 2006). Mainly Lamp-2 represents an important control step for autophagosome maturation. Indeed, Lamp-2 deficiency causes an accumulation of autophagic vacuoles in different tissues, leading to the onset of cardiomyopathy and myopathy in mice and patients suffering from Danon Disease (Tanaka *et al*., 2000; Saftig *et al*., 2008).

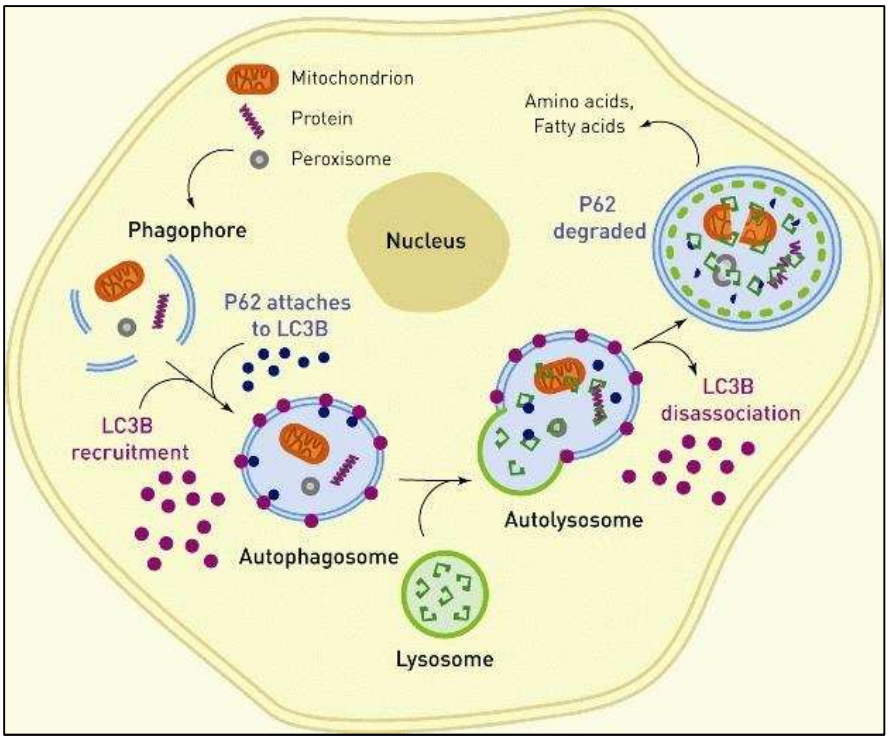

Fig.4
(picture from:
Premo™ Autophagy Tandem Sensor
RFP-GFP-LC3B Kit, datasheet).

Autophagy and skeletal muscle

Autophagy represents the major proteolitic pathway, together with the ubiquitin-proteasome system, activated in atrophying muscle (Sandri, 2010). However, an autophagic basal flux is always activated in skeletal muscle cells, to ensure the physiological turnover of protein and organelles.

Autophagosome accumulation has been found in almost all myopathies and dystrophies studied so far (Malicdan *et al*., 2008) but it is still unclear whether autophagy is part of the detrimental mechanism of muscle degeneration or a survival attempt (Sandri, 2010).

To better investigate the autophagic role in skeletal muscle, Masiero *et al*. (2009) generated a knockout mice for Atg7 to block autophagy and to gain more contractile proteins, improving muscle strength. Surprisingly, autophagy suppression didn't have beneficial effects; on the contrary it triggered atrophy, weakness and features of myopathy.

Raben et al. (2008) blocked autophagy by generating a muscle-specific Atg5 knockout mice, observing the same atrophic phenotype.

Autophagic suppression, according to other authors, causes the accumulation of abnormal mitochondria, protein aggregates and damaged organelles, leading to myofiber degeneration (Masiero *et al*., 2009).

Altogether these findings suggest that this mechanism is required to maintain muscle integrity and its modulation is crucial to avoid both atrophy and accumulation of damaged material within the cells.

Recent studies showed that autophagy is also important for nuclear stability. Inhibition of this mechanism, indeed, leads to the accumulation of nuclear abnormalities and reduces cell viability; so, this form of "nucleophagy" is required to hamper accumulation of nuclear damage. However, it is still unclear how portions of nuclei can be removed without affecting chromosome number and genes (Park *et al*., 2009).

Molecular mechanisms in the regulation skeletal muscle size

The highly regulated balance between synthesis and degradation of intracellular components determines the overall muscle fiber size (Mammucari *et al*., 2008). When synthesis rate is higher than the degradation one, fiber size increase occurs; nutrient abundance (Fujita *et al*., 2007), muscle stimulation by physical exercise and BCAAs (Branched-Chain Amino Acids) intake (Blomstrand *et al*., 2006) are needed for synthesis pathway stimulation. On the contrary, muscle atrophy takes place when the degradation rate is higher than synthesis, for instance during disuse (Reilly *et al*., 2016), fasting (Secor *et al*., 2016) or in pathological conditions such as diabetes (Kang *et al*., 2016) and cancer (Lu *et al*., 2013).

Hereafter we will discuss about the main molecular markers involved in protein synthesis and degradation, in skeletal muscle (***Fig.5***).

Akt. Akt is the most potent autophagic regulator in skeletal muscle. This kinase inhibits the autophagosome formation and the lysosomal degradation (Zhao *et al*., 2008), activating mTOR (mammalian Target of Rapamycin) a nutrient-sensitive kinase, critical for cell growth. Akt activation is induced by insulin and IGF1, which are responsible for PI3K activation in phosphatidylinositol-3,4,5,triphosphate (Sandri *et al*., 2008). Phosphorilation of Akt, via PI3K, occurs after muscle contractile activity (Nader *et al*., 2001; Sakamoto *et al*., 2002), or after hormonal and growth factor stimulation, triggering the protein synthesis pathway, involved in muscle hypertrophy.

Akt has three important downstream targets: *mTOR,* involved in protein synthesis, *GSK3β* (glycogen synthase kinase 3β) and *FoxO*, which controls protein degradation.

mTOR. The mammalian target of rapamycin (mTOR) is an important regulator of cell growth and protein synthesis (Hay *et al*., 2004). As suggested by the name, this kinase is rapamycin-sensitive; rapamycin is an immunosuppressant used in organ transplantation, which inhibits mTOR activity in a selective manner. mTOR is made of two complexes: mTORC1 and mTORC2. The first contains *raptor*, it is rapamycin sensitive and is required for *S6K activation* and *4EBP1* inhibition; the second, which contains *rictor*, is required for Akt-FoxO axis.

The S6K kinase is an important effector of the Akt-mTOR axis, concerning protein synthesis; indeed, Ohanna *et al*. (2005) observed an atrophic phenotype in a model of S6K-null mice. It is upregulated by mTORC1 activity, which is also responsible for the inhibition of 4EBP1, a protein involved in synthesis pathway arrest, transcriptionally activated by FoxO (Mammucari *et al*., 2008).

mTOR kinase inhibits autophagic activation also through the phosphorilation of *Ulk1* (mammalian autophagy-initiating kinase) on Ser 757, disrupting the interaction between Ulk1 and AMPK, which, on the contrary, is responsible for Ulk1 phosphorilation on Ser 317 and Ser 777, directly promoting autophagy (Kim *et al*., 2011).

FoxO. The family of FoxO (forkhead box O) transcription factors is negatively regulated by Akt. This last, indeed, phosphorilates FoxO promoting its export from the nucleus to the cytoplasm, avoiding the upregulation of atrophic genes (Sandri, 2008). In skeletal muscle three isoforms of FoxO have been found: FoxO1, FoxO3 and FoxO4. FoxO3 isoform is necessary and sufficient to promote atrogin1/MAFbx expression (Sandri, 2008) and to control autophagic activation *in vivo* (Mammucari *et al*., 2007). MAFbx (Muscle Atrophy F box) and MuRF1 (Muscle RING Finger1), are muscle specific ubiquitin ligases, found in several models of muscle atrophy.

FoxO3 controls also the transcription of *LC3* and *Bnip3* autophagy-related genes, considered key markers for autophagy detection.

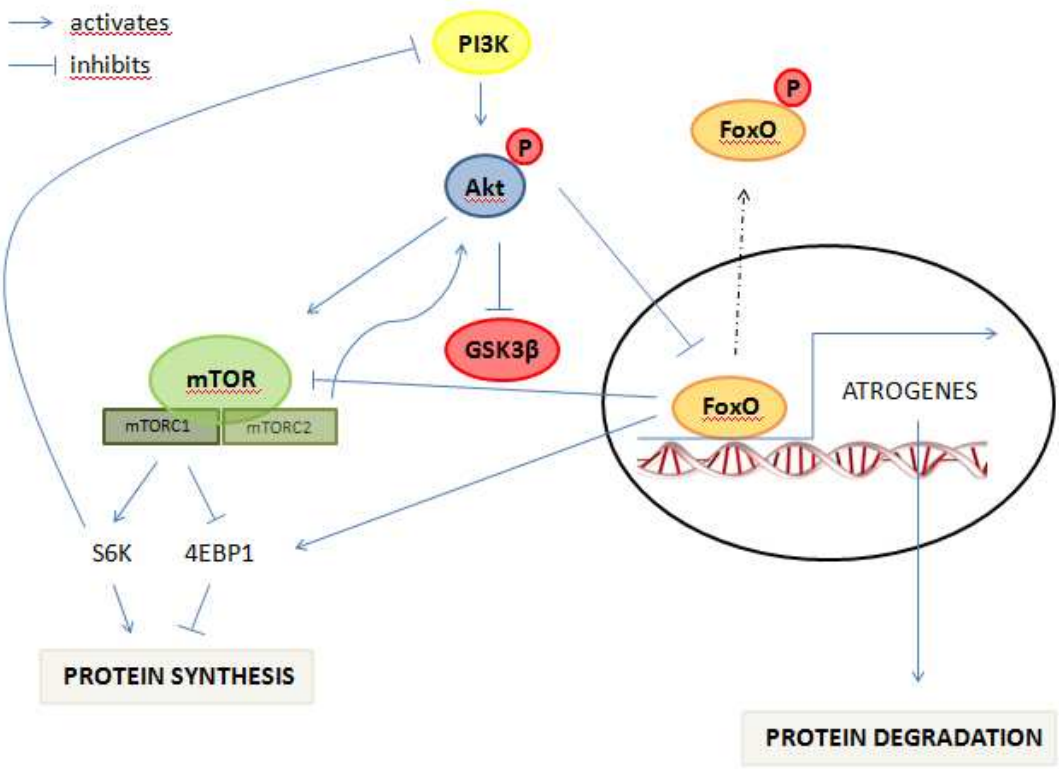

Fig.5

Protein synthesis and degradation pathways scheme.

Etoposide

Etoposide phosphate (VP-16) is an antineoplastic drug (Birandra *et al*., 2013) and its antitumor activity in several human malignancies, including lymphomas, germinal tumors and lung cancer, has been demonstrated by Hande already in 1992. Few papers in literature reported that its employment, together with other chemotherapeutic drugs, for instance cisplatin (cisPt), can hamper cancer cell growth, counteracting tumor progression (van der Burg *et al*., 2002; Meczes *et al*., 2002; Huw *et al*., 2002; William *et al*., 2015).

Etoposide (hereafter reported only as eto), is an inhibitor of the Topoisomerase II enzyme and some authors demonstrated its pro-apoptotic and pro-oxidant activity in different cell lines (Stasiłojć *et al*., 2013; Salucci *et al*., 2016). Topoisomerase II is a nuclear enzyme, responsible for both DNA replication and transcription; its inhibition causes the accumulation of double strand breaks within the nucleus, activating the apoptotic pathway. More in detail, etoposide triggers p53 phosphorylation and activation, resulting in the up-regulation of the pro-apoptotic protein Bax, which translocates from the cytosol to the mitochondria, causing the release of the *cytocrome c* and the beginning of the apoptotic cascade (Karpinich *et al*., 2002).

Previous studies, conducted by our group, demonstrated a peculiar behavior of C2C12 skeletal muscle cells after etoposide administration (Salucci *et al*., 2010; Salucci *et al*., 2013; Battistelli *et al*., 2014; Salucci *et al*., 2016).

In 2010, Salucci et al. studied the sensitivity of C2C12 myoblasts, undifferentiated skeletal muscle cells, to various chemical triggers. In this paper H_2O_2, staurosporine, etoposide and cisplatin have been used in different doses and exposure times (12h and 24h). All the triggers were able to induce apoptosis in different percentages. In particular, 25 μM etoposide exposure induced 15% of apoptotic cell death after 12h and 60% after 24h. Cell incubation with twice the concentration (50 μM) caused 14% and 31% of apoptosis after 12h and 24h respectively; in the latter, the reduced percentage

of apoptotic cells was explained, by the authors, with the coexistence of apoptosis and necrosis.

C2C12 myoblasts, exposed to 50 µM etoposide for 24 hours, showed a marked reduction in terms of cell proliferation and differentiation potential (***Fig.6***). Immunofluorescence staining for Ki67, a cell proliferation marker, suggests that this antineoplastic drug is able to impair cell cycle and to block cell proliferation. After the treatment, also the positivity to MyoD, a protein involved in muscle differentiation control, is reduced, explaining the impossibility to obtain myotubes from etoposide-treated myoblasts (***Fig.7***).

The different behavior of myoblasts and myotubes after chemical exposure, was investigated by Salucci *et al*. (2013) and Battistelli *et al*. (2014). Differentiated cells, thanks to their multinucleated nature, seemed to be more resistant to etoposide-induced cell death, in comparison with undifferentiated cells. TUNEL reaction, which allows the detection of nuclei with cleaved DNA, revealed, in syncytia, the coexistance of positive and negative nuclei within the same fiber. Myoblasts, as previously demonstrated (Salucci *et al*., 2010), were characterized by the peculiar features of apoptotic cell death (chromatin condensation and margination, blebs formation, detachment from the substrate), confirming their higher sensitivity to chemical-induced damage.

In myotubes, the formation of double membrane vescicles, suggestive of autophagy, was observable after etoposide and cisplatin exposure, according also to Fanzani *et al*. (2011). The perinuclear localization and the unusual accumulation of vesicles suggested a possible impairment of the mechanism, resulting in damaged organelle accumulation, metabolism alteration and cell stress.

Since the autophagic mechanism could protect cells from damage induction, but can also be involved in their final degradation , we though to better investigate the role of autophagy in C2C12 myotubes exposed to antineoplastic treatment.

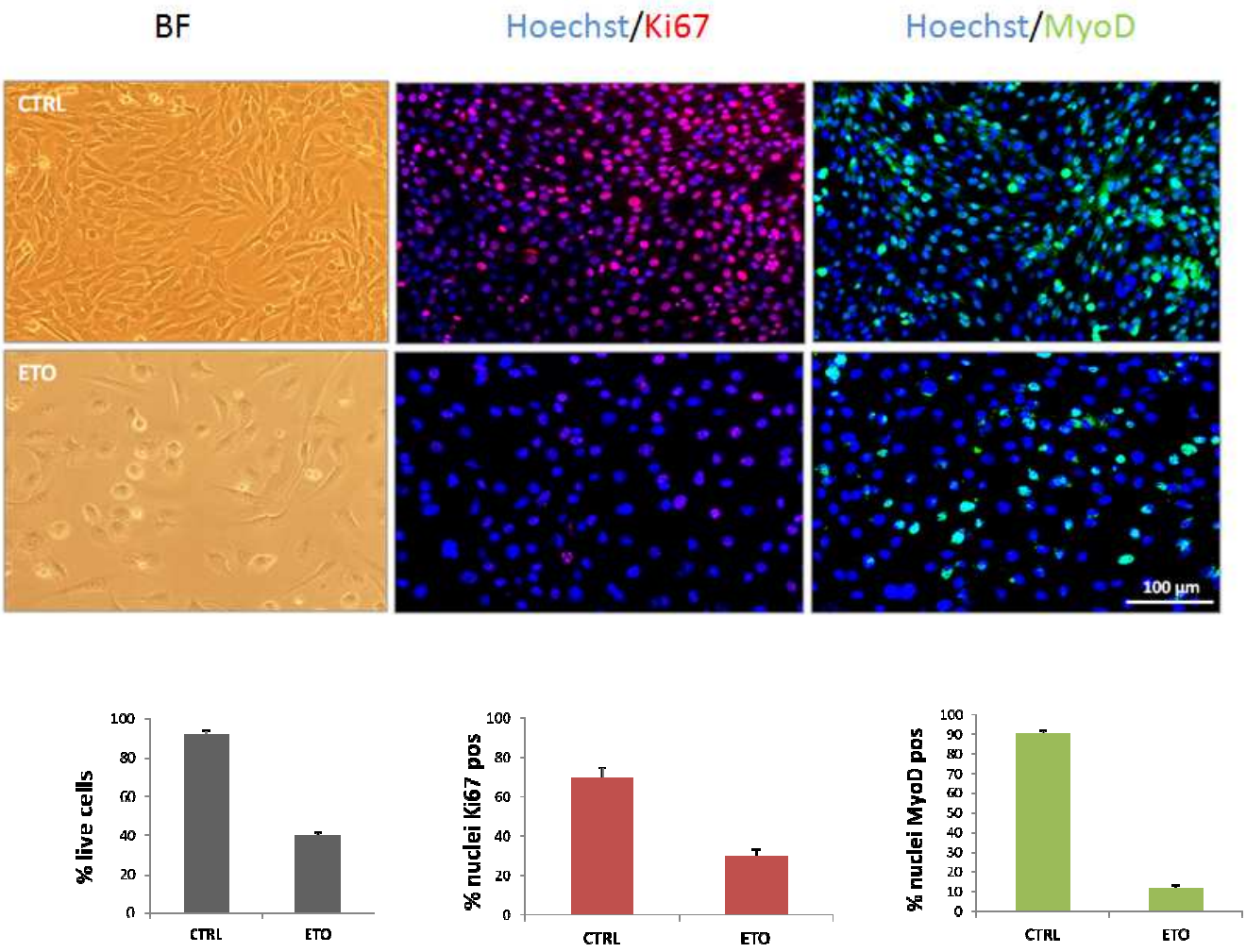

Fig.6

Brightfield shows live cell percentage after 50 µM, 24h eto exposure; IF double stainings show Ki67 (red) and MyoD (green) positivity after50 µM, 24h eto exposure.

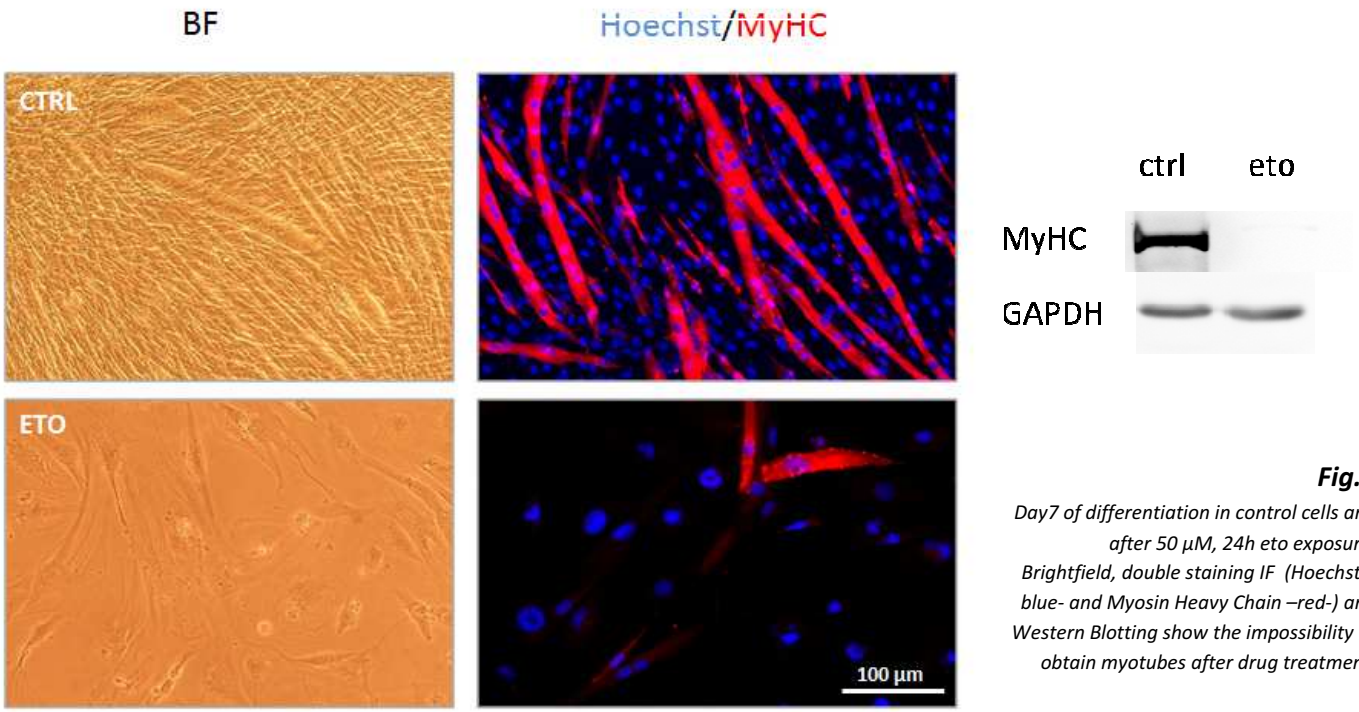

Fig.7

Day7 of differentiation in control cells and after 50 µM, 24h eto exposure. Brightfield, double staining IF (Hoechst – blue- and Myosin Heavy Chain –red-) and Western Blotting show the impossibility to obtain myotubes after drug treatment.

Glutamine

Glutamine is a non-essential branched-chain amino acid (Gaurav *et al*., 2012). It is abundant in the body and its level correlates with protein synthesis in muscles (Yoshida *et al*., 2001). Glutamine synthesis, in skeletal muscle and liver, occurs when its levels in the plasma are inadequate. In condition such as sepsis, trauma, major burns and often post-operatively, glutamine's concentration decreases; so, it seems to be required to give a supplementation in all these conditions as therapeutic and preventive approach (Gaurav *et al*., 2012).

Glutamine addition seems to have a protective role against various stress triggers. In fact, according to Gaurav *et al*., it is one of the three amino acids involved in glutathione (GSH) synthesis, an important intracellular antioxidant and an hepatic detoxifier.

Since skeletal muscle is a tissue extremely sensitive to various stress triggers, the possible protective and therapeutic role of glutamine supplementation drew attention, in recent years, of several researchers in muscle field.

Deldicque *et al*. (2008) studied the effects of several amino acid addition on C2C12 myotubes. In particular, they demonstrated the ability of these molecules to phosphorylate mTOR, enhancing the protein synthesis pathway. Surprisingly, glutamine was not only unable to promote mTOR phosphorylation, but also responsible for an antagonistic effect, in this sense. However it affected expression of desmin, myosin heavy chain and myogenin mRNAs in a time dependent manner and its role was not fully clarified.

Glutamine addition has been used to counteract *"in vitro"* the hyperexpression of atrogin-1 and the enhanced activity of the Ca^{2+}-dependent proteolytic system, induced by Tumor Necrosis Factor-α, a cytotoxic molecule (Bonetto *et al*., 2011).

C2C12 skeletal muscle cells, differentiated with a glutamine-enriched medium (8mM instead of the standard 2mM in differentiation medium), were then exposed to the trigger. Glutamine-differentiated cells showed, in comparison

with control, an higher resistance to TNF-α exposure, which usually reduces Myosin Heavy-Chain (MyHC) expression in existing myotubes (Li *et al*., 1998; Li and Reid, 2000; Girven *et al*., 2016). Authors demonstrated that MyHC expression, myostatin upregulation and atrogin-1 mRNA hyperexpression, normally induced by TNF-α, were contrasted by amino acid intake.

Moreover, TNF-α, which is cronically elevated in conditions where skeletal muscle loss occurs, as well as inducing atrophy in existing myotubes, is able to impair myoblast differentiation (Girven *et al*., 2016). According to Girven and colleagues, glutamine administration rescues myotubes from catabolic system, reducing FoxO3 activation, and improves skeletal muscle cell differentiation.

This apparent *protective role* of glutamine for skeletal muscle cells against stress triggers, suggested to better investigate the possibility to assume this treatment as a therapy for some drug side effects.

Cancer represents a worldwide health problem, responsible for one in four death (Pigna *et al*., 2016) and chemotherapy, although its efficacy, has a lot of side effects in normal tissues, including skeletal muscle. For this reason, several authors focused their attention on finding possible strategies to limit and prevent chemotherapy side effects.

Skeletal muscle is a tissue relatively sensitive to the effects of cancer and its therapy: not surprisingly, cachexia is a common feature in cancer patients (Penna *et al*., 2010) and drugs used in tumor treatment are often responsible for muscle atrophy occurrence (Fanzani *et al*., 2011; Chen *et al*., 2016).

Glutamine supplementation in cancer patients and during chemotherapy, had a central role in recent researches concerning muscle and tumor (Yoshida *et al*., 2001; Mora *et al*., 2002; Gaurav *et al*., 2012; Pesarini *et al*., 2014).

Glutamine is usually taken up by the growing tumor, causing a deficiency in the host which leads to cancer cachexia (Yoshida *et al*., 2001). Then, external intake could be dangerous for cancer patient; however, Klimberg *et al*. (1990)

demonstrated that glutamine supplementation supports only glutamine metabolism in muscles, replacing glutamine's plasma levels and without stimulating tumor growth.

Yoshida *et al*., in 2001, tried to give external glutamine to tumor-bearing rats undergoing chemotherapy. First of all, they noticed a decrease in rats body weight, due to a decrease in protein synthesis as a consequence of the disease; moreover, plasma and skeletal muscle glutamine levels were reduced. Amino acid supplementation increased glutamine levels and protein synthesis. Furthermore, as previously described by Klimberg *et al*., tumor protein synthesis, DNA synthesis and tumor weight were not stimulated by external glutamine administration. Altogether these findings suggested that glutamine supplementation avoids tumor-bearing rats weight loss, enhancing protein synthesis and counteracting tumor-induced protein breakdown.

Even if glutamine and its role in human and animal cancer are conflicting, as well as *in vitro* and *in vivo* studies, there are increasing evidences supporting that glutamine administration could be beneficial for patients undergoing chemotherapy.

Gaurav *et al*. (2012) report that glutamine consumption by the tumor is responsible for Natural Killer (NK) activity depression, due to a decrease in GSH (an intracellular antioxidant and hepatic detoxifier) concentration in these cells; dietary supplementation of glutamine restores GSH levels and NK activity, hampering tumor growth. Moreover, since tumors behave as a "glutamine trap", host glutamine depletion becomes a problem and pharmacological administration may be beneficial. In model of sarcoma from rats receiving methotrexate (an anti-tumoral drug), glutamine administration causes a depletion in GSH content in tumor cells, enhancing their susceptibility to chemotherapy. These interesting data suggest that oral glutamine supplementation enhances the selectivity of antitumor agents, protecting normal tissues from drug side effects.

Pesarini *et al*. (2014) studied the protective role of glutamine supplementation in preventing genotoxic and mutagenic effects induced by cisplatin (cisPt) in mice. cisPt is an antineoplastic agent, which reacts with many cellular structures, in particular DNA, and its use is limited because of its toxicity in normal tissues (Mora *et al*., 2002). Previous studies demonstrated its effects on both undifferentiated and differentiated C2C12 skeletal muscle cells: Salucci *et al*. (2010) studied the pro-apoptotic activity of this molecule on myoblasts, while Fanzani *et al*. (2011) evaluated its effect on mature C2C12 myotubes, focusing the attention on Akt signal impairment, with the consequent myotube atrophy.

The genotoxic and mutagenic effects of cisplatin were investigate on male Swiss mice peripheral blood through the micronucleus assay (Pesarini *et al*., 2014); glutamine administration (more than one concentrations were tested) was able to prevent DNA damages induced by cisPt.

The effects of oral glutamine administration to counteract cisplatin-induced genotoxicity were investigated also by other authors (Mora *et al*., 2002). In this study Wistar rats were used and researchers focused their attention on bone marrow cells. Cisplatin-treated rats showed an increased number of bone marrow cells with chromosome aberration, while just a single dose of glutamine, 24h before drug treatment, was able to reduce the percentage of cisplatin-induced chromosome aberrations.

Altogether, these results suggest that glutamine supplementation has beneficial effects in conditions of cell stress, genotoxicity, and against several side effects induced by commonly used drugs.

However, further investigations are needed to clarify its mechanism of action and to assume, in future, glutamine supplementation as part of drug therapy.

Starvation

The term starvation indicates nutrient deprivation. It has been already described in literature as a potent autophagy inducer, since the condition of nutrient absence is a known stimulus for cells to find alternative energy to survive. The autophagic mechanism is able, among others, to provide nutrients in stress conditions.

Several authors used starvation to conduct studies on autophagy, both *in vivo* and *in vitro* (Grumati *et al*., 2010; Desgeorges *et al*., 2014; De Palma *et al*., 2014).

Grumati *et al*. (2010) focused their attention on the defective autophagy in a model of Collagen VI-knockout mice, which develops muscular dystrophy. In this Col6a1(-/-) mice, the persistence of dysfunctional organelles and the appearance of spontaneous apoptosis induced myofiber degeneration. The absence of autophagosome formation, even after starvation, confirmed a defect in the autophagic machinery. However, autophagic induction through diet modulation and pharmacological therapy seemed to ameliorate the dystrophic phenotype.

Starvation was used by Desgeorges and colleagues (2014) to investigate autophagic regulation and C2C12 myotube behavior in condition of nutrient deprivation and restoration. In this paper, molecular studies suggest that 4h of starvation are associated with a decrease in mTOR activity and Ulk1 phosphorylation, confirming the autophagic activation; moreover LC3BII/LC3BI ratio, LC3B-positive punctate and LC3BII total levels were increased. Finally also the content of autophagy-related protein, such as Atg5-Atg12 conjugate, PI3K and Ulk1, was higher. Nutrient deprivation represents, furthermore, a strong stimulus for the atrophic pathway activation, since FoxO transcription promotes the increase of Atrogin1/MaFbx and MuRF1 mRNA levels; these two transcriptional target of FoxO are involved in the ubiquitine-proteasome system and are strongly expressed in atrophic condition. Concerning myotube

behavior, 4h of starvation were sufficient to induce a strong reduction in myotube area (almost 15%) and in protein content, which were restored after the subsequent nutrient restoration with normal medium, in a situation highly comparable to control.

De Palma *et al*. (2014) focused their attention on the possibility to assume autophagy reactivation as a therapeutic target in a murine model of muscular dystrophy. *mdx* mice showed the accumulation of damaged organelles and a general ultrastructural disorganization, due to a defect in the autophagic mechanism. The persistent activation of mTOR, via phosphorilation of Akt, was the main responsible of the inhibition of autophagy and the down-regulation of autophagy-related proteins. A long term exposure to a low-diet protein led to a normalization in mTOR activation, as well as a to a reduction in muscle inflammation, fibrosis and myofiber damage.

Thus, on the basis of previous works in literature, we hypothesized to assume starvation as an autophagic enhancement, to counteract etoposide-induced damage.

Moreover, since medium restoration after 4h of starvation is able to enhance myotube area up to a condition comparable to control (Desgeorges *et al*., 2014), as confirmed by Immunofluorescence staining below (***Fig.8***), we treated myotubes with etoposide in normal medium after starvation, trying to prevent the excessive cell shrinkage.

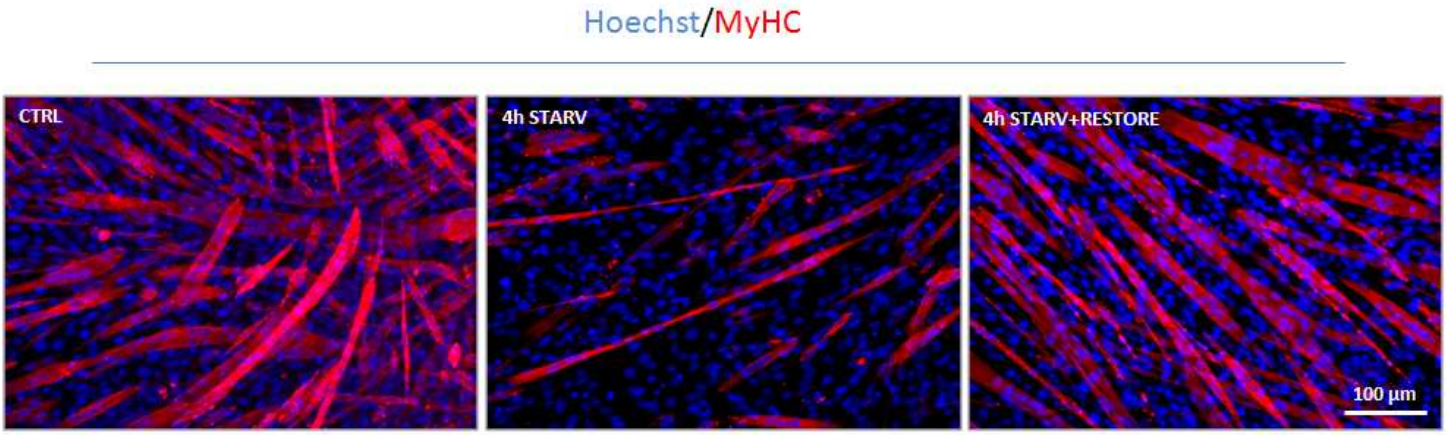

Fig.8

IF double staining for Hoechst (blue) and Myosin Heavy-Chain (red) shows myotube atrophy after 4h of starvation and the following restoration after rich-in-nutrient medium replacement.

Project experimental design

On the basis of the reported evidences, concerning the role of etoposide, glutamine supplementation effects and the pro-autophagic activity of starvation, we designed our experimental model.

We divided our project into two branches, using glutamine or starvation as pre-treatment before etoposide administration (***Fig.9***).

Concerning glutamine supplementation, we decided to induce myoblast differentiation with twice the concentration of the amino acid in DM (differentiation medium), relying on glutamine's beneficial effects found in literature, mentioned above. At the end of the 6th day of differentiation, 50 µM etoposide was administered to myotubes for 24 hours; the experiment ended the day after.

Starvation, on the contrary, was performed during the 6th day of differentiation; cells underwent 4 hours of nutrient deprivation, using DMEM without glutamine neither serum, added only with antibiotics (penicillin/streptomycin) to avoid contamination. The purpose was to activate the survival autophagy against cell

stress and damage induced by the drug. Etoposide administration (50 µM, 24 hours) was then performed in normal DM (see *Materials and Methods*).

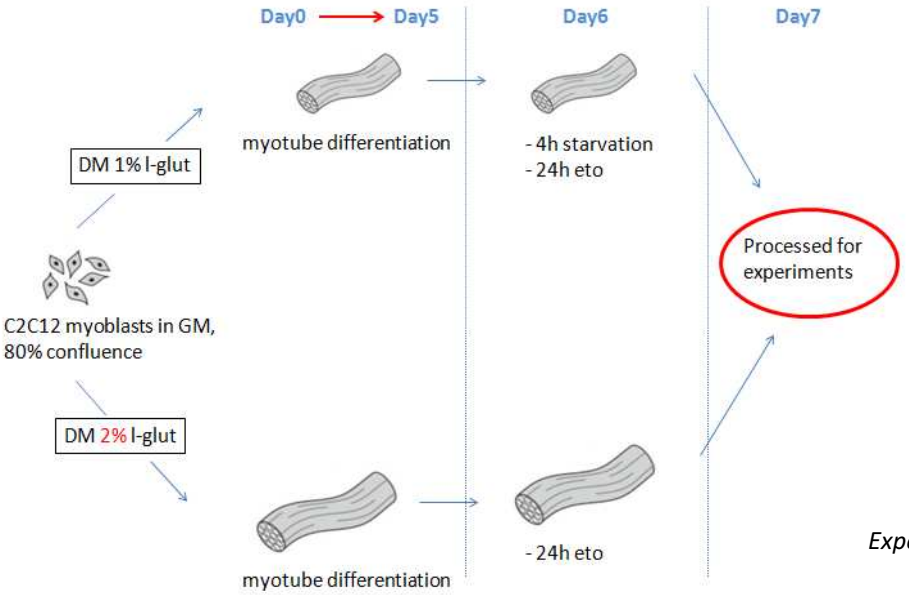

Fig.9
Experimental design scheme.

Materials and Methods

Cell culture

C2C12 cells, a primary line of murine myoblasts (Burattini *et al*., 2004), was used in this study.

Myoblasts were grown in flasks or on coverslips in dishes with growth medium (GM) composed by DMEM (Dulbecco's Modified Eagle Medium; Gibco™ by Life Technologies) added with 10% FBS (Fetal Bovine Serum; Gibco™, Termo Fisher Scientific), 1% L-Glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (pen/strep; PAA, Cell Culture Company). Once achieved approximately 80% of confluence, myotube formation was induced with differentiation medium (DM), composed by DMEM 1% FBS, 1% pen/strep and 1% l-glutamine or 2% l-glutamine, according to the experimental condition.

Cells were maintained at 37 °C in humidified air with 5% CO_2, daily monitored with a Nikon Eclipse TE 2000-S inverted microscope (IM) and photographed with a digital DN 100 Nikon system (Burattini *et al*., 2013). Cells were processed at the end of the 7th day of differentiation (Salucci *et al*., 2015).

Cell treatments

- Glutamine supplementation was carried out with twice the glutamine percentage in DM (2% instead of the standard 1%).
- 4h Starvation were performed with DMEM added only with pen/strep, to avoid cell contamination.
- 50 µM etoposide was administered to cells in DM for 24h at the end of the 6th day. Drug concentration was chosen on the basis of previous paper published by our group (Salucci *et al*., 2010).

Transmission Electron Microscopy (TEM)

Both pellets and monolayers were analysed by means of TEM.

Samples were grown in flasks to get pellets and on coverslips in dishes to obtain monolayers. In both cases, specimens were rinsed with PBS (Phosphate Buffer Saline; Gibco™ by Life Technologies) and immediately fixed "in situ" 2.5% glutaraldeyde in 0.1M phosphate buffer for a total of 45 minutes; cells designed to form pellets were gently scraped after 15 minutes and then centrifuged at 1200 rpm for an additional time of 30 minutes (Salucci *et al*., 2013). Post-fixation in 1% OsO_4 (osmium) for 1 hour, alcohol dehydratation and araldite inclusion were performed, as previously described (Salucci *et al*., 2010). Monolayers embedding was carried out inverting capsules full of resin on samples; coversplips were then crashed and removed in liquid nitrogen.

Thin section (~ 0.5 μm) analyses were preceded by the observation of toluidine blue-staining semithin sections (~ 1 μm), which allow an overall specimen view (Burattini *et al*., 2004). Sample sections were cut using an LKB ULTROTOME®V and collected on 200 mesh nickel grids. After uranyl acetate and lead citrate stainings, specimens were observed with a Philips CM10 electron microscope.

Confocal Laser Scanning Microscopy (CLSM)

Cells were grown, treated and processed for observations on coverslips in a 6well plate.

Acridine Orange (AO) (Immunological Sciences) is a pH-sensitive dye, useful to detect acidic vacuolar organelles (Salucci *et al*., 2013). 500 ng/ml AO were added directly to culture medium for 15 minutes at 37°C.

Premo™ Autophagy Sensor LC3-GFP (Thermo Fisher Scientific) allows to detect the LC3 protein localization; cells were transduced following the protocol provided by the datasheet and observed the day after.

Observations were performed with a Leica TCS-SP5 confocal microscope, connected to a DMI 6000 CS Inverted Microscope (Leica Microsystems CMS Gmbh); pictures were analyzed using the Leica Application Suite Advanced Fluorescence (LAS AF) software (Salucci *et al*., 2015).

Immunofluorescence analysis (IF)

Cells were grown in a 12well plate, fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes in the dark, rinsed with PBS and stored in the cold room (+4°C) until the immunostaining.

Samples were permeabilized with 0.2% Triton X-100 in PBS, containing 1% bovine serum albumin (BSA). Donkey serum (1:10 dilution in PBS) was applied as blocking solution and specimens were incubated with primary antibodies (reported hereafter) overnight in PBS+1% BSA (Costamagna *et al*., 2016). Secondary Alexa Fluor donkey antibodies were used 1:500 in PBS supplemented with 1% BSA and the nuclei were stained with Hoechst (Sigma-Aldrich, 1:3000 in PBS). Images were collected using an Eclipse Ti inverted microscope (Nikon).

Primary antibodies and their dilutions were as follows: 1:4 mouse anti-MyHC (Developmental Studies Hybridoma Bank, DSHB, clone MF20), 1:300 mouse anti-ki67 (Abcam), 1:50 rabbit anti-MyoD (Abcam).

Flow Cytometry (FC)

Acridine Orange (AO) and Nonyl Acridine Orange (NAO) dyes were used to evaluate the acidic compartment activity and the cardiolipin peroxidation respectively (Thibodeau *et al*., 2004; Manna *et al*., 2015).

AO is a pH-sensitive dye, used to detect acidic vesicular organelles formation (Chen *et al*., 2010; Canonico *et al*., 2014). It is a cell-permeable fluorescent dye which, at high concentrations, stains DNA in red and cytoplasm in bright green. Concerning the acidic compartments, such as lysosomes and autolysosomes, it

becomes protonated and sequestered; at low concentrations AO emits red fluorescence with an intensity proportional to the acidity degree and acidic compartment volume. (Tragonos *et al*., 1994).

Cells were washed and resuspendend in 0.5 ml of medium and then stained with AO 75 ng/ml, 15 minutes at 37°C. Red lysosomal and green cytoplasmatic fluorescences of 10,000 cells per sample were acquired by flow cytometry using the FL3 and FL1 channels respectively.

The cardiolipin-sensitive probe Nonyl Acridine Orange (NAO; Sigma-Aldrich) is able to monitor changes in mitochondrial lipids (Canonico *et al*., 2014; Luchetti *et al*., 2007; Salucci *et al*., 2013) and used at low concentrations, in living cells, is an efficient fluorescent indicator for the peroxidation of cardiolipin, an inner mitochondrial membrane lipid. Cells were incubated with 150nM NAO for 15 min at 37 °C in the dark and then acquired by flow cytometry using the appropriate fluorescence channels. 10,000 events for each condition were analyzed.

Results

Immunofluorescence

IF staining was performed to evaluate Myosin Heavy-Chain (MyHC) changes in our experimental model, in order to understand if pre-treatments were able to counteract the atrophic pathway activation, induced by etoposide administration, preventing fiber size reduction (***Fig.10***).

Control fibers were well organized and had the typical elongated shape (*Fig.10*, CTRL). Fusion index (***Fig.11***), calculated on the basis of the ratio between the number of nuclei inside myotubes and the total number of nuclei in a field, was about 60%, according to other papers present in literature, regarding C2C12 f.i. (Gabillard *et al*., 2010).

Eto-treated cells displayed a strong reduction in terms of myotube number and even the few existing syncitia significantly changed (*Fig.10*, ETO). All the considered field had little and thin myotubes, with a fusion index reduced to 20% or even less.

Glutamine-supplemented myotubes were similar to control (*Fig.10*, +GLUT). Moreover, myotube area seemed moderately higher, as well as the fusion index (~66%). Since glutamine alone produced some cell stress, as observed by means of TEM and FC (see below), few suffering myotubes were also detectable.

In nutrient starvation plus medium restoration condition (*Fig.10*, STARV+RESTORE), myotubes presented well preserved morphology and organization. In agreement with Desgeorges and colleagues (2014), 4 hours of nutrient deprivation were sufficient to induce protein loss but restoring cells with rich-in-nutrient medium seemed to reactivate the protein synthesis pathway, leading to re-establish myotube area, which could be even larger than the starting point. Calculated f.i. was about 54%.

Eto administration, after both pre-treatments, didn't produce the same negative effects observed after the treatment alone.

STARV+ETO condition, indeed, showed first of all the presence of a greater number of myotubes, in all examined fields; moreover, even if some thin

syncitia were present, cell area and fusion index (~47%) were preserved against the drug atrophic induction.

Nevertheless, the best way to preserve myotube area from the drug catabolic activity, was glutamine supplementation. Cells showed a good differentiation stage (*Fig.10*, GLUT+ETO) and the calculated fusion index was about 58%.

IF analyses suggested that etoposide administration induced fiber shrinkage, probably because of the activation of the atrophic pathway. Starvation and glutamine supplementation both improved myotube morphology and viability; moreover, amino acid supply seemed to promote myotube area preservation, counteracting drug-induced atrophy *in vitro*.

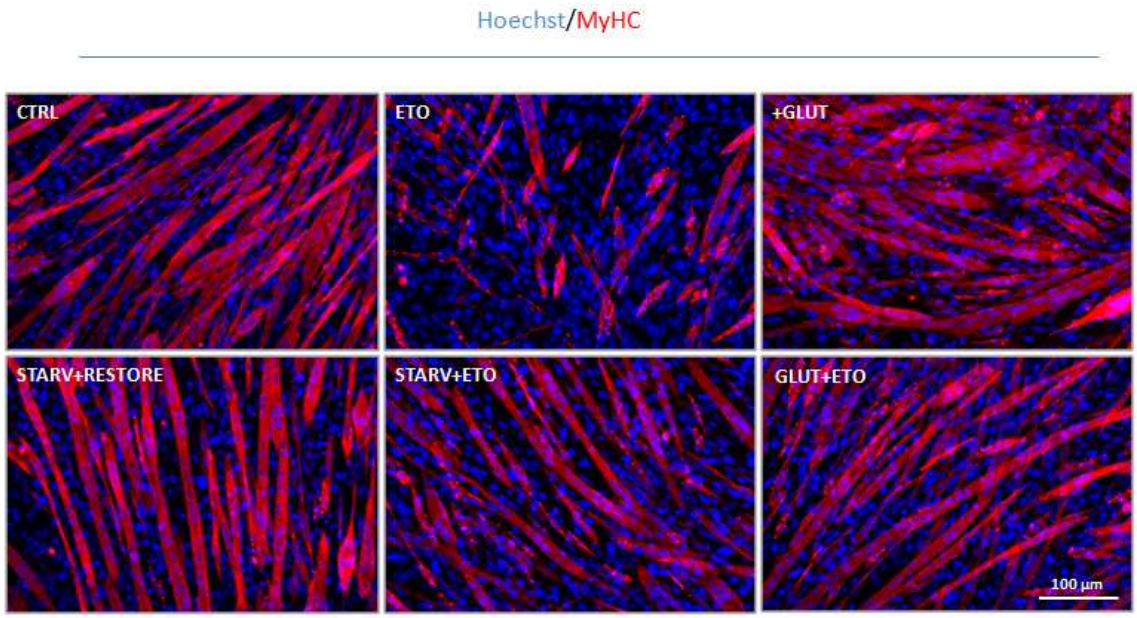

Fig.10

IF double staining for Hoechst (blue) and Myosin Heavy- Chain (red).

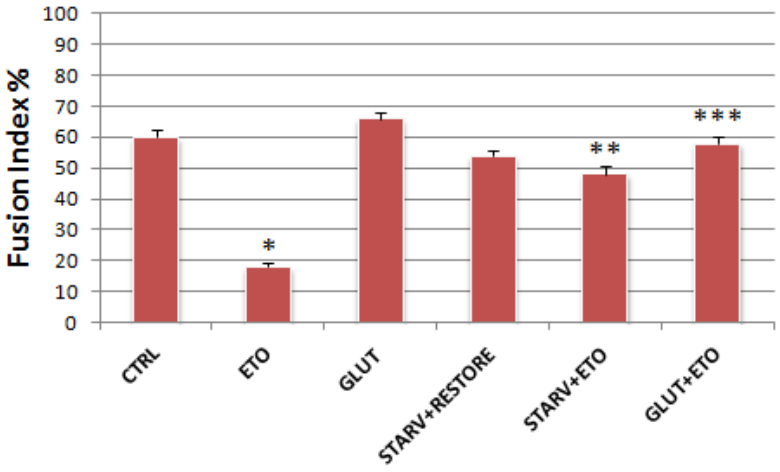

Fig.11

Fusion Index calculated through number of nuclei inside myotubes and total number of nuclei ratio. Data are from 3 different experiments and furnished as mean±SD.

**p≤ .05 vs CTRL;*
***p≤ .05 vs ETO;*
****p≤ .05 vs ETO.*

Transmission Electron Microscopy

TEM observations (***Fig.12-13***) allowed to monitor the treatment effect within cells. We focused our attention on cell stress and viability, vacuole formation and degradation, general organelle status, in all our experimental conditions.

Control myotubes (***Fig.12***, CTRL) were multinucleated (not shown) and ultrastructurally in a general good condition. At higher magnification, diffuse myofilaments were detectable and organelles, mitochondria and endoplasmic reticulum in particular, presented a well preserved morphology, suggesting a good cell viability and functionality. Since autophagy is important for organelle turnover and it occurs also in basal conditions, we detected the activation of the autophagic basal flux, as indicated by white arrows.

Glutamine supplementation (***Fig.12***, +GLUT) was able to promote a moderate increase in myotube size, confirming IF observations (see above, *Fig.10*). Despite mitochondria showed a preserved morphology and viability (***Fig.12***, high magnification), glutamine supply induced a bit of stress, in comparison with control: large and complex vacuoles were sometimes observable, especially in the perinuclear area.

Starvation induced, as expected, an increased cytoplasmic vacuolization, suggestive for the autophagic activation (***Fig.12***, STARV+RESTORE). Nutrient deprivation, indeed, is a common strategy used to promote autophagic activation and few hours have been demonstrated to be sufficient to enhance

the catabolic activity (Desgeorges *et al*., 2014). In our experiment we decided to "re-feed" myotubes after the starvation period, to induce autophagic activation without compromising cell integrity. Cells appeared well organized, with a diffuse cytoplasmic vacuolization, due to the presence of a large number of autophagosomes (***Fig.12***, high magnification). It was interesting to note that often vacuoles were close to damaged organelles, mainly mitochondria; mitophagy events, indeed, were observable.

Etoposide was already described as an apoptotic and oxidative stress inducer in C2C12 cells (Salucci *et al*., 2013). Due to their multinucleated nature, myotubes have been demonstrated more resistant to the pro-apoptotic activity of this drug, compared with myoblasts. In a previous work conducted by our group (Salucci *et al*., 2013), TUNEL reaction (performed by means of CLSM) showed the presence, within the same syncitia, of positive and negative nuclei. According to our observations, etoposide seemed to impair autophagy. Drug treatment, indeed, produced a diffuse cytoplasmic vacuolization, clearly different from the one induced by starvation (***Fig.13***, ETO). Large vacuoles, localized near the nucleus and often bigger than it (***Fig.13***, high magnification), characterized this condition. Perinuclear accumulation of complex vacuoles (***Fig.13***, ETO-inset), made us think about a break in the autophagic pathway: autophagosomes, probably, were not able to fuse with lysosomes for the final stage of the process, resulting in a cytoplasmic accumulation. However, we performed further analyses by means of FC and CLSM, to confirm this hypothesis. Deeply changed mitochondria were also observable at higher magnification, confirming the pro-oxidant activity of etoposide.

Ultrastructural analyses suggested that both pre-treatments were able to preserve myotube morphology against etoposide-induced damage.

Glutamine supplemented myotubes (***Fig.13***, GLUT+ETO) showed an ultrastructure comparable to control, although some stress markers, such as vacuoles and empty mitochondria, were observable. Nuclei and mitochondria

viability was largely preserved (*Fig.13*, GLUT+ETO-high magnification). Although few complex vacuoles, similar to the ones detected in etoposide-treated cells, were detectable, morphology of glutamine supplemented myotubes was generally comparable to control..

Even starvation attenuated antineoplastic drug effects (***Fig.13***, STARV+ETO). Forced pathway activation avoided vacuole accumulation, maintaining the general good morphology of myotubes. However, signs of stress were evident such as expanded tubules of endoplasmatic reticulum and some mitochondria with dilated cristae (*Fig.13*, STARV+ETO-high magnification).

Finally, we can assume that, on the basis of ultrastructural analyses, both pre-treatments were able to counteract etoposide-induced damage. Glutamine supplementation was the best way to counteract the atrophying activity of the drug and, apparently, to reduce its pro-oxidant effect. 4 hours of starvation, through the forced activation of the “protective autophagy”, seemed to keep safe cells from the subsequent etoposide administration, occurred in normal medium.

Anyhow, further techniques were performed to confirm these morphological observations.

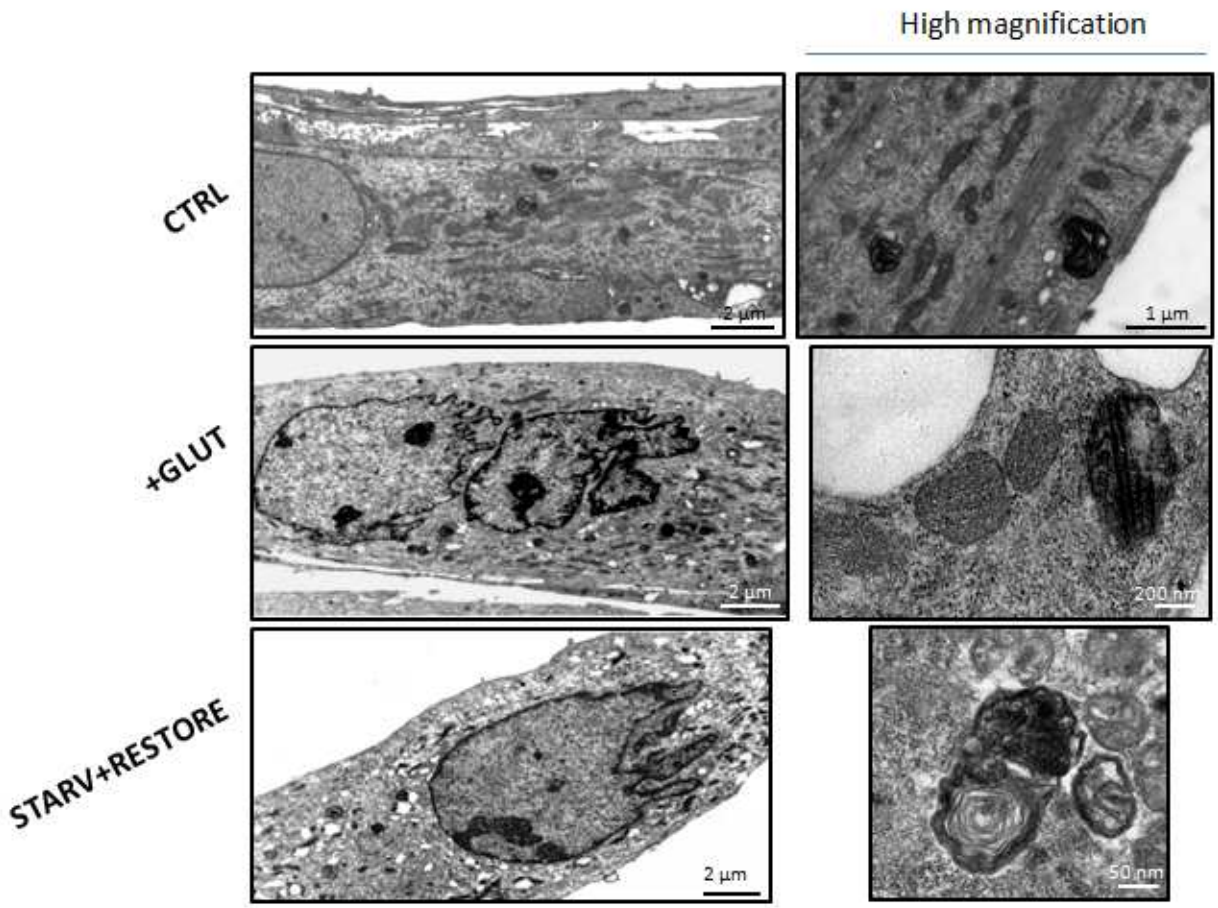

Fig.12

TEM analyses at low and high magnification of control, glutamine supplementation and starvation conditions.

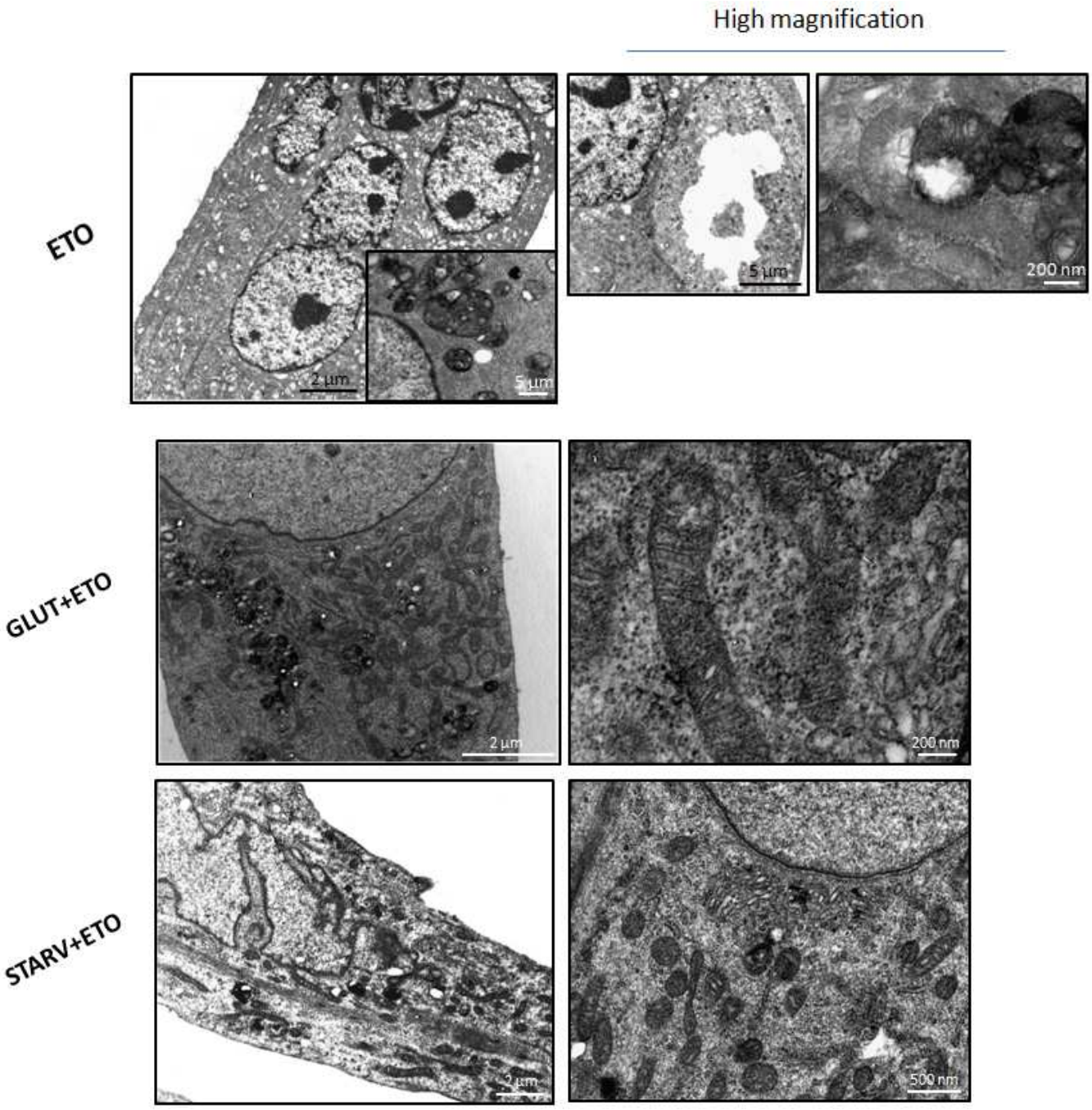

Fig.13

TEM analyses at low and high magnification of etoposide treated myotubes and eto administration after glutamine supplementation and 4 hours of starvation.

Confocal Laser Scanning Microscopy

LC3 is a protein localized on the autophagosome membrane. It is thought to be a good marker for autophagy and then monitoring its behavior could be useful to detect the status of the autophagic flux (Kimura et al., 2009). Thus, we monitored the protein behavior by means of CLSM (***Fig.14***).

Control cells (*Fig.14*, CTRL) showed a slight presence of green LC3 spots, confirming the constant activation of the autophagic basal flux.

Etoposide-treated cells showed an accumulation of LC3 protein as large green spots (*Fig.14*, ETO). In this condition we noticed that LC3 was mainly localized near the nucleus, confirming TEM observations. The blockage of the autophagic pathway, induced by drug administration, probably occurred just before the fusion between autophagosomes and lysosomes, causing the cytoplasmic vacuole accumulation.

Glutamine-differentiated myotubes (*Fig.14*, +GLUT) displayed a periferic localization of little LC3 dots. This enhanced autophagic activation is not a surprise, since TEM observation already showed a bit of stress induced by glutamine supply. Therefore, the presence of damaged cytoplasmic material presumably stimulated autophagosome formation involved in its removal.

On the contrary, starved cells (*Fig.14*, STARV+RESTORE) showed a diffuse cytoplasmic localization of LC3 protein, confirming the forced pathway activation.

Both pre-treatments seemed to have beneficial effects on etoposide-treated myotubes.

LC3 localization, in STARV+ETO condition, was similar to the control and the large accumulation noticed after drug exposure, was avoided. Glutamine supplementation (*Fig.14*, GLUT+ETO), before etoposide administration, limited vacuole accumulation, although autophagosome presence is not totally comparable to control.

Our idea was that starvation enhanced autophagic pathway activation, avoiding LC3 accumulation, while glutamine supplementation seemed to have a beneficial effect in preserving lysosomal activity, allowing autophagolysosome formation.

To confirm these last idea we needed to analyze acid compartment behavior; thus, we investigated the lysosomal activity performing Acridine Orange (AO) staining (***Fig.15***).

AO dye emits red/orange fluorescence in the presence of an active lysosomal compartment; in case of an impairment of this mechanism, fluorescence turns into green (Canonico *et al*., 1969).

During our observations, we noticed that control cells had a well preserved acid compartment activity, suggested by the presence of little red dots in the cytoplasm. Lysosomes were mainly localized at the periphery on nuclei (*Fig.15*, CTRL).

Etoposide exposure induced the appearance of big green dots, suggestive of an impairment of lysosomal activity (*Fig.15*, ETO). These observations were in agreement with both LC3 Confocal assay and TEM analyses, also on the basis of AO stained vacuoles dimensions and localization.

Glutamine supplementation alone (Fig.15, +GLUT) induced some cell stress at cytoplasmic level, as confirmed by the presence of green stained vacuoles; nevertheless, glutamine administration before etoposide exposure (Fig.15, GLUT+ETO), showed a reactivation of acid compartment , as demonstrated by the presence of red little dots.

Since starvation is a well known autophagic inducer, bright red vacuoles were detectable, as expected (*Fig.15*, STARV+RESTORE); starvation used as a pre-treatment against drug administration, improved lysosomal activity, avoiding the break in the autophagic machinery (*Fig.15*, STARV+ETO).

These AO data were further validate by means of FC.

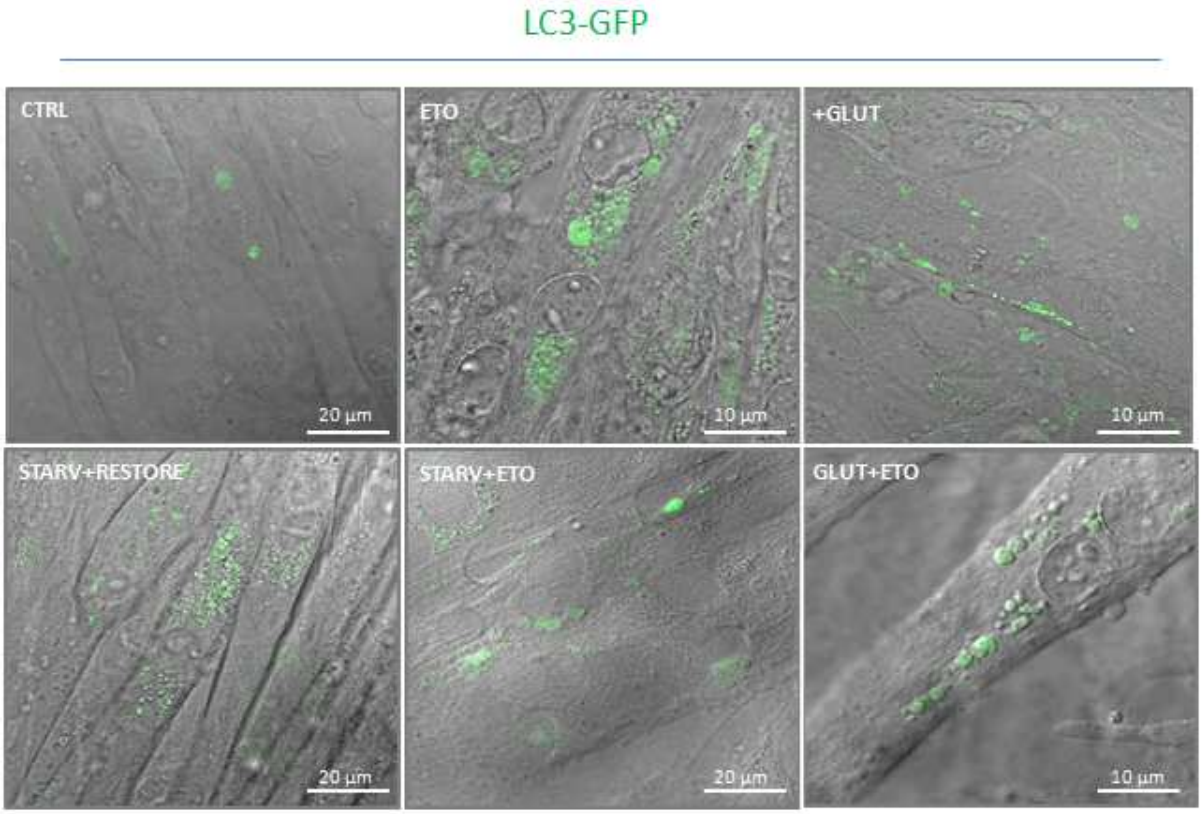

Fig.14

LC3 protein, conjugated with GFP, behavior monitored by means of CLSM.

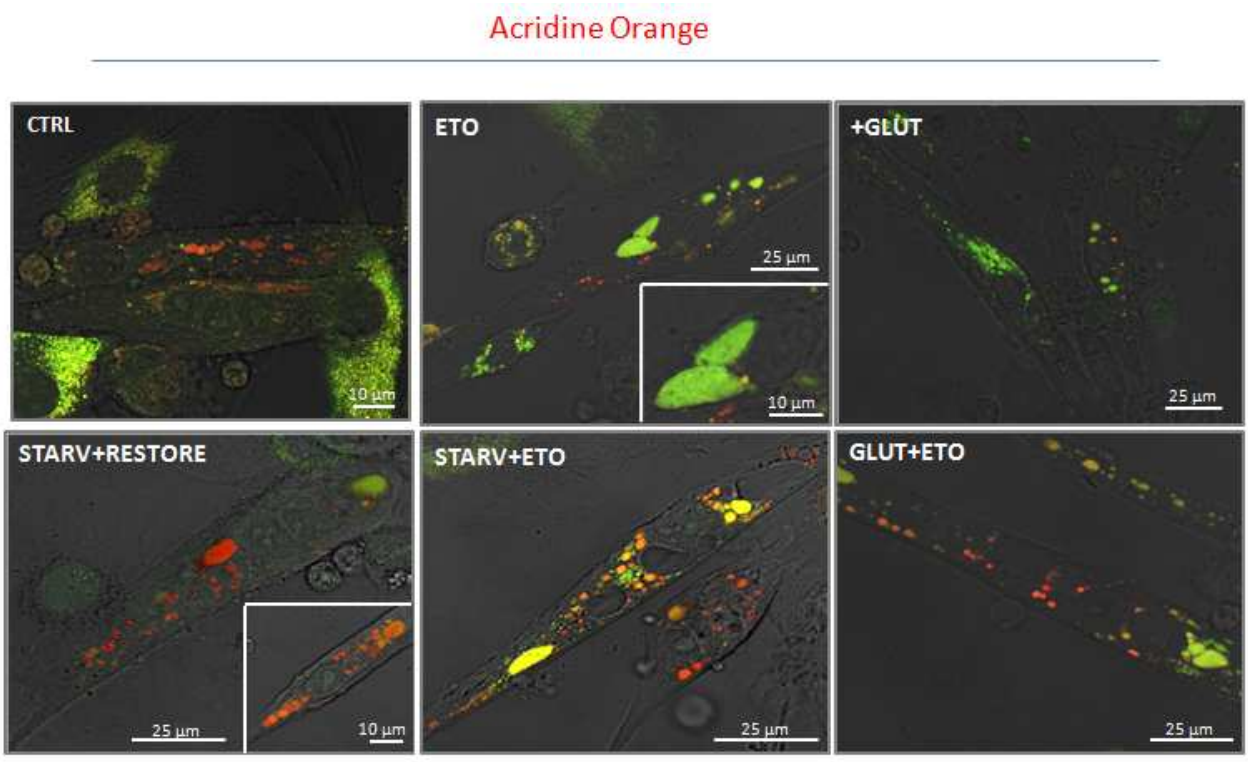

Fig.15

AO staining for lysosomal compartment activity evaluation, by means of CLSM.

Flow Cytometry

Acid compartment evaluation, by means of FC (Acridine Orange), confirmed CLSM analyses (***Fig.16***).

Etoposide administration, as well as glutamine supplementation, reduced lysosomal activity (*Fig16*, ETO; +GLUT). Starvation and nutrient restoration markedly activated lysosomal compartment, as confirmed by the calculated number of events (*Fig.16*, STARV+RESTORE). Drug exposure, after 4h of nutrient deprivation didn't affect in the same way lysosomal compartment which seemed to be preserved (*Fig.16*, STARV+ETO). Glutamine supplementation as pre-treatment against etoposide-induced damage, significantly reactivated acid compartment if compared to etoposide treated myotubes (*Fig.16*, GLUT+ETO).

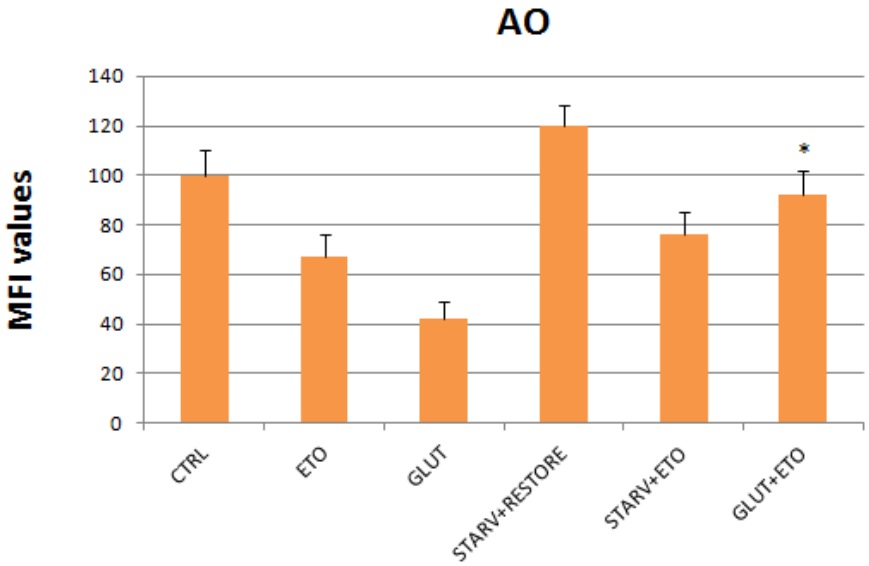

Fig.16

Statistical histogram of MFI (Mean Fluorescence Intensity)variation of AO.
Each value is expressed as a mean±SD (results from 3 independent experiments).

Cardiolipin is an inner mitochondrial membrane lipid, and it is responsible for the *cytocrome c* anchorage to the membrane. Thus, in order to investigate its peroxidation, we performed NAO assay to acquire informations on the mitochondrial pool status (***Fig. 17***).

FC analyses showed that an increased oxidation occurred after etoposide administration (*Fig.17*, ETO); according to other assays already performed, also glutamine supplementation alone seemed to produce mitochondrial stress (*Fig.17*, +GLUT), in comparison with control.

Although starvation is beneficial for the general cell health, it basically represents a stress; FC analysis, indeed, showed an increase in cardiolipin peroxidation level also in this condition (*Fig.17*, STARV+RESTORE).

Nutrient deprivation, before drug exposure, didn't have any beneficial effect (*Fig.17*, STARV+ETO); mitochondrial lipid oxidation, indeed, was at the same levels of etoposide administration alone.

Glutamine, on the contrary, seemed to protect myotubes against lipid peroxidation (*Fig.17*, GLUT+ETO). The number of events revealed after eto exposure, was significantly reduced after amino acid supplementation.

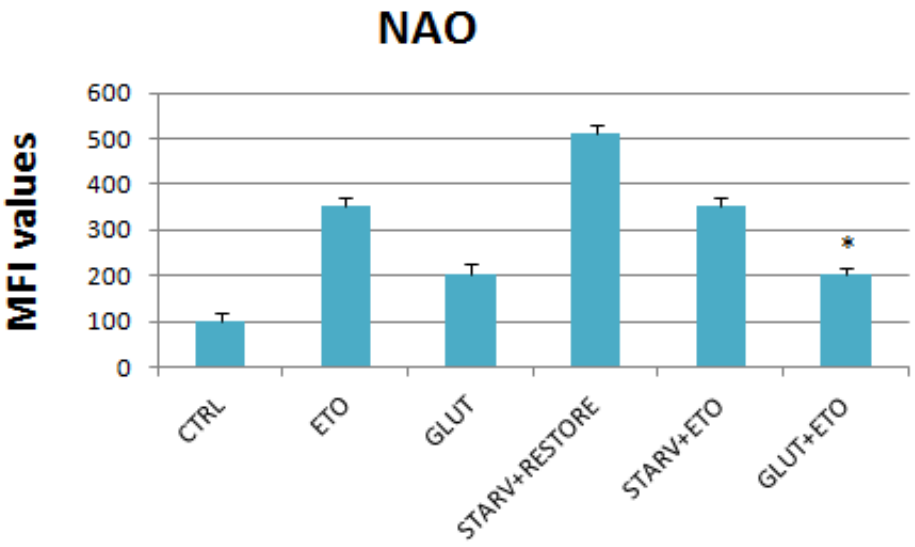

Fig.17

Statistical histogram of MFI (Mean Fluorescence Intensity)variation of NAO.
Each value is expressed as a mean±SD (results from 3 independent experiments).

Discussion

This project had the purpose to find a strategy to counteract damage induced on muscle cells by an antineoplastic treatment. Chemotherapy, indeed, represents a valid means to counteract tumor progression, but it is well known that side effects of the treatment are harmful for patients. Skeletal muscle is an extensive constitutive tissue of human's body and it is very susceptible to cancer related therapy. Cancer cachexia, is a common feature in patients affected by tumor (Penna *et al*., 2010) and one of the most frequent side effects of antineoplastic treatment, among others, is muscle atrophy (Fanzani *et al*., 2011; Chen *et al*., 2016).

In our *in vitro* model, we administered etoposide (50 µM for 24 h; Salucci *et al*., 2010), a chemotherapeutic drug, to mature C2C12 myotubes, to reproduce the harmful effects of this drug on muscles. We observed, by means of IF and TEM, that etoposide induced first of all a marked reduction of cell area; then, oxidative stress, general organelle disorganization as well as an accumulation of autophagic vacuoles were detectable. Acridine Orange assay, analyzed by means of CLSM and FC, suggested that drug administration induced an alteration at lysosomal level, compromising the autolysosome formation. These data supported observations of LC3 protein behavior, which was accumulated after etoposide exposure, suggesting a break in the autophagic machinery.

In order to prevent the hyper-activation of the catabolic pathway, we tried to induce cell differentiation introducing twice the glutamine concentration in cell culture medium. Moreover, a few papers in literature already demonstrated the beneficial effect of glutamine supplementation after TNF-β administration (Bonetto *et al*., 2011) and as a conditioning therapy for several chemotherapic side effects (Yoshida *et al*., 2001; Mora *et al*., 2002; Gaurav *et al*., 2012; Pesarini *et al*., 2014). IF and TEM analyses, showed that amino acid supplementation is able to preserve myotube area, counteracting the cell shrinkage promoted by 24 hours of etoposide administration.

Since TEM observations suggested an autophagic impairment, in a parallel experiment we administered etoposide in normal medium, after 4 hours of starvation, which is a common strategy used to induce autophagy (Desgeorges *et al*., 2014). IF staining for Myosin Heavy-Chain showed that myotubes didn't undergo the same harmful effect in terms of cell area shrinkage; moreover, TEM analyses suggested that organelles, especially mitochondria, were preserved, as well as cell viability.

Etoposide, moreover, is an oxidative stress inducer (Salucci *et al*., 2013), and NAO assay, analyzed by means of FC, demonstrated an increased cardiolipin peroxidation after the administration. Surprisingly, amino acid supplementation reduced lipid peroxidation, suggesting, in this model, a role in preventing oxidative stress events. In this sense, starvation didn't produce beneficial effects.

Seeing that drug administration produced an unusual cytoplasmic autophagosome accumulation, we hypothesize that etoposide impaired autophagy in muscle cells, probably at the end of its steps: the fusion between autophagosomes and lysosomes, to form autolysosomes, didn't occur. To validate our idea we monitored LC3 protein behavior and lysosomal activity by means of CLSM.

LC3-GFP assay showed, as expected, a slight presence of autophagosomes in control cells, suggestive of an always active autophagic basal flux. In etoposide treated cells, large green spots were accumulated and localized in the perinuclear area, partially confirming the block in the autophagic machinery. Both starvation and glutamine produced beneficial effects: starvation, enhancing autophagy, generated a control-like condition, with just a slight and diffuse LC3 presence. Glutamine supplementation hindered uncontrolled

autophagosome accumulation, showing the presence of some LC3 positive dots, smaller than the ones detected in etoposide-treated myotubes.
Through Acridine Orange assay we tried to better explain what reported above. Red fluorescence, emitted by control and starvation conditions, suggested a preserved and active lysosomal compartment; lysosomal activity, as confirmed by AO assay by means of FC, was highly down-regulated after etoposide administration and after glutamine supplementation. Surprisingly, etoposide was able to generate a slight impairment of lysosomal activity also after starvation (orange/yellow fluorescence), while amino acid supply preserved acid compartment also after drug administration (red fluorescence).

Finally we can assume that in a *in vitro* model of skeletal muscle, antineoplastic treatment had a detrimental effect. Nutrient modulation, through amino acid supplementation and deprivation, produced some beneficial results in terms of fiber area preservation, oxidative stress limitation and autophagic machinery progression.
Since the two chosen pre-treatments had some contrasting effects, it could be interesting to merge amino acid supply and deprivation focusing on the timing of treatment in relation to drug administration.

Further molecular studies are already ongoing to further validate our data and give us more information on the effects of our treatments on the autophagic pathway.
Once achieved exhaustive results *in vitro*, it could be interesting to shift our model *in vivo*, evaluating effects of diet modulation on tumor-bearing mice undergoing chemotherapy.

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"se sapessimo esattamente quello che stiamo facendo,

non si chiamerebbe ricerca, no?"

A. Einstein