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ACID SPHINGOMYELINASE AS A NEW PHARMACOLOGICAL TARGET IN THE ACUTE AND CHRONIC MUSCLE DAMAGE: AN ALTERNATIVE STRATEGY FOR MUSCULAR DYSTROPHIES THERAPY

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SUMMARY

Skeletal muscle inflammation plays a critical role in bridging early muscle injury responses and timely muscle injury recovery (Yang and Hu, 2018). In this study, we investigated the functional role of the sphingolipid-metabolizing enzyme Acid Sphingomyelinase (A-SMase) in the pathophysiology of acute and chronic muscle damage in order to elucidate its role in the establishment of inflammation and in the subsequent muscle regeneration process so that this protein may be proposed as possible therapeutic target. A-SMase is a critical mediator of cell signaling since it is able to generate ceramide from the membrane lipid sphingomyelin thus modulating membrane fluidity, which is determinant in triggering many cellular processes. Several recent studies report the strong relation between high levels of A-SMase expression and inflammatory-associated disorders (Schissel et al., 1998; Devlin et al., 2008; Garcia-Ruiz et al., 2015).

In this study, we found that A-SMase expression increases upon induced-acute muscle damage suggesting its involvement in skeletal muscle inflammation. We also demonstrated the importance of A-SMase in regulating the muscle regeneration process following acute muscle damage. Our results showed that A-SMase deficiency leads to an increase of muscle satellite cells, essential for skeletal muscle regeneration, soon after injury, accompanied by a higher number of regenerating myofibers within the injured site. Moreover, two important muscle transcription factors, MyoD and Myogenin, responsible for a correct regeneration were much higher in the absence of A-SMase suggesting that muscle regeneration is accelerated without the hydrolase. In addition, IGF-1, a potent enhancer of tissue regeneration, showed much higher expression levels in absence of A-SMase, consistently with our finding that A-SMase deficiency accelerates the regeneration process. Furthermore, we provide the first evidence of a novel role of A-SMase in regulating macrophage subsets during muscle regeneration demonstrating that A-SMase is able to regulate the polarization of macrophages towards an inflammatory M1 phenotype since its absence leads to an impairment in the expression of M1 macrophage markers.

Noteworthy, investigating the role of A-SMase in *mdx* mice, a mouse model of Duchenne Muscular Dystrophy (DMD), we found an up-regulation of A-SMase in expression and activity in muscles of these mice, that implies its involvement in the pathogenesis of DMD with a particular effect on inflammation. Several studies demonstrated a predominant role of inflammation in the pathogenesis of DMD (Villalta et al., 2009; Radley et al., 2008). Of notice, we observed that the increase of A-

SMase in *mdx* mice paralleled with the increase of muscle inflammatory state. This finding has been further corroborated by the use of the anti-inflammatory drug Naproxcinod that reduced inflammation in *mdx* muscle and at the same time significantly decreased A-SMase expression and activity.

Altogether, our findings open new vistas in the identification of a new potential pharmacological target, A-SMase, in the development and regulation of skeletal muscle inflammation and regeneration process by raising the possibility that the modulation of A-SMase expression levels could bring therapeutic benefits not only in DMD pathology but also in various muscle-wasting diseases.

RIASSUNTO

L'infiammazione del muscolo scheletrico rappresenta la prima risposta protettiva conseguente ad un danno muscolare e svolge un ruolo fondamentale nel recupero funzionale del muscolo (Yang and Hu, 2018). In questo studio, abbiamo investigato il ruolo dell'enzima-idrolasi Sfingomielinasi Acida (A-SMase) nella patofisiologia del danno muscolare acuto e cronico con lo scopo di comprendere al meglio il suo ruolo funzionale nell'insorgenza del processo infiammatorio e nella successiva fase di rigenerazione muscolare affinché possa essere considerato come possibile bersaglio terapeutico. L'A-SMase è un mediatore critico nella segnalazione cellulare; la sua capacità di generare ceramide a partire da un importante lipide di membrana, la sfingomielina, lo rende abile nel modulare la fluidità del doppio strato lipidico di membrana, determinante per l'innesco di molti processi cellulari. Recenti studi, riportano una evidente relazione tra alti livelli di espressione dell'A-SMase e numerose patologie caratterizzate da una forte componente infiammatoria (Schissel et al., 1998; Devlin et al., 2008; Garcia-Ruiz et al., 2015). I nostri risultati rivelano che l'espressione dell'A-SMase aumenta significativamente in seguito a danno muscolare acuto, dimostrando il suo coinvolgimento nell'infiammazione del muscolo scheletrico. Inoltre, abbiamo anche dimostrato l'importanza dell'enzima nella regolazione del processo di rigenerazione muscolare conseguente al danno acuto. In seguito a danno muscolare, abbiamo scoperto che l'assenza di A-SMase causa un incremento sia del numero delle cellule satelliti, essenziali per la rigenerazione muscolare, che del numero delle miofibre rigeneranti nella sede di lesione. Inoltre, MyoD e Myogenin, due importanti fattori trascrizionali, responsabili di una corretta rigenerazione muscolare, mostrano livelli di espressione più alti in assenza di A-SMase, indicando un processo di rigenerazione accelerato. Anche IGF-1, un fattore di crescita cruciale nel promuovere la rigenerazione muscolare, mostra livelli di espressione più alti in assenza dell'A-SMase, coerentemente con la nostra evidenza precedente in cui abbiamo riportato che in assenza dell'enzima il processo di rigenerazione è accelerato. Molto importante, i nostri risultati forniscono la prima evidenza sperimentale di un nuovo ruolo dell'A-SMase nella regolazione del fenotipo dei macrofagi durante la rigenerazione muscolare. Mediante esperimenti *in vitro,* infatti, abbiamo dimostrato la capacità dell'enzima di regolare la polarizzazione dei macrofagi verso un fenotipo pro-infiammatorio (M1): l'assenza di A-SMase comporta una riduzione significativa dell'espressione di marcatori specifici per il fenotipo M1. Inoltre, investigando il ruolo dell'A-SMase nei muscoli del topo *mdx*, modello animale per la Distrofia muscolare di Duchenne (DMD), abbiamo individuato un incremento significativo sia dell'espressione che dell'attività dell'idrolasi

dimostrando il coinvolgimento dell'enzima nella patogenesi della DMD, in particolar modo nell'insorgenza dell'infiammazione. Diversi studi riportano il ruolo predominante dell'infiammazione nella patogenesi della DMD (Villalta et al., 2009; Radley et al., 2008). I nostri dati dimostrano che l'aumento dei livelli di A-SMase osservato nel topo *mdx* coincide con l'incremento dello stato infiammatorio presente nel muscolo degli stessi. Questa evidenza è stata corroborata dall'utilizzo del farmaco anti-infiammatorio Naproxcinod in grado di ridurre l'infiammazione muscolare nel topo distrofico e nel contempo diminuire significativamente l'espressione e l'attività dell'A-SMase.

In conclusione, i nostri risultati aprono a nuove prospettive nell'identificazione dell'A-SMase come possibile bersaglio farmacologico durante lo sviluppo e la regolazione del processo infiammatorio e di rigenerazione muscolare contemplando la possibilità che la modulazione dei livelli di espressione dell'enzima possa apportare benefici terapeutici non solo nella distrofia di Duchenne ma anche in altre malattie da deperimento muscolare.

INTRODUCTION

Chapter 1: Skeletal muscle development

Skeletal muscle is a complex and heterogeneous tissue fundamental for several functions in the organism. Myogenesis is the process responsible for the muscle generation and it can be divided in several distinct phases (Tajbakhsh et al., 1997). Skeletal muscle is originated during embryonic myogenesis from somites, which are transitory structures fundamental in organizing the segmental plan of vertebrate embryos (Aulehla; Pourquiè, 2010). The somites are detached from one another and subsequently due to local molecular signals, they differentiate into three portions: *sclerotome, myotome and dermatome*. During the development of skeletal muscle the myotome cells separate, migrate in the appropriate areas and generate myoblasts. Some progenitor cells do not differentiate in order to give rise to the myogenic precursors, Satellite Cells (SCs), located in a niche on the surface of the muscle fibre, beneath the basal lamina of the muscle cells. Skeletal muscle development during embryonic, fetal and neonatal periods requires successive waves of muscle stem cell activation, proliferation and fusion that involve different types of myoblasts (Zecchini et al., 2018).

A hierarchy of transcription factors regulates the progression of myogenesis. The basic helix-loop-helix factors such as Myogenic Differentiation (MyoD), Myogenic factor 5 *(*Myf5*),* Myogenin, and MRF4 (Myf6) are expressed in the skeletal muscle lineage and are considered as *myogenic regulatory factors* (MRFs) (Weintraub et al., 1991; Rudnicki and Jaenisch, 1995). Myf5 and MyoD act upstream of Myogenin and MRF4 to specify myoblasts for terminal differentiation. Myogenin and MRF4 are involved in the differentiation process and trigger the expression of myotube-specific genes (Bentzinger et al., 2012). The next degree of genetic hierarchy regulating myogenic process is occupied by the paired-homeobox transcription factors, Pax3 and Pax7. All vertebrates have at least one of these genes. Pax3 positive cells are founder cells forming a template of initial fibers in the limb to which Pax7 positive cells then contribute by forming secondary fibers and establishing the satellite cell pool (Maqbool and Jagla, 2007).

SCs are responsible for generating myoblasts in postnatal skeletal muscle (Katz, 1961; Mauro 1961); indeed, they initially provide myoblasts for muscle growth, before becoming mitotically quiescent as the muscle matures. In adults, SCs can be recruited to supply myoblasts for muscle fiber homeostasis, or for the myofiber repair (Zammit, 2008).

Moreover, SCs are able to maintain their own population by self-renewal, satisfying the criteria of a stem cell (Collins et al., 2005).

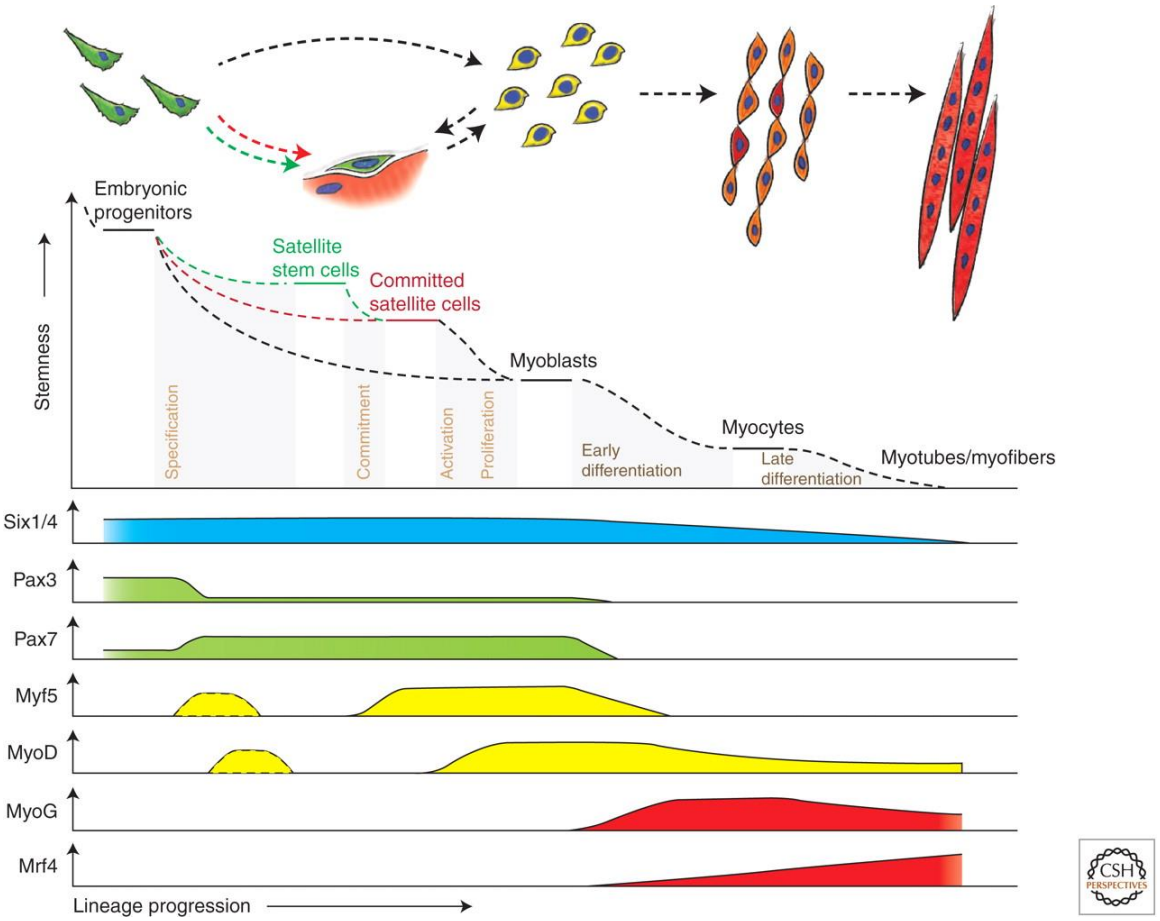


Figure 1: Genetic hierarchy of transcription factors regulating myogenic progression (Bentzinger et al., 2012)

1.1 Structure and function

Skeletal muscle is one of the most dynamic and plastic tissues of the body. Muscle mass depends on the balance between protein synthesis and degradation and both processes are sensitive to factors such as nutritional status, hormonal balance, physical activity/exercise, and injury or disease, among others (Frontera and Ochala, 2015). Skeletal muscle is a key component of several bodily functions such as locomotion, contraction, maintenance of posture, respiration, constriction of organs and vessels and production of body heat. Long cylindrical muscle fiber, also called myofiber, is the functional unit of skeletal muscle, which is able to generate force by contraction.

The skeletal muscle architecture consists of three different connective tissue layers: *epimysium*, *perimysium* and *endomysium*. The *epimysium* is the connective tissue, which surrounds the entire skeletal muscle; from epimysium originates the *perimysium,* which surrounds groups of muscle fibers and the last but not least, the *endomysium* a reticular connective tissue surrounding each muscle fibre. Each myofiber is packed with myofibrils composed of thousands of sarcomeres that contain the actin and myosin filaments that interact to produce the force (Relaix and Zammit, 2012) (Fig.2).

Muscle fibers are syncytial and multinucleated cells that often containing hundreds of myonuclei within a continuous cytoplasm.

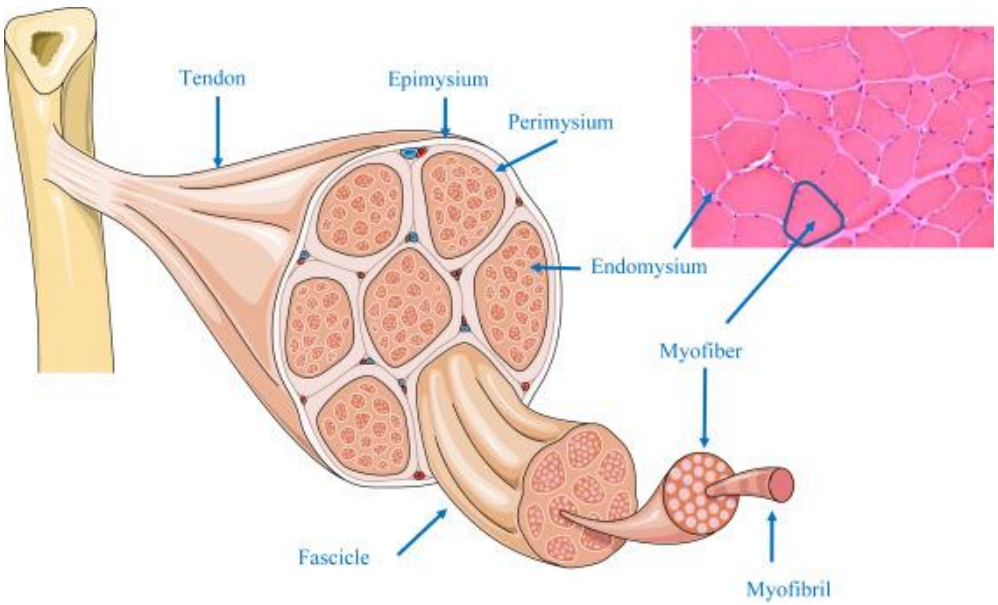


Figure 2: Representation of muscle fiber structure

1.2 Skeletal muscle regeneration

Skeletal muscle has a great capacity to regenerate new muscle fibers after severe damage by injury or by degenerative diseases such as muscular dystrophy (Tedesco et al., 2010). The regeneration process is very efficient. Indeed, the muscular function is restored even when a muscle is removed and replaced back in situ (Studitsky, 1964).

This regenerative capacity relies on muscle progenitor cells SCs that have stem cell properties and are responsible for generating myoblast in both postnatal growth of skeletal muscle and possible repair processes (Mitchell et al., 2010). Muscle regeneration is a highly coordinated process involving a variety of cell types and signaling molecules that work systematically to repair the damaged myofibers (Brack et al., 2012). Muscle regeneration recapitulates many stages of muscle development because the molecular program that underlines prenatal development is reactivated for tissue reconstruction; however, during adult regeneration after injury, extrinsic factors are different from those during embryonic development (Nie et al., 2016).

Skeletal muscle regeneration consists of five and time-dependent phases: degeneration (necrosis), inflammation, regeneration, remodelling, and maturation/functional repair (Fig.3) (Musarò, 2014).

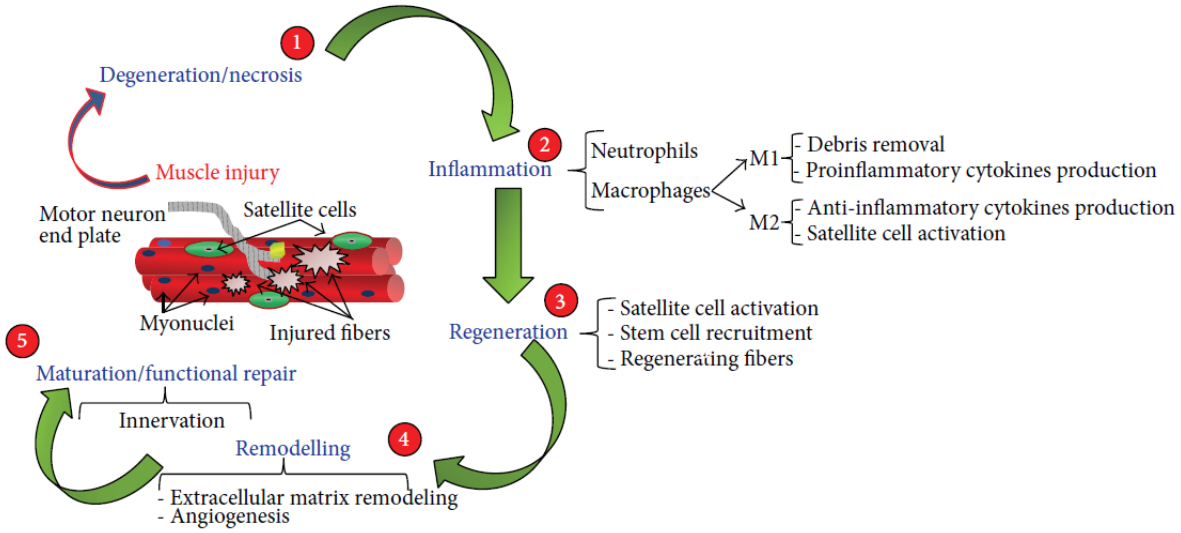


Figure 3*:* Schematic model of the muscle regeneration process (Advances in Biology, Vol.2014, Review, Musarò)

The *degeneration* phase is characterized by necrosis of myofibers and involves the influx of calcium ions, the loss of the plasmalemma, myonuclear, contractile material and cellular organelles dissolution. Necrotic fibers show an altered internal architecture with the presence of internal nuclei, which may reflects the invasion by macrophages. Necrotic cell death stimulates a host inflammatory response.

Inflammation of injured skeletal muscle plays a critical role in muscle homeostasis and regeneration and it is responsible for the recruitment of specific myeloid cell populations within the injured area. The immune system plays a crucial role during skeletal muscle regeneration: neutrophils are the first inflammatory myeloid cells recruited at the site of muscle injury by interacting with adhesion molecules of vascular endothelial cells. The phagocytic activity of neutrophils is responsible for the release of high concentrations of free radicals and the secretion of pro-inflammatory cytokines that stimulate the homing of other inflammatory cell populations such as monocyte and macrophages (Tidball et al., 2005). Macrophages are a heterogeneous population of innate myeloid cells and are considered the predominant inflammatory cell types. They rapidly increase within 24 hours after injury and are detectable at perimysium and epimysium. The role of macrophages is very important because they are able to remove tissue debris and activate stem cell populations (Pimorady-Esfahani et al., 1997).

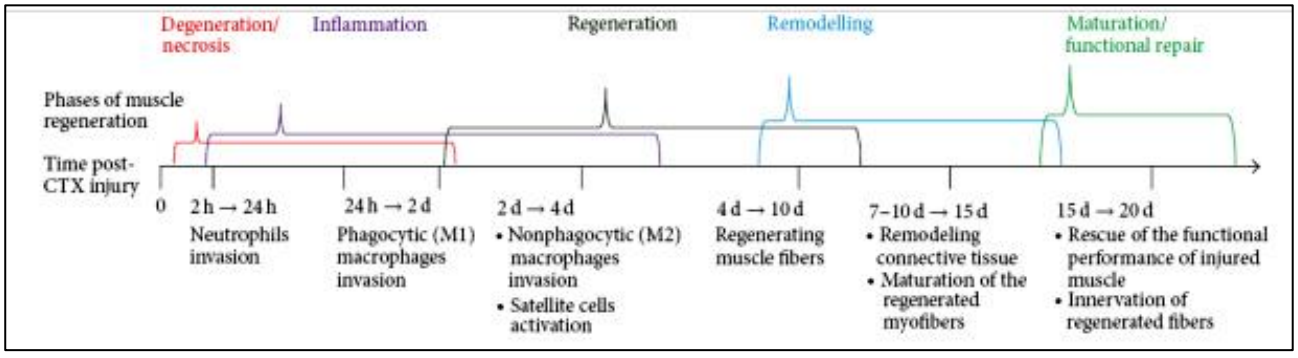


Figure 4: Schematic diagram of time-dependent cellular response following muscle injury (Advances in Biology, Vol.2014, Review, Musarò)

The central role of macrophages in regulating the inflammatory process depends on their capacity to polarize in different phenotypes that can orchestrate or counteract inflammation in response to trauma. Interestingly, different inflammatory processes mediated by macrophages play a significant role in the pathogenesis of several muscular dystrophies such as Duchenne Muscular Dystrophy.

The first steps of the inflammatory response are associated with the "classically activated" (M1) macrophages, which produce pro-inflammatory cytokines. In contrast, the resolution of inflammation is associated with the "alternatively activated" anti-inflammatory (M2) macrophages (Xuan et al., 2014). Macrophages can undergo dynamic transitions between M1 and M2 states called "*polarization skewing*" and sequentially, change their functional phenotype in response to changes in microenvironmental influences (Stout et al., 2005). M1 macrophages are activated by T-helper (Th) 1 cytokines interferon-gamma (IFNγ), Tumor Necrosis Factor-alpha (TNF-α), Lipopolysaccharide (LPS), Interleukin-6 (IL-6) and IL-1. The M2 population can be divided into three possible subtypes known as M2a, M2b and M2c macrophages. Their activation changes according to the type of subpopulation: M2a cells are induced by exposure to IL-4 and IL-13, M2b types are activated by combined exposure to immune complexes and toll-like receptors (TLR) or IL-1R agonists and M2c classes are induced via IL-10. Each class has a specific function. M2a and M2b macrophages exert immunoregulatory functions whereas M2c types are involved in the suppression of immune responses and tissue remodelling (Mantovani et al., 2004). The transition from pro-inflammatory (M1) to anti-inflammatory (M2) phenotype is necessary for proper and efficient muscle growth and regeneration (Lawrence and Natoli, 2011). Nevertheless, until now, the signaling pathway responsible for the *polarization skewing* is not entirely clear.

Subset of macrophages	Factors driving macrophages polarization	Molecules expressed by polarized macrophages	Functional properties of polarized macrophages
M1	IFN-γ + LPS, TNFα	TLR2, TLR4, CD16, CD32, CD64, CD80, CD86, IL-12, IL-23, TNF-α, IL-1, IL-6, Type I IFN, IL-1RI, IL-15Ra, IL-7R, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL16, CCR7, COX2, iNOS, and miR-26-2	Proinflammatory properties, phagocytosis, and cytotoxic and antitumoral properties
M2a	IL-4, IL-13	CD163, CD23, CD302, CD209, IL-10 Decoy IL-1RII, CCL17, CCL22, CCL24, CXCR1, CXCR2, miR-193b, and miR-222	Anti-inflammatory properties and immunoregulatory functions drive type II responses
M2b	IC + TLR	CD163, IL-10, CCL1, miR-27a, miR-222, miR29b-1, and miR-132	Anti-inflammatory properties and immunoregulatory functions drive type II responses
M2c	IL-10	CD163, scavenger receptor A and B, CD14, CCR2, CCL16, CCL18, CXCL13, CD204, and CD206	Suppression of immune responses and tissue remodeling

IC: immune complexes; IFN-g: interferon-g; LPS: lipopolysaccharide; TLR: toll-like receptor; COX2: cyclooxygenase 2; TNFα: tumour necrosis factor; iNOS: inducible nitric oxide synthase; Interleukin-: IL-.

Table 1: Characteristics of macrophage subtypes (Advances in Biology, Vol.2014, Review, Musarò)

M2 macrophages play an important role in deactivating M1 macrophages, in fact, in terms of a functional regeneration, M2 appear soon after M1. Sometimes, under specific pathologic conditions such as muscular dystrophy disease, it is possible that the M1 macrophages are accompanied by the contemporaneous invasion of M2a influx. This simultaneous recruitment of macrophage populations is responsible for the reduction of muscle damage caused by M1 subset (Tidball and Villalta, 2010; Villalta et al., 2009). The inflammatory response is a coordinated process finely regulated and able to promote an efficient regenerative process. It is well known that the perturbed spatial distribution of inflammatory cells can alters identity of the inflammatory infiltrate by disrupting temporal sequence that leads to a persistent inflammatory phase (Douglas et al., 2002).
The muscle *regeneration* phase is guaranteed by the role of SCs. Normally, they reside between the basal lamina and sarcolemma of myofibers. Upon physiological stimuli such as growth, exercise or pathological states represented by muscle injury or degenerative diseases they are activated (Mauro, 1961). When activated, SCs come out from their quiescence state and start repairing damaged muscle fiber by fusing with the existing myofibers or to each other in order to form new myofibers (Gayraud et al., 2009). However, not all the SCs differentiate, in fact, a little minority reenters into the quiescence state to preserve the stem cell pool (Zammit et al., 2004). During the different stages of skeletal muscle differentiation, SCs express different markers. The quiescent SCs are recognized for the presence of Pax3, CD34, Integrin α7 and other specific markers showed in the picture below (Fig.5).

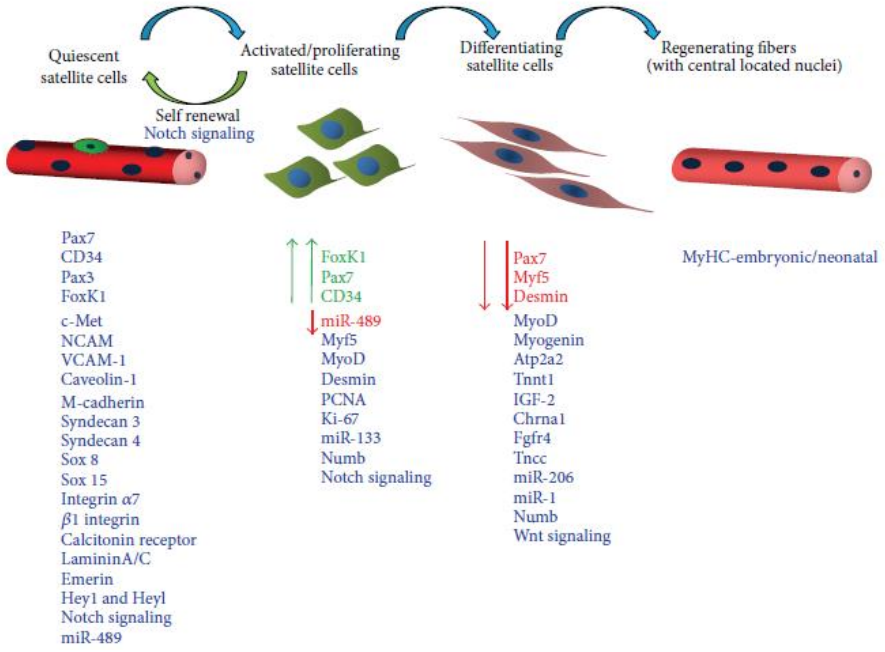


Figure 5: The molecular markers of satellite cells during the different stages of skeletal muscle regeneration (Advances in Biology, Vol.2014, Review, Musarò)

The main markers that characterize the proliferating SCs state are Pax7, MyoD, Ki67 and Myf5. Once activate SCs can down-regulate MyoD in order to self-renew and to preserve the pool of quiescent $Pax7^{+}$ positive cells. Otherwise, SCs can keep the MyoD expression down-regulating Pax7 and activating Myogenin expression by committing to differentiation of SCs (Boldrin et al., 2010; Relaix et al., 2012).

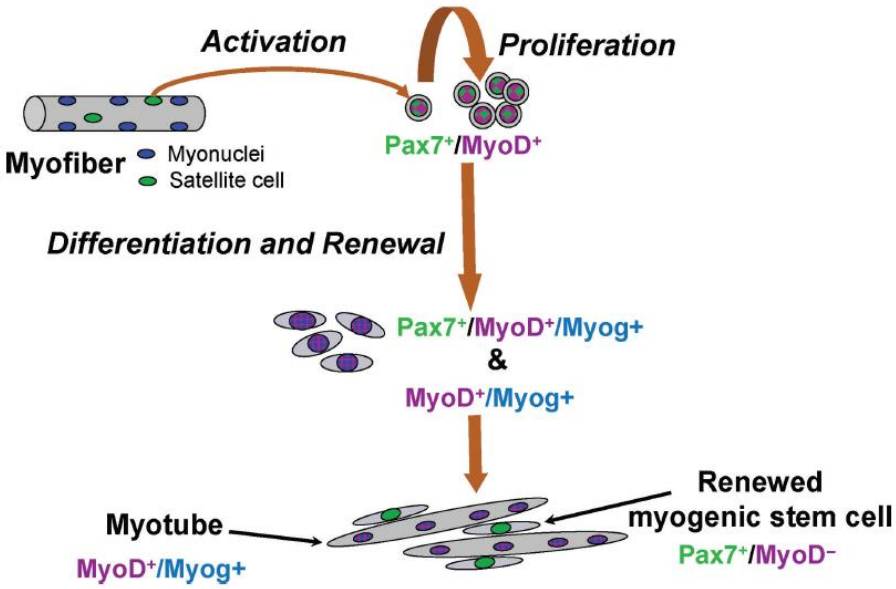


Figure 6: Schematic representation of satellite cells activation after injury (Yablonka–Reuveni et al., 2008)

In this context, the sequential presence of M1 and then M2 macrophages accompany the entire process of muscle regeneration. In fact, M1 subset is able to stimulate the SCs proliferation whereas M2 macrophages can promote their differentiation (Arnold et al., 2007; Saclier et al., 2013).

The *remodeling* phase represents the fourth stage of muscle regenerative process in which the extracellular matrix plays an important role in the production of different types of Collagens, Elastin, Laminin, Fibronectin and Proteoglycans (Mutsaers et al., 1997). All these components are necessary to stabilize the tissue, to lead the formation of neuromuscular junctions and to serve as scaffold for the new fibers (Lluri et al., 2006).

The *repair* of injured myofibers is the last phase of the process; it is completed when the myofibers recover their functional activity and the regenerated muscles are re-vascularized and re-innervated (Pelosi et al., 2007).

Chapter 2: Muscular Dystrophies

Muscular dystrophies are a genetically, biochemically and clinically heterogeneous group of inherited disorders with clinical and dystrophic pathological features on muscle biopsy (Emery, 2002). Progressive muscle weakness that interests limb, axial and facial muscles is the prominent feature of the muscular dystrophies profile. Moreover, other muscles such as respiratory, swallowing and cardiac smooth muscles can be affected. In specific forms of disorder, there is also the involvement of other different organs or tissues such as the brain, eyes, skin or inner ear. The severity and the age of onset of these disorders change largely with the different form of the variant. As a rule, congenital muscular dystrophies have clinical signs at birth or in the first few months of life. Many other forms manifest their symptoms in early or late childhood or adolescence unlike other muscular dystrophies that not manifest clinical signs until adulthood. The distribution of the skeletal muscle weakness is quite important because it can help to distinguish between different forms of muscular dystrophy and in several cases allows to the disorder to be suspected rapidly (Emery, 2002; Guglieri et al., 2008; Quinlivan et al., 2010). Muscle weaknesses can be associated with the presence of either muscle atrophy or hypertrophy, or both. Joint contractures and scoliosis are other symptoms that arise in specific variants. The progression of each form depends on the different dystrophy variants. In patients affected by congenital muscular dystrophy, ambulation is never achieved, whereas in the childhood onset forms the ambulation is achieved but will be lost during the rapidly progression of variant; generally, it occurs by the early or middle teenage years. In most cases of muscular dystrophies, the ambulation can be maintained and the wheelchair assistance is needed only later in life. Moreover, respiratory impairment is frequent but its severity is not always related to the degree of motor impairment. Cardiac involvement is also common and variable in many muscular dystrophies but is not the main feature (Mercuri and Muntoni, 2013).

The mechanisms underlying the muscular dystrophies have been thoroughly studied in order to give rise of specific criteria about their classification. Indeed, the classification cannot be based only on the previous suppositions indicating that every clinical phenotype is associated to a distinct genetic defect. In fact, individual phenotypes are often associated with mutations in different proteins that share similar cellular functions (Mercuri and Muntoni, 2012; Guglieri et al., 2008; Muntoni and Voit, 2008).

Progression of the disease is controlled by the severity of the individual mutation affecting each gene. The main class of proteins involved in the muscular dystrophy context including external membrane and extracellular matrix proteins, enzyme or protein with putative enzymatic function, sarcolemma-associated proteins, sarcomeric proteins and nuclear membrane proteins.

Abnormalities in the external and extracellular proteins such as Laminin and Collagen VI are responsible for congenital onset of weakness, which suggest an important role of these proteins in prenatal skeletal muscle development and function (Mercuri and Muntoni, 2012).

The enzyme or protein with putative enzymatic function can be divided in the proteins involved in the glycosylation of α-dystroglycan and those that are not involved in this process. The glycosylation of α-dystroglycan is very important; in fact, a defected process is the major cause of the congenital and Limb-Girdle muscular dystrophies (Muntoni et al., 2010; Muntoni et al., 2002). The proteins that are not involved in the glycosylation of α-dystroglycan are represented mainly by Calpain3, which belongs to a family of calcium-activated neutral proteases, and interacts with several proteins important for muscle functions. This protein is involved in the Limb-Girdle A, characterized by a dysfunction in calcium-calmodulin protein kinase II signaling (Kramerova et al., 2012).

Proteins belong to the sarcolemma are responsible for the most common forms of muscular dystrophies in childhood, the Duchenne (DMD) and Becker (BMD). The main sub-complex, called dystrophin-associated-glycoprotein (DGC) is fundamental for the stabilisation of the muscle fibre against the mechanical stress of muscle contraction by representing an important connection between the cytoskeleton and the extracellular matrix (Khairallah et al., 2007). With the destabilisation of the DGC, muscle fibers become more sensitive to stretch-induced damage and necrosis leads to weakness and muscle regeneration.

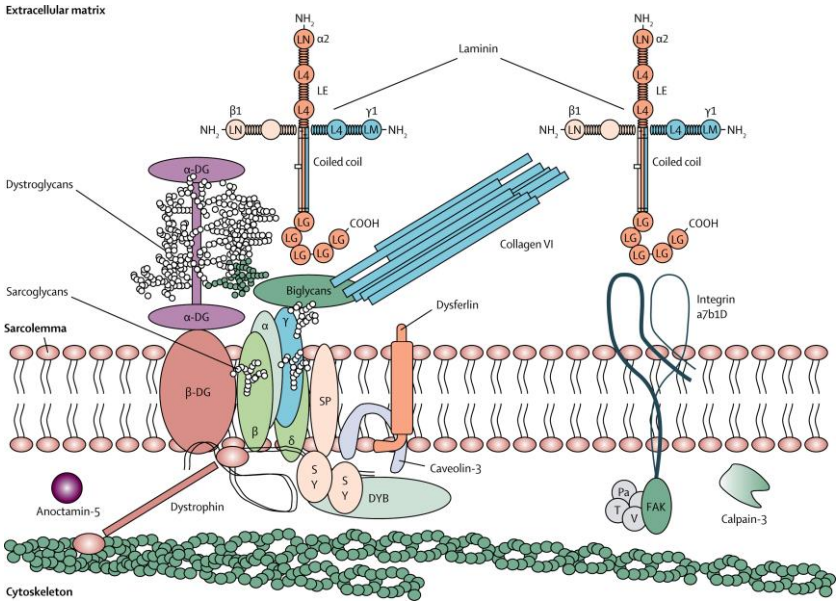


Figure 7: Sarcolemma and proteins involved in muscular dystrophies (The Lancet; Mercuri and Muntano, 2013).

Defects in sarcomeric proteins are responsible for dystrophic phenotypes with mainly distal distribution of weakness. Mutations in the proteins located in the outer or in the inner-nuclear membrane such as Lamin A/C, Emerin, Nesprin 1/2 and LUMA result in disorders with progressive muscular dystrophy phenotype, originally described by Emery and Dreifuss with humeroperoneal weakness and coexistent cardiac involvement (Emery and Dreifuss, 1966).

For the diagnosis of specific form of muscular dystrophy the combination of clinical signs and an analysis of the mode of inheritance are fundamental although the overlaps between genetically distinct variants makes identification more difficult. Often, patients affected by muscular dystrophy showed up to ten times higher than normal values of serum creatinine kinase, a chemical waste molecule generated from muscle metabolism. Moreover, *electromyography* can help in the diagnosis of several dystrophies but it has low value in the identification of a specific disorder in patients with elevated serum creatinine kinase. Other important diagnostic methods are *muscle biopsy and Western blotting analysis*. The former allows assessing the morphology excluding disorders with overlapping features such as myofibrillar myopathies. The latter, uses antibodies to find the level and localisation of different muscle proteins in order to calculate their abundance and identify the primary protein defect to direct genetic testing (Mercuri and Muntoni, 2013).

The identification of genetic defects is the gold-standard diagnostic method because not all dystrophies are caused by protein deficiency. In many diagnoses of neuromuscular dystrophies muscle imaging can be used in order to identify disease-specific patterns of muscle involvement (Mercuri et al., 2007).

2.1 Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is the most severe and diffuse type of dystrophies group with a high incidence (1out of 3.500 boys) affecting mainly skeletal muscle. DMD is a fatal X-linked disease caused by a wide range of mutations in the dystrophin gene, the largest gene inside the human genome (Govoni et al., 2013). At present, more than 4.700 mutations on dystrophin gene have been detected: deletions (65.8%) and duplications (13.6%) are responsible for the majority of gene mutations. Point mutations such as micro-insertions, micro-deletions, non-sense, missense and splicing mutations represent the 20.6% only (Magri et al., 2011).

In muscles of patients affected by DMD the dystrophin mutations accounting for premature stop codons or change the reading frame of the gene resulting in the complete loss of the protein. Generally, the onset of DMD is before the third year of age. Typically, the disease implies ambulation loss between 10 and 14 years of age with the die of patient around 20- 30 years of age caused by hypoxemia, hypercapnia and hypoventilation associated with diaphragm wasting, which is the most affected muscle in DMD and responsible for respiratory failure (Davies et al., 1988; Fayssoil et al., 2010). The DMD disorder also involves cardiac and smooth muscles. More than 90% of DMD patients show cardiomyopathy, which is responsible for a progressive impairment of the ejection fraction leading to arrhythmias and heart failure (Mosqueira et al., 2013; Jefferies et al., 2005).

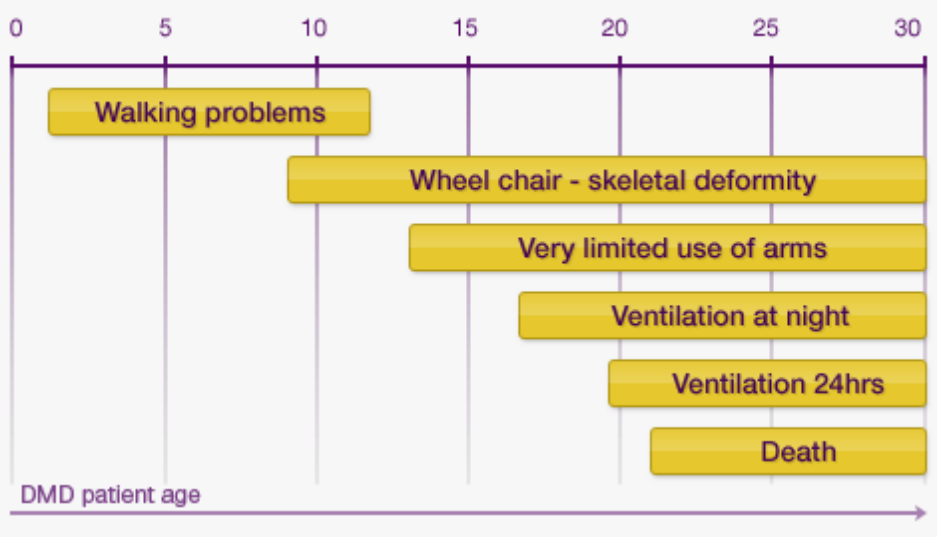


Figure 8: Timeline Duchenne Muscular Dystrophy patients.

In healthy muscle, dystrophin is located at the subsarcolemma and interacts with many membrane proteins forming the dystrophin glycoprotein complex (DGC), which is responsible for the stabilization of muscle cell membrane during cycles of contraction and relaxation. The DGC complex is also involved in the regulation of mechano-sensitive calcium channels, by influencing calcium homeostasis and signaling (Willmann et al., 2008). In the DGC structure, dystrophin represents the main cytoskeletal scaffolding protein and it is able to recruit crucial structural and signaling factors to the muscle membrane by creating a highly organized network.

The DAG complex contains many tissue specific transmembrane proteins such as β-dystroglycan, α, β-, γ-, δ-,ε-,and ζ-sarcoglycans, sarcospan and caveolin 3, extracellular proteins which are α-dystroglycan and Laminin, and also several sarcoplasmic proteins such as α- and β-dystrobrevins, α1, β1-,β2-,γ1-, γ2 syntrophins and neuronal nitric oxide synthase (nNos).

The proteins in the DGC complex are tightly connected to each other, therefore, every single modification in one of these proteins, also affects the others and hence it is responsible for different muscular defects. In DMD disorder, the disintegration of the dystrophin associated to DGC structure is responsible for the relocation of the nNOS in the sarcolemma by generating low NO production. The reduction of NO reduces muscle bulk and force generation, causing increased fatigability (De Palma and Clementi, 2012).

Therefore, the absence of dystrophin protein makes the muscle fibres more sensitive to mechanical contraction than the healthy fibres (Lynch et al., 2010). In fact, in response to contractile stress, they undergo continued cycles of necrosis and regeneration during which the multipotent myogenic precursor pool, into skeletal muscle fibres, the satellite cells, lead to muscle regeneration process (Le Grand and Rudnicki, 2007; Biressi and Rando, 2010). The repeated cycles of necrosis and regeneration exhaust the skeletal muscle regenerative potential resulting in an inefficient process characterised by gradual substitution of muscle with fat and connective tissue. These are the reasons why the loss of dystrophin leads to chronic and local inflammation, progressive muscle wasting and weakness, exhaustion of satellite cell pool and an impaired muscle regenerative process.

2.2 Mouse models for DMD

The physiopathology of DMD has been widely studied and the most of knowledge on the molecular mechanisms underlying the disease derived from studies in different mammalian models for DMD. The first dystrophin-deficient mutant was described in 1984 in a colony of C57BL/10 mice (C57BL/10ScSnJ) (Bulfield et al., 1984) referred as the "*mdx*-mouse". This mouse, also called C57BL/10ScSn-*Dmd*^{mdx}/J, is widely used in basic and translational research; it carries a point mutation in exon 23 of the mouse *dystrophin* gene introducing a premature stop codon, which leads to the absence of full-length dystrophin. This type of mutation is responsible for approximately one third of the mutations found in DMD patients (Willmann et al., 2008).

The *mdx* mice have a shorter life compared to wild type controls. A prominent degeneration and regeneration of muscle fibers is observed in the young age between 2 and 4 weeks by resulting in an increase in the number of newly differentiating myofibers characterized by centralized nuclei and an increased heterogeneity in myofiber size (McGeachie et al., 1993). At the same early age, *mdx* muscles show necrosis that decrease after 60 days (Bulfield G. et al., 1984). Among *mdx* muscles, the diaphragm is the only one with a marked fibrosis unlike to the other muscles which show less fibrosis also compared to DMD patients. The *mdx* mice have an absolute muscle force unaffected but a reduction in the relative force normalized to body weight. At 16 months of age *mdx* mice show an evident worsening of respiratory capacity in parallel to the increased histological damage of respiratory muscle (Gayraud et al., 2007). Another pathological feature of *mdx* muscles is an abnormal cardiac function characterized by myocardial fibrosis, necrosis and inflammation. Therefore, this model is considered a valid preclinical model to better understand cardio-protective pharmacological approaches (Quinlan et al., 2004). Moreover, since the subsarcolemma compartment and sarcoplasmic reticulum of *mdx* muscles show increased levels of calcium, it represents a valid model to investigate the calcium homeostasis and its possible alterations (De Backer et al., 2002).

- mdx variants

Other models for DMD are the *mdx* variants defined as *mdx*^{2Cv}, *mdx*^{3Cv}, *mdx*^{4Cv}, and *mdx*^{5Cv} that not differ from the original *mdx* mutant; in fact, the mice show muscle dystrophic condition and elevated serum creatine kinase levels as well as the first *mdx* generated. The muscle immunohistochemistry revealed degeneration and regeneration in muscle fibers, centrally nucleated fibers, cellular infiltration and necrosis. The diaphragm shows fibrosis as well as the diaphragm of the original *mdx* mice. The muscle fiber size of the *mdx* variants is larger than in *mdx* mice and at 4 weeks of age the *mdx*^{2Cv} and *mdx*^{3Cv} models show an abnormal slow contraction/relaxation of

muscles and some fibrosis in the heart. The *mdx*^{3Cv} variant has a reduced neonatal survival (Chapman et al., 1989; Cox et al., 1993).

The *mdx* variant mdx52 was created in order to get a mouse model with large deletions in the dystrophin gene like those observed in two thirds of human patients. To this end, the exon 52 was disrupted on a C57BL/6J background. Tibialis Anterior and Extensor Digitorum-longus muscles of mdx52 are 1.5 times larger than wild type muscles at 4 months of age. In this age, variation of fiber size, necrotic sites and central-nucleated fibers predominate. Until 18 months of age in these mice, there is no evidence of skeletal muscle weakness. Fibrosis only affects diaphragm, Soleus and Extensor Digitorum-longus muscles. Heart and brain do not show pathological changes (Araki et al., 1997).

- *mdx double mutants*

In order to better understand the mechanism underlying the DMD pathology, double mutant mice models have been created. They have different and additional knockout genes and are termed as *mdx*; utr$^{-/-}$, *mdx*; MyoD$^{-/-}$, *mdx*; adbn$^{-/-}$, *mdx*; α-7integrin$^{-/-}$, *mdx*: PV$^{-/-}$. Each double mutant represents a specific model able to resemble specific features of DMD pathology. For example, *mdx* mice knockout for utrophin, a dystrophin related proteins, (*mdx*; utr$^{-/-}$) loses both utrophin and dystrophin by showing a dystrophic phenotype that remains severe without any improvements. As these mice show interstitial fibrosis of skeletal muscle, this model is useful to assess the efficacy of anti-fibrotic treatment.

The muscle regeneration is an ineffective process in DMD patients. This is the reason that leads to the creation of a mouse model with a reduced capacity to regenerate given by the *mdx*; MyoD$^{-/-}$.

MyoD is involved in the muscle differentiation and mutants for this gene show evident muscle dystrophy phenotype with cardiomyopathy. Therefore, this model has been proposed as a model of DMD associated to cardiomyopathy.

Moreover, the *mdx* mice knockout for α-dystrobrevin, a protein of DGC complex, has been created in order to investigate the role of this protein in signaling and in muscle stability. These animals display a more severe dystrophic phenotype than *mdx* but not as severe as the *mdx* knockout for utrophin.

The main integrin expressed in skeletal muscle is α7β1. Data from literature suggest its capacity to act in synergy with DGC in order to connect the extracellular matrix to the cytoskeleton. To test this evidence a double mutant model for α7-integrin and dystrophin has been created. These mice show an aggravated phenotype suggesting the importance of α7-integrin levels in different clinical severity of dystrophin deficiency in human and mouse (Guo et al., 2006).

Since *mdx* mice show a relatively mild pathology when compared to the DMD patients, has been investigate whether this difference might be due to the high levels of the calcium-buffer parvalbumin (PV) in the skeletal muscle of rodents. So *mdx* mice were crossed with PV knockout mice ($PV^{-/-}$). The *mdx;* $PV^{-/-}$ are similar to *mdx* mice but they show a more number of central-nucleated fibers compared to *mdx*. Furthermore, muscles from 90 day old of double mutants are weaker in comparison to *mdx in vitro* and *in vivo*. Currently, although the PV levels are high, they are not able to explain the difference to DMD phenotypes (Willmann et al., 2008).

2.3 Pharmacological approaches to muscular dystrophy

Corticosteroids treatment

At the moment, despite much is known about the underlying molecular mechanism of muscular dystrophies, they are still considered the most difficult diseases to treat (Cossu and Sampaolesi, 2004). The current pharmacological treatments are not able to cure the different forms of muscular dystrophy but can delay disease progression and relieve symptom by improving the quality of life in order to extend the lifespan. The musculoskeletal system of patients affect by DMD undergoes the lack of structural protein dystrophin that cause a chronic influx of calcium in muscle fibers leading to cell death and inflammatory state (Hamid and Clemens, 2012). For these reasons corticosteroids treatment has been the first pharmacological therapy suggested in the 1974, in order to prevent muscle necrosis by stabilizing the muscle membrane and counteract the inflammation (Drachman et al., 1974). Currently, corticosteroids administration represents the gold standard therapy for DMD and other forms of muscular dystrophy. Corticosteroids are tested in different trials in order to define the optimal dose, frequency and age of initiation (Griggs et al., 1993; Escolar et al., 2011). Until now, they are considered the only pharmacological treatment able to give benefits by delaying the progression of the disease and prolonging at least two years the time before the loss of ambulation (Hamid and Clemens, 2012; Goemans et al., 2014). Unfortunately, corticosteroids are associated with severe side effects. DMD patients treated with steroids are also at risk of loss of bone density, hypertension, metabolic disorder, long bone fractures, and vertebral compression fractures. Sometimes, steroid-induced behavioural problems forced a limited long-administration (Mendell et al., 2012). The corticosteroids mainly used for DMD are Prednisone/Prednisolone and Deflazacort, an alternative, sodium-sparing glucocorticoid. The efficacy of these drugs has been established by performing a randomized, double blind controlled trial in boys with DMD. After 6 months of Prednisone treatment, boys showed an improvement in the muscle strength and function,

as well as the time needed to rise from supine to standing, to walk 9m and to climb four stairs ($p < 0.001$ for all comparisons) when compared to boys treated with placebo. Moreover, from 6 months patients had an increase in urinary creatinine excretion, which suggested an increase in total muscle mass (Mendell et al., 1989). Similar results were then documented using Deflazacort, which is able to prolong ambulation ($p < 0.005$) (Biggar et al., 2006). The *mdx* mouse model has been used to investigate the mechanism of action of corticosteroids.

Several studies indicate that Prednisone and Prednisolone have an anti-inflammatory effect, increase the force of skeletal muscle but have no functions on muscle fibers regeneration (Wehling-Henricks et al., 2004). In contrast, Deflazacort reduces the skeletal muscle pathology by acting on muscle regeneration and differentiation (Anderson et al., 2000). Furthermore, other studies demonstrated that the long-term corticosteroids treatment in *mdx* mice is responsible for the increase of fibrosis on cardiac muscle suggesting that this mouse model could not be an appropriate positive control in long-term pharmacological studies (Sali et al., 2012).

Histone deacetylase inhibitors as an alternative approach to corticosteroids treatment

Data from literature demonstrated that in dystrophic condition there is a high activity of histone deacetylases (HDAC), a class of enzymes that modulates the gene expression.

HDACs allow the histones to wrap DNA more tightly in condenses DNA structure preventing genetic transcription (Colussi et al., 2008; Illi et al., 2009). So, another pharmacological strategy is based on the use of histone deacetylase inhibitors (HDACi) in order to modify chromatin structure and allows the genetic transcription. Givinostat, originally ITF2357, is a histone deacetylase inhibitor with anti-inflammatory, anti-angiogenic and anti-neoplastic activities. In *mdx* mice, HDACi are able to slow disease progression, reduce fibrotic tissue, fatty infiltration and increase cross-sectional area of myofibers (Colussi et al., 2008; Consalvi et al., 2013).

Gene therapy

Duchenne and Becker muscular dystrophies are characterized by the lack or alteration of dystrophin protein. Gene replacement approach arises with the purpose of counteracting the dystrophin lack and replacing the defective *dmd* gene by using delivery vehicles such as adeno-associated viruses (AAV) and lentivirus based vectors. Gene replacement technologies should be applicable regardless the patients genetic mutation. Further, the use of AAV is advantageous given its capacity to stably persist in muscle cells for years without arise of pathogenicity (Wright et al., 2008).

Since dystrophin gene size is very big, this represents a limitation of AAV vectors, as they are not able to delivery large genes. An attempt to overcome this limit is to realize micro but functional dystrophin gene to insert into the vector (Seto et al., 2014). Another alternative is given by *trans splicing*, a method able to reconstitute the whole gene. This reconstitution consists in co-delivery of two or more AAVs with the gene of interest split between them, in order to reform the entire gene and deliver it into muscle cells (Li et al., 2008). Albeit this strategy could seem an extraordinary finding, it has a big limit given by the host immune response against both the viral vector and the delivered protein. For this reason, different AAV serotypes have been tested in order to reduce the host immune response. AAV2/8, chimeric vectors, has been tested on *mdx* mice and has been demonstrated its ability to restore dystrophin expression in heart and skeletal muscle without immune response (Van Westering et al., 2015).

Exon Skipping and Antisense Oligonucleotides

In DMD and BDM, the dystrophin gene undergoes to single or multiple exons deletion that cause a shift of the reading frame. The result is a non-functional dystrophin protein (Kole et al., 2015). From these data the idea to develop the antisense-mediated exon skipping strategy arises in order to increase dystrophin expression in dystrophic muscle by restoring the reading frame (Douglas et al., 2013). The antisense oligonucleotides (AONs) do not contain more than 20-30 nucleotides. They are able to bind specific pre-mRNA sequences (mutated exons) in order to allow their skipping at the moment in which the gene is translated by restoring the reading frame (Harding et al., 2007).

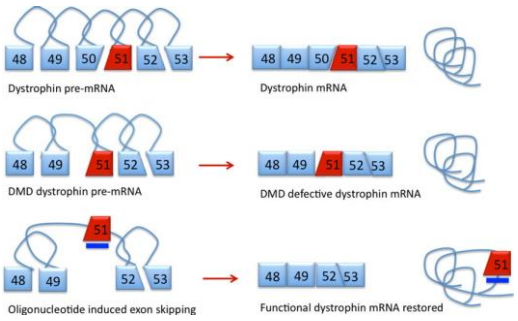


Figure 9: Example of anti-sense oligonucleotides activity (Kole and Krieg, 2015; Advanced Drug Delivery Reviews).

Many AONs were developed and tested both *in vivo* and *in vitro* (Benedetti et al., 2013). The most AONs studied have been morpholino phosphorodiamidate oligomers (PMOs) and phosphorothioate oligonucleotides (PS). The AAV vectors have been used as a vehicle to deliver AONs by resulting

in a restored dystrophin expression and in an improvement in the pathology of dystrophic muscles mice (Goyenvalle et al., 2004; Goyenvalle et al., 2012). As the dystrophin levels restoration decline in the time and the AAV are lost, repeated injections are needed. Currently, AAV mediated exon skipping is in preclinical stages to treat DMD patients (Le Hir et al., 2013). Unfortunately, until now there are no clinical benefits from antisense therapy in DMD because of low tissue uptake and recovery of dystrophin expression (Tedesco et al., 2012). Therefore, modifications in AONs chemistry are necessary to ameliorate cellular uptake and reduce the clearance by the organs such as liver and kidney (Miura et al., 2006). Interestingly, tricycle-DNA (tcDNA), a new class of AONs and an analogue of DNA, has a good uptake from many tissues in two mouse models of DMD. After systemic administration of tcDNA, skeletal muscles and heart showed high dystrophin rescue with an ameliorated phenotype of dystrophic mouse. These new AONs represent a precious tool not only for DMD pathology but also for other diseases in which can be possible apply antisense oligonucleotides by systemic treatment (Miura et al., 2006).

Cell therapy

As the loss or alteration of dystrophin is the main cause of the DMD pathology, new pharmacological approaches have been designed in order to replace dystrophin by transplantation of cells carrier a functional dystrophin gene. This strategy called *cell therapy* aims to restore the lost muscle mass and arrest the disease progression (Seto et al., 2014). Satellite cells play an essential role in muscle regeneration after injury. In DMD muscles, continuous cycles of damage and regeneration caused a reduction in the capacity of satellite cells to self-renew and restock muscle damaged. The first cell sources for DMD cell therapy have been myoblasts harvested from healthy donors and then transplanted in DMD patients by local intramuscular injection in order to reintroduce the dystrophin expression. This approach was not successful owing to the low migration, survivability and immune rejection of transplanted cells (Fan et al., 1996; Skuk et al., 2006; Skuk et al., 2011).

Satellite cells are not only the cell sources of myofibers; in fact, other stem cell types able to differentiate into myofibers such as, the bone marrow derived stem cells, mesenchymal cells and mesoangioblasts can be used for cell therapy. Mesoangioblast, the myogenic vessel-associated stem/progenitor cells, influence muscle regeneration thanks to their high migration ability to injured site (Minasi et al., 2002; Sampaolesi et al., 2003). Intra-arterial delivery of mesoangioblasts leads to a significant amelioration of muscle structure and function by representing a promising cell therapy for muscular dystrophy (Sampaolesi et al., 2003).

Recently, data from literature emphasized the therapeutic use of pluripotent stem cells such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPS). They could be exceptional potential clinical tools useful for any muscle degenerations treatment even though further studies might be required in order to avoid any risk (Berardi et al., 2014).

Read-through of Stop-Codon strategy

Another pharmacological strategy for DMD pathology has been developed as that the 10-15% of patients showed a non-sense mutation (premature stop-codon) responsible for the release of a truncated, and a non-functional dystrophin protein. To overcome this obstacle, small molecules called aminoglycosides have been used as their capacity to bind the decoding site of ribosomal RNA. Aminoglycosides change the mRNA conformational structure by allowing the substitution of mutation-induced stop codon with a single amino acid by ribosomal subunit at the aminoacyl transfer RNA acceptor site. The result is the production of a full-length dystrophin protein and an increase read-through of the premature stop-codon (Partridge, 2011; Finkel, 2010). Different aminoglycosides are synthetized with the purpose to create molecules poor of side effects. *In vitro* and *in vivo* studies carried out in *mdx* mice by using aminoglycoside Gentamicin showed its ability to suppress stop- codon and to enhance the dystrophin expression (Barton-Davis et al., 1999). Thus, Gentamicin has been tested on DMD patients carrying a stop-codon. They were treated weekly or twice weekly for six months. After 6 months, 3 out 12 patients showed 13 to 15% of normal dystrophin levels (Malik et al., 2010). Another compound developed was Ataluren (PTC124), an orally bioavailable drug with read-through activity. Pre-clinical studies in *mdx* mice revealed its ability to restore dystrophin up to 20% of normal levels but for a limited time (Welch et al., 2007). The safety of Ataluren has been established in humans by phase I study in healthy volunteers (Hirawat et al., 2007). This result leads to a phase IIa proof-concept study in patients affected by DMD and BMD followed by the IIb phase.

Utrophin treatment

Utrophin, an orthologue of dystrophin, has a structural organization and binding properties similar to dystrophin. After birth, when muscle becomes mature, Utrophin is replaced by dystrophin. The expression of Utrophin depends on the promoter A and the promoter B (Burton et al., 1999). The promoter A allows the Utrophin expression in skeletal muscle; instead the B promoter is responsible for the expression of Utrophin in endothelial cells (Weir et al., 2002). The up-regulation

of Utrophin in adult and dystrophic muscle represents one of the first strategies realized in order to replace dystrophin protein. Several studies indicated that in muscle fibers of *mdx* mice the increase of Utrophin could ameliorate the dystrophic condition by restoring the sarcolemma expression of the dystrophin-associated protein complex (DAPC) (Tinsley et al., 1996; Gilbert et al., 1999). Therefore, by high-throughput screening studies, small molecules have been created with the purpose to increase Utrophin expression. An example of these molecules is Hergulin. This molecule acts by Utrophin promoter A via the N-box motif and L-arginine so that it is able to increase the production of nNOS followed by an increase in Utrophin expression (Miura et al., 2006).
Thus, other approaches such as the overexpression of the small GTP-ase Rho and the inhibition of Calpain have been adopted in *mdx* mice, but they are not able to increase the protein levels in order to give the complete recovery of the dystrophic phenotype (Gauthier and Kerrache, 2009). Moreover, also the glucocorticoids have been tempted to stabilize the Utrophin in muscle (Lu et al., 2008).

Nitric Oxide therapy in Muscular Dystrophies

The intracellular messenger Nitric oxide (NO) is expressed in skeletal muscle and represents an important regulatory signal for different physiological effects in the muscle such as force generation, muscle mass, muscle repair from injury, fatigue, blood flow and oxidative stress (Stamler and Meissner, 2001; De Palma and Clementi, 2012). In skeletal muscle NO synthesis is catalyzed by the nNOSμ enzyme, which belongs to the DGC complex (Miglietta et al., 2012). In DMD dystrophy, the absence of dystrophin is responsible for the loss of nNOSμ enzyme and hence for NO, contributing significantly to dystrophic pathology (Lai et al., 2009; Li et al., 2011). In this scenario, various pharmacological approaches aimed at regulating NO supply to the skeletal muscle have arisen (Miglietta et al., 2012). Results from therapies based on the administration of NO donors only, did not show persistent beneficial effects (Voisin et al., 2005; Marques et al., 2005). In particular, two NO donors, the organic nitrate isosorbide and molsidomine have been investigated. While the former alone was no effective, the latter showed a moderate long-term efficacy (Buono et al., 2012). The reasons of these different effects are still poorly understood so the realization of alternative strategies for an effective pharmacological therapy has been necessary. The use of NO donation in combination with anti-inflammatory drugs has been the subsequent approach tested. The first study on *mdx* mice provided the use of L-arginine (NO donor) associated with the corticosteroid Deflazacort. Results showed functional recovery on muscle voluntary exercise (Archer et al., 2006). Then, other variants of this approach by using non-steroidal anti-inflammatory

agents (NSAIDs) have been tested. An example is given by the use of isosorbide dinitrate in combination with ibuprofen, in which the NO-donating agent is a different molecular entity respect to the anti-inflammatory drug; another example consists in a strategy in which the two activities are associated in a single molecular entity represented by a new class of NO-donating drugs called cyclooxygenase (COX)-inhibiting NO donors (CINODs) (Wallace et al., 2009). Both these approaches reported beneficial effects in mouse models of muscular dystrophies without significant side effects (Uaesoontrachoon et al., 2014). The combination of isosorbide dinitrate with ibuprofen was also tested in a cohort of adult patients affected by DMD, Becker and Limb-Girdle dystrophies. The results demonstrated good safety and tolerability profiles of the long-term co-administration of the drugs and of notice, the side effects reported were few and transient. Moreover, other results revealed also a good efficacy of the drugs demonstrated by an amelioration of motor function measure scale (D'Angelo et al., 2012). Until now, the best compound belonging to the CINOD class is Naproxcinod that has both anti-inflammatory and analgesic effects (Schnitzer et al., 2005). Initially, it was developed for the osteoarthritis. Upon absorption, Naproxcinod is metabolized to Naproxen and the NO-donating moiety, which is able to release NO by enzyme bioactivation. In 2015, Miglietta et al., demonstrated that Naproxcinod treatment improves skeletal muscle force after 1 month of administration in *mdx* sedentary mice. Of importance, they also reported that the same effect of Naproxcinod was maintained after 6 months of treatment also in animals forced to exercise. Moreover, long-term treatment with Naproxcinod reduces diaphragm inflammatory infiltrates. For all these reasons, Naproxcinod has been currently considered the ideal therapeutic option among CINODs for DMD treatment (Miglietta et al., 2015).

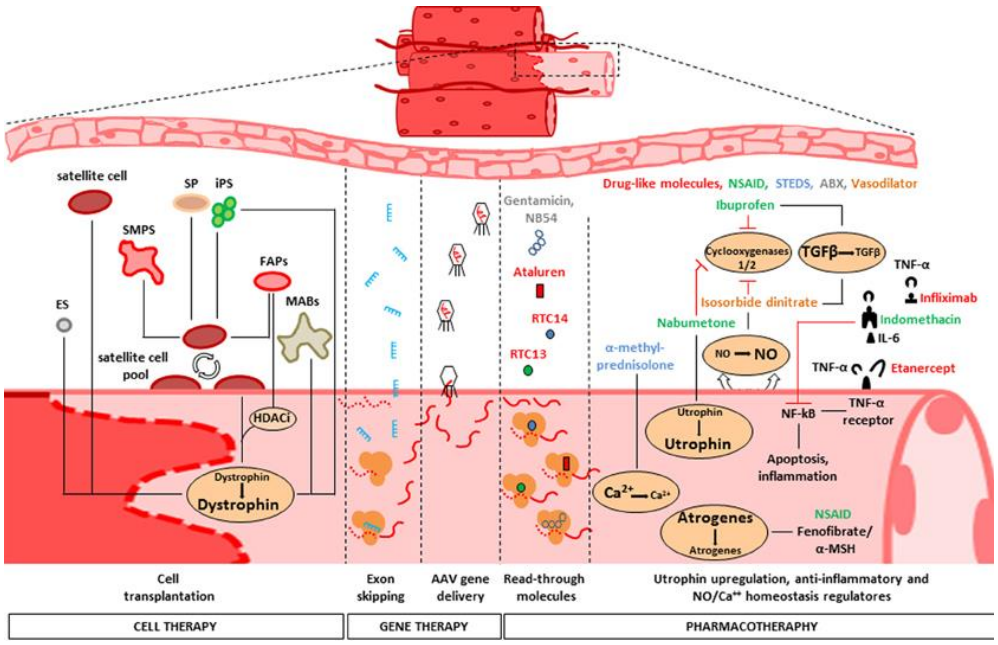


Figure 10: Approaches for muscular dystrophies (Berardi et al., 2014; Frontiers in Physiology).

Chapter 3: Sphingolipids and Acid Sphingomyelinase

3.1 Sphingolipids in cell signaling

Sphingolipids (SLs) are ubiquitous, membrane components of all eukaryotic cells and the major constituents of lipoproteins. The term *'sphingolipid'* refers to a number of lipids synthesized in the endoplasmic reticulum (ER) from non-sphingolipid precursor and defined by their long-chain sphingoid base backbone (e.g. sphingosine), an amide-linked, long chain fatty acid and one of various polar head groups. Modification of these head groups is what gives rise to the vast family of sphingolipids. Sphingolipids are mainly present at the level of the plasma membrane, where they play an important role in defining its physical and chemical properties. Although the diversity of structure and function the creation and destruction of sphingolipids are regulated by common synthetic and catabolic pathways (Deigner et al., 2007; Gault et al., 2010).

First discovered by *J. L. W. Thudichum* in 1876 for a long time SLs were considered to play primarily structural roles in membrane formation (Bartke and Hannun, 2008). However, SLs do not just play a structural role in membrane bi-layer with rather inert metabolism, in contrast, some of the intermediate molecules of its metabolism (i.e. Ceramide, Glucosil-ceramide and Sphingosine-1-phosphate) are important signaling molecules involved in the modulation of various and fundamental key cellular processes such as proliferation, differentiation and induction of apoptosis (Bleicher and Cabot 2002; Payne, Milstien et al., 2002; Hannun and Luberto, 2004).

Hence, structure, recognition and signal transduction can be considered the three crucial aspects in the sphingolipid actions. The mechanisms of sphingolipids-mediated signal transduction are characterized by an extreme complexity (Riboni, Prinetti et al., 1995; Huwiler, Kolter et al., 2000). Furthermore, a significant body of experimental evidence points to an important role for sphingolipid mediators in the inflammatory process (Chalfant and Spiegel, 2005; El Alwani et al., 2006).

Enzymes in the sphingomyelin cycle

Sphingomyelin (SM) is the most abundant sphingolipid with an important structural role in the integrity of the plasma membrane (Villani et al., 2008).

Sphingomyelin synthase (*phosphatidylcholine/Ceramide–PCh transferase*) is the enzyme responsible for the production of SM through the transfer of a phosphocholine head group from

phosphatidylcholine on to the primary hydroxyl group of Ceramide, an important bioactive molecule, generating diacylglycerol (DAG) as an additional product of the reaction (Ullman et al., 1974; Voelker et al., 1982). Therefore, the biological importance of Sphingomyelin synthase resides not only in the biosynthesis of SM but also in the regulation, in opposing directions, of the levels of Ceramide (Luberto et al., 1998). Biochemical studies reported the Sphingomyelin synthase activity in the Golgi and residual activity in other cellular compartments such as plasma membrane (Marggraf et al., 1981; Futerman et al., 1991). The major substrates of Sphingomyelin synthase are Ceramide and Phosphatidylcholine; however, the rate-limiting factor in the Golgi apparatus seems to be the availability of ceramide substrate.

An alternative pathway to the formation of Sphingomyelin can take place through the addition of phosphoethanolamine to Ceramide followed by single methyl transfer to form the choline head group. Most notable component of Sphingomyelin cycle is Ceramide. Ceramide is a bioactive sphingolipid that can be considered as a metabolic hub in the sphingolipid biosynthesis and catabolism (Hannun and Obeid, 2008). Multiple metabolic pathways are responsible for Ceramide formation and among these *de novo* biosynthetic pathway and the Sphingomyelinase (SMase) pathway have been deeply studied (Kitatani et al., 2008).

De novo sphingolipid synthesis begins at the cytosolic leaflet of the endoplasmic reticulum where a coordinately set of four enzyme groups generate ceramides of different acyl chain lengths from non-sphingolipid precursors (Gault et al., 2010). The first step is catalyzed by the *serine palmitoyl transferase* (SPT) complex which condenses the amino acid serine and the fatty acid palmitate to form *3-keto-dihydrosphingosine*. The carbonyl group of 3-keto-dihydrosphingosine is then reduced to form *dihydrosphingosine,* and the enzyme (dihydro) ceramide synthase (CerS) then adds a fatty acid chain (the acyl chain) by N-acylation to form *dihydroceramide*. Finally, desaturation of the 4, 5 carbon-carbon bond on the sphingoid backbone generates Ceramide (Airola et al., 2014). Ceramide, generated is transported to the Golgi complex, where it serves as a substrate for the production of complex sphingolipids (Gault et al., 2010).

Ceramide can be also generated by the hydrolysis of sphingomyelin by the activation of the catabolic enzymes sphingomyelinases (SMases) (Marchesini et al., 2004). Five distinct enzymes have been identified based upon their pH optima, cellular localization and cation dependence. The neutral SMase (N-SMase) membrane-bound Mg^{++} independent sphingomyelinase and the lysosomal acid pH-optima sphingomyelinase (A-SMase) have been the most studied for their roles in ceramide synthesis. These enzymes break down sphingomyelin to produce *ceramide* and *phosphorylcholine*. Accumulation of ceramide in cellular membranes results in the formation of

lipid rafts and functional clustering of surface receptors (Deigner et al., 2007). Currently, evidence addresses the importance of A-SMase to the transmembrane signaling.

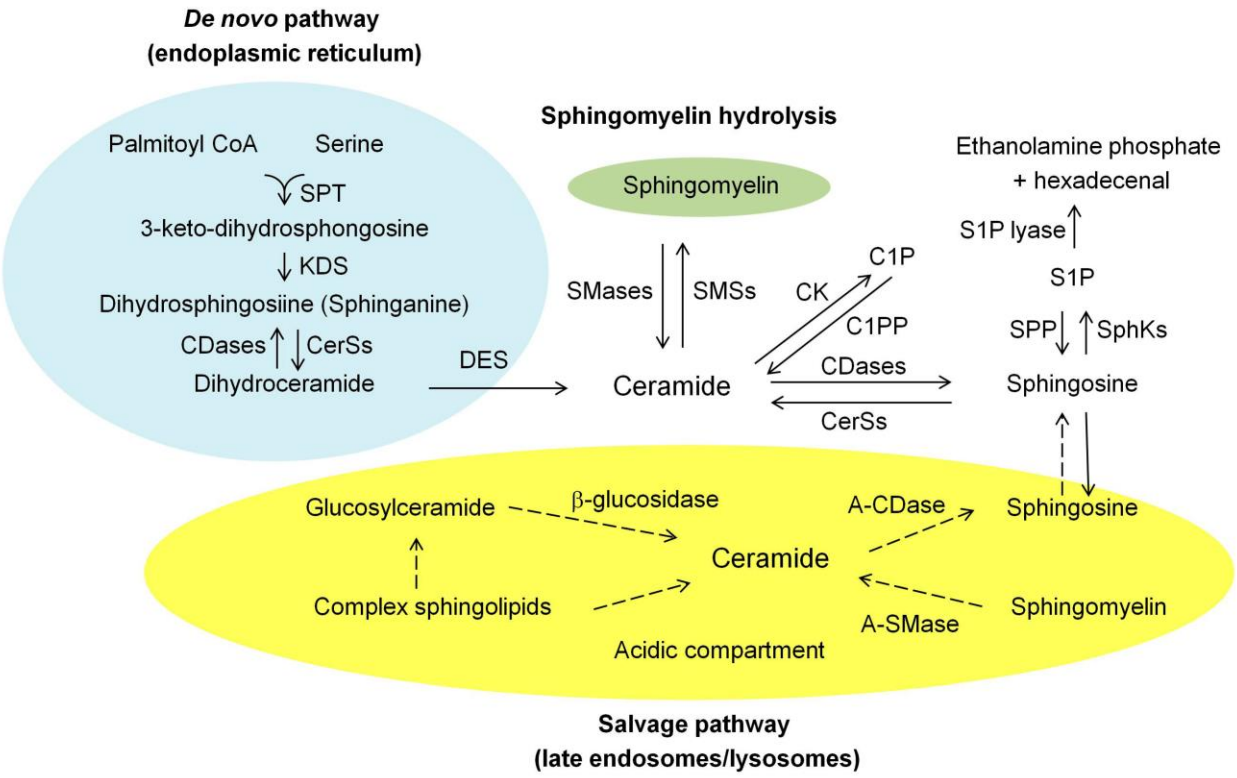


Figure 11: Metabolism of sphingolipids (Norishi Ueda, 2014; International Journal of Molecular Sciences).

3.2 Sphingolipids in inflammation

As sphingolipids are not considered the inert molecules, they have been extensively studied and recent data from literature suggest their importance in complex processes such as the inflammatory response. The involvement of a specific sphingolipid mediator or another depends on the cellular context but this new finding allows new pharmacological interventions. Each sphingolipid has a specific mode of action so that each one influences the inflammation in different ways (Chalfant and Spiegel, 2005; El Alwani et al., 2006). Sphingolipids are involved in specific phases of acute inflammation such as migration of immune cells, recognition of exogenous agents, activation and differentiation of immune cells (Grösch et al., 2018).

Among sphingolipids, ceramide, ceramide-1 phosphate (C1P) and sphingosine-1 phosphate (S1P) represent the sphingolipid metabolites mainly implicated in the inflammatory pathways.

The role of ceramide in the inflammatory process has been reported for the first time from studies carried out on the intracellular effects of the inflammatory cytokine tumor necrosis factor α (TNF-α) (Kim et al., 1991; Mathias et al., 1991). TNF-α activates Acid Sphingomyelinase, which is able to produce ceramide. The ceramide production results in the activation of the inflammatory pathway by nuclear factor- κβ (NF-κβ) gene transcription (Nixon, 2009). NF-κβ is a pro-inflammatory transcription factor responsible for the induction of different genes that encode cytokines and chemokines such as interleukin 1β (IL-1β), IL-6, IL-8, monocyte chemoattractant protein-1 (MCCP1) and pro-inflammatory enzymes such as COX-2 (Xiao and Ghosh, 2005). Up to now, the capacity of ceramide in regulating the inflammatory process in all cell types is still unclear but data from literature indicate its possible in *vivo* role. Indeed, Teichgärber et al., demonstrated the importance of ceramide in the transgenic mouse model of cystic fibrosis in which an accumulation of membrane ceramide is responsible for the pulmonary inflammation.

The role of C1P in the inflammatory signaling is less clear compared to ceramide action. Data from studies carried out in macrophages and in lung cancer cells revealed the involvement of C1P in the activation of cytosolic phospholipase A2 (cPLA2) (Pettus et al., 2003; Pettus et al., 2004). Moreover, ceramide kinase and C1P are able to inhibit TNF-α post-translational modification by TNF-α converting enzyme (TACE) and hence the TNF-α production (Jozefowski, 2010; Loamour et al., 2011).

The Sphingosine 1-phosphate is involved in different inflammatory processes and its effects depend on the cell type specificity. It is well known that S1P has been a pro-survival signal and a promoter of chemotaxis for lymphocytes (Rutherford et al., 2013; Messias et al., 2016). Hughes et al.

demonstrated that the treatment of LPS-stimulated primary macrophages with S1P is able to inhibit the development of pro-inflammatory macrophages subset (M1).

Moreover, several recent studies highlighted the importance of S1P and S1P receptor signaling in pathological inflammation such as diabetic retinopathy and acute lung injury. S1P receptors are G protein coupled receptors and are able to mediate various functions (Hla et al., 2001). For example, in mast cells activation $SP1_1$ receptor is responsible for the mast cells migration and $SP1_2$ for degranulation (Jolly et al., 2004). A fungal metabolite FTY720 represents the potential therapeutic intervention able to regulate the sphingolipid effects on inflammatory process. The phosphorylation of FTY720 by Sphingosine Kinase (SK) has been assessed by in *vivo* experiments. FTY720-P is an agonist of S1P receptors. After binding to the S1P receptor, FTY720-P exerts its immunosuppressant function by sequestering lymphocytes to lymph nodes (Billich et al., 2003). This mechanism of action is responsible for the reduction of atherosclerosis in mice that display low monocyte infiltration to artery walls and inflammation (Nofer et al., 2007; Keul et al., 2007). In other inflammatory diseases including ulcerative colitis and inflammatory bowel disease, both in mouse model and in patients increased activity levels of S1P/ Sphingosine Kinase 1 (SK1) were found (Snider et al., 2009). In *vivo* studies showed that the deletion of SK protects mice from hepatic steatosis (Mauer et al., 2017). Indeed, high levels of SK1 were observed in mouse model and human patients affected by nonalcoholic fatty liver disease which correlates with the activation of NF-κB and the increase of pro-inflammatory cytokines and the infiltration of immune cells (Geng et al., 2015).

3.3 Acid sphingomyelinase (A-SMase)

The sphingolipid-metabolizing enzyme Acid Sphingomyelinase (A-SMase) is a key enzyme in sphingolipid metabolism because it catalyzes the hydrolysis of sphingomyelin (SM) to ceramide and poshorylcholine. A-SMase plays an important role in the ceramide-mediated signaling pathway (Zhou et al., 2016). Mammalian cells contain the gene for A-SMase, SMPD1, which is responsible for the generation of the enzyme. A-SMase is highly conserved in animals, from *C. elegans* to human, with over 35% protein sequence identities.

The A-SMase structure is characterized by a multi-domain protein including a saposin domain, a proline-rich linker, a metallo-dependent phosphatase catalytic domain, and an ill-defined C-terminal domain (Fig. 12) (Jenkins et al., 2009). Saposin proteins are sphingolipid activator proteins that present lipids from membranes to the active sites of various enzymes in an acidic cellular compartment (Kolter et al., 2005). The saposin domain in A-SMase is sufficient to support the

hydrolysis of sphingomyelin without external saposin proteins (Paton et al., 1992; Bradovà et al., 1993). A-SMase was originally identified as a cation–independent hydrolase contributing to the catabolism of sphingomyelin in lysosome compartment. Subsequently, studies have indicated an enzyme metallo-dependence and a localization limited not only to lysosomes. Indeed, the enzyme is also secreted from vascular endothelial cells and macrophages through the Golgi secretory pathway as a Zn^{++} dependent enzyme (Marathe, Schissel et al., 1998). Upon its localization, two forms of A-SMase originating from one gene have been reported, an intracellular lysosomal form and an extracellular secreted form. The lysosomal A-SMase is preloaded with zinc, while the secreted form requires exogenous zinc (Schissel SL, et al., 1996).

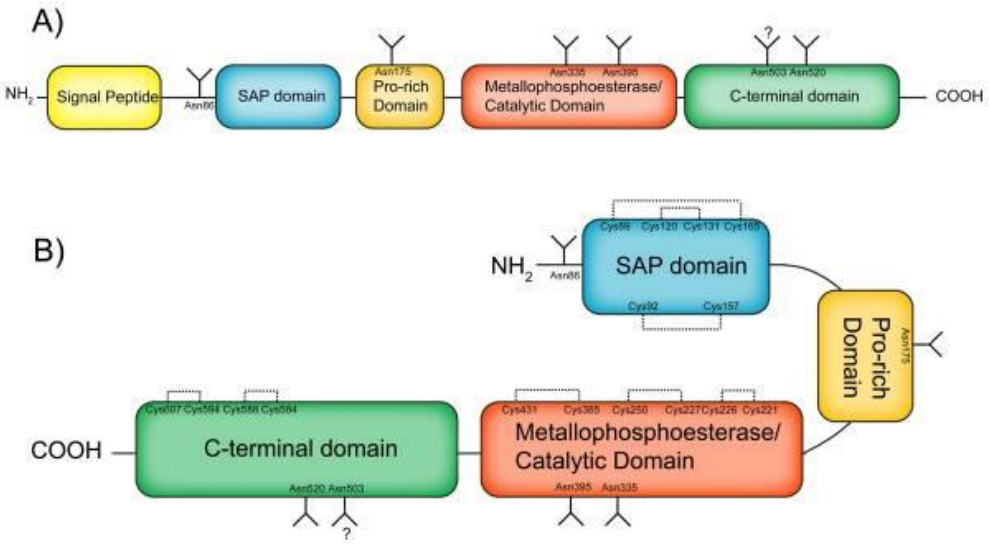


Figure 12: A) Primary structure of A-SMase polypeptide. B) Stylized conformation of mature A-SMase (Russel W., 2009; Cell Signal.).

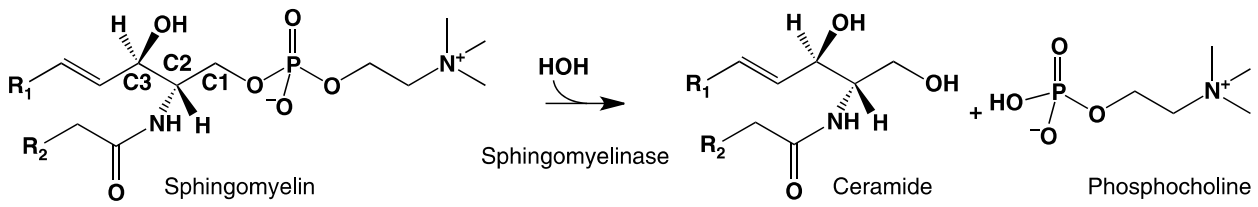


Figure 13: Sphingomyelin degradation catalyzed by Acid Sphingomyelinase activity (Zhou et al., 2015; Nature Communications).

The cDNA and complete gene-encoding mouse A-SMase has been isolated. Comparison with the human sequence shows 81% sequence identity at cDNA level and 82% at the protein level. Homology is reduced in the N-terminal region, especially in the presumed signal peptide. The six-exon gene structure is similar to the human one; the murine enzyme has six potential N-glycosylation sites, which prevent the enzyme from proteolytic degradation in the lysosome (Newrzella and Stoffel, 1996). Moreover, A-SMase has a mannose 6-phosphate residue that is required for lysosomal targeting of the enzyme via the mannose 6-phosphate receptor (Newrzella and Stoffel, 1992).

Studies performed in COS-1 cells transfected with A-SMase cDNA, revealed three major forms of 75, 72 and 57 kDa generated from the same nascent polypeptide derived from a single processed transcript (Ferlinz, Hurwitz et al., 1994). After deglycosylation they are reduced in size of 64, 61 and 47 kDa, respectively. The 75kDa form is the pre-polypeptide, which is found in the endoplasmic reticulum. The enzyme precursor is represented by the second form of 72kDa obtained by cutting a possible signaling sequence of 4kDa. After cutting, the precursor is transported to the endo/lysosomal compartments where it is processed to its mature form of 70kDa (Hurwitz, Ferlinz et al., 1994). Then, the mature enzyme is degraded to an inactive form of 52kDa.

Upon stimulation, A-SMase is relocated from intracellular organelles to the outer leaflet of the plasma membrane, where it is able to hydrolase the SM, localized within lipid raft micro domains, to ceramide. Lipid rafts are dynamic structures formed by clustering of sphingolipids and cholesterol by functioning as platforms for signal transduction and protein sorting (Simons and Ikonen, 1997).

Stimulation with CD95 (Fas) ligand rapidly induces the translocation of A-SMase from lysosomal to the outer leaflet of the plasma membrane. Moreover, ceramide produced by A-SMase is an important component of the lipid micro domains and it is essential for CD95 signaling and the induction of apoptosis (Grassme, Jekle et al., 2001).

Furthermore, A-SMase on the cell surface is essential for the clustering of CD40 (Grassme, Jendrossek et al., 2002), Fcy receptor II (Abdel Shakor, Kwiatkowska et al., 2004), CD20 and TRAIL receptor (Martin, Philips et al., 2005). Genetic alterations in A-SMase lead to A-SMase deficiency (ASMD) and have been linked to *Niemann–Pick* disease types A and B (Zhou Y-F et al., 2016). The mapping of mutations from *Zhou Y et al*. study revealed that 82% of the mutations are located in the catalytic domain, resulting in 21.6% mutation rate in protein sequence. In contrast, the mutation rates in the saposin, proline-rich linker, and C-terminal domains are no >11% (Fig. 14).

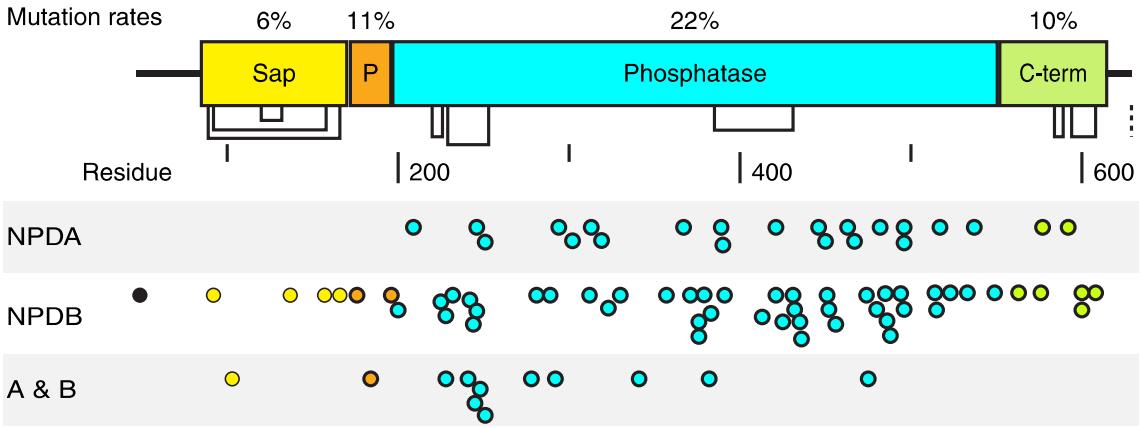


Figure 14: Distribution of mutations on hA-SMase sequence (Zhou et al., 2015; Nature Communications).

3.4 Acid Sphingomyelinase and inflammation

The involvement of A-SMase in the lysosomal storage disorder Niemann-Pick disease (NPD) is well known since 1960, when for the first time researchers reported that A-SMase deficiency was responsible for the accumulation of sphingomyelin in lysosomes and late endosomes (Brady et al., 1966). Subsequent investigations highlighted the important role of the hydrolase in mediating cell signaling by generating ceramide from the membrane lipid sphingomyelin. As ceramide is a bioactive lipid and a well-known mediator of stress signals and pro-inflammatory response, the A-SMase activation has been considered the key event in a variety of pathophysiological processes including inflammation, oxidative stress and cytokines release in common human diseases (Schuchman, 2010). In recent years, it has become increasingly evident that in the pathophysiology of many human diseases the effect of the imbalance of A-SMase activity may be related to inflammation. High levels of A-SMase activity have been found in various diseases characterized by a strong inflammatory component such as Alzheimer's and Parkinson's diseases (Haughey, 2010), diabetes (Opreanu et al., 2011), atherosclerosis (Deulin et al., 2008), chronic heart failure (Schissel et al., 1998) and steatohepatitis (Garcia-Ruiz, 2015).

Since A-SMase is responsible for the ceramide production at the cell surface, it is responsible for the plasma membrane reorganization. This ability gives rise to the important role of the enzyme in cell signaling. Moreover, A-SMase is a mediator of different stimuli, such as cell death receptor activation, radiations and chemotherapeutic drugs. An increase of A-SMase activity has also been found in cancer, a disease characterized by abnormal cell growth. Interestingly, Assi et al., demonstrated the role for A-SMase in the modulation of tumour immune microenvironment.

A-SMase plays also a role in other common pathologies such as inflammatory bowel diseases (IBD) and pulmonary diseases.

In patients affected by IBD, macrophages play a critical role in the pathology and they are responsible for the production of inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (Rogler et al., 1998). These cytokines as well as other stress stimuli are able to activate A-SMase and hence to increase the ceramide levels. High levels of ceramide lead to an increase of expression of various inflammatory genes by the activation of pro-inflammatory NF-κβ transcription factor (Spiegel et al., 1996). So, the attenuation of inflammatory response by suppressing A-SMase activity and hence the ceramide signaling, has been tested, but further studies are needed in order to better understand the ceramide metabolic pathways involved.

In the inflammatory context, the hydrolase A-SMase has been considered a potential therapeutic target also in lung injury and in other conditions related to the pulmonary functions (Uhlig et al., 2013). Studies carried out in lung injury model by Okuro et al., showed that the inhibition of A-SMase suppressed the up-regulation of NF-κβ activity with a reduction of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6. These findings suggest that the inflammatory response could be regulated by A-SMase via NF-κβ.

Although this evidence requires further studies they represent the beginning of a complex study related to the inflammatory process and its new possible targets belonging to the sphingolipids class.

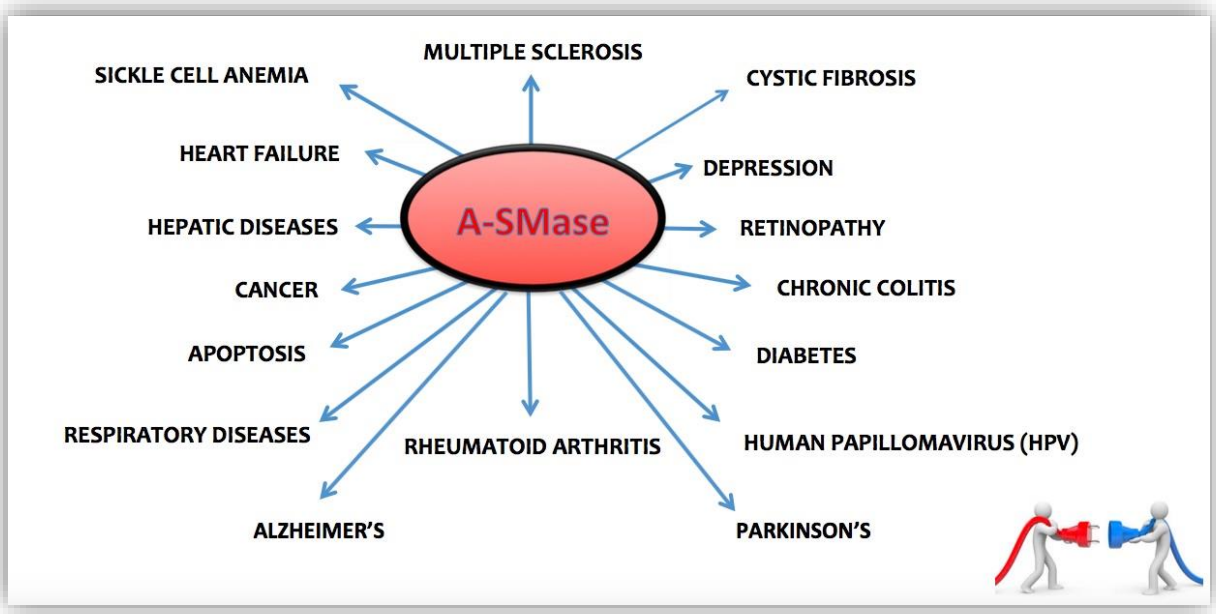


Figure 15: Schematic representation of Acid Sphingomyelinase involvement in inflammatory related diseases

AIM OF THE STUDY

This project arise from the idea to investigate the functional role of the sphingolipid-metabolizing enzyme A-SMase in acute and chronic muscle damage with the purpose to better understand its role in the establishment of muscle inflammation and in the subsequent skeletal muscle regeneration process so that this protein may be proposed as possible therapeutic target.

The first aim of the study is the investigation of A-SMase expression in the intricate inflammatory process following cardiotoxin induced-acute muscle damage. To this end, we use wild type and A-SMase knockout mice in order to elucidate its role in the signaling of muscle regeneration. Thus, we focus our attention on the action of A-SMase on the proliferation and differentiation of muscle stem cells (satellite cells) before and after muscle damage. The inflammatory response in muscle damage is a coordinated process in which macrophages play a critical role in the resolution of inflammation (Mounier et al., 2013) through a delicate biphasic transition from pro-inflammatory (M1) to anti-inflammatory phenotype (M2). Currently, the signaling pathways responsible for the macrophages polarization are poorly understood (Mounier et al., 2013) so we investigate whether A-SMase might influence the balance between the M1 and M2 macrophage subsets and hence the muscle repair after injury.

The second aim of the study is the evaluation of the possible role of A-SMase in the pathogenesis of Duchenne Muscular Dystrophy (DMD). In particular, we focus our attention on the role of A-SMase in the establishment of inflammation during the progression of the disease.

All the experiments are carried out comparing wild-type mice to the *mdx* mice, a mouse model of DMD, used in order to deepen the involvement of A-SMase in DMD pathogenesis.

Overall, we aim at identifying A-SMase as a potentially targetable player not only in DMD but also in various muscle-wasting diseases.

MATERIALS AND METHODS

Mice and animal care

Experiments were performed on (C57J/6N) A-SMase knockout (A-SMase-KO), *mdx* (C57BL/10-*mdx)* and wild type (WT) (C57BL/6J) mice. Animals were handled in accordance with the Italian law on animal care (D.L. 26/2014) and the European Directive (2010/63/UE). The mice were housed in an environmentally controlled room (23°C ± 1°C, 50% ± 5% humidity) with a 12-hour light/dark cycle and provided food and water ad libitum. The Ethics Committee of the University of Milan also approved the experimental protocols. All efforts were made to reduce both animal suffering and the number of animal used.

Acute muscle damage

A-SMase-KO and WT mice were anesthetized and subsequently injected with Cardiotoxin (CTX) from *Naja Mossambica mossambica* (Sigma- Aldrich). CTX was dissolved in sterile saline to a final concentration of 15μM and then injected in *Tibialis Anterior* (TA) muscles from 2-months-old. In total, 50μl of CTX were injected with a 27 Gauge needle into one TA muscle. Mice were sacrificed at 1, 3, 5, 7,14 and 21 days after injury and the TA muscles were harvested and analyzed at different time points after injury.

Naproxcinod treatment

Male *mdx* mice (n=5) were treated with Naproxcinod (30 mg/Kg) for 6 months starting at 5 weeks of age. The compound was administered daily in the diet (Mucedola, Milano, Italy), and the same diet without drug was given to control *mdx* mice (n=5). Following 6 months of treatment Acid Sphingomyelinase mRNA expression and activity have been detected in *Tibialis Anterior* of *mdx* animals.

Quantitative Real-Time PCR analysis

Total RNA was isolated by phase separation in PureZOL™ reagent (Bio-Rad) according to the manufacturer's instructions. After solubilisation in RNase-free water, total RNA was quantified by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA (1µg) was retro-transcribed by using iScript™ Reverse Transcription Supermix (Bio-Rad). PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725271) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All reactions were run as triplicates by using 2µl of cDNA and the GoTaq Green Master Mix (Promega). RPL38 (Ribosomal Protein L38) and RPL32 (Ribosomal Protein L32) have been used as housekeeping for normalization by using the $2^{-\Delta\Delta CT}$ formula. The primers pairs designed for RTqPCR analysis are listed in the table below.

NAME	FORWARD PRIMER	REVERSE PRIMER
CCL2	5'-AGGTGTCCCAAAGAAGCTGTA-3'	5'-ATGTCTGGACCCATTCCTTCT-3'
CCL7	5'-GCTGCTTTCAGCATCCAAGTG-3'	5'-CCAGGGACACCGACTACTG-3'
CCR2	5'-ATCCACGGCATACTATCAACATC-3'	5'-CAAGGCTCACCATCATCGTAG-3'
SMPD1	5'-TGGGACTCCTTTGGATGGG-3''	5'-CGGCGCTATGGCACTGAAT-3'
CD80	5'- AGTTTCTCTTTTTCAGGTTGTGAA-3'	5'-CACCCGGCAGATGCTAAAGA-3'
CD163	5'-CTCCTGTGGACTCTGAAGCG-3'	5'-CTCTGAATGACCCCCGAGGA-3'
CD206	5'- ATGGATTGCCCTGAACAGCA-3'	5'-TGTACCGCACCCTCCATCTA-3'
IL-1β	5'-CCCTGCAGCTGGAGAGTGTGGA-3'	5'- TGTGCTCTGCTTGTGAGGTGCTG-3'
CD86	5'-CTTACGGAAGCACCCACGAT-3'	5'-TGTAAATGGGCACGGCAGAT-3'
IL-10	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
TNF α	5'-CCCACGTCGTAGCAAACCACC-3'	5'-TCGGGGCAGCCTTGTCCCTT-3'
TGF β	5'-AAACGGAAGCGCATCGAA-3'	5'-GGGACTGGCGAGCCTTAGTT-3'
IGF 1	5'-GTGTGGACCGAGGGGCTTTTACTTC-3'	5'- GCTTCAGTGGGGCACAGTACATCTC-3'
F4/80	5'-TGACTCACCTTGTGGTCCTAA-3'	5'-CTTCCCAGAATCCAGTCTTTCC-3'
iNOS	5'GTTCTCAGCCCAACAATACAAGA-3'	5'-GTGGACGGGTCGATGTCAC-3'
Myogenin	5'-GACCCTACAGACGCCCACAATC-3'	5'-ACACCCAGCCTGACAGACAATC-3'
MyoD	5'- CTGGCGCCGCTGCCTTCTAC-3'	5'- GGCCGCTGTAATCCATCATGCCA-3'
Arginase 1	5'-CTCCAAGCCAAAGTCCTTAGA-3'	5'-AGGAGCTGTCATTAGGGACAT-3'
RPL38	5'-GAAGAATGCCAAGTCTGTCAA-3'	5'-GAGGGCTGGTTCATTTCAGA-3'
RPL32	5'-TTAAGCGAAACTGGCGGAAAC-3'	5'-TTGTTGCTCCCATAACCGATG-3'
IL-6	5'-TAGTCCTTCCTAGCCCAATTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'
MyHC II	5'AAGCGAAGAGTAAGGCTGTC3'	5'GTGATTGCTTGCAAAGGAAC3'
MyHC IV	5'ACAAGCTGCGGGTGAAGAGC3'	5'CAGGACAGTGACAAAGAACG3'

Tab 1: Primers used for quantitative RT real-time analyses

Protein Isolation and Western Blotting

For protein analysis, muscles from mice were harvested and homogenized with Ultra-Turrax (Ika Werke GmbH & Co. KG, Staufen Germany / Deutschland) in a protein lysis buffer containing 20mM Tris-HCl (pH 7.4), 10 mM EGTA, 150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich, X100), 10% glycerol, 1% SDS (Sigma Aldrich, L3771) supplemented with a cocktail of protease and phosphatase inhibitors (cOmplete and PhosSTOP; Sigma-Aldrich, 04693116001 and PHOSS-RO).
Proteins concentration was determined by Bio-Rad protein assay (Bio-Rad, 500-0006). Thirty to fifty µg of total protein were loaded on 4-20 % polyacrylamide precast gels (Criterion TGX Stain-free precast gels; Bio-Rad, 5678094). Proteins were transferred onto a nitrocellulose membrane using a Trans-Blot Turbo System™ (Bio-Rad, Hercules, CA) and Transfer pack™ (Bio-Rad, 1704156). The antibodies used for the analysis were rabbit polyclonal anti-GAPDH (1:3000; Santa Cruz Biotechnology), rabbit anti A-SMase (1:500) and mouse anti Myogenin (1:100).
Bands were visualized using HRP-linked secondary antibodies (Bio-Rad) and the Clarity Western ECL substrate (Bio-Rad, 1705061), with ChemiDoc MP imaging system (Bio-Rad, Hercules, CA). Image Lab software (Bio-Rad, Hercules, CA) was used for bands quantification and densitometry analyses.

Histological analysis of skeletal muscle

Skeletal muscle damage and repair were evaluated by Hematoxylin and Eosin (H&E) staining. H&E stains the cytoplasm in pink and the nuclei in dark purple and gives information on the morphology of the analysed muscle tissue in order to detect qualitative and quantitative alterations.
The fresh muscles were mounted on cork circles using *tragacanth gum* and routinely frozen in liquid N_2-cooled isopentane (Sigma-Aldrich). Muscles were sectioned on a cryostat at 10μm thickness and placed on Superfrost™ glass slides. Frozen sections of TA muscle were fixed in 4% paraformaldehyde (Sigma-Aldrich, P6148) (pH 7.4) for 10 minutes at 4°C, washed for three times with deionized water and stained with Eosin 1% for 10 minutes. After three washes with deionized water, sections were stained with Hematoxylin for 5 minutes followed by three washes with deionized water at room temperature. Then, muscle sections were dehydrated in series of Ethanol dilutions from 70% to 100% (2 minutes for each dilutions), passed though Xylene for 20 minutes, mounted with Eukitt ® (Sigma- Aldrich) and coverslip.

Images were acquired using a bright-light microscope; digital camera and image capture software. Histological features were identified manually and quantified using *Image J* analysis software. For quantitative analysis, cross-sectional area (CSA) was measured in Laminin-Hematoxylin stained by drawing around the entire cross-section area of the muscle fibre; central nucleation analysis and the number of fibers were counted in H&E stained (Bio-Optica) by using Image J software.

Masson's Trichrome

Masson's Trichrome is used to discriminate collagen fibers from muscular tissues. To this end, cryostatic sections (10μm) of untreated TA muscles from WT and A-SMase-KO mice were placed on Superfrost™ glass slide and stained with Masson Trichrome Kit (Bio-Optica Milano s.p.a). This staining demonstrates:

- Nuclei and Gametes (black)
- Cytoplasm, keratin, muscle fibres, acidophil granules (red)
- Collagen, mucus, basophil granules of hypophysis (blue)
- Delta cells granules of hypophysis (blue)
- Erythrocytes (yellow)

Non-specific Esterase Assay

Non-specific esterase assay (NSA kit; *30-30122LY*; Bio-Optica Milano s.p.a.) is used to identify macrophages infiltration in mouse skeletal muscle. To this end, cryostatic sections (10μm) of TA and Diaphragm muscles from WT and *mdx* mice were placed on Superfrost™ glass slide and stained with NSA kit (Bio-Optica Milano, s.p.a). This staining demonstrates:

- Angular atrophic fibers (beige)
- Neuromuscural junction (brown)
- Lipofuscin brown Lysosomal activity (brown)

Immunofluorescence

For immunofluorescence, frozen 10µm cross-sections of muscle were fixed with paraformaldehyde 4% for 20 min at 4 °C, blocked and permeabilized with 10% goat serum (NGS; Vector Laboratories, S-1000), 0.1 % Triton X-100 (Sigma-Aldrich, X100) in DPBS (EuroClone) for 1hour at room temperature. Then, sections were incubated with primary antibody Laminin (1:250, Abcam) for 1 hour at 4°C. The primary antibody was diluted in PBS, 0.1% Triton X-100 and 10% goat serum. After incubation with the appropriate fluorescent-labelled secondary antibodies (donkey anti-rabbit IgG H+L conjugated with Alexa Fluor® 488, 1:5000), nuclei were counterstained with Hoechst 33342 and slides were finally mounted with the ProLong Gold antifade reagent (all products from ThermoFisher Scientific, Walthan, MA, USA).

For myogenic precursor cells staining, cells were fixed with 4% PFA for 10 min at room temperature and permeabilized by 0.1% TritonX-100 in DPBS for 5 minutes at room temperature. Cells were blocked in blocking solution (10% NGS in DPBS) and then, labeled with specific primary antibodies prepared in blocking solution, and incubated at 4°C overnight. Then, cells were incubated with Alexa Fluor-conjugated antibodies in blocking solution for 1 h at room temperature.

Flow Cytometry

Fluorescence activated cell sorting (FACS) is an assay in which single cell properties either physical or chemical are simultaneously analysed in a fluid stream system and used to separate a heterogeneous sample into distinct groups of cells.

In order to isolate muscle cell from immune cell populations, injured TA muscles from WT and A-SMase-KO mice were harvested, minced and digested in a freshly prepared Dispase and Collagenase (type II) for 40 minutes at 37°C. Disaggregation was stopped with 10% fetal bovine serum and cells filtered through a 70µm cell strainer (Miltenyi Biotec). To determine the expression of cell surface markers in both mouse model, a fluorescence-activated cell sorter analysis was performed: collected cells were washed with PBS supplemented with 2% FBS and incubated with different antibodies for 30 minutes at 4°C. After washing, the samples were analysed by using Gallios Flow Cytometer (Beckman-Coulter, Brea, CA, USA) and the software FCS Express 4 (De Novo System, Portland, OR, USA).

Satellite cells were identified as an enriched population of α7-Integrin-PE (AbLab) and CD34-Alexa Fluor (BD Pharmingen™) double positive cells and CD45-PE-Cy7, CD31-PE-Cy7, CD80-FITC, CD86-FITC, CD14-FITC (eBioscience) and Sca1-FITC (BD Pharmingen™) negative cells. *For macrophage* analysis cells were stained with CD45-PE-Cy7, CD80-FITC (eBioscience), F4/80-PE (Miltenyi Biotec) and CD206 Alexa Fluor647 (BioLegend).

Myogenic precursor cells isolation and culture conditions

Murine myogenic precursor cells (MPCs) were obtained from fore limbs and hind limbs of neonatal A-SMase-KO and WT mice (5-7d old). Muscle mass was minced and digested in Dispase® (1.867 units/mg, Gibco®) and collagenase II (0.2% Sigma-Aldrich) for 40 minutes at 37°C. Satellite cells were generated by culture in Dulbecco's Modified Eagle's Medium (DMEM) (EuroClone ECB7501L) supplemented with 20% fetal bovine serum (EuroClone, ECS0180L), 3% chick embryo extract (custom made), 10 ng/ml basic fibroblast growth factor (PeproTech, 100-18B) and 1% penicillin-streptomycin (EuroClone, ECB3001D/1) in culture dishes coated with Matrigel (BD Biosciences, 354234) at 37°C with 5% CO_2. To observe in vitro differentiation, MPCs were seeded 30000 cell/cm^2 and induced in DMEM containing 2% horse serum (HS) (EuroClone, ECS0090L) and 1% penicillin-streptomycin for 3 days. Then, cells were incubated with antibodies against skeletal muscle myosin and DAPI (ThermoFisher Scientific, 62248). Fusion index, diameter of myotubes, number of nuclei/myotubes and myotubes ≥ 5 nuclei were calculated from three to five randomly chosen microscopic fields. Fusion index was calculated as the percentage of number of nuclei within myotubes over the total number of nuclei.

Macrophage Primary Culture

Macrophages were obtained from bone marrow (BM) precursor cells of WT and A-SMase-KO mice. Total BM was obtained from mice by flushing femur and tibiae BM with DMEM medium. Cells were isolated and propagated for 8 days in α-minimum essential medium (Gibco®) (37°C, 5% CO_2 in humidified atmosphere) containing 10% fetal bovine serum (FBS) in the presence of 100ng/mL macrophage-specific colony-stimulating factor (M-CSF, Miltenyi Biotec) to generate macrophages. Macrophages were activated with cytokines in order to obtain various activation states. Hence, adherent cells were collected and cultured in the presence of 10ng/mL M-CSF to form monolayers of differentiated, unpolarized macrophages. Cells were cultured for two additional

days in the presence of 10ng/mL IFN-γ (Miltenyi Biotec) to generate activated, polarized M1 cells and for 4 additional days with 10 ng/mL IL-4, IL-10 and M-CSF (Miltenyi Biotec) in order to generate activated, polarized M2a and M2c macrophages, respectively. Purity of differentiated macrophages was estimated by flow cytometry after F4/80 - PeCy7 labelling (eBioscience). Cells were used for various analyses.

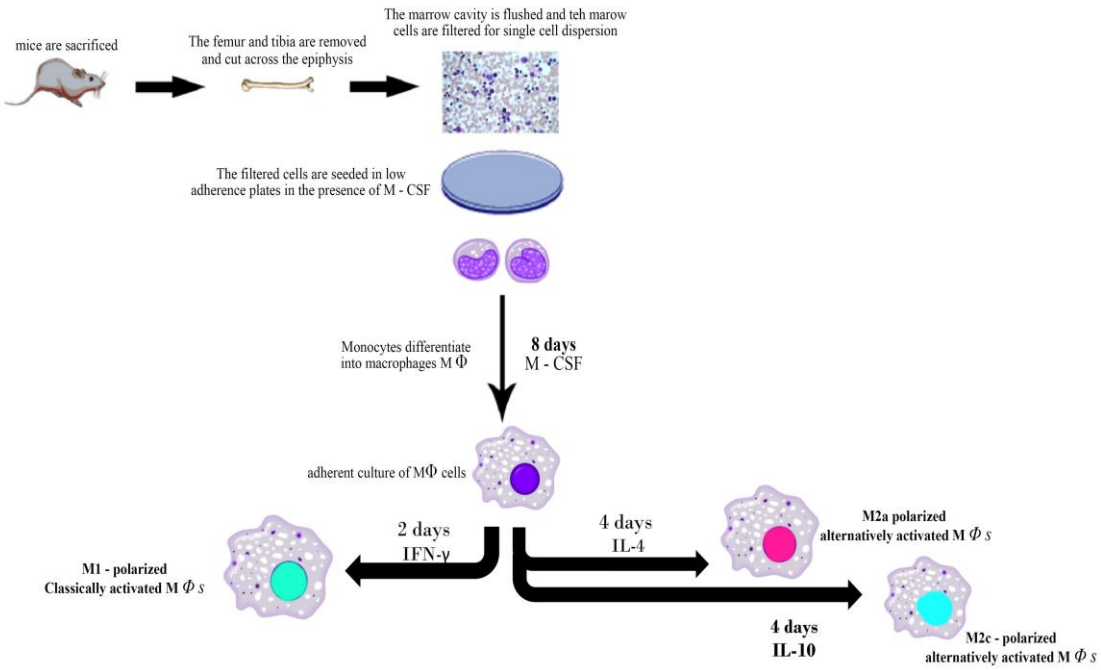


Figure 1: Isolation of Bone Marrow-Derived Cells, Differentiation and activation of Macrophage Primary Cultures (The American Journal of Pathology, Perrotta et al., 2014).

- *Macrophage-conditioned medium*

Macrophages were obtained by following the aforementioned standard protocol. Once the polarization of macrophages has been achieved, cells were washed. After washing steps, DMEM serum free medium was added for 24h, recovered, filtered and centrifuged in order to obtain macrophage-conditioned medium. Co-culture experiments were performed in which macrophage-conditioned medium was added to MPCs: for proliferation studies, macrophage-conditioned medium + 2.5% FBS was added to MPCs (seeded at 10000 cell/cm^2 on Matrigel).

Then, cells were incubated with rabbit anti *ki-67* antibody (1:200) which were visualized using secondary antibody. For differentiation studies, MPCs were seeded 30000 cell/cm^2 on Matrigel and

incubated for three days with macrophage-conditioned medium containing 2% horse serum. Cells were then incubated with anti-skeletal muscle myosin and subsequently visualized by using secondary antibody.

A-SMase activity assay

The A-SMase activity was determined using an acid sphingomyelinase assay kit (Echelon Biosciences Inc. Salt Lake City, UT) following company's instructions.

- *Preparation of Cell Lysate*

Cells were disrupted in ice-cold lysis buffer [150 mM NaCl, 50 mM Tris pH 7.4, 0.6% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich)] for 20 min. Cellular debris was removed after centrifugation at 10,000 × g for 5 min, and 20 μg protein was used to determine A-SMase activity. Reaction was stopped after incubation at 37°C for 2 hrs and analyzed using a fluorescence plate reader at 260 nm excitation and 460 nm emission.

- *Preparation of Tissue Homogenate*

To the muscle tissue has been added 8x volume of ddH2O and then it was keeped on ice. Immediately, tissue was homogenized 3x for 15 seconds each, on medium-high power and keeped on ice. The homogenate was freezed-thawed once on dry ice and immediately, the tissue was sonicated at medium-high power for 30 seconds, allow 10 seconds break, and resume for 30 more seconds (total 1 minute). After sonication, 14μg total protein was used to determine A-SMase activity. Reaction was stopped after incubation at 37°C for 2 hrs and analyzed using a fluorescence plate reader at 260 nm excitation and 460 nm emission.

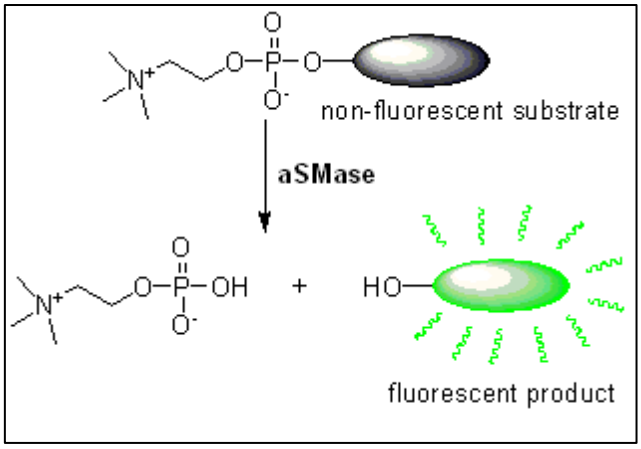


Figure 2: The Echelon Acid Sphingomyelinase Assay Kit uses a fluorogenic substrate, specific for acid Sphingomyelinase, to provide a sensitive and homogenous method to measure the activity of A-SMase in vitro

Statistical Analyses

Results were expressed as means ± SEM (standard error of the mean) of the indicative n values. All statistical analyses were performed using Student's t-tests for single comparison or by one-way ANOVA followed by the Newman-Keuls post-test (multiple comparisons). When data are not normally distributed, the Mann Whitney test was used. The GraphPad Prism software package (Graph Software, San Diego, CA, USA) was used.

RESULTS

Acid Sphingomyelinase is increased in cardiotoxin-induced acute damage

Several lines of evidence indicate the strong relation between A-SMase expression and diseases with a strong inflammatory component (Haughey, 2010; Opreanu et al., 2011; Devlin et al., 2008). Therefore, we assessed the possible connection between muscle inflammation and A-SMase expression by inducing muscle injury by the injection of cardiotoxin (CTX), a useful model for sterile inflammation.

Tibialis Anterior (TA) of 2-month-old wild type (WT) mice were injected with CTX and harvested at 1, 3, 5 and 7 days after damage (Fig. 1A). The injected TA muscles and their uninjected controls were processed for quantitative Real Time PCR (RTqPCR), western blot, and immunofluorescence analysis.

The RTqPCR analysis showed an increase of *Smpd1* mRNA, the gene that encodes for A-SMase, in TA muscles from WT after CTX treatment compared to those of untreated mice (Fig. 1B). Western blot confirmed with the RTqPCR data: the expression levels of A-SMase were much higher in injured-TA muscles than that in the untreated controls (UT) (Fig. 1C). Finally, the expression of A-SMase was also evaluated by immunofluorescence on histological sections from TA muscle damaged in which we found a different expression pattern of A-SMase in injured muscle fibers compared to healthy muscle fibers (Fig. 1D).

Taken together these findings indicate that A-SMase expression in muscle is up regulated upon cardiotoxin-induced acute damage and suggest a role for the protein in the process of muscle regeneration.

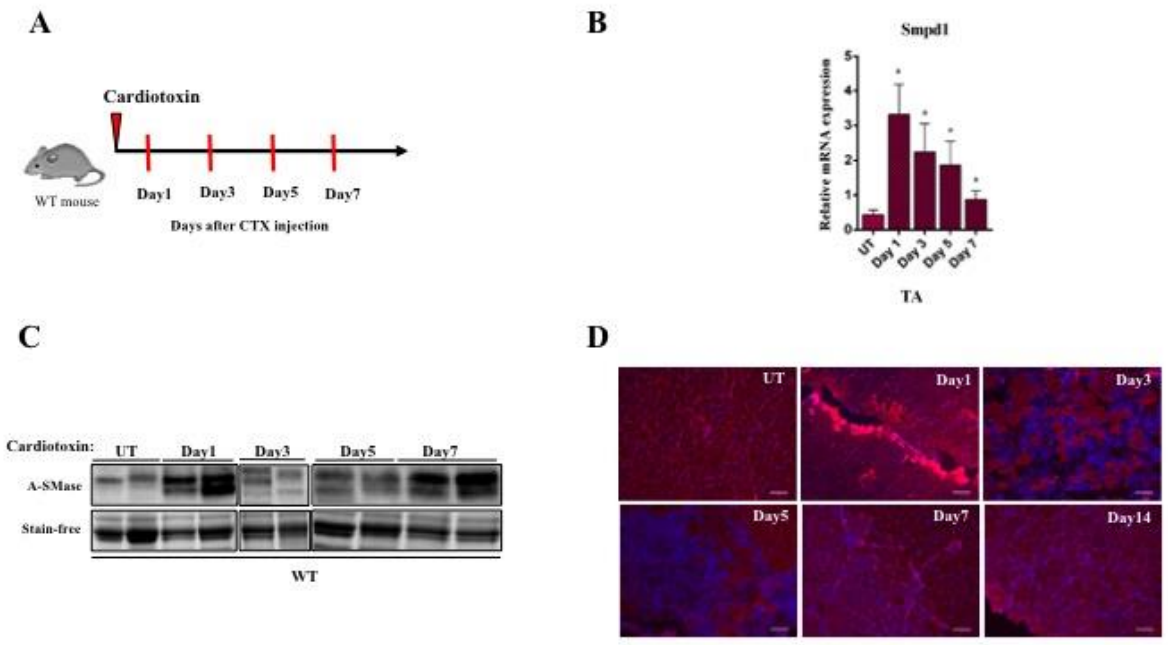


Figure 1: Acid Sphingomyelinase is increased in cardiotoxin-induced acute damage

A) Schematic representation of CTX injury experiment. Tibialis Anterior of WT mice (2-month-old) were injected with CTX and then harvested at 1, 3, 5, and 7 days after muscle damage. **B)** RTqPCR analysis of the *Smpd1* in TA of WT mice at 2-month-old. TA muscles were harvested and analyzed at 1, 3, 5, and 7 days after CTX injury. Values are expressed as mean ± SEM (n=6); *p<0.05. **C)** Western blot analysis of A-SMase protein in TA from WT mice treated with CTX and untreated WT mice used as control (UT) (n=2 mice for each experimental time point). The stain-free gel of total proteins was used as loading control. **D)** Injured and uninjured TA muscles of WT mice were immunostained with anti-A-SMase (red) antibody; nuclei were stained with DAPI (blue). The uninjured (UT) muscles were used as control. *Scale bars,* 100 µm.

A-SMase$^{-/-}$ and WT mice show similar skeletal muscle morphology

To assess the possible role of A-SMase in the muscle regeneration process which follows the inflammatory response, we took advantage of the A-SMase knockout mice (A-SMase$^{-/-}$).

First, TA of WT and A-SMase$^{-/-}$ mice of 2-month-old have been harvested and analyzed by immuno-histological assays in order to compare the two skeletal muscle characterizations. We observed that the TA gross morphology and the dimensions of A-SMase$^{-/-}$ mice were similar to those of WT (Fig. 2A).

Histological analysis by Hematoxylin & Eosin staining (H&E) did not show defects in the skeletal muscle of A-SMase$^{-/-}$ mice; Masson's Trichrome staining also did not reveal fibrosis or degenerating muscle fibers in A-SMase$^{-/-}$ as well as in control WT muscle (Fig. 2B). Then, we examined muscle fiber size by measuring the cross-sectional area (CSA) of TA from WT and A-SMase$^{-/-}$ mice and we did not found significant differences between the two types of mice (Fig. 2C).

Lastly, we assessed the number of satellite cells in muscles of WT and A-SMase$^{-/-}$ mice. By Flow Cytometry analysis (FACS) a similar percentage of satellite cells was found in both types of mice (Fig. 2D).

These data indicate that the loss of A-SMase neither affect the normal skeletal muscle morphology nor satellite cells number.

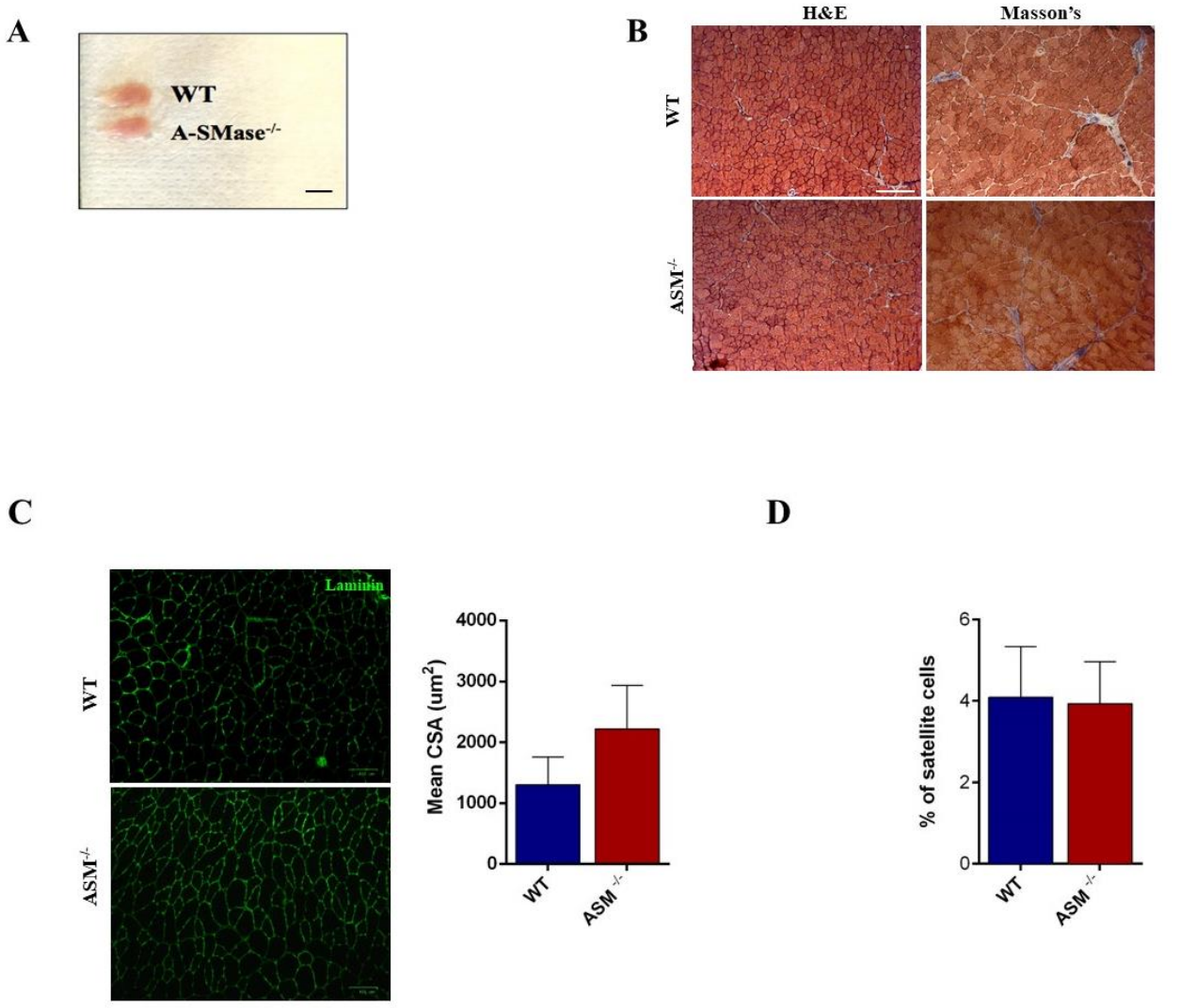


Figure 2: A-SMase$^{-/-}$ and WT mice show similar skeletal muscle morphology

A) Representative image of TA of WT and A-SMase$^{-/-}$ mice. Scale Bar, 2μm. **B)** H&E and Masson's Trichrome stainings of cryosections of 8-week-old WT and A-SMase$^{-/-}$ mice TA muscle (n=3). Scale Bar, 100 μm. **C)** TA muscle from WT and A-SMase$^{-/-}$ mice were immunostained with Laminin (green). The right graph indicates the mean CSA of TA muscle myofibers measured by using Image J software based on Laminin staining. Four animals of each genotype and at least 500 fibers for each animal were measured. **D)** The graph shows the percentage (%) of satellite cells number ($CD45^-/CD31^-/CD11b^-/Sca1^-/\alpha7^+/CD34^+$ CD45 cells) in TA from WT and A-SMase$^{-/-}$ mice measured by FACS analysis (n=3).

Muscle satellite cells from A-SMase$^{-/-}$ and WT mice show similar grow and differentiation ability *in vitro*

As satellite cells are able to proliferate and differentiate in response to injury giving rise to regenerated muscle, we wondered whether the absence of A-SMase could affect these two capacities of muscle progenitor cells.

To test this, we isolated the muscle satellite cells from WT and A-SMase$^{-/-}$ mice and compared their proliferation and differentiation ability *in vitro*. Proliferation of myoblasts was measured after 24 hours of culture by immunostaining with the cell cycle marker *Ki67*. The total number of *Ki67*$^{+}$ positive cells was similar in myoblasts derived from WT and A-SMase$^{-/-}$ mice (Fig. 3A). Moreover, satellite cells were also stained for MyoD in order to better define the proliferating myoblasts (Fig. 3B).

Then, we analyzed the differentiation of myoblasts into myotubes in both types of mice in the presence of 2% horse serum (Fig. 3C). Myotubes identified by Myosin-Heavy-Chain (MF20) staining generated in WT and A-SMase$^{-/-}$ mice did not show any differences in the morphology assessed by the parameters such as diameter, fusion index, myotubes with more five nuclei and number of myonuclei per myotube (Fig. 3C).

Moreover, also the transcript expression levels of MyoD and Myogenin fundamental for myogenic process and of Myosin Heavy Chain II and IV, responsible for muscle contraction, did not show any differences in satellite cells derived from both types of mice (Fig. 3D).

Therefore, this evidence suggests that *in vitro*, muscle satellite cells isolated from A-SMase$^{-/-}$ mice show a profile of proliferation and differentiation similar to that of WT control mice. Hence, our findings indicate that the loss of A-SMase does not affect satellite cell proliferation and differentiation ability.

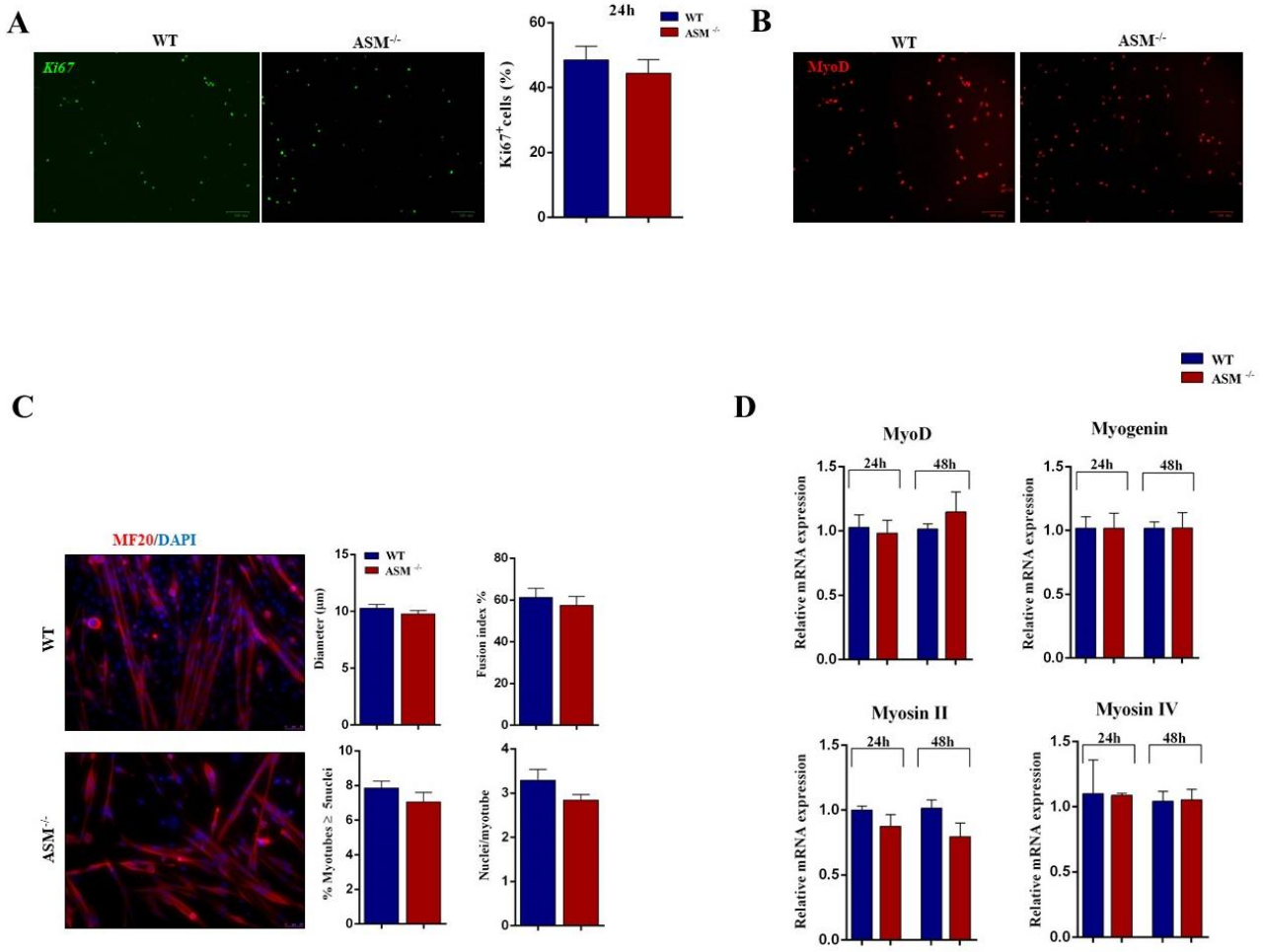


Figure 3: Muscle satellite cells from A-SMase$^{-/-}$ and WT mice show similar grow and differentiation ability *in vitro*

A) Immunostaining for *Ki67*$^{+}$ on WT and ASM$^{-/-}$ mice satellite cells after 24 hours of proliferation assay. *Scale Bar*, 100µm. Ki67^{+} positive satellite cells of WT and ASM$^{-/-}$ mice are quantified (right graph) by using Image J software and represented using GraphPad software analysis. Values are expressed as mean ± SEM (n=3). **B)** Immunostaining for MyoD on WT and ASM$^{-/-}$ satellite cells. *Scale Bar*, 100µm. **C)** *In vitro* differentiation of myoblasts into myotubes (48h) of WT and ASM$^{-/-}$ mice. In red MF20 staining identifies the differentiated myoblasts; in blu, DAPI staining, for the nuclei identification. The right graphs show the quantitative analysis of differentiated myotubes parameters, such as: Diameter (µm); Fusion Index (the percentage of Myosin-Heavy-Chain positive cells in myotubes over the total number of Myosin-Heavy-Chain positive cells); Myotubes with more than 5 nuclei and the number of Nuclei per Myotube. For quantification, 5 images per experiment across 3 independent experiments (n=15 images) were analyzed by Image J software and represented using GraphPad software analysis. Values are expressed as mean ± SEM. **D)** RTqPCR analysis of MyoD and Myogenin (upper panel) and of Myosin Heavy Chain II/IV (lower panel) in myotubes of WT and ASM$^{-/-}$mice after 24 and 48 hours of *in vitro* differentiation. Values are expressed as a mean ± SEM (n=8).

A-SMase affects muscle regeneration

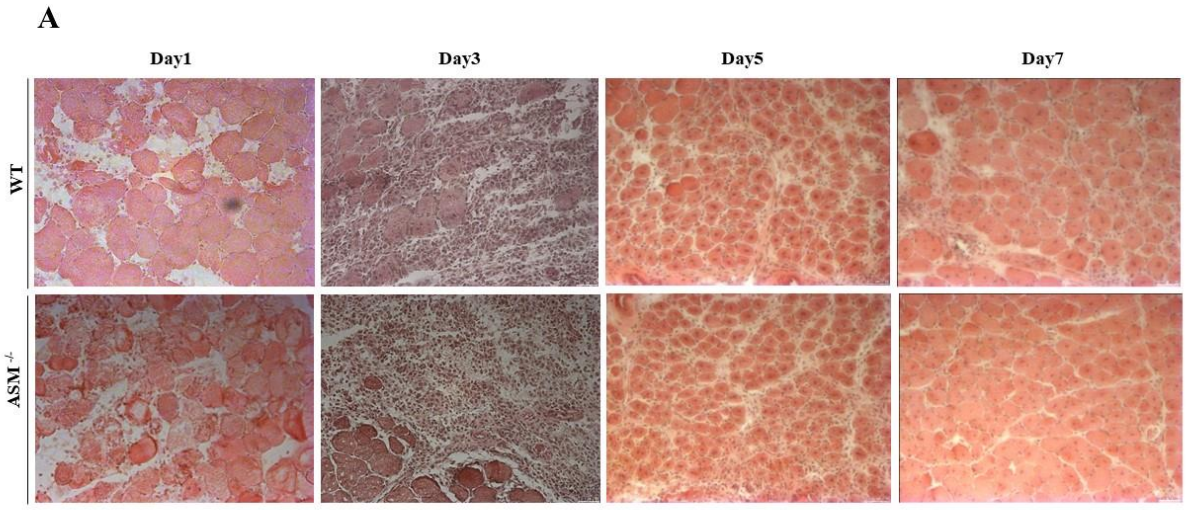
To explore whether A-SMase plays a role in the skeletal muscle regeneration process after induced damage, we performed an experiment by using the well characterized CTX induced injury model. To this end, TA of WT and A-SMase$^{-/-}$ mice were injected with CTX and then, harvested at different time points.

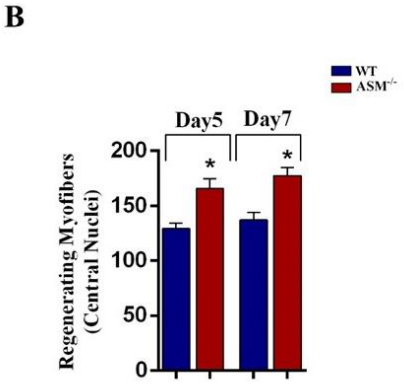
Histological analysis showed that the skeletal muscle repair was enhanced in the absence of A-SMase (Fig. 4A). Indeed, the analysis of newly formed myofibers in A-SMase$^{-/-}$ and WT mice after CTX treatment obtained by counting the centrally nucleated fibers (i.e. fibers with nuclei in the center of cytoplasm) showed that the the number of centrally nucleated fibers was enhanced in A-SMase$^{-/-}$ compared to WT mice at both 5 and 7 days postinjury (Fig. 4B).

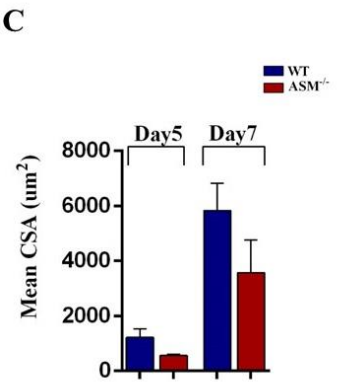
Likewise, we did not found differences between the two types of mice at both 5 and 7 days postinjury regarding the CSA of regenerating myofibers (Fig. 4C).

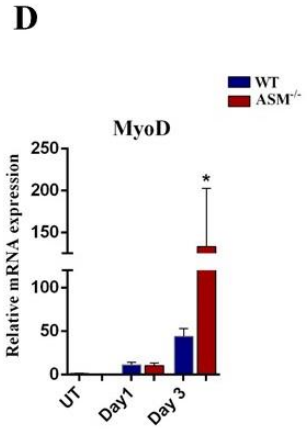
Interestingly, the expression mRNA levels of MyoD, the early myogenic transcription factor which is responsible for myogenic initiation and able to mark proliferating myoblast, were significantly higher in A-SMase$^{-/-}$ mice than that in WT mice 3 days after cardiotoxin injury (Fig. 4D). At the same time point, also the mRNA expression levels of the late myogenic transcription factor, Myogenin, were significantly increased in the absence of A-SMase compared to WT (Fig. 4E). This result was further confirmed by western blot analysis (Fig. 4F).

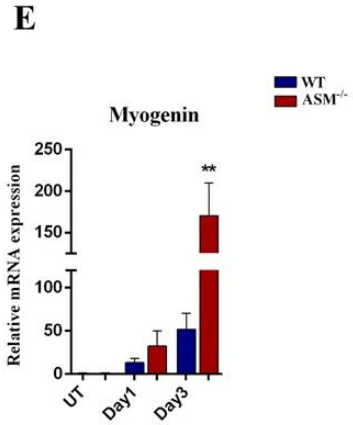
Taken together these data indicate that the muscle regeneration is accelerated in A-SMase$^{-/-}$ mice.


A
Day1
Day3
Day5
Day7
WT
ASM-/-


B
WT
ASM-/-
Day5
Day7
Regenerating Myofibers (Central Nuclei)
200
150
100
50
0
*
*


C
WT
ASM-/-
Day5
Day7
Mean CSA (um2)
8000
6000
4000
2000
0


D
WT
ASM-/-
MyoD
Relative mRNA expression
250
200
150
100
50
0
*
UT
Day1
Day 3


E
WT
ASM-/-
Myogenin
Relative mRNA expression
250
200
150
100
50
0
**
UT
Day1
Day3

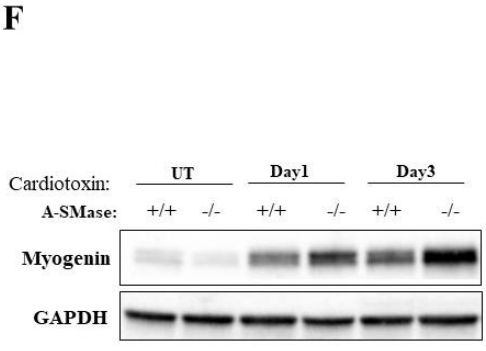

F
Cardiotoxin:
UT
Day1
Day3
A-SMase:
+/+
-/-
+/+
-/-
+/+
-/-
Myogenin
GAPDH

Figure 4: A-SMase affects muscle regeneration

A) H&E staining on transverse sections of TA muscles treated with CTX at 1, 3, 5 and 7 days from WT and A-SMase$^{-/-}$ mice, 2-month old; (n=2 mice for each experimental time point). Scale Bars, 100µm. **B)** Quantification of centrally nucleated total fibers number carried out on TA muscles at 5 and 7 days after CTX treatment in WT and A-SMase$^{-/-}$ mice. For quantification, 5 images per experiment across 3 independent experiments (n=15 images) were analyzed by Image J software. Values are expressed as mean ± SEM. Asterisks indicate statistically significant difference between WT and A-SMase$^{-/-}$ mice at each time point considered (*p=<0.05). **C)** CSA of TA muscle fibers, at 5 and 7 days after CTX damage were measured by using Image J based on H&E staining. Data are shown as mean value of CSA of the two types of mice. Four animals of each genotype and at least 500 fibers for each animal were measured. **D-E)** RTqPCR analysis of muscle transcription factors MyoD and Myogenin in untreated and CTX treated TA muscles (at 1 and 3 days) from WT and A-SMase$^{-/-}$ mice.Values are expressed as mean ± SEM (n=8 per group). **F)** Representative western blot of Myogenin protein in CTX treated TA muscles from WT and A-SMase$^{-/-}$ mice (untreated TA used as control). GAPDH used as loading control.

Macrophage infiltration at the injury site is increased in A-SMase$^{-/-}$ compared to WT mice

The skeletal muscle regeneration process is realized through the activation, proliferation and differentiation of muscle progenitor cells. Since *in vitro* analysis revealed no differences in muscle satellite cells derived from A-SMase$^{-/-}$ and WT mice in terms of number, proliferation and differentiation ability, we hypothesized that after muscle injury, the muscle niche of A-SMase$^{-/-}$ mice might be different from that WT and thus influences the muscle progenitor cells response to muscle damage.

First, to test this hypothesis we assessed the number of satellite cells in TA of A-SMase$^{-/-}$ and WT mice post CTX injury. Flow Cytometry analysis showed that the number of satellite cells significantly increased in A-SMase$^{-/-}$ mice at 1 and 3 days after damage in comparison to WT mice (Fig. 5A).

Since inflammatory cells and their extrinsic factors play a significant role in the local environment at the injury site, we investigated whether A-SMase might be involved in the signaling of immune response which naturally occurs after muscle damage.

We thus assessed the macrophages infiltration occuring after CTX injury. By Flow Cytometry analysis we found a higher number of macrophages ($CD45^+/F4/80^+$ positive cells) infiltrating the damaged muscle of A-SMase$^{-/-}$ compared to WT mice at 1, 3, and 5 days after injury (Fig. 5B).

This data was further confirmed by RTqPCR analysis of F4/80 and of cytokines involved in the recruitment of inflammatory cells to the injured site. After day 1 of CTX damage we found that F4/80, MCP-1, CCL7 and CCR2 were significantly higher in A-SMase$^{-/-}$ mice compared to WT (Fig. 5C).

This evidence suggests that the enhanced regeneration in A-SMase$^{-/-}$ mice should be due to a definite niche at the damaged site, strongly influenced by the presence or absence of A-SMase which could be the key factor determining the speed of regeneration.

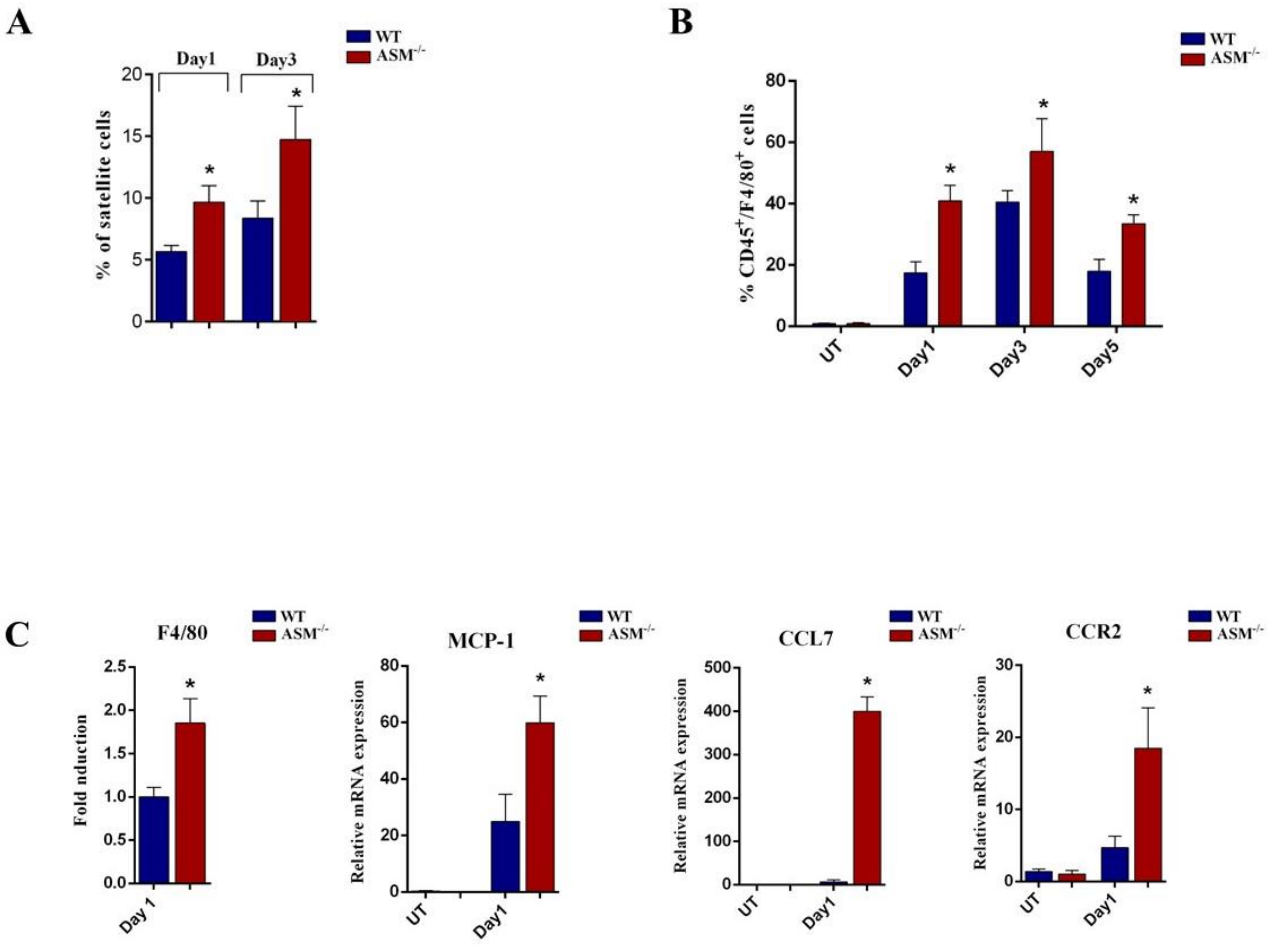


Figure 5: Macrophage infiltration at the injury site is increased in A-SMase$^{-/-}$ compared to WT mice

A) The graph shows the percentage (%) of satellite cells number ($\alpha7^+$cells) in TA from WT and A-SMase$^{-/-}$ mice measured by Flow Cytometry after 1 and 3 days of CTX treatment. **B)** Percentage (%) of CD45$^+$/F4/80$^+$ positive cells measured by Flow Cytometry in untreated and treated TA muscles with CTX from WT and A-SMase$^{-/-}$ mice at 1, 3 and 5 days.. **C)** RTqPCR analysis of F4/80 (fold induction), MCP-1, CCL7 and CCR2 on mRNA extracted from TA of WT and A-SMase$^{-/-}$ mice after 1 day of CTX treatment. Values are expressed as mean ± SEM (n=8 per group). Asterisks indicate statistically significant difference between WT and A-SMase$^{-/-}$ mice at each time point considered (*$p<0.05$).

Anti-inflammatory cytokines expression is enhanced in A-SMase$^{-/-}$mice after cardiotoxin injury

Inflammatory reaction plays a key role in satellite cells activation and proliferation in injured muscle. Thus we measured the pro- and anti-inflammatory cytokines profile of TA from WT and A-SMase$^{-/-}$ mice after CTX injection.

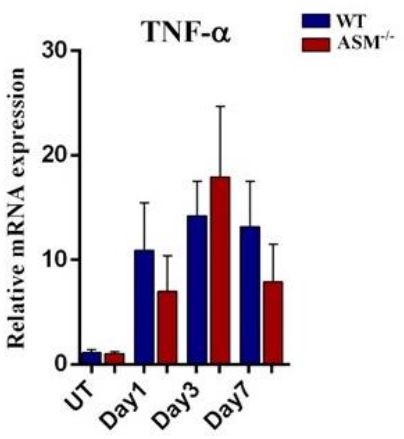
We did not found any significant difference in the expression levels of the pro-inflammatory cytokine TNF-α between WT and A-SMase$^{-/-}$ mice, noteworthy, the pro-inflammatory cytokine IL-1β was present in lower levels over time in A-SMase$^{-/-}$ mice after CTX injury compared to WT, suggesting an enhanced clearence of M1-type pro-inflammatory macrophages at the injured site (Fig. 6A).

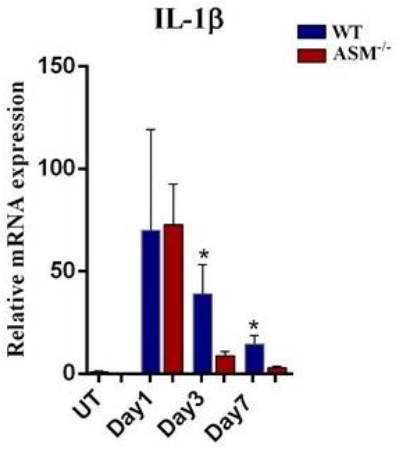
Contrariwise, 1 and 3 days after CTX injection, the anti-inflammatory cytokines IL-10 and TGF-β, showed significantly higher expression levels in A-SMase$^{-/-}$ when compared to WT mice, suggesting an enhanced activation of M2-type anti-inflammatory macrophages (Fig. 6B).

Interestingly, Insulin-like growth factor 1 (IGF-1), a well-known growth factor and a potent enhancer of tissue regeneration, showed much higher expression levels in A-SMase$^{-/-}$ mice compared to WT, at each time point from day 1 to 7 after CTX injury (Fig. 6C). This is a relevant finding since IGF-1 is able to promotes the proliferation and differentiation of myoblasts and its over expression in muscle injury leads to faster resolution of the inflammatory phase (Tonkin et al., 2015).

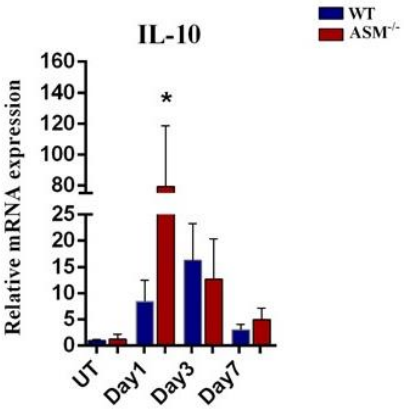
These results suggest that the switch from pro- to anti-inflammatory macrophages is enhanced in A-SMase$^{-/-}$ mice after muscle damage and so this might be an explanation of our data demonstrating that the absence of A-SMase leads to an accelerated regeneration after CTX injury.

A


TNF-α
WT
ASM-/-
Relative mRNA expression
30
20
10
0
UT
Day1
Day3
Day7


IL-1β
WT
ASM-/-
Relative mRNA expression
150
100
50
0
*
*
UT
Day1
Day3
Day7

B


IL-10
WT
ASM-/-
Relative mRNA expression
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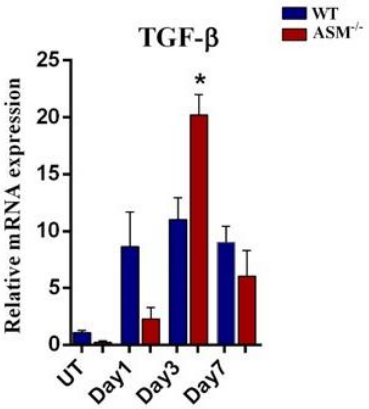
140
120
100
80
25
20
15
10
5
0
*
UT
Day1
Day3
Day7


TGF-β
WT
ASM-/-
Relative mRNA expression
25
20
15
10
5
0
*
UT
Day1
Day3
Day7

C

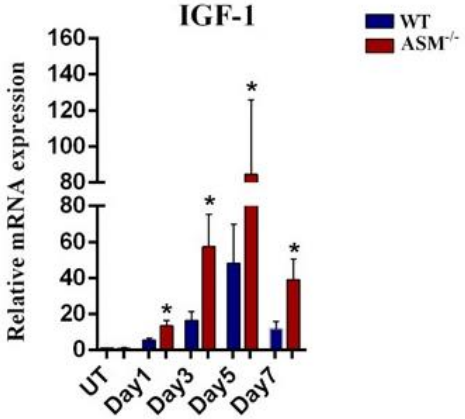

IGF-1
WT
ASM-/-
Relative mRNA expression
160
140
120
100
80
80
60
40
20
0
*
*
*
UT
Day1
Day3
Day5
Day7

Figure 6: Anti-inflammatory cytokines expression is enhanced in A-SMase$^{-/-}$ mice after cardiotoxin injury

A) RTqPCR analysis of TNF-α and IL-1β on mRNA extracted from TA of WT and A-SMase$^{-/-}$ mice after 1, 3 and 7 days of CTX treatment. Values are expressed as mean ± SEM (n=8 per group) *p<0.05. **B)** RTqPCR analysis of IL-10 and TGF-β on mRNA extracted from TA of WT and A-SMase$^{-/-}$ mice after 1, 3 and 7 days of CTX treatment. Values are expressed as mean ± SEM (n=8 per group)) *p<0.05. **C)** RTqPCR analysis of IGF-1 on mRNA extracted from TA of WT and A-SMase$^{-/-}$ mice after 1, 3, 5 and 7 days of CTX treatment. Values are expressed as mean ± SEM (n=8 per group) *p<0.05. Asterisks indicate statistically significant difference between WT and A-SMase$^{-/-}$ mice at each time point considered. For each graph the UT indicates the untreated state of TA muscle analyzed.

A-SMase$^{-/-}$ mice show an altered balance between pro- and anti-inflammatory macrophages after muscle damage

To understand whether the balance between pro- and anti-inflammatory macrophages could be altered by A-SMase expression after muscle injury, we examined and quantified by Flow Cytometry both M1 and M2 macrophage types from the first day after CTX injection in TA of A-SMase$^{-/-}$ and WT mice.

As cell surface markers for M1 and M2 macrophages we used CD80 and CD206, respectively.

At day 1 and 3 after muscle injury, we found that the distribution of the two macrophage subsets was altered in A-SMase$^{-/-}$ regenerating muscle with fewer CD80-positive macrophages in comparison to WT muscle. Accordingly, the M1/M2 ratio indicates that the M1 macrophage proportion was significantly lower in the absence of A-SMase compared to WT (Fig. 7A). This finding indicates a short M1 phenotype retention phase and corresponding enhanced M2 activation in A-SMase$^{-/-}$ mice.

Thus, in order to confirm the role of A-SMase in the alteration of macrophage subsets balance in muscle we analyzed the polarization to M1/M2 macrophages, *in vitro*. To this end, bone marrow derived macrophages (BMDM) from WT and A-SMase$^{-/-}$ mice were treated with different cytokines in order to trigger various pro-inflammatory and anti-inflammatory profiles: IFN-γ treatment has been used to elicited M1 pro-inflammatory macrophages, IL-4 and IL-10 have been used to stimulate the anti-inflammatory M2 state. By RTqPCR we analyzed genes normally expressed by M1 subsets such as CD80, iNOS and IL-1β and by M2 types such as CD206, Arginase1 and CD163. The results show that A-SMase$^{-/-}$ M1 macrophages have a significantly lower expression levels of transcripts that normally are elevated in M1 macrophages (CD80, iNOS, IL-1β) in comparison to M1 population of WT. Conversely, A-SMase$^{-/-}$ M2 macrophages showed no differences compared to WT macrophages (Fig. 7B).

Together, our findings indicate that after muscle injury the number of M1 macrophages is lower in the absence of A-SMase compared to WT; furthermore, our *in vitro* results indicate that A-SMase is fundamental for M1 macrophage polarization since its absence leads to an impairment in the expression of M1 macrophage markers.

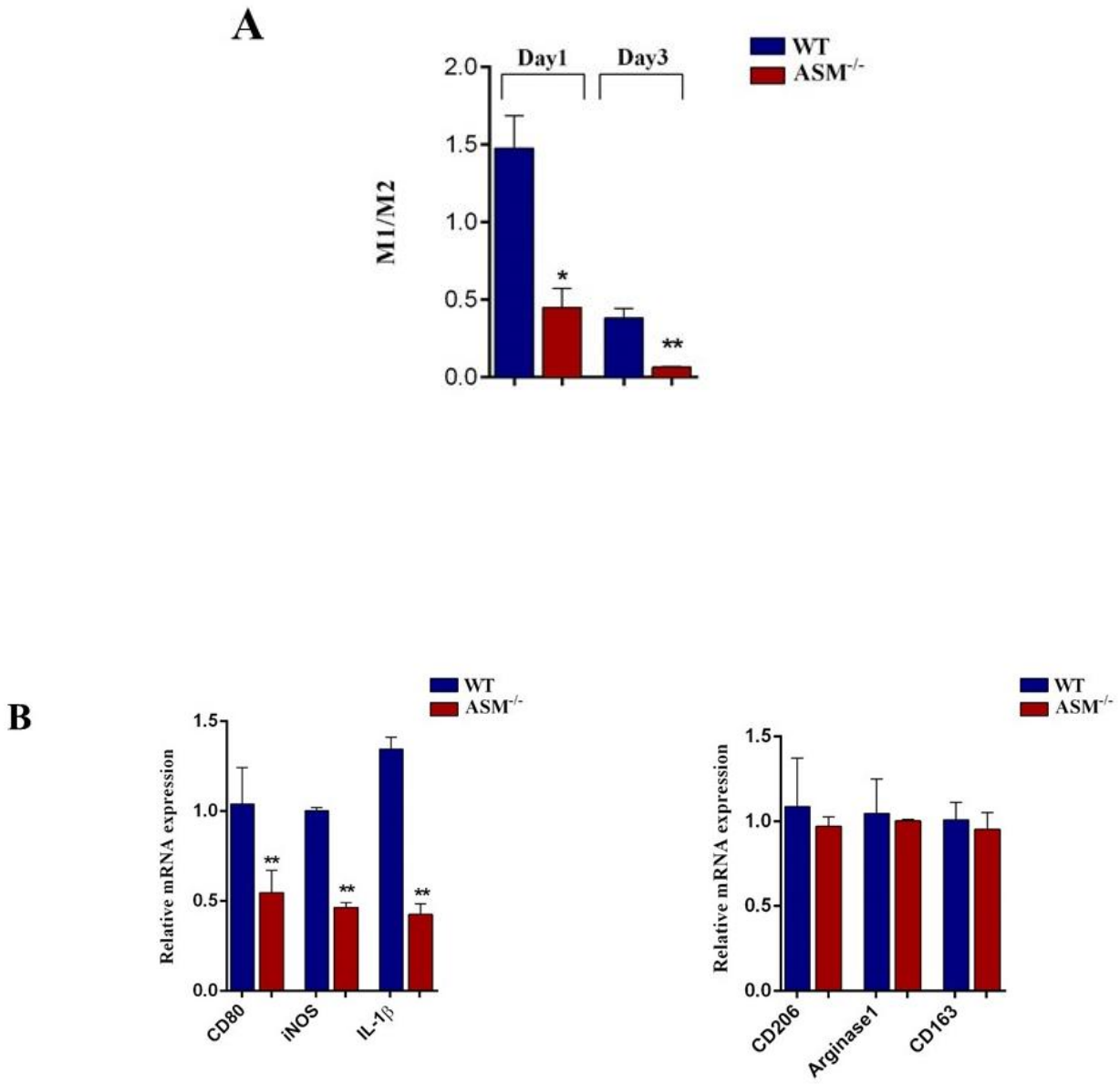


Figure 7: A-SMase$^{-/-}$ mice show an altered balance between pro- and anti-inflammatory macrophages after muscle damage

A) The graph shows the ratio of M1/M2 macrophages in TA from WT and A-SMase$^{-/-}$ mice measured by Flow Cytometry analysis after 1 and 3 days of CTX treatment (n=4 per group). **B)** Left panel: RTqPCR analysis of M1 macrophage markers, CD80, iNOS and IL-1β on mRNA extracted from bone marrow derived macrophages polarized to M1 macrophages of WT and A-SMase$^{-/-}$ mice. Right panel: RTqPCR analysis of M2 macrophage markers CD206, Arginase1 and CD163 on mRNA extracted from bone marrow derived macrophages polarized to M2 phenotype, of WT and A-SMase$^{-/-}$ mice. Values are expressed as mean ± SEM (n=4 per group). Asterisks indicate statistically significant difference between WT and A-SMase$^{-/-}$ mice (*$p<0.05$; **$p<0.001$).

A-SMase is over expressed in *mdx* Tibialis Anterior muscle

We then decided to investigate the functional role of A-SMase in the pathogenesis of Duchenne muscular dystrophy (DMD), a disease characterized by a strong activation of components of the innate immune system (Rosenberg AS et al., 2015) in order to better understand its involvement in the establishment of inflammation during the progression of the disease. To this end, we took advantage of the *mdx* mice, a mouse model for DMD pathology assessing A-SMase expression in TA of WT and *mdx* mice at 4 and 12 weeks of age that represent the stage of peak muscle necrosis and muscle regeneration, respectively.

By RTqPCR analysis we found a significant increase in the mRNA expression of A-SMase in TA of *mdx* mice both at 4 and 12 weeks compared to WT muscles (Fig. 8A). This result was then confirmed by immunofluorescence analysis (Fig. 8B). Also the activity of A-SMase was found to be higher in in TA of *mdx* mice both at 4 and 12 weeks of age compared to WT mice (Fig. 8C). Taken together, these findings suggest the involvment of the enzyme A-SMase in the pathogenesis of DMD.

Interestingly, we found that the increase of A-SMase in expression and in activity, paralleled with the increase of muscle inflammatory state. To test this evidence, we used CD45 as a marker to identify the activated inflammatory cells in TA muscles of WT and *mdx* mice at 4 and 12 weeks. By immunofluorescence staining we detected a higher number of $CD45^+$ positive cells in TA of *mdx* mice both at 4 and 12 weeks of age compared to WT (Fig. 8D). The muscle inflammatory state was also assessed by RTqPCR in order to evaluate the mRNA expression of inflammatory cytokines such as IL-6, IL-1β and TNF-α. Both *mdx* TA muscles at 4 and 12 weeks showed a significant increase in the mRNA expression of the all inflammatory cytokines compared to WT muscles. (Fig. 8E). These data were confirmed by Nonspecific Esterase (NSE) staining that depicted myophagocytosis: we observed the presence of macrophage activity in regions of *mdx* TA sections both at 4 and 12 weeks of age, completely absent in TA of WT muscles (Fig. 8F).

No relation was found between A-SMase expression/activity and fibrosis. The fibrosis state evaluated by the Masson's Trichrome staining reveals traces of collagen and interstitial fibrosis in TA of both mice at 4 and 12 weeks of age (Fig. 8F).

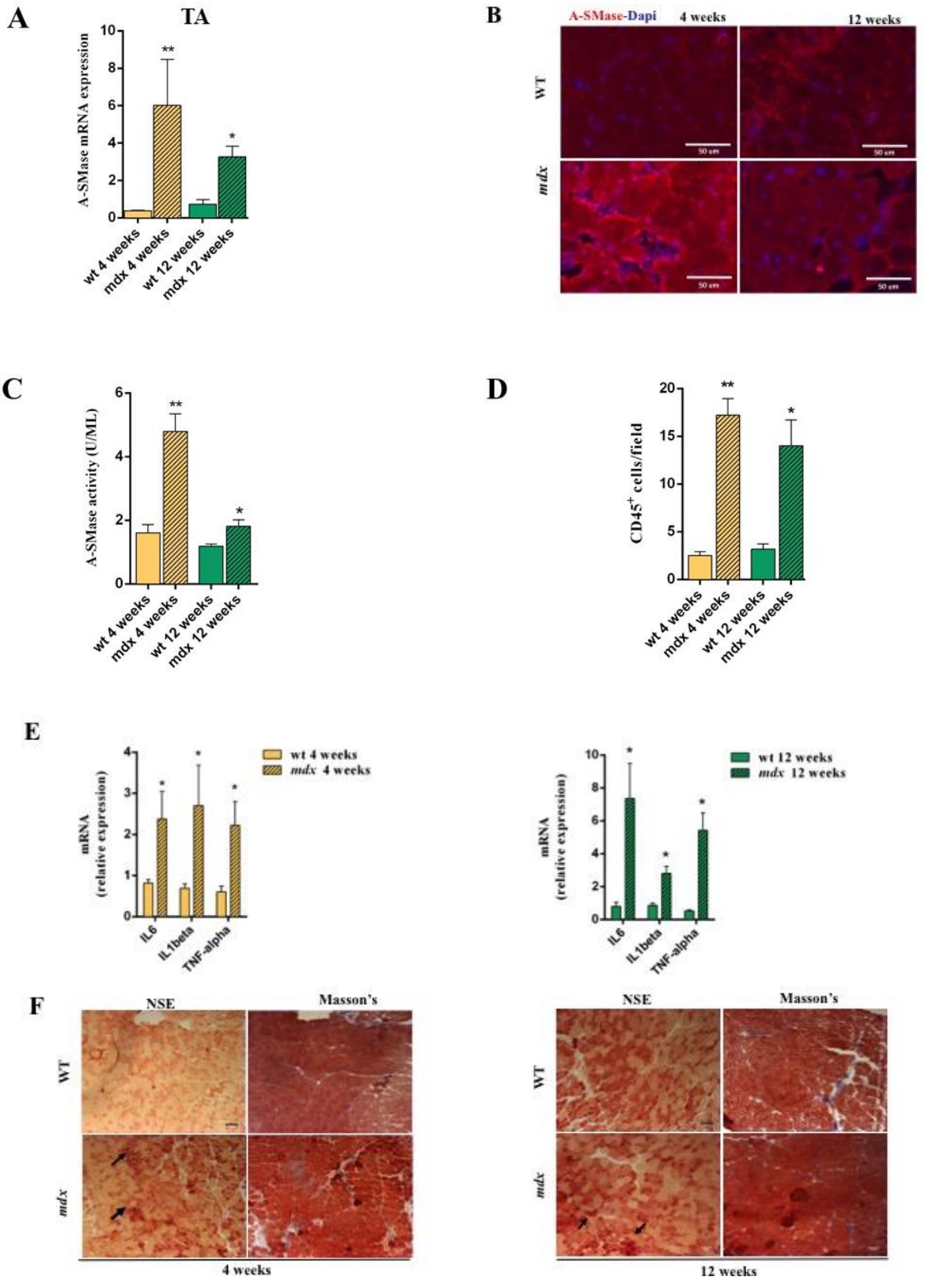

A
TA
A-SMase mRNA expression
wt 4 weeks
mdx 4 weeks
wt 12 weeks
mdx 12 weeks
B
A-SMase-Dapi
4 weeks
12 weeks
WT
mdx
C
A-SMase activity (U/ML)
D
CD45+ cells/field
E
wt 4 weeks
mdx 4 weeks
mRNA (relative expression)
IL6
IL1beta
TNF-alpha
wt 12 weeks
mdx 12 weeks
F
NSE
Masson's
4 weeks
12 weeks

Figure 8: A-SMase is over expressed in *mdx* Tibialis Anterior muscle

A) RTqPCR analysis of A-SMase expression in TA of WT and *mdx* mice at 4 and 12 weeks of age. Values are expressed as mean ± SEM (n=4 per group) *p<0.05; **p<0.001. **B)** Immunofluorescence staining of A-SMase expression in muscle fibers of TA from WT and *mdx* mice at 4 and 12 weeks of age. *Scale bars,* 50µm. **C)** A-SMase activity (U/ML) detected by colorimetric assay and GraphPad software for quantification. The detection was carried out on 2 independent experiments with 3 animals per each time point (n=24 animals). **D)** Quantification of $CD45^+$ positive cells in TA muscles of WT and *mdx* mice both at 4 and 12 weeks analyzed by Image J software (n=3 per each genotype). **E)** RTqPCR analysis of inflammatory cytokines IL-6, IL-1β and TNF-α on mRNA extracted from TA muscles of WT and *mdx* mice both at 4 and 12 weeks of age. Asterisks indicate statistically significant difference between WT and *mdx* mice; *p<0.05; **p<0.001. **F)** NSE and Masson's Trichrome stainings on transverse sections of TA muscles from WT and *mdx* mice at 4 weeks (left panel) and 12 weeks of age (right panel) (n=3 mice per each genotype). *Scale bars*, 100µm.

A-SMase is over expressed in *mdx* Diaphragm muscle

The diaphragm of *mdx* mouse undergoes a progressive degeneration and impairment in structure and function that reproduce the degenerative changes of Duchenne Muscular Dystrophy (Stedman et al., 1991; Lynch et al 2001). Therefore, we decide to explore the A-SMase expression in diaphragm of old *mdx* and WT mice at 12 weeks of age.

By immunofluorescence analysis, we found that at 12 weeks of age, A-SMase is mainly distributed at muscle fiber periphery in *mdx* mice by suggesting a translocation of the enzyme to the sarcolemma where it is able to produce ceramide from sphingomyelin degradation (Fig. 9A). As the translocation of A-SMase is usually associated to an increase of the enzyme activity, we detected also A-SMase activity in diaphragm of old *mdx* and WT mice at 12 weeks of age. The colorimetric assay for A-SMase indicated a significantly increased of the enzyme activity in *mdx* diaphragm when compared to that of the WT mice (Fig. 9B). Thus, as for the TA, also for the diaphragm we investigated the muscle inflammatory state by using CD45 as marker and NSE staining. We observed a higher number of $CD45^+$ positive cells in *mdx* mice than in WT and regions of myophagocytosis/inflammation in *mdx* diaphragm at both 4 and 12 weeks of age (Fig. 9C-D). These findings agree with the above data carried out on *mdx* TA muscles in which we found that the increase of A-SMase is related to the increase of the muscle inflammatory state.

Also in diaphragm, we did not found any relation between A-SMase expression/activity and fibrosis. The Masson's Trichrome revealed the presence of collagen and interstitial fibrosis in diaphragm of *mdx* mice but not in diaphragm of WT animals at both 4 and 12 weeks of age (Fig. 9D).

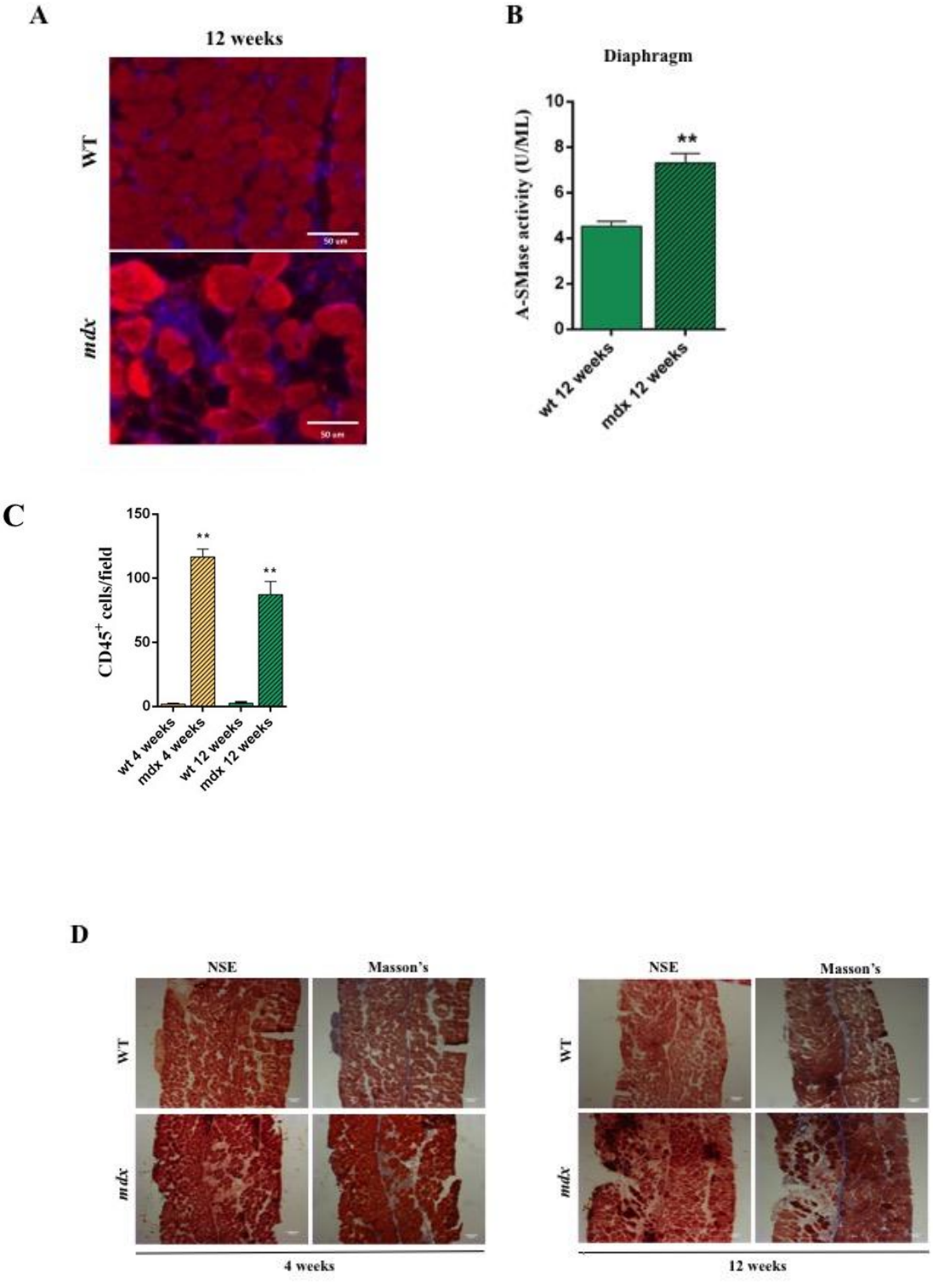

A
12 weeks
WT
mdx
50 um
50 um
B
Diaphragm
A-SMase activity (U/ML)
10
8
6
4
2
0
**
wt 12 weeks
mdx 12 weeks
C
CD45+ cells/field
150
100
50
0
**
**
wt 4 weeks
mdx 4 weeks
wt 12 weeks
mdx 12 weeks
D
NSE
Masson's
NSE
Masson's
WT
mdx
WT
mdx
4 weeks
12 weeks

Figure 9: A-SMase is over expressed in *mdx* Diaphragm muscle

A) Immunofluorescence staining of A-SMase (red) expression in muscle fibers of diaphragm from WT and *mdx* mice at 12 weeks of age; nuclei were stained with DAPI (blu). Images are representative of immunofluorescence performed in muscles from n=3 mice per each genotype. *Scale bars,* 50 µm. **B)** A-SMase activity (U/ML) in diaphragm of WT and *mdx* mice at 12 weeks of age detected by colorimetric assay and GraphPad software for quantification. The detection was carried out on 2 independent experiments with 3 animals per each genotype (n=12 animals). Asterisks indicate statistically significant difference between WT and *mdx* mice, **$p<0.001$. **C)** Quantification of $CD45^+$ positive cells in diaphragm of WT and *mdx* mice at 4 and 12 weeks of age analyzed by Image J software (n=3 mice per each genotype) **D)** NSE and Masson's Trichrome stainings on diaphragm from WT and *mdx* mice at 4 weeks (left panel) and 12 weeks of age (right panel) (n=3 mice per each genotype). *Scale bars,* 100µm.

A-SMase activity is over expressed in *mdx* immune and macrophage derived cell populations

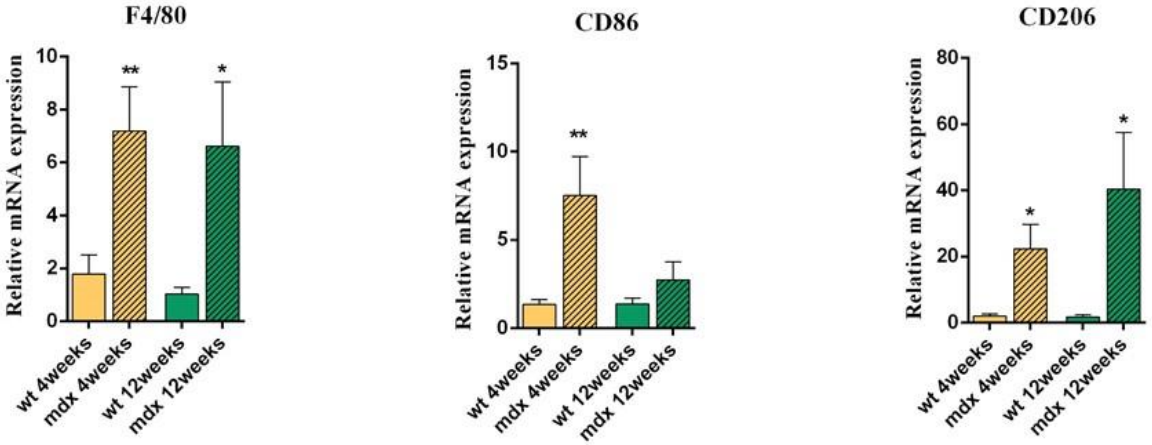
Muscle regeneration is a complex process characterized by a synchronized cross-talk between skeletal muscle and immune cells (Pillon et al.,2012). Neverthless, dystrophic muscle undergoes to an irregular interaction between muscle cells and immune system so that we wondered whether A-SMase might have a role in the balance of this cross-talk and hence in the DMD pathogenesis.

For this reason, we assessed A-SMase expression and activity in muscle and immune system-derived cells from *mdx* and WT at both the necrosis peak (4 weeks) and at the regeneration phase (12 weeks).

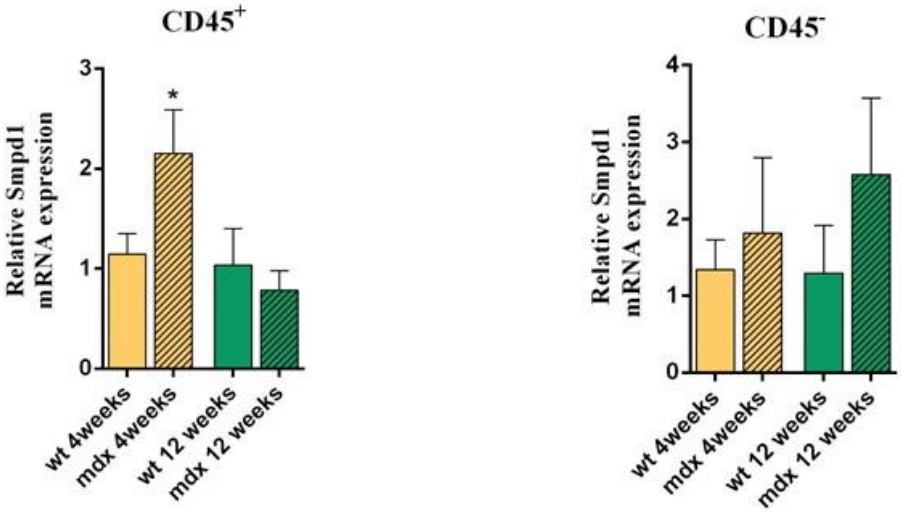
By magnetic separation we isolated muscular and immune cells from muscles of both *mdx* and WT mice. Thus, immune cells, identified as $CD45^+$ positive cells, were analyzed by RTqPCR finding that in *mdx* mice they showed a higher positivity for F4/80, CD86 and CD206 macrophage markers, compared to those found in WT muscles (Fig. 10A). Moreover, as expected, we observed that while the M1 marker CD86 was highly expressed in *mdx* at 4 weeks of age and then returns to level similar to that of WT at 12 weeks, in line with necrotic phase of DMD, the M2 marker CD206 was always increased in *mdx*, with a much higher expression at 12 weeks of age, consistently with the regenerative phase of DMD (Fig. 10A).

Then, A-SMase expression/activity were evaluated in immune and muscular cells in dystrophic context. So, immune system cell ($CD45^+$) and muscle derived cell ($CD45^-$) populations were analyzed by RTqPCR. A-SMase expression and activity were significantly higher in *mdx* immune cells at 1 month of age compared to WT (Fig. 10B-C); this event paralleled with the increased M1 macrophages (Fig. 10A) that we demonstrated being dependent on A-SMase expression (Fig. 7B). Moreover, A-SMase activity was found to be significantly increased in muscle cells of *mdx* mice in comparison to WT at 4 weeks of age (Fig. 10C).

A


F4/80
Relative mRNA expression
10
8
6
4
2
0
**
*
wt 4weeks
mdx 4weeks
wt 12weeks
mdx 12weeks
CD86
Relative mRNA expression
15
10
5
0
**
wt 4weeks
mdx 4weeks
wt 12weeks
mdx 12weeks
CD206
Relative mRNA expression
80
60
40
20
0
*
*
wt 4weeks
mdx 4weeks
wt 12weeks
mdx 12weeks

B


CD45+
Relative Smpd1 mRNA expression
3
2
1
0
*
wt 4weeks
mdx 4weeks
wt 12 weeks
mdx 12 weeks
CD45-
Relative Smpd1 mRNA expression
4
3
2
1
0
wt 4weeks
mdx 4weeks
wt 12 weeks
mdx 12 weeks

C

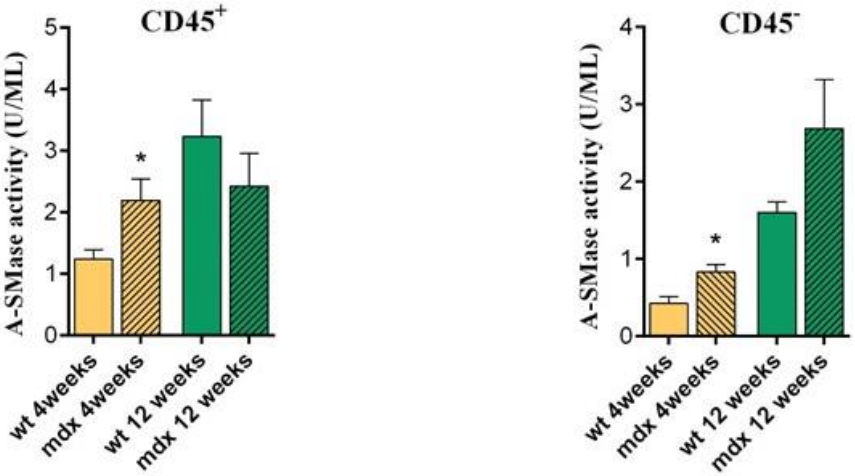

CD45+
A-SMase activity (U/ML)
5
4
3
2
1
0
*
wt 4weeks
mdx 4weeks
wt 12 weeks
mdx 12 weeks
CD45-
A-SMase activity (U/ML)
4
3
2
1
0
*
wt 4weeks
mdx 4weeks
wt 12 weeks
mdx 12 weeks

Figure 10: A-SMase activity is over expressed in *mdx* immune and macrophage derived cell populations

A) RTqPCR analysis of immune system cells ($CD45^+$) for macrophage markers F4/80, CD86 and CD206 in *mdx* and WT mice at 4 and 12 weeks of age. Values are expressed as mean ± SEM (n= 5 per each group). **B)** RTqPCR analysis of immune system ($CD45^+$) and muscular cells ($CD45^-$) for A-SMase expression (*Smpd1* gene) in *mdx* and WT mice at 4 and 12 weeks of age. Values are expressed as mean ± SEM (n=5 per each group). **C)** A-SMase activity (U/ML) measured in immune system ($CD45^+$) and muscular cells ($CD45^-$) by colorimetric assay and GraphPad software for quantification in *mdx* and WT mice at 4 and 12 weeks of age (n=3 per each group). Asterisks indicate statistically significant difference between WT and *mdx* mice, *$p<0.05$; **$p<0.001$.

Naproxcinod significantly decreases A-SMase expression and activity in Tibialis Anterior of *mdx* mice

Since our results showed that the increase of A-SMase in expression and activity paralleled with the increase of muscle inflammatory state (Fig.8D-9C) we evaluated the expression and the activity of the enzyme in TA of *mdx* mice after long-term treatment (6 months) with the anti-inflammatory drug Naproxcinod, starting at 1 month of age.

Naproxcinod has both anti-inflammatory and analgesic effects (Schintzer et al., 2005). The effects of Naproxcinod have been investigated on the *mdx* skeletal muscle. Uaesoontrachoon et al., demonstrated that Naproxcinod-treated *mdx* mice showed a significant reduction of inflammation in both fore- and hind limb muscles.

After 6 months, we found that TA of Naproxcinod-treated *mdx* mice showed the mRNA expression levels of A-SMase significantly decreased compared to vehicle-treated *mdx* animals (Fig. 11A). More importantly, we also found that in TA, Naproxcinod significantly reduced A-SMase activity compared to vehicle-treated *mdx* mice (Fig. 11B).

This finding corroborates our previous results in which we demonstrated a strong connection between A-SMase increase in expression/activity and muscle inflammatory state observed in a chronic damage such as that DMD.

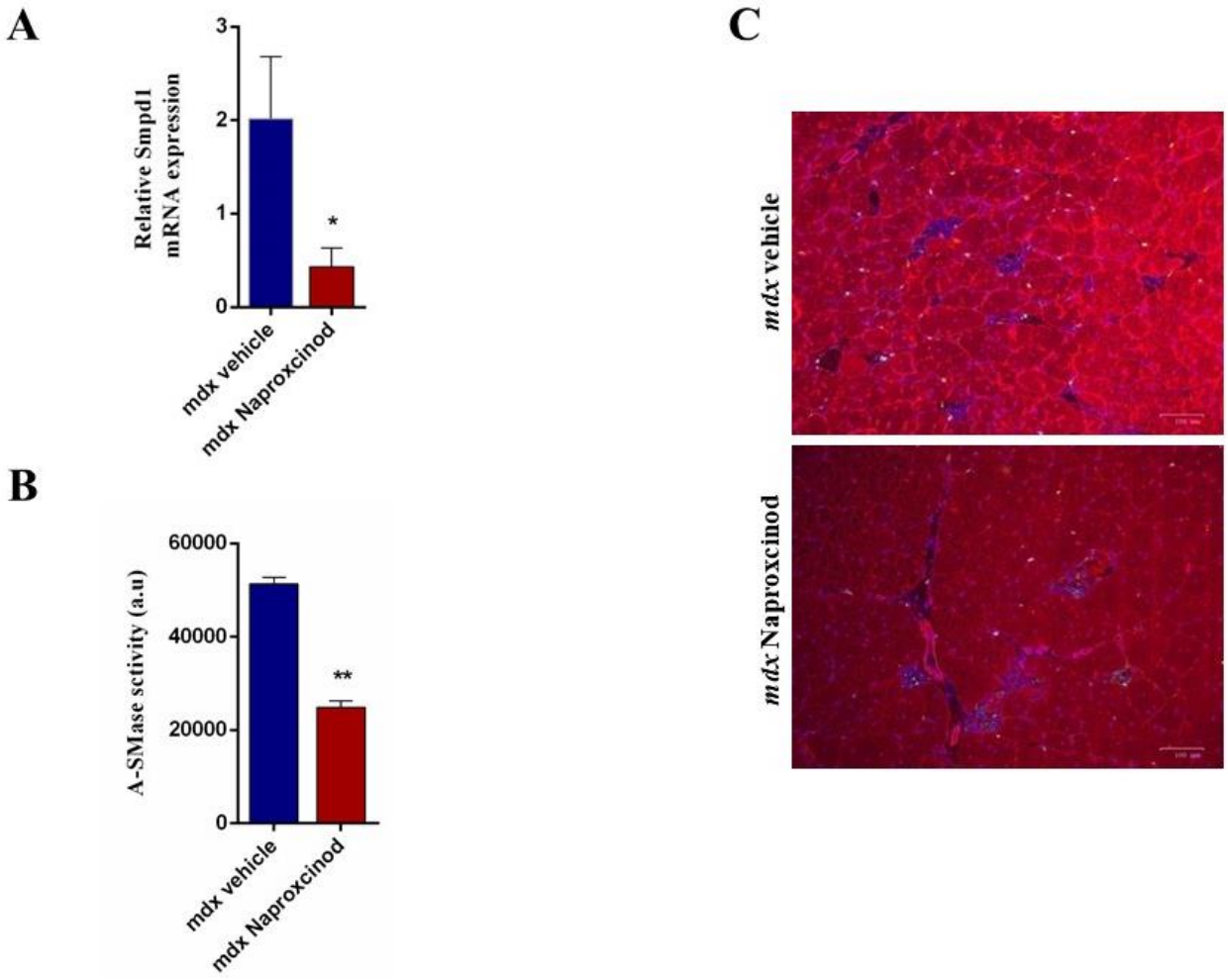


Figure 11: **Naproxcinod significantly decreases A-SMase expression and activity in Tibialis Anterior of *mdx* mice.**

A) RTqPCR analysis of A-SMase expression (*Smpd1* gene) in *mdx*-vehicle and and *mdx*-treated with Naproxcinod. Values are expressed as mean ± SEM (n=5 per each group). **B)** A-SMase activity (a.u) measured in *mdx* vehicle and *mdx* treated with Naproxcinod by colorimetric assay and GraphPad software for quantification (n=5 per each group). Asterisks indicate statistically significant difference between *mdx*-vehicle and *mdx*-Naproxcinod-treated mice (*$p<0.05$, **$p<0.01$). **C)** Immunofluorescence staining of A-SMase (red) expression in muscle fibers of TA from *mdx*-vehicle and and *mdx*-treated with Naproxcinod; nuclei were stained with DAPI (blu); in green $CD45^+$ positive cells. Images are representative of immunofluorescence performed in muscles from n=5 mice per each group. *Scale bars,* 100 μm**.**

DISCUSSION

In the present study, we investigated, for the first time, the functional role of the sphingolipid metabolizing-enzyme A-SMase in the pathophysiology of acute and chronic muscle damage both characterized by the activation of the immune system and by a strong inflammatory status. A-SMase is a critical mediator of cell signaling since it is able to generate ceramide from the membrane lipid sphingomyelin modulating membrane fluidity, which is determinant in triggering many cellular processes such as inflammatory pathways. Several studies reported the strong relation between high levels of A-SMase expression and inflammatory-associated disorders such as Alzheimer's, Parkinson's diseases and atherosclerosis (Haughey, 2010; Opreanu et al., 2011; Devlin et al., 2008).

A-SMase in acute muscle damage

Our results reveal an up regulation of A-SMase expression upon cardiotoxin (CTX)-induced acute damage in Tibialis Anterior (TA) of WT mice that implies its involvement in muscle inflammation/regeneration. Taking advantage of A-SMase $^{-/-}$ mice, we demonstrated that A-SMase is important in regulating skeletal muscle regeneration following acute muscle damage. As regard the skeletal muscle morphology of WT and A-SMase$^{-/-}$ mice before injury, no difference were found between the two murine models. This finding reveals that the loss of A-SMase does not affect the normal skeletal muscle development.

Skeletal muscle has a great capacity to regenerate new muscle fibers after severe damage (Tedesco et al., 2010). This regenerative capacity relies on muscle progenitor cells, namely, SCs. As SCs are able to proliferate and differentiate in response to injury, we investigated whether the absence of A-SMase could affect these two capacities of muscle progenitor cells. The assessment of SC populations *in vivo* and *in vitro* isolated from WT and A-SMase$^{-/-}$ mice before injury did not reveal differences both in terms of percentage inside the muscle and in terms of proliferation and differentiation ability. We hypothesized that after muscle injury the muscle niche of A-SMase$^{-/-}$ mice might be different from that of WT and thus could influence the muscle progenitor cells response to muscle damage. The analysis of SCs in TA of A-SMase$^{-/-}$ and WT mice post CTX injury, showed a significant increase in SCs number in A-SMase$^{-/-}$ mice compared to WT at 1 and 3 days after damage. This increase in SCs paralleled with an enhanced muscle repair upon acute muscle injury. After muscle damage, myofibers undergo degeneration and regeneration (Gao et al., 2012) events. At 5 and 7 days post-injury, more regenerating myofibers were found in A-SMase$^{-/-}$

mice than that in WT animals and the early and the late muscle transcription factor, MyoD and Myogenin, were much higher in A-SMase$^{-/-}$ mice compared to WT suggesting that the regeneration process was accelerated in the A-SMase$^{-/-}$animals.

After muscle damage, interactions between resident SC and infiltrating inflammatory cells are fundamental to restore tissue homeostasis (Mann et al., 2011). The inflammatory response is a coordinated process finely regulated and able to promote an efficient regenerative process. Functional inflammation necessitates well-regulated infiltration of inflammatory cells and cytokines production. For instance, MCP1 is responsible for the mobilization of macrophages from the bone marrow to the blood stream and from the blood to the damaged muscle (Lu et al., 2011). Moreover, Insulin-like growth factor 1 (IGF-1), a well-known growth factor, is not only important in the cell recruitment to the injured muscle (Mourkioti and Rosenthal, 2005) but it is also considered a potent enhancer of tissue regeneration since it is able to modulate specific inflammatory mediators accelerating skeletal muscle regeneration (Pelosi et al., 2007).

The analysis of the immune infiltrate at 1-day post-injury showed that the number of infiltrating macrophages was significantly higher in A-SMase$^{-/-}$ mice compared to WT suggesting a possible role for the enzyme in the recruitment of inflammatory cells after injury. At the same time, several cytokines responsible for the recruitment of inflammatory cells, such as MCP-1, CCL7 and CCR2 found to be enhanced at the injured site in A-SMase$^{-/-}$ muscle compared to WT. This evidence suggests that the differences in muscle regeneration might be due to a definite niche at the damaged site, strongly influenced by the presence or absence of A-SMase, which could be the key factor determining the speed of regeneration.

Inflammatory reaction plays a key role in SCs activation, proliferation and differentiation in injured muscle through the release of cytokines from inflammatory cells (Charge and Rudnicki, 2004; Hawke and Garry, 2001; Philippou et al., 2007). Many cytokine genes are up regulated at the inflammatory stage (1-3 days) and at the stage of differentiation into myotubes (5 days) (Hirata et al., 2003). The first steps of the inflammatory response are associated with the "classically activated" (M1) macrophages, which produce pro-inflammatory cytokines. In contrast, the resolution of inflammation is associated with the "alternatively activated" anti-inflammatory (M2) macrophages (Xuan et al., 2014). M1 macrophages are activated by T-helper (Th)1 cytokines interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNF-α), Lipopolysaccharide (LPS), Interleukin-6 (IL-6) and IL-1; M2 cells are induced by exposure to cytokines such as IL-4, IL-13, IL-10 (Mantovani et al., 2004). The analysis of the expression levels of pro- and anti-inflammatory cytokines in WT and A-SMase$^{-/-}$ mice after injury showed the decrease of IL-1β in A-SMase$^{-/-}$ mice compared to WT suggesting an enhanced clearance of M1-type pro-inflammatory macrophages at

the injured site. Contrariwise, the anti-inflammatory cytokines, IL-10 and TGF-β, showed significantly higher expression levels in A-SMase$^{-/-}$ mice compared to WT suggesting an enhanced activation of M2-type anti-inflammatory macrophages. Macrophages control the resolution of inflammation through the dynamic transition between M1 and M2 phenotype (Mounier et al., 2013). Our results suggest that the switch from pro- to anti-inflammatory macrophages is enhanced in A-SMase$^{-/-}$ mice after injury. This evidence reveals a novel role for A-SMase in regulating the delicate balance between M1 and M2 macrophage phenotypes during skeletal muscle regeneration. Indeed, upon muscle injury, we found that the M1 macrophage portion was significantly lower in the absence of A-SMase indicating a short M1 phenotype retention phase and corresponding enhanced M2 activation in A-SMase$^{-/-}$mice. This evidence was confirmed by A-SMase$^{-/-}$ macrophage polarization *in vitro,* further demonstrating that in the absence of A-SMase M1 subsets have significantly lower expression levels of transcripts that normally are elevated in M1 macrophages in comparison to M1 population of WT mice. Our *in vitro* results indicate that A-SMase can regulate the polarization of macrophages towards an inflammatory M1 phenotype since its absence leads to impairment in the expression of M1 macrophage markers. IGF-1, a potent enhancer of tissue regeneration, critical for muscle growth and able to promote the proliferation and differentiation of SCs (Tonkin et al., 2015), showed a much higher expression in A-SMase$^{-/-}$ mice compared to WT, at each time point considered after CTX injection. This is consistent with our previous finding that regeneration process was accelerated in A-SMase$^{-/-}$ mice. Overall, our findings provide A-SMase as a possible target for modulation of muscle regeneration.

A-SMase in chronic muscle damage: data from a model of Duchenne Muscular Dystrophy

In the second part of this project we investigated the functional role of A-SMase in the pathogenesis of DMD, a disease characterized by a strong activation of component of the innate immune system (Rosenberg et al., 2015), in order to understand its involvement in the progression of the disease. To this end, we took advantage of the *mdx* mice, a mouse model for DMD. A significant increase of A-SMase in expression and activity in TA of *mdx* mice compared to WT, at both 4 and 12 weeks of age (necrosis and muscle regenerative peak, respectively) suggesting the involvement of the enzyme in the pathogenesis of Duchenne dystrophy.

Consistent with previous findings, in our study, we found that the increase of A-SMase, paralleled with the increase of muscle inflammatory state that was confirmed by the higher levels of inflammatory cells (CD45^{+}) and inflammatory cytokines such as IL-6, IL-1β and TNF-α found in muscles of *mdx* mice compared to WT.

In DMD pathology, respiratory activity is significantly impaired. It is well known that the diaphragm of *mdx* mouse undergoes a progressive degeneration and impairment in structure and function that reproduce the degenerative changes of DMD (Stedman et al., 1991; Lynch et al., 2000). For these reasons, we decide to explore A-SMase expression in diaphragm of old *mdx* and WT mice at 12 weeks of age.

The increased activity of A-SMase in diaphragm of *mdx* mice compared to WT controls at 12 weeks of age, paralleled with A-SMase localization at muscle fiber periphery. Indeed, the translocation of A-SMase on the periphery of the cells is usually associated to an increase of enzyme activity (Perrotta et al., 2010; Perrotta et al., 2018). Also in *mdx* diaphragm at both 4 and 12 weeks of age, inflammation was observed. These findings agree with the data obtained in *mdx* TA muscles in which we found that the increase of A-SMase is related to the increase of the muscle inflammatory state. Contrariwise, both in TA and in diaphragm of *mdx* mice, we did not find relation between the increase of A-SMase expression/activity and fibrosis suggesting that A-SMase does not influence this event.

During DMD progression, dystrophic muscle undergoes an irregular interaction between muscle cells and immune system. The analysis of *mdx* muscles showed high levels of M1 marker CD86 at 4 weeks of age that returns to levels similar to those of WT at 12 weeks of age. The M2 marker, CD206, was always increased in *mdx* with a much higher expression at 12 weeks of age, consistently with the regenerative phase of DMD. Consistent with the data about the role of A-SMase in M1 polarization, A-SMase expression and activity in immune cells ($CD45^+$) derived from mice (4 weeks old) was increased in *mdx* mice compared to WT. Furthermore, we also found a significant increase of A-SMase activity in muscle cells ($CD45^-$) of *mdx* mice in comparison to WT at 4 weeks of age.

Finally, the involvement of A-SMase in skeletal muscle inflammation in DMD has been corroborated by the use of anti-inflammatory drug Naproxcinod. Long-term treatment with Naproxcinod is able to reduce the inflammatory infiltrates and improve skeletal muscle phenotype in the *mdx* mouse model (Miglietta et al., 2015; Uaesoontrachoon et al., 2014). The analysis of TA of *mdx* Naproxcinod-treated mice showed that A-SMase mRNA expression levels decrease compared to vehicle-treated *mdx* animals. More importantly, Naproxcinod also reduced significantly A-SMase activity.

Conclusions

In conclusion, our results revealed the strong relation between A-SMase expression and skeletal muscle inflammation upon muscle injury. We provide the first evidence that A-SMase affects skeletal muscle regeneration after muscle damage and modulates muscle repair by controlling the delicate balance of pro- and anti-inflammatory macrophage activation. Of notice, we demonstrated that the A-SMase deficiency leads to a defect in the acquisition of the M1 pro-inflammatory macrophages phenotype and to an accelerated skeletal muscle regeneration after acute damage. Moreover, the results of this present study also showed the upregulation of A-SMase in *mdx* muscles, implying its involvement in the pathogenesis of DMD with a particular effect on inflammation. The active role of A-SMase in regulating macrophage homeostasis makes it a potential pharmacological target by raising the possibility that modulation of A-SMase expression levels could bring therapeutic benefits in DMD pathology but also in various muscle-wasting diseases.

BIBLIOGRAPHY

1. Abdel Shakor, A. B., K. Kwiatkowska, et al., (2004) "Cell surface ceramide generation precedes and controls FcgammaRII clustering and phosphorylation in rafts." J Biol Chem 279 (35): 36778-36787.

2. Anderson, J.; Weber, M.; Vargas, C. (2000). "Deflazacort increases laminin expression and myogenic repair, and induces early persistant functional gain in mdx mouse muscular dystrophy." Cell Transplant, 9, 551-564.

3. Araki E., K. Nakamura, K. Nakao, et al. (1997). "Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy." Biochem Biophys Res Commun, 238 (2), pp. 492-497.

4. Archer JD, Vargas CC, Anderson JE, (2006). " Persistent and improved functional gain in mdx dystrophic mice after treatment with L-arginine and deflazacort." FASEB J 20(6):738-740.

5. Arnold L., A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis," Journal of Experi- mental Medicine, vol. 204, no. 5, pp. 1057–1069, (2007).

6. Aulehla, A.; Pourquiè, O. 2010. "Signaling gradients during paraxial mesoderm development." Cold Spring Harb. Perspect. Biol., 2.

7. Barton-Davis, E.R., Cordier, L., Shoturma, D.I., Leland, S.E. & Sweeney, H.L. (1999). "Aminoglycoside antibiotics restore dystrophin function to skeletal muscle of mdx mice." J Clin Invest 104, 375-381.

8. Benedetti, S., Hoshiya, H., and Tedesco, F. S. (2013). Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies. FEBS J. 280, 4263–4280. doi: 10.1111/febs.12178.

9. Bentzinger C.Florian,Yu Xin Wang, and Michael A.Rudnicki : Building muscle: Molecular Regulation of Myogenesis. Cold Spring Harb Perspect Biol (2012); 4: a 008342.

10. Berardi, E., Annibali, D., Cassano, M., Crippa, S., & Sampaolesi, M. (2014)." Molecular and cell based therapies for muscle degenerations: a road under construction." Front Physiol 5, 119.

11. Biggar,W.D., Harris, V.A.,Eliasoph, L.& Alman, B. (2006). "Long-term benefits of Deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade." Neuromuscul Disorder 16, 249-255.

12. Billich A, Bornancin F, De'vay P, Mechtcheriakova D, Urtz N, Baum- ruker T (2003). "Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases." J Biol Chem 278: 47408– 47415.

13. Biressi S, Rando TA (2010). “Heterogeneity in the muscle satellite cell population.” Semin Cell Dev Biol 21:845-854.

14. Bleicher, R.J. and M.C. Cabot (2002). “Glucosylceramide synthase and apoptosis.” Biochim Biophys Acta 1585(2-3): 172-178.

15. Boldrin L., F. Muntoni, and J. E. Morgan, “Are human and mouse satellite cells really the same?” Journal of Histochemistry and Cytochemistry, vol. 58, no. 11, pp. 941–955, 2010.

16. Brack AS, Rando TA. Tissue-specific stem cells: lessons from the skeletal muscle satellite cell. Cell Stem Cell 2012; 10: 504–514.

17. Bradová V, Smíd F, Ulrich-Bott B, Roggendorf W, Paton BC, Harzer K.(1993). "Prosaposin deficiency: further characterization of the sphingolipid activator protein-deficient sibs. Multiple glycolipid elevations (including lactosylceramidosis), partial enzyme deficiencies and ultrastructure of the skin in this generalized sphingolipid storage disease." Hum Genet.; 92(2):143-52.

18. Brady R O, Kanfer J N, Mock M B, Fredrickson D S. “The metabolism of sphingomyelin. II. evidence of an enzymatic deficiency in Niemann-Pick disease.” Proc Natl Acad Sci U S A.1966;55:366–369.

19. Brunelli S. & Rovere-Querini, P. “The immune system and the repair of skeletal muscle.” Pharmacological Research 58, 117–121 (2008).

20. Bulfield G., W.G.Siller, P.A.Wight, K.J.Moore (1984). “X chromosome-linked muscular dystrophy (mdx) in the mouse.” Proc Natl Acad Sci USA, 81(4) (1984), pp.1189-1192.

21. Buono R, Vantaggiato C, Pisa V, Azzoni E, Bassi MT, Brunelli S, Siorati C, Clementi E, (2012).“Nitric Oxide sustains long-term skeletal muscle regeneration by regulating fate of satellite cells via signaling pathways requiring Vangl2 and Cyclic GMP. “Stem Cells 30(2):197-209.

22. Burton, E.A., Tinsley, J.M., Holzfeind, P.,J., Rodrigues, N.R. & Davies, K.E.(1999). “A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy.” Proc Natl Acad Sci U S A 96, 14025-14030.

23. Campanha, F. G., Dias, R. C. E. & De Toledo Benassi, M “Discrimination of coffee species using kahweol and cafestol: Effects of roasting and of defects.” Coffee Sci. 5, 87–96 (2010).

24. Chalfant CE, Spiegel S (2005). Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. J Cell Sci 118:4605– 4612.

25. Charge SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84: 209–238.

26. Chapman V.M., D.R.Miller, D. Armstrong,C.T.Caskey (1989). “Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice.” Proc Natl Acad Sci USA, 86 (4), pp. 1292-1296.

27. Colussi, C., Mozzetta, C., Gurtner, A., Illi, B., Rosati, J. Straino, S., Ragone, G., Pescatori,

M., Zaccagnini, G., Antonini, A., et al. (2008). "HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment." Proc. Natl.Acad. Sci. USA 105, 19183-19187.

28. Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A and Morgan, J. E. (2005). "Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche." Cell 122, 289-301.

29. Consalvi, S., Mozzetta, C.; Bettica, P.; Germani, M.; Fiorentini, F.; del Bene, F.; Rocchetti, M.; Leoni, F.; V. Mascagni, P.; et al. (2013). "Preclinical studies in the mdx mouse model of duchenne muscular dystrophy with the histone deacetylase inhibitor givinostat." Mol. Med 19, 79-87.

30. Cordani,N.; Pisa, V.; Pozzi, L.; Sciorati, C.; Clementi, E. (2014)."Nitric oxide controls fat deposition in dystrophic skeletal muscle by regulating fibro-adipogenic precursor differentiation." Stem Cell 32,874-885.

31. Cossu G, Sampaolesi M. (2004). "New therapies for muscular dystrophy: cautious, optimism." Trends in Molecular Medicine. Vol.10; 516–520.

32. Cox G.A., S.F.Phelps, V.M.Chapman, J.S.Chamberlain (1993). "New mdx mutation disrupts expression of muscle and nonmuscle isoforms of dystrophin." Nat Genet, 4 (1) (1993), pp. 87-93.

33. D'Angelo MG, Grandossini S, Boneschi FM, Sciorati C, Bonato S, Brighina E, Comi GP, Turconi AC, Magri F, Stefanoni G, Brunelli S, Bresolin N, Cattaneo D, Clementi E (2012). "Nitric oxide donor and non steroidal anti-inflammatory drugs as a therapy for muscular dystrophies: evidence from a safety study with pilot efficacy measures in adult dystrophic patients." Pharmacolo Res 65:472-479.

34. Davies KE, SmithTJ, Bundey S, Read AP, Flint T, Bell M, Speer A (1998). "Mild and severere muscular dystrophy associated with deletions in Xp21 of the human X chromosome." J Med Genet 25:9-13.

35. De Backer F., C. Vandebrouck, P. Gailly, J.M. Gillis (2002). "Long-term study of Ca (2+) homeostasis and of survival in collagenase-isolated muscle fibres from normal and mdx mice." J Physiol, 542 (Pt 3), pp.855-865.

36. De Palma C, Clementi E (2012)."Nitric Oxide in Myogenesis and Therapeutic Muscle Repair." Molecular Neurobiology 1-11.

37. Deigner H.P., Gulbins E., Claus R.A. (2007) Sphingolipid Metabolism in Systemic Inflammation. In: Vincent JL. (eds) Intensive Care Medicine. Springer, New York, NY doi.org/10.1007/978-0-387-49518-7_23 Online ISBN978-0-387-49518-7.

38. Devlin, C. M. et al. Acid Sphingomyelinase Promotes Lipoprotein Retention Within Early Atheromata and Accelerates Lesion Progression. Arterioscler. Thromb. Vasc. Biol. 28, 1723–1730 (2008).

39. Douglas, A. G., and Wood, M. J. (2013). Splicing therapy for neuromuscular disease. Mol. Cell. Neurosci. 56, 169–185. doi: 10.1016/j.mcn.2013.04.005

40. Drachman, D.B.; Toyka, K.V.; Myer, E. (1974) "Prednisone in Duchenne muscular dystrophy." Lancet,2, 1409–1412.

41. Emery AEH, Dreifuss FE."Unusual type of benign x-linked muscular dystrophy." J Neurol Neurosurg Psychiatr (1966); 29: 338–42.

42. Emery, AE. (2002)."The muscular dystrophies."Lancet; 359: 687–95.

43. Escolar,D.M.; Hache, L.P.; Clemens, P.R.; Cnaan, A.; McDonald, C.M.; Viswanathan, V.; Kornberg, A.J.; Bertorini, T.E.; Nevo, Y.; Lotze, T.; et al. (2011). "Randomized, blinded trial of weekend vs. daily prednisone in Duchenne muscular dystrophy." Neurology, 77, 444-452.

44. Fan Y, Maley M, Beilharz M, Grounds M. Rapid death of injected myoblasts in myoblast transfer therapy. Muscle Nerve. 1996;19(7):853–860.

45. Fayssoil A, Nardi O, Orlikowski D,Annane D (2010). "Cardiomyopathy in Duchenne muscular dystrophy: pathogenesis and therapeutics." Heart Fail Rev 15:103-107.

46. Ferlinz K, Hurwitz R, Vielhaber G, Suzuki K, Sandhoff K. (1994). "Occurrence of two molecular forms of human acid sphingomyelinase."Biochemical Journal. 301(Pt 3):855-862.

47. Foteini Mourkioti and Nadia Rosenthal (2005). " IGF-1, inflammation and stem cells:interactions during muscle regeneration." Review , Trends in immunology, Vol 26, No.10.

48. Frontera, W. R. & J. Ochala (2015) Skeletal muscle: a brief review of structure and function Calcif Tissue Int, 96, 183-95.

49. Futerman AH, Pagano (1991). "Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver." RE Biochem J. 1 Dec 1; 280 (Pt 2) (): 295-302. :

50. Gao Y, Li Y, Guo X, Wu Z, Zhang W (2012) "Loss of STAT1 in Bone Marrow-Derived Cells Accelerates Skeletal Muscle Regeneration." PLoS ONE 7(5): e37656. doi:10.1371/journal.pone.0037656

51. Garcia-Ruiz, C., Mato, J. M., Vance, D., Kaplowitz, N. & Fernández-Checa, J. C. Acid sphingomyelinase-ceramide system in steatohepatitis: A novel target regulating multiple pathways. J. Hepatol 62, 219–233 (2015).

52. Gayraud-Morel B., F. Chr´etien, and S. Tajbakhsh, "Skeletal muscle as a paradigm for regenerative biology and medicine." Regenerative Medicine, vol. 4, no. 2, pp. 293–319, 2009.

53. Gayraud J., S. Matecki, K. Hnia,et al. (2007). "Ventilation during air breathing and in response to hypercapnia in 5- and 16-month-old mdx and c57 mice." J Muscle Res Cell Motil, 28(1), pp.29-37.

54. Gault CR, LM Obeid, and YA Hannun (2010). "An overview of sphingolipid metabolism: from synthesis to breakdown." dv Exp Med Biol. 688: 1–23.

55. Gauthier-Rouviere, C. & Bonet-Kerrache, A., (2009). "RhoA leads to up regulation and relocalization of utrophin in muscle fibers." Biochem Biophys Res Commun 384, 322-328.

56. Geng T., A. Sutter, M.D. Harland, B.A. Law, J.S. Ross, D. Lewin, A. Palanisamy, S.B. Russo, K.D. Chavin, L.A. Cowart (2015). "SphK1 mediates hepatic inflammation in a mouse model of NASH induced by high saturated fat feeding and initiates proinflammatory signaling in hepatocytes." J Lipid Res, 56, 2359-2371.

57. Gilbert, R., et al., (1999). "Adenovirus-mediated utrophin gene transfer mitigates the dystrophic phenotype of mdx mouse muscles." Hum Gene Ther 10, 1299-1310.

58. Goemans, N.; Buyse, G. (2014). "Current Treatment and Management of Dystrophinopathies." Curr. Treat.Options Neurol. 16, 287, doi: 10. 1007/s11940-014-0287-4.

59. Grösche S., Alessenko A.V., Albi E. "The Many Facets of Sphingolipids in the Specific Phases of Acute Inflammatory Response". Mediators Inflamm. (2018): 2018:5378284.

60. Govoni A, Magri F., Brajkovic S., Zanetta C., Faravelli I., Corti S., Bresolin N., Comi GP., (2013). "Ongoing therapeutic trials and outcome measures for Duchenne muscular dystrophy." Cell Mol Life Sci 70:4585-4602.

61. Goyenvalle A, Vulin A, Fougerousse F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science. 2004; 306(5702):1796–1799.

62. Goyenvalle A, Babbs A, Wright J, et al. Rescue of severely affected dystrophin/utrophin-deficient mice through scAAV-U7snRNA-mediated exon skipping. Hum Mol Genet. 2012; 21(11):2559–2571. PMCID: 3349427.

63. Grassme, H., A. Jekle, et al. (2001). "CD95 signaling via ceramide-rich membrane rafts." J Biol Chem 276(23): 20589-20596.

64. Grassme, H., V. Jendrossek, et al., (2002). " Ceramide -rich membrane rafts mediate CD40 clustering." J Immunol 168(1):298-307.

65. Griggs, R.C.; Moxley, R.T., 3rd;Mendell, J.R.; Fenichel, G.M.; Brooke, M.H.; Pestronk, A.; Miller, J.P.; Cwik, V.A.; Pandya, S.; Robison, J.; et al. (1993). "Duchenne dystrophy: Randomized, controlled trial of prednisone (18 months) and azathioprine (12 months)." Neurology, 43, 520-527.

66. Guglieri M, Straub V, Bushby K, Lochmuller H. (2008). "Limb-girdle muscular dystrophies." Curr Opin Neurol 21: 576–84.

67. Guo C., M. Willem, A.Werner, et al. (2006). "Absence of alpha 7 integrin in dystrophin deficient mice causes a myopathy similar to Duchenne muscular dystrophy." Hum Mol Genet, 15 (6), pp. 989-998.

68. Hamid, H.A., Clemens P.R. (2012). "Pharmacological therapies for muscular dystrophies." Current Opinion in Neurology, 25(5): 604-608.

69. Hannun, Y. A. and C. Luberto (2004). "Lipid metabolism: ceramide transfer protein adds a new dimension." Curr Biol 14(4): R163-165.

70. Hannun, Y. A., and Obeid, L. M. (2008). “Principles of bioactive lipid signalling: lessons from sphingolipids.” Nat. Rev. Mol. Cell Biol.9, 139–150. doi: 10.1038/nrm2329.

71. Harding, P. L.; Fall, A. M.; Honeyman, K; Fletcher, S; Wilton, S. D. (2007). "The influence of antisense oligonucleotide length on dystrophin exon skipping". Molecular Therapy.15 (1):157–66.

72. Haughey, N. J. Sphingolipids in Neurodegeneration. NeuroMolecular Med. 12, 301–305 (2010).

73. Hawke TJ, Garry DJ (2001) “Myogenic satellite cells: physiology to molecular biology.” J Appl Physiol 91: 534–551.

74. Hirata A., Masuda S., Tamura T., Kai K., Ojima K., Fukase A., Motoyoshi K., Kamakura K., Miyagoe-Suzuki Y., Takeda S. “Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: A role for osteopontin.” Am. J. Pathol.2003; 163:203–215. doi: 10.1016/S0002-9440(10)63644-9.

75. Hirawat, S., et al., (2007). “Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers.” J Clin Pharmacol 47, 430-444.

76. Hla, T. “Sphingosine1-phosphatereceptors.” Prostaglandins Other Lipid Mediat.2001, 64, 135-142.

77. Hoffman EP, Brown RH Jr, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell (1987); 51: 919–928.

78. Holland, W. L. et al. (2011). “Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid–induced ceramide biosynthesis in mice.” J. Clin. Invest. 121, 1858–1870

79. Hughes, J.E.; Srinivasan, S.; Lynch, K.R.; Proia, R.L.; Ferdek, P.; Hedrick, C.C. “Sphingosine-1-phosphate induces an anti-inflammatory phenotype in macrophages.” Circ. Res. 2008, 102, 950–958.

80. Hurwitz R, Ferlinz K, Vielhabe G, Moczall H, Sandhoff K. (1994). “Processing of human acid sphingomyelinase in normal and in I-cell fibroblasts.” J Biol Chem 269(7): 5440-5.

81. Huwiler, A., T. Kolter, et al. (2000). “Phisiology and pathophysiology of sphingolipid metabolism and signaling.” Biochim Biophys Acta 1485(2-3): 63-99.

82. Illi, B., Colussi, C., Grasselli, A., Farsetti, A., Capogrossi, M.C., and Gaetano, C. (2009). “NO sparks off chromatin:tales of a multifaceted epigenetic regulator.” Pharmacol. Ther. 123,344-352.

83. Jefferies JL, Eidem BW, Belmont JW, Craigen WJ, Ware SM, Fernbach SD, Neish SR, Smith EO, Towbin JA (2005). “Genetic predictors and remodeling of dilated cardiomyopathy in muscular dystrophy.” Circulation 112:2799-2804.

84. Jenkins RW, Canals D, Hannun YA (2009). “Roles and regulation of secretory and

lysosomal acid sphingomyelinase." Cell Signal.; 21(6): 836-46.

85. Jolly PS, Bektas M, Olivera A, Gonzalez-Espinosa C, Proia RL, Rivera J et al. (2004). "Transactivation of sphingosine-1 phosphate receptors by Fc_RI triggering is required for normal mast cell degranulation and chemotaxis." J Exp Med 199: 959–970.

86. Jozefowski, S.; Czerkies, M.; Lukasik, A.; Bielawska, A.; Bielawski, J.; Kwiatkowska, K.; Sobota, A. "Ceramide and ceramide 1-phosphate are negative regulators of TNF-alpha production induced by lipopolysaccharide." J. Immunol. (2010), 185, 6960–6973.

87. Juhas M, Bursac N. (2013). "Engineering skeletal muscle repair." Curr Opin Biotechnol; 24:880–886.

88. Katz, B. (1961). "The terminations of the afferent nerve fibre in the muscle spindle of the frog." Philos. Trans. R. Soc. Lond. B Biol. Sci. 243, 221-240.

89. Kazuyuki Kitatani, Jolanta Idkowiak-Baldys, and Yusuf A. Hannun (2008). "The sphingolipid salvage pathway in ceramide metabolism and signalling." Cell Signal; 20(6): 1010–1018.

90. Keul, P.; Tolle, M.; Lucke,S.; von WnuckLipinski,K.; Heusch, G.; Schuchardt, M.; vanderGiet, M.; Levkau, B. "The sphingosine-1-phosphate analogue FTY720 reduces atherosclerosis in apolipoprotein E-deficient mice." Arterioscler. Thromb. Vasc. Biol. 2007, 27, 607–613.

91. Khairallah M, Khairallah R, Young ME, Dyck JR, Petrof BJ, Des Rosiers C., (2007) "Metabolic and signaling alterations in dystrophin-deficient hearts precede overt cardiomyopathy." J Mol Cell Cardiol ; 43: 119–29.

92. Kim MY, Linardic C, Obeid L, Hannun Y (1991). "Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation." J Biol Chem 266: 484–489.

93. Kole R., Krieg A.M., (2015). "Exon skipping therapy for Duchenne muscular dystrophy." Advanced Drug Delivery Reviews , Vol.87 ,104-107

94. Kolter T, Sandhoff K Annu Rev Cell Dev Biol. 2005; 21():81-103. Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids.

95. Kramerova I, Kudryashova E, Ermolova N, et al. Impaired calcium calmodulin kinase signaling and muscle adaptation response in the absence of calpain 3. Hum Mol Genet 2012; 21: 3193–204.

96. Lai, Y., Thomas, G.D.,Yue, Y., Yang, H.T., Li D., Long, C., Judge, L., Bostick, B., Chamberlain, J.S., Terjung, R.L. et al (2009). "Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. J Clin Invest, 119,624-635.

97. Lamour, N.F.; Wijesinghe, D.S.; Mietla, J.A.; Ward, K.E.; Stahelin, R.V.; Chalfant, C.E. "Ceramide kinase regulates the production of tumor necrosis factor alpha (TNFα) via inhibition of TNFα-converting enzyme." J. Biol. Chem. 2011, 286, 42808–42817.

98. Lawrence T. and G. Natoli, "Transcriptional regulation of macrophage polarization: enabling diversity with identity," Nature Reviews Immunology, vol. 11, no. 11, pp. 750–761, 2011.

99. Le Grand F, Rudnicki MA (2007). Skeletal muscle satellite cells and adult myogenesis." Curr Opin Cell Biol 19:628-633.

100. Le Hir M, Goyenvalle A, Peccate C, et al. "AAV genome loss from dystrophic mouse muscles during AAV-U7 snRNA-mediated exon-skipping therapy." Mol Ther. 2013;21(8):1551–1558. PMCID: 3734654.

101. Li, D.,Yue Y., Lai Y., Hakin, C.H. and Duan, D. (2011). "Nitrosative stress elicited by nNOSmicro delocalization inhibits muscle force in dystrophin-null mice. J Pathol, 223,88-98.

102. Li J, Sun W, Wang B, Xiao X, Liu XQ. Protein trans-splicing as a means for viral vector-mediated in vivo gene therapy. Hum Gene Ther. 2008;19(9):958–964. PMCID: 2940629.

103. Lluri G., G. D. Langlois, B. McClellan, P. D. Soloway, and D. M. Jaworski, "Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates neuromuscular junction development via a beta1integrin-mediated mechanism," Journal of Neurobiology, vol. 66, no. 12, pp. 1365–1377, 2006.

104. Luberto C, Hannun YA (1998). "Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation. Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase C? ." J Biol Chem.; 273(23):14550-9.

105. Lu, H, Huang D., Saederup N., Charo IF., Ransohoff (2011). "Macrophages recruited via CCR2 produce insulin-like growth factor-1 to repair acute skeletal muscle injury." FASEB J., 25(1): 358-69

106. Lu Y., et al., (2008). "Targeting artificial transcription factors to the utrophin A promoter: effects on dystrophic pathology and muscle function." J Biol Chem 283, 34720-34727.

107. Lynch, GS, Rafael JA Chamberlain JS, Faulkner JA (2000). "Contraction-induced injury to single permeabilezed muscle fibres from mdx, transgenic mdx, and control mice." Am J Physiol Cell Physiol 279: C1290-1294.

108. Lynch, G.S., Hinkle R.T., Faulkner J.A. (2001). "Force and power output of diaphragm muscle strips from mdx and control mice after clenbuterol treatment." Neuromuscular Disorders, Vol.11, Issue 2, 192-196.

109. Magri, F., Govoni A., D'Angelo MG., Del Bo R., Ghezzi S., Sandra G., Turconi AC., Sciacco M., Ciscato P.,Bordoni A., Tedeschi S., Fortunato F.,Lucchini V., Bonato S., Lamperti C, Coviello D., Torrente Y., Corti S., Moggio M., Bresolin N., Comi GP (2011).

"Genotype and phenotype characterization in a large dystrophinopathic cohort with extended follow-up." J Neurol 258: 1610-1623.

110. Malik, V., et al. (2010). " Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy." Ann Neurol 67,771-780.

111. Mann et al. (2011)Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Munoz-Canoves P. Aberrant repair and fibrosis development in skeletal muscle.Skeletal Muscle.2011;1(1) doi: 10.1186/2044-5040-1-21.Article 21

112. Mantovani. A, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M.Locati, (2004). "The chemokine systemin diverse forms of macrophage activation and polarization." Trends in Immunology, vol. 25, no. 12, pp. 677–686.

113. Maqbool T, Jagla K. 2007. Genetic control of muscle development: Learning from Drosophila. J Muscle Res Cell Motil 28: 397–407.

114. Marathe, S., S. L.Schissel, et al. (1998). "Human vascular endothelial cells are a rich and regulatable source og secretory sphingomyelinase. Implications for early atherogenesis and ceramide-mediated cell signaling."J Biol Chem 273(7): 4081-4088.

115. Marchesini N, Hannun YA. Biochem. "Acid and neutral sphingomyelinases:roles and mechanisms of regulation". Cell Biol 2004;82:27–44.

116. Marggraf WD, Anderer FA, Kanfer The formation of sphingomyelin from phosphatidylcholine in plasma membrane preparations from mouse fibroblasts. JN Biochim Biophys Acta. 1981 Apr 23; 664(1):61-73.

117. Marques MJ, Luz MA, Minatel E, Neto HS, (2005). "Muscle regeneration in dystrophic mdx mice is enhanced by isosorbide dinitrate." Neurosci Lett 382(3):342-345.

118. Martin, S., D. C. Phillips, et al. (2005). "Cyclooxygenase-2 inhibition sensitizes human colon carcinoma cells to TRAIL-induced apoptosis through clustering of DR5 and concentration death-inducing signaling complex components into ceramide-enriched caveolae." Cancer Res 65(24): 11447-11458.

119. Mathias S, Dressler KA, Kolesnick RN (1991). "Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor alpha." Proc Natl Acad Sci USA 88: 10009–10013.

120. Mauer, A.S.; Hirsova, P.; Maiers, J.L.; Shah,V.H.; Malhi,H. "Inhibitionofsphingosine1-phosphate signaling ameliorates murine nonalcoholic steatohepatitis." Am. J. Physiol. Gastrointest. Liver Physiol. 2017, 312, G300–G313.

121. Mauro A., (1961). Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. 9, 493-495.

122. McGeachie J.K, M.D.Grounds, T.A Partridge, J.E.Morgan (1993). "Age-related changes in replication of myogenic cells in mdx mice: quantitative autoradiographic studies." J Neurol Sci, 119 (2), pp. 169-179.

123. Mendell, J.R., et al (1989). "Randomized, double-blind six month trial of prednisone in Duchenne's muscular dystrophy." N England J Med 320,1592-1597.

124. Mendell, J.R.; Rodino-Klapac, L.; Sahenk, Z.,;Malik, V.; kaspar, B.K.; Walker, C.M.; Clark, K.R.; (2012). "Gene therapy for muscular dystrophy: Lesson learned and path forward." Neurosci.Lett. 527, 90-99.

125. Mercuri E, Pichiecchio A, Allsop J, Messina S, Pane M, Muntoni F., (2007). "Muscle MRI in inherited neuromuscular disorders: past, present, and future." J Magn Reson Imaging; 25: 433–40.

126. Mercuri E, Muntoni F. (2012)."The ever expanding spectrum of congenital muscular dystrophies." Ann Neurol 72: 9–17.

127. Mercuri E,Muntoni F., (2013). "Muscular dystrophies." Lancet, 381: 845–60.

128. Messias, C.V.; Santana-Van-Vliet, E.; Lemos, J.P.; Moreira, O.C.; Cotta-de-Almeida, V.; Savino, W.; Mendes-da-Cruz, D.A."Sphingosine-1-phosphate induces dose-dependent chemotaxis or fugetaxis of t-all blasts through S1P1 activation." PLoS ONE 2016, 11, e0148137.

129. Miglietta et al., (2015). " Naproxcinod shows significant adavantages over Naproxen in the mdx model of Duchenne Muscular Dystrophy." Orphanet Journal of Rare Diseases 10:101.

130. Minasi, M.G., et al., (2002). "The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues." Development 129,2773-2783.

131. Mitchell, K.J.; Pannerec, A.; Cadot, B.; Parlakian, A.; Besson, V.; Gomes, E.R.; Marazzi, G.; Sassoon, D.A. Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat. Cell Biol. 2010, 12, 257–266.

132. Miura, P. & Jasmin, B.J. (2006). "Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we?." Trends Mol Med 12,122-129.

133. Mosqueira M, Zeiger U, Forderer M, Brinkmeier H, Fink RH (2013). "Cardiac and respiratory dysfunction in Duchenne muscular dystrophy and the role of second messangers." Med Res Rev 33:1174-1213.

134. Mounier R., Théret M., Arnold L., Cuvellier S., Bultot L., Göransson O., Sanz N., Ferry A., Sakamoto K., Foretz M., Viollet B., and Chazaud B., (2013). " AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration." Cell Metab 18:251-264.

135. Mourkioti F., and Rosenthal N., (2005). " IGF-1, inflammation and stem cells: interactions during muscle regeneration." Cell Press, Trends in immunology; Vol.26.10:535-542.

136. Muntoni F, Brockington M, Blake DJ, Torelli S, Brown SC., 2002. "Defective glycosylation in muscular dystrophy." Lancet; 360: 1419–21.

137. Muntoni F, Torelli S, Wells DJ, Brown SC., 2010. "Muscular dystrophies due to glycosylation defects: diagnosis and therapeutic strategies." Curr Opin Neurol; 24: 437–42.

138. Mutsaers S. E., J. E. Bishop, G. McGrouther, and G. J. Laurent, "Mechanisms of tissue repair: from wound healing to fibrosis." The International Journal of Biochemistry & Cell Biology, vol. 29, no. 1, pp. 5–17, 1997.

139. Muntoni F, Voit T. "The congenital muscular dystrophies in 2004: a century of exciting progress." Neuromuscul Disord 2004; 14: 635–49.

140. Nie M, J Liu, Q Yang, HY Seok2, X Hu2, Z-L Deng and D-Z Wang (2016). "MicroRNA-155 facilitates skeletal muscle regeneration by balancing pro- and anti-inflammatory macrophages Cell Death and Disease." 7, e2261; doi: 10.1038/cddis.2016.165.

141. Newrzella, D. and W. Stoffel (1992)."Molecular cloning of the acid sphingomyelinase of the mouse and the organization and complete nucleotide sequence of the gene." Biol Chem Hoppe Seyler 373(12):1233-1238.

142. Newrzella, D. and W. Stoffel (1996). "Functional analysis of the glycosylation of murine acid sphingomyelinase." J Biol Chem 271(50):32089-32095.

143. Nixon G.F. (2009). "Themed section : mediators and receptors in the resolution of inflammation. Review. Sphingolipids in inflammation: pathological implications and potential therapeutic targets." British J of Pharmacology 158, 982-993.

144. Nofer,J.R.;Bot,M.;Brodde,M.;Taylor,P.J.;Salm,P.;Brinkmann,V.;vanBerkel,T.;Assmann,G. Biessen,E.A. "Fty720, a synthetic sphingosine 1 phosphate analogue, inhibits development of atherosclerosis in low-density lipoprotein receptor-deficient mice." Circulation 2007, 115, 501–508.

145. Opreanu, M. et al. (2011). "The Unconventional Role of Acid Sphingomyelinase in Regulation of Retinal Microangiopathy in Diabetic Human and Animal Models." Diabetes 60, 2370–2378.

146. Okuro, R.T. et al., (2018). "The role of sphingolipid metabolism distruption on lipopolysaccharide-induced lung injury in mice." Pulmonary Pharmacology & Therapeutics 50. 100-110.

147. Paton BC, Schmid B, Kustermann-Kuhn B, Poulos A, Harzer K (1992)."Additional biochemical findings in a patient and fetal sibling with a genetic defect in the sphingolipid activator protein (SAP) precursor, prosaposin. Evidence for a deficiency in SAP-1 and for a normal lysosomal neuraminidase." Biochem J.; 285 (Pt 2) (): 481-8.

148. Partridge, T.A. (2011)."Impending therapies for Duchenne muscular dystrophy." Curr Opin Neurol 24, 415-422.

149. Pelosi L., C. Giacinti, C. Nardis et al., (2007). "Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines," The FASEB Journal, vol. 21, no. 7, pp. 1393–1402,

150. Perrotta C., D. Cervia, I. Di Renzo, C. Moscheni, MT Bassi, L. Campana, C. Martelli, E. Catalani, M. Giovarelli, S. Zecchini, M. Coazzoli, A. Capobianco, L. Ottobrini, G. Lucignani, P. Rosa, P. Rovere-Querini, C. De Palma and Emilio Clementi (2018). "Nitric Oxide Generated by Tumor-Associated Macrophages Is Responsible for Cancer Resistance to Cisplatin and Correlated With Syntaxin 4 and Acid Sphingomyelinase Inhibition." Frontiers in Immunology, 9:1186.

151. Perrotta C, Buonanno F, Zecchini S, et al. "Climacostol reduces tumour progression in a mouse model of melanoma via the p53-dependent intrinsic apoptotic programme".Jun 07 (2016); 6: 27281. doi: 10.1038/srep2728.

152. Perrotta C., Buldorini M., Assi E., Cazzato D., De Palma C., Clementi E., Cervia D.,(2014). "The Thyroid Hormone Triiodothyronine controls macrophage maturation and functions: protective role during inflammation." The American Journal of Pathology, Vol. 184, Issue 1, Pages 230-247.

153. Perrotta C., Bizzozero L., Cazzato D., Marlocchi S., Assi E., Simbari F., Zhang Y., Gulbins E., MT Bassi, Rosa P., and Emilio Clementi (2010). "Syntaxin 4 is required for acid sphingomyelinase activity and apoptotic function." The Journal of Biological Chemistry 285, 40240-40251.

154. Pettus,B.J.;Bielawska,A.;Spiegel,S.;Roddy,P.;Hannun,Y.A.;Chalfant,C.E."Ceramidekinase mediates cytokine- and calcium ionophore-induced arachidonic acid release." J. Biol. Chem. 2003, 278, 38206–38213.

155. Pettus, B.J.; Bielawska, A.; Subramanian, P.; Wijesinghe, D.S.; Maceyka, M.; Leslie, C.C.; Evans, J.H.; Freiberg, J.; Roddy, P.; Hannun, Y.A.; et al. "Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2." J. Biol. Chem. 2004, 279, 11320–11326.

156. Philippou A, Halapas A, Maridaki M, Koutsilieris M (2007) Type I insulin-like growth factor receptor signaling in skeletal muscle regeneration and hypertrophy. J Musculoskelet Neuronal Interact 7: 208–218.

157. Pillon NJ,Bilan PJ, Fink LN, Klip A. (2013)."Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications." Am J Physiol Endocrinol Metab 304:E453-E465.

158. Pimorady-Esfahani A., M. D. Grounds, and P. G. McMenamin, "Macrophages and dendritic cells in normal and regenerating murine skeletal muscle,"Muscle Nerve, vol. 20, pp. 158–166, 1997.

159. Pyne, S. G., S. Milstien, et al. (2002)."Sphingosine-1-phosphate: dual messenger functions." FEBS Lett 531(1): 54-57.

160. Quinlan J.G., H.S. Hahn, B.L. Wong, J.N. Lorenz, A.S. Wenisch, L.S. Levin (2004). "Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings." Neuromuscul Disord, 14(8–9) (2004), pp. 491-496.

161. Quinlivan R, Shaw N, Bushby K. 170th ENMC International Workshop: bone protection for corticosteroid treated Duchenne muscular dystrophy. 27–29 November 2009, Naarden,The Netherlands. Neuromuscul Disord 2010; 20: 761–69.

162. Radley, H. G., Davies, M. J. & Grounds, M. D. Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment. Neuromuscul. Disord. 18, 227–238 (2008).

163. Rai M, Nongthomba U, Grounds MD (2014).“Skeletal muscle degeneration and regeneration in mice and flies.” Curr Top Dev Biol ; 108: 247–281.

164. Relaix, F. & P. S. Zammit (2012) “Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage.” Development, 139, 2845-56.

165. Riboni. L., A. Prinetti, et al (1995)."A mediator role of ceramide in the regulation of neuroblastoma Neuro2a cell differentiation." J Biol Chem 270(45): 26868-26875.

166. Rogler G, Andus T. “Cytokines in inflammatory bowel disease.” World J Surg 1998; 22:382–9.

167. Rosenberg Amy S., Montserrat Puig Kanneboyina Nagaraj,Eric P. HoffmanS. Armando Villalta V. Ashutosh Rao, Lalage M. Wakefield and Janet Woodcock (2015). “Immune-mediated pathology in Duchenne muscular dystrophy Science Translational Medicine 05 Aug 2015:Vol. 7, Issue 299, pp. 299rv4.

168. Rudnicki MA, Jaenisch R. 1995. “The MyoD family of transcription factors and skeletal myogenesis.” Bioessays 17: 203–209.

169. Rutherford, C.; Childs, S.; Ohotski, J.; McGlynn, L.; Riddick, M.; MacFarlane, S.; Tasker, D.; Pyne, S.; Pyne, N.J.; Edwards, J.; et al. “Regulation of cell survival by sphingosine-1-phosphate receptor S1P1 via reciprocal ERK-dependent suppression of BIM and PI-3-kinase/protein kinase C-mediated upregulation of Mcl-1” Cell Death Dis. 2013, 4, e927.

170. Saclier, M., Yacoub-Youssef, H., Mackey, A.L., Arnold, L., Ardjoune, H., Magnan, M., Sailhan, F., Chelly, J., Pavlath, G.K., Mounier, R., et al. (2013). “Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration. Stem Cells 31, 384–396.

171. Sali, A.; Guerron, A.D.; Gordish-Dressman, H.; Spurney, C.F.; Iantorno, M.,;Hoffman, E.P.;Nagaraju, K (2012). "Glucocorticoid-treated mice are an inappropriate positive control for long-term preclinical studies in the mdx mouse." PloS ONE, 7, e34204.

172. Sampaolesi, M. et al., (2003).“Cell therapy of alpha-sarcoglycan null dystrophic mice throughi intra-arterial dlivery of mesoangioblasts.” Science 301, 487-492.

173. Schnitzer, T.J., Kivitz, A.J., Lipetz, R.S.,Sanders N. and Hee, A. (2005). “Comparisono of the COX-inhibiting nitric oxide donator AZD3582 and rofecoxib in treating the signs and symptomps of osteoarthritis of the knee.” Arthritis Rheum, 53. 872-837.

174. Schissel S. L, Schuchman EH, Williams KJ, Tabas I. (1996). "Zn2+-stimulated sphingomyelinase is secreted by many cell types and is a product of the acid sphingomyelinase gene." J Biol Chem.; 271(31): 18431-6.

175. Schissel, S. L. et al. Secretory sphingomyelinase, a product of the acid sphingomyelinase

gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. J. Biol. Chem. 273, 2738–46 (1998).

176. Schuchman, E.H. (2010) "Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease." FEBS Lett., 584, 1895-1900.

177. Seto, J.T.; Bengtsson, N.E.;Chamberlain,J.S. (2014). "Therapy of Genetic Disorders-Novel Therapies for Duchenne Muscular dystrophy." Curr. Pediatr. Rep. 2,102.112.

178. Simons, S. and E. Ikonen (1997). "Functional rafts in cell membranes." Nature 387(6633): 569-572.

179. Skuk D, Goulet M, Roy B, et al. (2006). "Dystrophin expression in muscles of Duchenne muscular dystrophy patients after high-density injections of normal myogenic cells. J Neuropathol Exp Neurol.; 65(4): 371–386.

180. Skuk D, Goulet M, Tremblay JP (2011). "Transplanted myoblasts can migrate several millimeters to fuse with damaged myofibers in nonhuman primate skeletal muscle. J Neuropathol Exp Neurol.; 70(9): 770–778.

181. Snider A.J., T. Kawamori, S.G. Bradshaw, K.A. Orr, G.S. Gilkeson, Y.A. Hannun, L.M. Obeid "A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis." FASEB J, 23 (2009) 143-152.

182. Spiegel S, Foster D, Kolesnick R. Signal transduction through lipid second messengers. Curr Opin Cell Biol 1996; 8:159–67.

183. Stamler JS, Meissner G. (2001). "Physiology of nitric oxide in skeletal muscle." Physiol Rev. 81(1):209-37.

184. Stedman,HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, Narusawa M, Leferovich JM, Sladky J,Kelly AM (1991)." The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy." 8;352(6335):536-9.

185. Stout R.D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles, "Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences,"The Journal of Immunology, vol. 175, no. 1, pp. 342–349, 2005.

186. Studitsky, A.N. Free auto-and homografts of muscle tissue in experiments on animals. Ann.N.Y. Acad.Sci.120, 789-801.

187. Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M.1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89:127–138.

188. Tedesco, F.S., Arianna Dellavalle, Jordi Diaz-Manera, Graziella Messina and Giulio Cossu (2010). "Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells J.Clin.Invest. 120:11–19.

189. Tedesco, F.S., et al (2012). "Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystroph." Sci Transl Med 4, 140ra 189.

190. Teichgräber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC et al. (2008). "Ceramide accumulation medi- ates inflammation, cell death and infection susceptibility in cystic fibrosis." Nat Med 14: 382–391.

191. Tidball J.G., "Inflammatory processes in muscle injury and repair," The American Journal of Physiology: Regulatory Integrative and Comparative Physiology, vol. 288, no. 2, pp. R345–R353, 2005.

192. Tidball J.G. and S. A. Villalta. (2010). "Regulatory interactions between muscle and the immune system during muscle regeneration," The American Journal of Physiology—Regulatory Integrative and Comparative Physiology, vol. 298, no. 5, pp. R1173–R1187.

193. Tinsley, J.M., et al., (1996). "Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature 384, 349-353.

194. Tonkin Temmerman J., L., Sampson R.D., Gallego-Colon E., Barberi L., Bilbao D., Schneider M.D., Musarò A. and Rosenthal N. (2015). "Monocyte/Macrophage-derived IGF-1 Orchestrates Murine Skeletal Muscle Regeneration and Modulates Autocrine Polarization." Molecular Therapy vol. 23 no.7, 1189-1200.

195. Trovato F.M., Rosa Imbesi Nerys Conway and Paola Castrogiovanni (2016). "Morphological and Functional Aspects of Human Skeletal Muscle." J. Funct. Morphol. Kinesiol. 1, 289–302; doi: 10.3390 /jfmk1030289.

196. Uaesoontrachon K., Quinn JL., Tatem KS., Van Der Meulen JK., Yu Q., Phadke A., Miller BK., Gordish-Dressman H., Ongini E., Miglietta D., Nagaraju K. (2014). "Long-term treatment with naproxcinod significantly improves skeletal and cardiac disease phenotype in the mdx mouse model of dystrophy." Human Molecular Genetics, Vol.23, No.12.

197. Uhlig, S., Yang, Y., (2013). " Sphingolipids in acute lung injury". Handb.Exp.Pharmacol. 216. 227-246.

198. Ullman MD, Radin NS The enzymatic formation of sphingomyelin from ceramide and lecithin in mouse liver. J Biol Chem. 1974 Mar 10; 249(5):1506-12.

199. Vandanmagsar, B. et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat. Med. 17, 179–188 (2011).

200. Van Westering, T.L.E., Betts, C.A.; Wood, M.J.A. Current understanding of molecular pathology and treatment of cardiomyopathy in Duchenne Muscular Dystrophy. Molecules 2015, 20, 8823–8855.

201. Villalta S.A., H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," Human Molecular Genetics, vol. 18, no. 3, pp. 482–496, 2009.

202. Villani M, Subathra M, Im Y-B, et al. Sphingomyelin synthases regulate production of diacylglycerol at the Golgi. The Biochemical journal. 2008;414(1):31-41. doi: 10.1042/BJ20071240.

203. Voelker DR, Kennedy EP Biochemistry. Cellular and enzymic synthesis of sphingomyelin.

1982 May 25; 21(11):2753-9.

204. Voisin V, Sebrie C, Matecki S, Yu H, Gillet B, Ramonatxo M, Israel M, De la Porte S (2005). "L-arginine improves dystrophic phenotype in mdx mice." Neurobiol Dis 20(1):123-130.

205. Wallace JL, Vippiani S, Bolla M, (2009). "Cyclooxigenase inhibiting nitric oxide donators for osteoarthritis." Trends Pharmacol Sci 30(3):112-117.

206. Wehling-Hemricks, M.; Lee, J.J.,Tidball, J.G.(2004). "Prednisolone decreases cellular adhesion molecules required for inflammatory cell infiltration in dytrophin-deficient skeletal muscle." Neuromuscul.Disord. 14.483-490.

207. Weintraub H, Davis R, Tapscott S, ThayerM,Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S, et al. 1991.The myoD gene family: "Nodal point during specification of the muscle cell lineage. Science 251: 761–766.

208. Welch, E.M., et al. (2007). "PTC124 targets genetic disorders caused by nonsense mutations. Nature 447,87-91.

209. Weir, A.P., Burton E.A., Harrod, G., & Davies, K.E. (2002). "A-and B-utrophin have different expression patterns and are differentially up-regulated in mdx muscle." J Biol Chem 277, 45285-45290.

210. Willmann R, Possekel S, Dubach-Powell J, Meier T and Ruegg MA (2008)."Mammalian animal models for Duchenne muscular dystrophy. Neuromuscul Disord 19,241–249.

211. Wright JF. Manufacturing and characterizing AAV-based vectors for use in clinical studies. Gene Ther. 2008;15(11):840–848.

212. Xiao C, Ghosh S (2005). "NF-kB, an evolutionarily conserved mediator of immune and inflammatory responses." Adv Exp Med Biol 560: 41–45.

213. Xuan Wenjuan, Qing Qu, Biao Zheng, Sidong Xiong, and Guo-Huang Fan, 2 DOI: 10.1189/jlb.1A0314-170R, 2014. "The chemotaxis of M1 and M2 macrophages is regulated by different chemokines."

214. Yablonka-Reuveni, Z., Day, K., Vine, a & Shefer, G. Defining the transcriptional signature of skeletal muscle stem cells. Journal of animal science 86, E207– 16 (2008).

215. Yan, W., Hu Ping, (2018). "Skeletal muscle regeneration is modulated by inflammation." Journal of Orthopaedic Translation, Vol.13, Pages 25-32.

216. Zammit P. S, J. P. Golding, Y. Nagata, V. Hudon, T. A. Partridge, and J. R. Beauchamp, "Muscle satellite cells adopt divergent fates: a mechanism for self-renewal?" The Journal of Cell Biology, vol. 166, no. 3, pp. 347–357, 2004.

217. Zammit, P. S. (2008). All muscle satellite cells are equal, but are some more equal than others ? J. Cell Sci. 121, 2975-2982

218. Zecchini Silvia, Matteo Giovarelli, Cristiana Perrotta, Federica Morisi, Thierry Touvier, Ilaria Di Renzo, Claudia Moscheni, Maria Teresa Bassi, Davide Cervia, Marco Sandri,

Emilio Clementi & Clara De Palma (2018) “Autophagy controls neonatal myogenesis by regulating the GH-IGF1 system through a NFE2L2- and DDIT3-mediated mechanism” Autophagy, DOI: 10.1080/15548627.2018.1507439.

219. Zhou Y-F, Metcalf MC, Garman SC, Edmunds T, Qiu H, Wei RR (2016). "Human acid sphingomyelinase structures provide insight to molecular basis of Niemann–Pick disease." Nature Communications; 7:13082. doi: 10.1038/ncomms13082.