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THE ACID SPHINGOMYELINASE PATHWAY AS A PROMISING TARGET OF INFLAMMATION AND OXIDATIVE STRESS IN DUCHENNE MUSCULAR DYSTROPHY

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ABSTRACT

The enzyme Acid Sphingomyelinase (ASMase) plays a major role in the metabolism of sphingolipids by generating ceramide, a lipid second messenger involved in a wide range of physiological and pathological processes (Zeidan & Hannun, 2010). ASMase is activated by multiple stress stimuli, and in turn induces the production of key mediators of inflammation and oxidative stress, which are hallmarks of Duchenne Muscular Dystrophy (DMD) (Petrillo et al., 2017; Tidball et al., 2018). Since we recently demonstrated the involvement of ASMase in the progression of DMD, we here explored whether targeting this enzyme by administering the functional inhibitors of ASMase (FIASMAs), i.e. Fluoxetine and Sertraline (Kornhuber et al., 2010), and/or antioxidant molecules, i.e. Plumbagin, Quercetin, and N-Acetylcysteine (NAC) to *mdx* mice, the murine model of DMD, would be a pertinent therapeutic strategy.

Both Plumbagin and Quercetin improved the muscle function compared to the *mdx* vehicle and reached a recovery score versus wild-type (WT) similar to NAC. This is promising as several studies support the benefits of NAC in *mdx* mice (de Senzi Moraes Pinto et al., 2013; Whitehead et al., 2008), here employed as a control of the efficacy of our treatment. FIASMAs failed to improve muscle function, however, the potential improvement was possibly hidden due to the behavioral effect of Fluoxetine and Sertraline, two selective serotonin reuptake inhibitors, as suggested by administering the drugs to WT mice who had a poor performance compared to the WT vehicle. All the treatments however improved muscle morphology and significantly decreased muscle damage. In parallel, the muscle regeneration was enhanced.

We found that both the tested FIASMAs and Plumbagin were able to decrease ASMase activity in the diaphragm while Quercetin did not.

Both FIASMAs and antioxidants reduced and modulated inflammation towards an anti-inflammatory state as evidenced by the decreased expression of the M1 macrophage markers, worsening muscle damage, in parallel with the increase in M2 macrophage markers promoting tissue repair (Madaro et al., 2019; S. A. Villalta et al., 2009). All the treatments also attenuated oxidative stress by increasing the expression of several enzymes involved in the antioxidant defense system. Only Fluoxetine and Plumbagin induced Nrf2 expression, which plays a pivotal role in both oxidative stress and inflammation, thus holding great potential in DMD therapy (Kourakis, Timpani, de Haan, et al., 2021).

Similar results were obtained in an acute muscle injury model by using ASMase-KO mice, thus suggesting that the events observed in treated *mdx* mice are related to the decrease of ASMase activity. Indeed, ASMase ablation induced a surge of Nrf2, alleviated and modulated inflammation towards an anti-inflammatory state, leading to an improved regeneration by acting on the niche of satellite cells (Roux-Biejat et al., 2021).

We provide evidence that acting on the ASMase pathway is a promising alternative therapeutic strategy to the incurable DMD. Being antidepressants, these FIASMAs could alleviate the depressive symptoms often displayed by the patient. Moreover, drug repurposing allows time- and cost-savings as the drugs are already commercially available with a known safety profile. Finally, the efficacy could potentially be improved in a synergistic/additive

manner by combining a FIASMA with an antioxidant. Our results show that the most efficient ones in their class are Fluoxetine and Plumbagin.

SINTESI

L'enzima sfingomielinasi acida (ASMase) svolge un ruolo importante nel metabolismo degli sfingolipidi generando ceramide, un secondo messaggero lipidico coinvolto in un'ampia gamma di processi fisiologici e patologici (Zeidan & Hannun, 2010). ASMase è attivato da molteplici stimoli di stress e, a sua volta, induce la produzione di mediatori chiave dell'infiammazione e dello stress ossidativo, che sono caratteristiche distintive della distrofia muscolare di Duchenne (DMD) (Petrillo et al., 2017; Tidball et al., 2018). Dato che abbiamo recentemente dimostrato il coinvolgimento di ASMase nella progressione della DMD, abbiamo quindi investigato se inibire l'attività di questo enzima, somministrando degli inibitori funzionali di ASMase (FIASMA), ovvero Fluoxetina e Sertralina (Kornhuber et al., 2010), e/o molecole antiossidanti, ovvero Plumbagina, Quercetina e N-Acetilcisteina (NAC), ai topi *mdx*, il modello murino della DMD, possa essere una valida strategia terapeutica.

Sia la Plumbagina che la Quercetina hanno migliorato la funzione muscolare dei topi *mdx* rispetto al veicolo e hanno raggiunto un recovery score, rispetto ai wild-type (WT), simile a quello di NAC. Ciò è promettente poiché molti studi supportano i benefici di NAC nei topi *mdx* (de Senzi Moraes Pinto et al., 2013; Whitehead et al., 2008), qui usato come controllo dell'efficacia del nostro trattamento. FIASMA non ha avuto successo nel miglioramento della funzione muscolare, che comunque, è stato probabilmente mascherato dall'effetto del comportamento di Fluoxetina e Sertralina, che appartengono alla classe degli inibitori selettivi della ricaptazione della serotonina, come suggerito dalla somministrazione degli stessi farmaci ai topi WT, che presentavano una ridotta performance rispetto ai topi WT non trattati. Tutti i trattamenti hanno comunque migliorato la morfologia muscolare e ridotto significativamente il danno muscolare. Parallelamente, è stata potenziata la rigenerazione muscolare.

Abbiamo inoltre trovato che sia i FIASMA testati che la Plumbagina sono in grado di ridurre l'attività di ASMase nel diaframma, mentre la Quercetina non ha prodotto nessun effetto in tal senso.

Sia i FIASMA che gli antiossidanti hanno ridotto e modulato l'infiammazione verso uno stato antinfiammatorio, come evidenziato dalla ridotta espressione di marcatori dei macrofagi M1, che peggiorano il danno muscolare, e parallelamente dall'aumento dei marcatori dei macrofagi M2, che promuovono la riparazione dei tessuti (Madaro et al., 2019; S. A. Villalta et al., 2009). Tutti i trattamenti hanno inoltre attenuato lo stress ossidativo, aumentando l'espressione di diversi enzimi coinvolti nel sistema di difesa antiossidante. Solo Fluoxetina e Plumbagina sono state capaci di indurre l'espressione di Nrf2, proteina che svolge un ruolo fondamentale nella regolazione dello stress ossidativo e dell'infiammazione con un grande potenziale nella terapia della DMD (Kourakis, Timpani, de Haan, et al., 2021).

Risultati simili sono stati ottenuti in un modello di danno muscolare acuto, utilizzando topi ASMasi-KO, suggerendo così che gli eventi osservati nei topi *mdx* trattati farmacologicamente possano essere correlati alla diminuzione dell'attività di ASMasi. Infatti, in assenza di ASMase si è osservato un aumento di Nrf2 a seguito del danno muscolare ed una riduzione dell'infiammazione con conseguente miglioramento della rigenerazione da parte delle cellule satelliti muscolari (Roux-Biejat et al., 2021).

In questo lavoro forniamo delle prove sull'inibizione della ASMase come una terapia alternativa promettente contro l'incurabile DMD. Essendo degli antidepressivi, questi FIASMA possono alleviare i sintomi depressivi spesso osservati nei pazienti. Inoltre, il riposizionamento di farmaci già approvati e con un noto profilo di sicurezza consentirebbe di risparmiare tempo e denaro per la loro nuova approvazione. Infine, l'efficacia delle molecole testate potrebbe potenzialmente essere migliorata in modo sinergico/additivo combinando un FIASMA con un'antiossidante. I nostri risultati mostrano che i più efficaci nelle loro rispettive classi sono Fluoxetina e Plumbagina.

INTRODUCTION

Chapter 1: Skeletal Muscle Physiology

1.1 Structure & Function

Accounting for ~ 40% of our body mass, skeletal muscle is the largest organ in our body allowing us to move, maintain our posture, breath and protect our internal organs. Beyond its mechanical function, skeletal muscle plays a central role in the metabolism by serving as a reservoir for amino acids to maintain protein synthesis in the entire body, by being the primary site for glucose uptake and storage, by producing and releasing cytokines and other peptides named «myokines» upon contraction to mediate a crosstalk between the muscle itself and other organs, and by generating heat (Frontera & Ochala, 2015; Pedersen, 2013). Muscle mass relies on nutritional status, hormonal balance, physical activity as well as injury and disease as these factors can alter the equilibrium between protein synthesis and degradation. Loss of muscle is associated with a wide range of harmful health effects including impaired recovery from illness or wound, and chronic disease. The skeletal muscle should be seen as an important actor of our health, as physical activity decreases the risk of numerous diseases and exercise may be included in the therapeutic management for many disorders such as type 1 diabetes, cardiovascular diseases, cancer, and dementia (Severinsen & Pedersen, 2020; Wolfe, 2006).

An individual muscle is surrounded by a layer of connective tissue called the *epimysium*, which branches into the *perimysium* to envelope fascicles composed of bundles of muscle fibers. A single myofiber is around 100 μm in diameter, 1 cm in length and is surrounded by the *endomysium*, its reticular connective tissue. The sarcoplasm is packed with myofibrils consisting of myofilaments arranged in a well-defined manner to form contractile units known as sarcomeres. Myofibers are syncytial cells formed by the fusion of myogenic progenitor cells (Trovato et al., 2016) (*Figure 1*).

Although physiologically, skeletal muscle is a stable post-mitotic tissue with infrequent turnover of myonuclei, it has a robust regenerative capacity upon injury. The resident muscle stem cells, named Satellite Cells due to their peripheral localization: beneath the endomysium and outside the myofiber plasma membrane, play a pivotal role during muscle regeneration (Mauro, 1961; Relaix & Zammit, 2012).

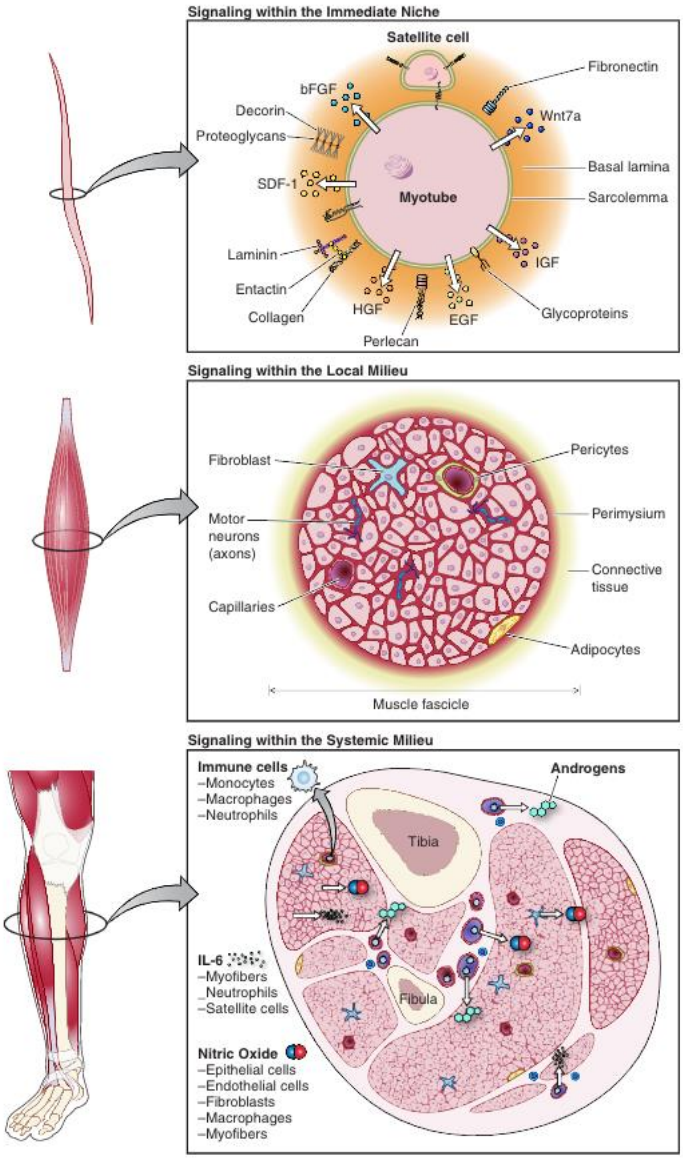

Figure 1: The satellite cell and its niche (Yin et al., 2013)

1.2 Skeletal muscle regeneration

Skeletal muscles originate embryologically from paraxial mesoderm arranged in bilateral paired clusters, named somites, which are crucial transitory structures for organization of the segmental plan of vertebrates. Local molecular signals from neighboring tissues including Sonic Hedgehog (SHH), Wnt and Insulin-like growth factor-1 (IGF-1) regulates the expression of Myf5 and MyoD, committing cells to myogenesis (Cossu et al., 2000; Pownall et al., 2002).

Myofiber formation is a multiphasic process with an early and a late wave. First, during primary (embryonic) myogenesis, proliferation and fusion of embryonic myoblasts form primary fibers. Then, during the secondary (fetal) myogenesis, the fetal myoblasts fuse to form secondary fibers. They are enveloped with the same basal lamina as the primary fiber they

merge with, and they gradually elongate and become independent fibers (Biressi et al., 2007; Duxson et al., 1989; Kelly & Zacks, 1969).

Skeletal muscle regeneration is a well-orchestrated process that recapitulates the embryonic developmental program in many aspects, as the molecular program that underlines prenatal myogenesis is reactivated upon injury. However, there are important distinctions between developmental and regenerative myogenesis, notably regarding the extrinsic factors with the important inflammatory infiltrate after injury which influences the activity of satellite cells but also in the inherent signaling pathways as the embryonic positional signals Wnt, SHH and bone morphogenetic proteins (BMP) are not activated in muscle regeneration (Zhao & Hoffman, 2004).

With a turnover rate of at most 1-2% myonuclei per week, adult skeletal muscle retains nonetheless a remarkable capacity to regenerate itself when needed (Schmalbruch & Lewis, 2000). This process relies on satellite cells, which are sublaminal and mitotically quiescent muscle stem cells in intact muscle. Their interaction with their environmental niche influences their activation, proliferation, migration, and differentiation (Relaix et al., 2021; Scharner & Zammit, 2011) (*Figure 2*).

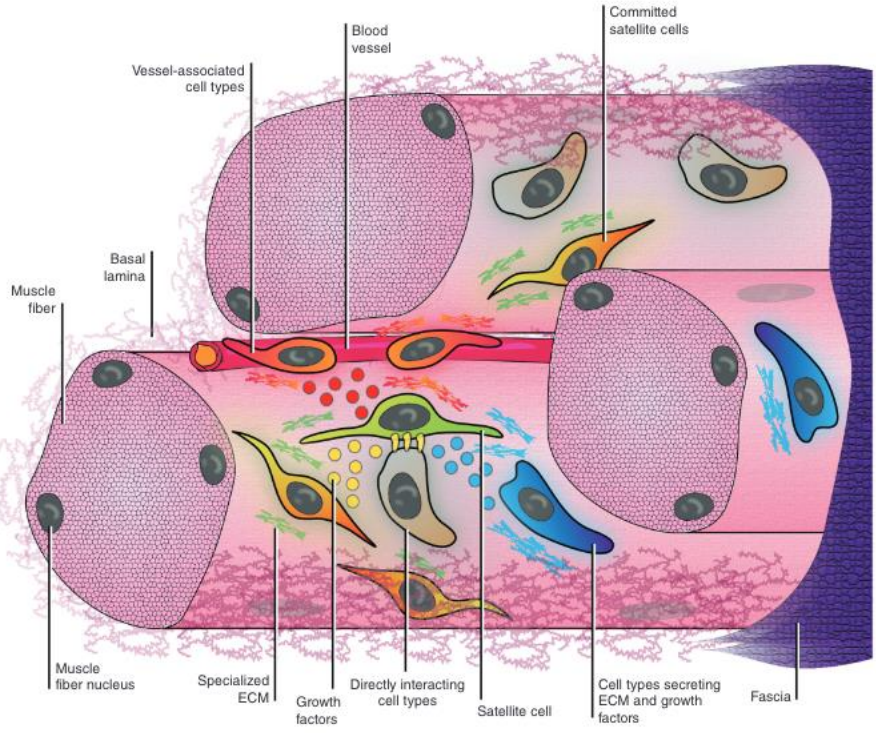

Figure 2: Schematic representation of the interaction of the satellite cell with its microenvironment including inflammatory cells and stromal cells. (Dumont, Bentzinger, et al., 2015)

Muscle regeneration can be outlined in five correlated and time-dependent phases: degeneration (necrosis), inflammation, regeneration, maturation/remodeling, and functional recovery (Forcina et al., 2020) (*Figure 3*).

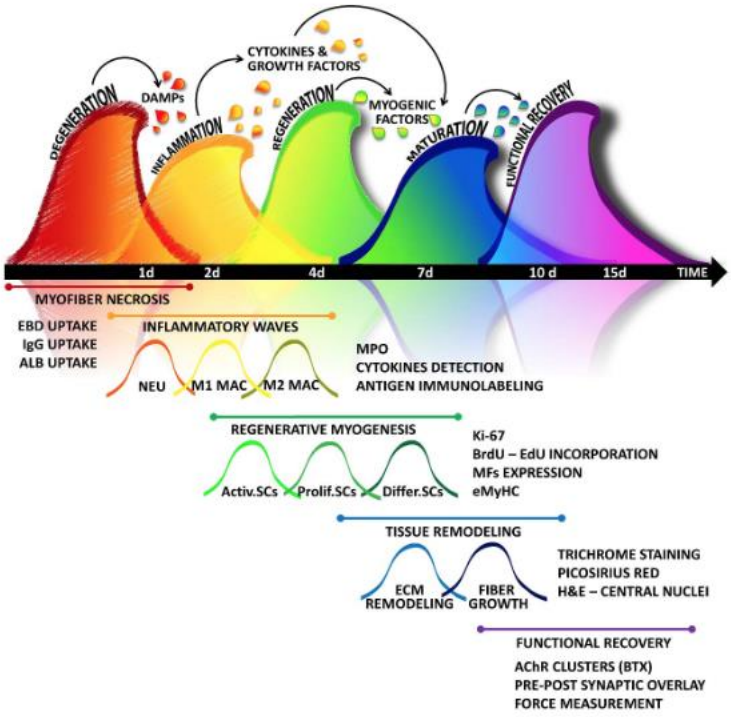

Figure 3: A simplified model of the skeletal muscle regeneration process (Forcina et al., 2020)

The degeneration phase is characterized by necrosis of irreversibly damaged muscle fibers and is initiated by dissolution of sarcolemma. Disruption of myofiber integrity increases its permeability and leads to increased plasma levels of muscle proteins and microRNAs normally restricted to the sarcoplasm such as creatine kinase (Angelini et al., 1968) and miR-133a (Laterza et al., 2009). Additionally, there is an increased calcium influx/release from the sarcoplasmic reticulum which in turn activates calcium-dependent proteolysis (Alderton & Steinhardt, 2000; Belcastro et al., 1998), loss of the plasmalemma, myonucleus, contractile material and swelling of organelles. Necrotic fibers appear enlarged with an altered internal architecture where the presence of internal nuclei may reflect the invasion of macrophages. Necrotic cell death activates the complement cascade and induces inflammatory responses (Orimo et al., 1991).

Inflammation of injured skeletal muscle plays a crucial role in muscle homeostasis and regeneration. The dispersed intracellular components throughout the extracellular space can act as signals called damage-associated molecular patterns (DAMPs) which trigger inflammatory reactions (Roh & Sohn, 2018). The complement system, activated only within seconds after injury, is the first defense of innate immunity and promotes infiltration of inflammatory cells to the wound site (Frenette et al., 2000) (*Figure 4*).

The first immune cells to be activated are resident neutrophils which along with mast cells release pro-inflammatory cytokines such as Tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ) and Interleukin-1 beta (IL-1β), inducing the recruitment of peripheral neutrophils. This process allows infiltration of an abundant amount of neutrophils around the injured fibers rapidly as their number peaks within 6-24h after damage (Duque & Descoteaux, 2014).

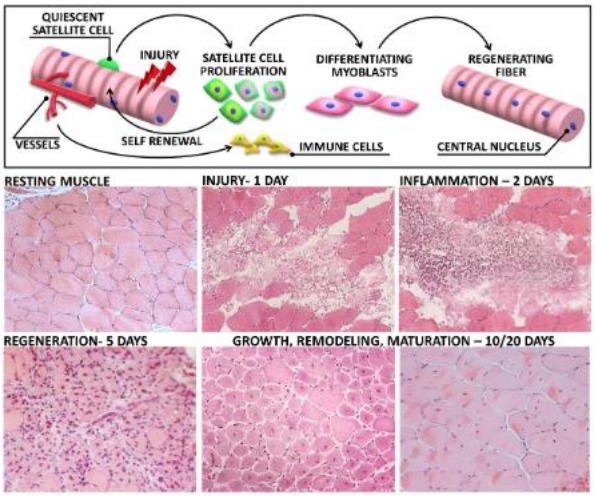

Figure 4: Skeletal muscle regeneration upon acute damage. The upper panel represents the main biological responses activated in acute muscle damage. The lower panel reports Hematoxylin and Eosin images of muscle sections following cardiotoxin (CTX) injection. (Forcina et al., 2020)

The phagocytic activity of neutrophils generates the release of high concentrations of reactive oxygen species (ROS), proteases and the secretion of pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-8 (IL-8) and interleukin-6 (IL-6), which stimulates the recruitment of other inflammatory cell populations: monocytes and macrophages (Tidball, 2005; Yang & Hu, 2018).

Within 2 days after damage, while the population of neutrophils decline, the number of macrophages significantly increases, and they become the predominant inflammatory cell type within the lesioned area. Macrophages play a pivotal role in muscle regeneration as they are responsible for the removal of tissue debris and for the activation of stem cell populations (Chazaud et al., 2009). Distinct subpopulations of macrophages sequentially invade the wound site: first the pro-inflammatory M1 macrophages and then the anti-inflammatory M2 macrophages. Macrophages constitute a heterogenous population in which their polarization confer them different receptor expression, cytokine production, effector function and chemokine repertoire (*Figure 5*).

If there is the possibility that macrophages undergo dynamic transitions between M1 and M2 states named «*polarization skewing*» (Stout et al., 2005), it is however debated as the microenvironment within the inflammatory site prevents local conversion to an M2 subtype (Shechter et al., 2009) and it has also been reported more recently that M1 and M2 monocytes/macrophages originate from different sources (Shechter et al., 2013).

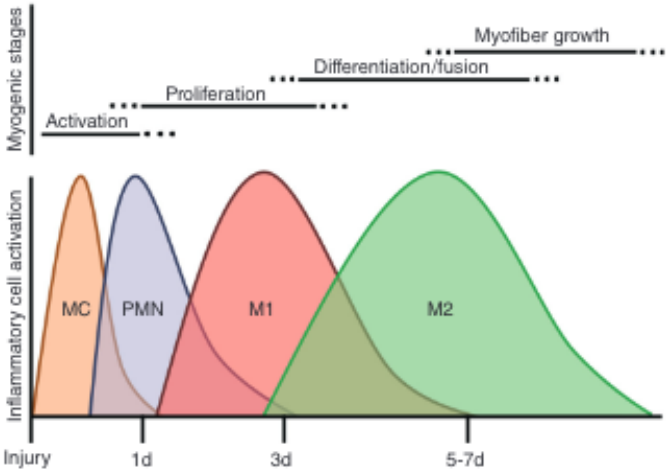

Figure 5: The parallel between inflammation and myogenesis (Dumont, Bentzinger, et al., 2015)

M1 or «*classically activated*» macrophage polarization is driven by the exposure to IFN-γ, TNF-α and lipopolysaccharide (LPS). M1 macrophages remove the cellular debris generated by the trauma, secrete a large amount of cytokines such as TNF-α, IL-6 and IL-1β and highly express inducible Nitric Oxide Synthase (iNOS), which leads to the generation of the reactive free radical nitric oxide (NO) (S. A. Villalta et al., 2009). While high concentrations of NO can induce apoptosis of the damaged cells to promote debris removal (Yoshioka et al., 2003), low concentrations of NO protect against oxidative damage (Wink et al., 1994). M1 macrophages can attract muscle stem cells to the wound site, stimulate the proliferation of satellite cells and myogenic precursors, while repressing their differentiation (Arnold et al., 2007).

The M2 population can be further divided into three main subsets: M2a, M2b and M2c, each of which requires specific polarization cues and exerts diverse physiological roles.

The M2a subtype is defined as «*alternatively activated*» and arises in presence of IL-4 and IL-13. M2b, also known as type 2 macrophages, are polarized by combined exposure to immune complexes and toll-like receptors (TLR) or IL-1R agonists. The M2c phenotype is induced via IL-10, TGF-β, and glucocorticoids and is referred to as deactivated macrophages. However, this terminology descending from *in vitro* model is difficult to translate *in vivo* as tissues can contain mixed macrophage populations with a spectrum of activation states (Mantovani et al., 2004; Murray et al., 2014; Rőszer, 2015). M2a and M2b macrophages have immunoregulatory properties and drive type II responses, whereas M2c cells rather suppress immune responses and support tissue remodeling. To repress the local inflammatory response, M2 macrophages produce anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13. M2 macrophages also promote the differentiation of muscle stem cells to myotubes, therefore supporting the late stage of myogenesis and regeneration.

In order to be beneficial, the accumulation of different subtypes of macrophages must be controlled, transient and sequential as the prolonged presence of M1 phenotype or the imbalance of M1 over M2 macrophages can lead to tissue damage and inhibit regeneration. The M1 macrophage-dominated aberrant inflammation is involved in the pathogenesis of many chronic inflammatory disorders such as multiple sclerosis, atherosclerosis, osteoarthritis, but

also muscular dystrophies (Dort et al., 2019; Fahy et al., 2014; Mantovani et al., 2009; Mikita et al., 2011).

The regenerative phase is mainly governed by satellite cells. The myogenic program is orchestrated by several transcription factors which have been identified as markers and key regulators of the progression from quiescence, activation, proliferation, differentiation, and self-renewal of satellite cells. Among them, the paired homeobox factors PAX7, used as a common marker of satellite cells and PAX3 which is co-expressed in a subgroup of satellite cells in adult muscle. The Myogenic Regulatory Factors – MRFs (Myf5, MyoD, Myogenin and MRF4 (Myf6)) are also fundamental for muscle formation, specification, homeostasis, and repair (Musarò, 2014). Quiescent satellite cells express Pax7 and Myf5 but neither MyoD nor Myogenin.

Damage to the area surrounding satellite cells leads to the deterioration of the basal lamina and activation of the muscle stem cells. Proliferating satellite cells and their progeny also called myogenic precursor cells or adult myoblasts are conferred an extensive cell migration capacity. Adult myoblasts express Myf5 and MyoD on which depend the fate of satellite cells: either they retain Myf5 expression while downregulating MyoD and self-renew, or they maintain MyoD expression and differentiate. Myf5 regulates MyoD activity, which in turn promotes the expression of Myogenin and MRF4. Myogenin expression initiates terminal differentiation, fusion, and promotes Myosin expression (*Figure 6*).

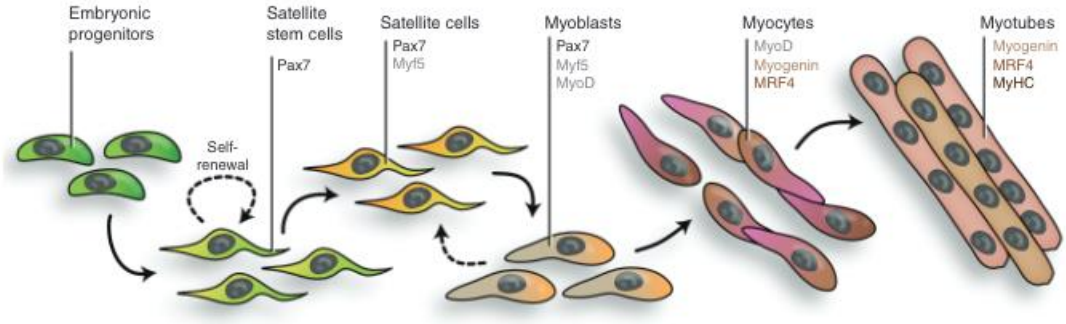

Figure 6: Myogenic lineage progression of satellite cells following muscle injury (Dumont, Bentzinger, et al., 2015)

Satellite cells constitute a heterogeneous population that may underlie their variability in cell fate and function, as they differ in many aspects including transcription factor, surface marker expression, clonal capacity, and functional response under environmental stress (Tierney & Sacco, 2016). Satellite cells can be classified into two groups: satellite stem cells, which have never expressed Myf5, preferentially repopulate the satellite cell population and contribute to long-term regeneration, and committed satellite cells, which express Myf5 and can proliferate and differentiate (*Figure 7*).

Stem cells can divide and self-renew in two modes: asymmetric division and symmetric (stochastic) division (relative to the myofiber). In asymmetric division, one parental stem cell gives rise to one daughter stem cell and one committed satellite cell, whereas in symmetric division, one parental stem cell divides into two daughter stem cells of equal stemness (Bentzinger et al., 2012; Dumont, Bentzinger, et al., 2015; Yin et al., 2013) (*Figure 8*).

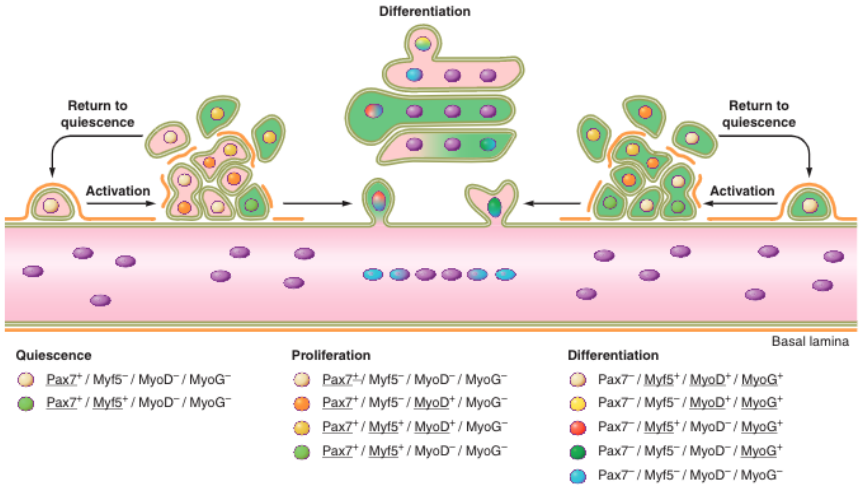

Figure 7: Schematic representation of the myogenic program of satellite cells (Yin et al., 2013)

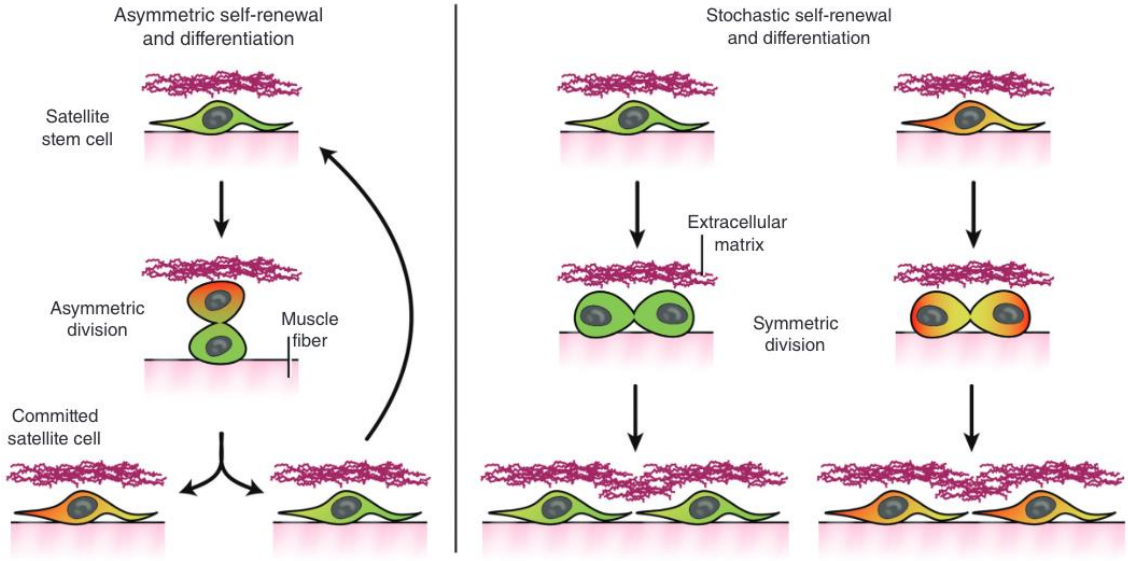

Figure 8 : Asymmetric and stochastic self-renewal of Satellite Cells (Dumont, Bentzinger, et al., 2015)

The remodeling of connective tissue and angiogenesis relies on the extracellular matrix (ECM), which serves as a scaffold for the invading cells, supports the damaged muscle fibers and guide the formation of new myofibers and neuromuscular junctions. In response to TGF-β1 among other local mediators, fibroblasts transform into myofibroblasts, which can also arise from different cell types and synthesize ECM proteins such as fibronectin, tenascin-C, and several types of collagens. The regeneration wave overlaps the ECM deposition which can be observed within a week after the damage and can carry on for several weeks. Nonetheless, exacerbation of this process can lead to scar tissue formation called fibrosis and loss of muscular function (Garg et al., 2015).

The muscle is considered fully healed when regenerated myofibers become effectively innervated in order to recover their functional performance.

Chapter 2: Duchenne Muscular Dystrophy

2.1 General Picture of DMD

Muscular dystrophy passed from being considered as a single disease in the 19th century to a superfamily of more than 50 genetically distinct diseases characterized by progressive weakness and degeneration of skeletal muscle (Kang & Griggs, 2015). Muscular dystrophies constitute a heterogeneous group, even in between themselves, with variabilities in the age of onset, the distribution of affected muscles, the involvement of cardiac, respiratory, or other organ systems, and therefore severity (Carter et al., 2018). Adding to the complexity, mutations in different genes may lead to the same outcome while mutations within a single gene may result in different phenotypes (Chinoy & Cooper, 2018).

Duchenne muscular dystrophy (DMD) is the most common and severe pediatric muscular dystrophy with an incidence of 1:3500-5000 male births (Mendell et al., 2012). This X-linked disease is a lethal progressive muscular-wasting disorder caused by mutations in the dystrophin gene which disrupt the translational reading frame or create a premature stop codon leading to the absence or structural defect of dystrophin. Dystrophin is predominantly expressed in skeletal and cardiac muscles and to a lesser extent in smooth muscles, brain, and retina. The size of the gene is the largest one known in human beings with more than 2.5 million base pairs contributing to its high rate of mutations. As such, there are more than 7000 different mutations identified in DMD patients and in one in three cases the disease is caused by a *de novo* mutation. The majority of the mutations are deletions (~68%) or duplications (~11%) of one or more exons although small mutations (~20% of patients) are also found (Aartsma-Rus et al., 2016; Luce et al., 2021; Muntoni et al., 2003).

The first symptoms become apparent at the age of 2-3 years. Patients left untreated become wheelchair dependent by 12 years of age and die in their late teens. Although there is still no cure for DMD, improvements in multidisciplinary care, notably with ventilation support have significantly improved quality of life and survival until their thirties or forties. Muscle weakness typically begins in the proximal lower limb and the trunk, gradually progressing in the upper limb and distal muscles. Clinical features include using the Gower maneuver to arise from the floor, a particular gate, delay in walking, difficulty in running, climbing stairs, or jumping. Patients also commonly show muscle enlargement, especially of the calves due to increased replacement of muscle with fibrous and fatty tissue leading to pseudohypertrophy. Although lagging behind their peers, boys with DMD gain strength and motor skills until they reach about 6 years of age, after which their force deteriorates markedly. The major determinants of morbidity are impaired respiratory and cardiac functions. Chronic respiratory insufficiency secondary to restrictive lung disease is ubiquitous and aggravated by scoliosis. Cardiac involvement, including dilated cardiomyopathy and arrhythmias, is often masked by physical inactivity. Cognition can also be impaired as the mean intelligence quotient (IQ) is 18 points below normal in 30% of DMD patients who therefore may have trouble with attention, memory, and verbal learning. This mild intellectual disability is likely caused by changes in isoforms of dystrophin expressed in the brain (Flanigan, 2014; Lovering et al., 2005; Yiu & Kornberg, 2015) (*Figure 9*).

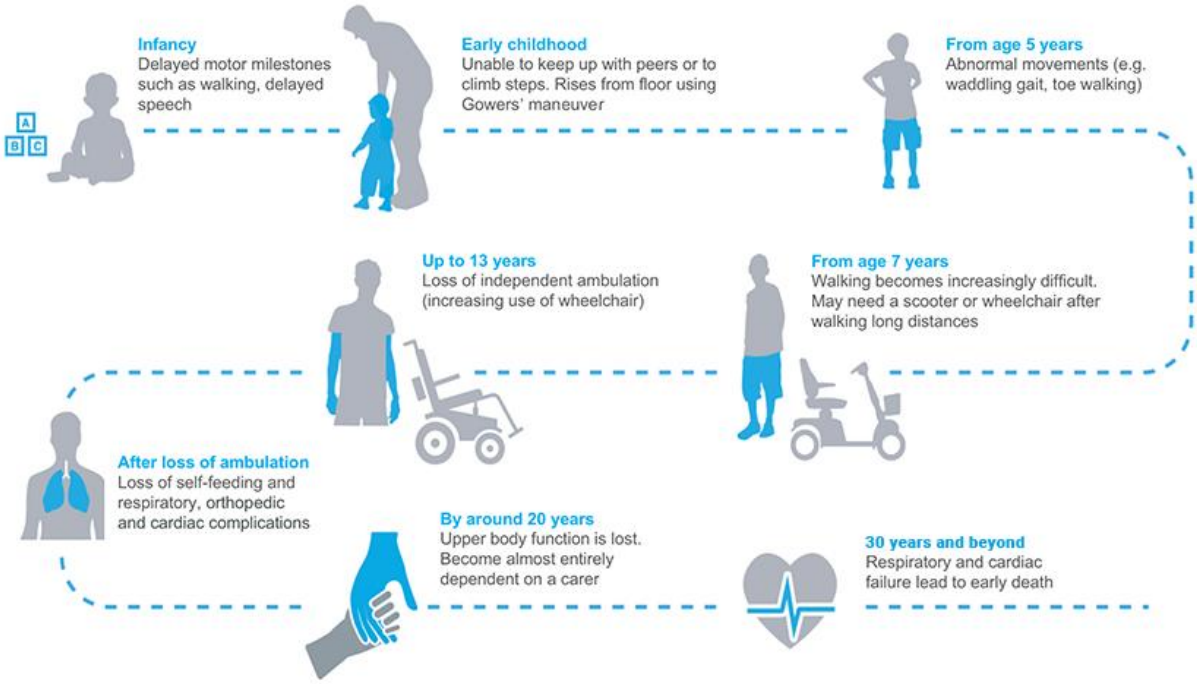

Figure 9: Progression of DMD (Duchenneandyou.com)

Dystrophin is located beneath the sarcolemma and is enriched at the costameres and myotendinous junctions. This protein is an essential component of the dystrophin-glycoprotein complex (DGC) which can be divided into three groups according to their cellular localization: extracellular (α-dystroglycan); transmembrane (β-dystroglycan, sarcoglycans, sarcospan); cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase (nNOS). Dystrophin connects the sarcomeric network, by binding to F-actin, to the extracellular matrix by interacting with other transmembrane and cytoplasmic members of the DGC including dystroglycan, dystrobrevin and syntrophin, which in turn binds to an extracellular matrix protein: laminin (*Figure 10*).

The DGC flexibly connects the inner cytoskeleton of the myofiber to the surrounding extracellular matrix through the sarcolemma. The mechanical role of the DGC is to stabilize the sarcolemma and to protect myofibers from contraction-induced damage, but its transmembrane signaling function renders it fundamental for cell survival. Genetic deletion of dystrophin not only disrupts the DGC but also leads to reduced quantities of other proteins of the complex or associated with, notably sarcoglycans, syntrophin and nNOS which can contribute to dystrophinopathies. Moreover, mutations in almost any of the DGC genes cause various types of muscular dystrophies.

In the absence of dystrophin, the impaired DGC weakens the myofibers which cannot withstand the contraction-induced stress and undergo constant cycles of degeneration and regeneration. The disruption of the myofibers membrane triggers a cascade of events including an influx of calcium and pro-inflammatory cytokines, subsequent activation of proteases and mitochondrial dysfunction resulting in progressive muscle degeneration. The constant repetition of myonecrosis increases oxidative stress and pro-inflammatory immune response which in turn exacerbate each other. This perpetuating «*vicious cycle*» impairs the regenerative

capacity leading to the accumulation of inflammation and fibrosis with loss of muscle mass and function (Gao & McNally, 2015; Grounds et al., 2020; Mah, 2016; Tidball et al., 2018).

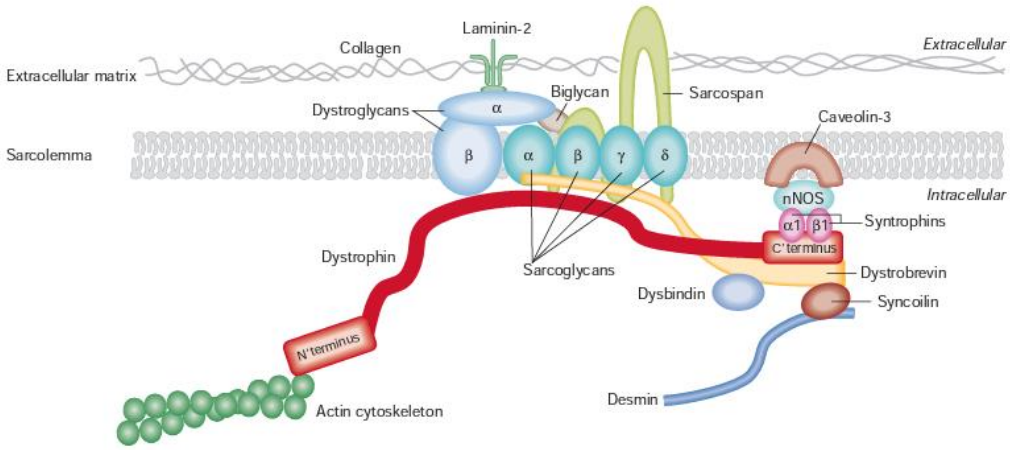

Figure 10: The Dystrophin glycoprotein complex in muscle bonding the internal cytoskeleton to the extracellular matrix (Nowak & Davies, 2004)

2.2 The role of Inflammation in DMD

Inappropriate immune response amplifying the pathology is evidenced in many muscular dystrophies in different ways. Among the numerous cells involved in chronic inflammation, macrophages play a crucial role as they are involved in myogenesis and fibrosis by modulating satellite cell and fibro/adipogenic precursor (FAP) proliferation/differentiation. Macrophages and other myeloid cells rapidly invade damaged tissue and generate free radicals to clear the debris, however, the high concentration and nonspecificity of the free-radical mediated cytolysis augment tissue injury. This has been demonstrated by the early depletion of macrophages as well as through the inhibition of key mediators of inflammation such as NF-κB which resulted in reduced cell lysis and improved pathology in *mdx* mice, the murine model of DMD (Wehling et al., 2001).

M1 macrophages express iNOS which utilizes L-arginine as a substrate to generate NO in high concentrations and for prolonged periods leading to complete alteration of the redox environment and tissue damage. On the contrary, nNOS, a member of the DGC, produces low levels of NO important for cell signaling. After tissue damage in a healthy muscle iNOS, nNOS together with arginase of M2 macrophages compete for their substrate arginine. However, in dystrophic muscle, the secondary loss of nNOS increases arginine availability for iNOS and arginase thus increasing myofiber lysis by M1 macrophages and fibrosis mediated by M2 macrophages. Nonetheless, the M2a subtype expressing arginase, plays an important immunomodulatory role by downregulating the cytolytic activity of iNOS expressing macrophages (De Palma & Clementi, 2012; Tidball & Wehling-Henricks, 2014).

Furthermore, over-activation of M1 macrophages producing higher levels of cytokines such as IL-1β, TNF-α and IFN-γ impedes on myogenic precursor cell proliferation. Additionally, the continuous stimulation of myoblasts via IFN-γ suppresses the genes

responsible for terminal differentiation (Cappellari et al., 2020). M1 macrophages inhibit IL-4 and block M2 proliferation, which in turn downregulates the pro-myogenic hormone Klotho involved in the expansion of myogenic cell populations. Moreover, the hybrid macrophages found in dystrophic muscle, with high expression of both TNF-α and TGF-β, are unable to reduce the buildup of FAPs in the damaged muscle (Lemos et al., 2015).

The muscles of *mdx* mice undergo a period of successful muscle regeneration before the continuous degradation. This could be explained by the transition in the macrophage phenotype in *mdx* mice from the cytolytic M1 to the repair promoting M2 between the phase of muscle necrosis at 4 weeks of age and muscle regeneration at 12 weeks of age (*Figure 11*). This switch is orchestrated by increased expression of IL-4 and IL-10, which deactivate the M1 subtype while promoting activation of M2c phenotype via CD163. Pro-inflammatory TNF suppresses CD163 expression, but the anti-inflammatory IL-10 induces it. In turn, the binding of hemoglobin-haptoglobin, present in high quantity in damaged tissue, to CD163 elicits IL-10 release, which is involved in the induction of the antioxidant heme oxigenase 1 (HO-1) (Abraham & Drummond, 2006). The increased levels of IL-10 resulting from this feedback system can dampen the M1 phenotype and inhibit NF-κB signaling thus decreasing iNOS expression. This role played by IL-10 as a switch from M1 to M2 macrophages is determinant for efficient regeneration, notably to promote satellite cell proliferation.

The mechanisms governing the shift from M1 to M2 macrophages are still not clear, however, a candidate recently emerged: regulatory T cells (Treg). These cells were found elevated in DMD and *mdx* muscle and have the capacity to modulate the immune response by secreting IL-10. Depletion of Treg cells worsens muscle damage and enhances IFN-γ response along with activation of M1 Macrophages (A. S. Villalta et al., 2014).

These findings bring to light the potential of therapeutic interventions aimed at modulating the balance between M1 and M2 macrophages, notably by deactivating the pro-inflammatory phenotype to influence the course of muscular dystrophy.

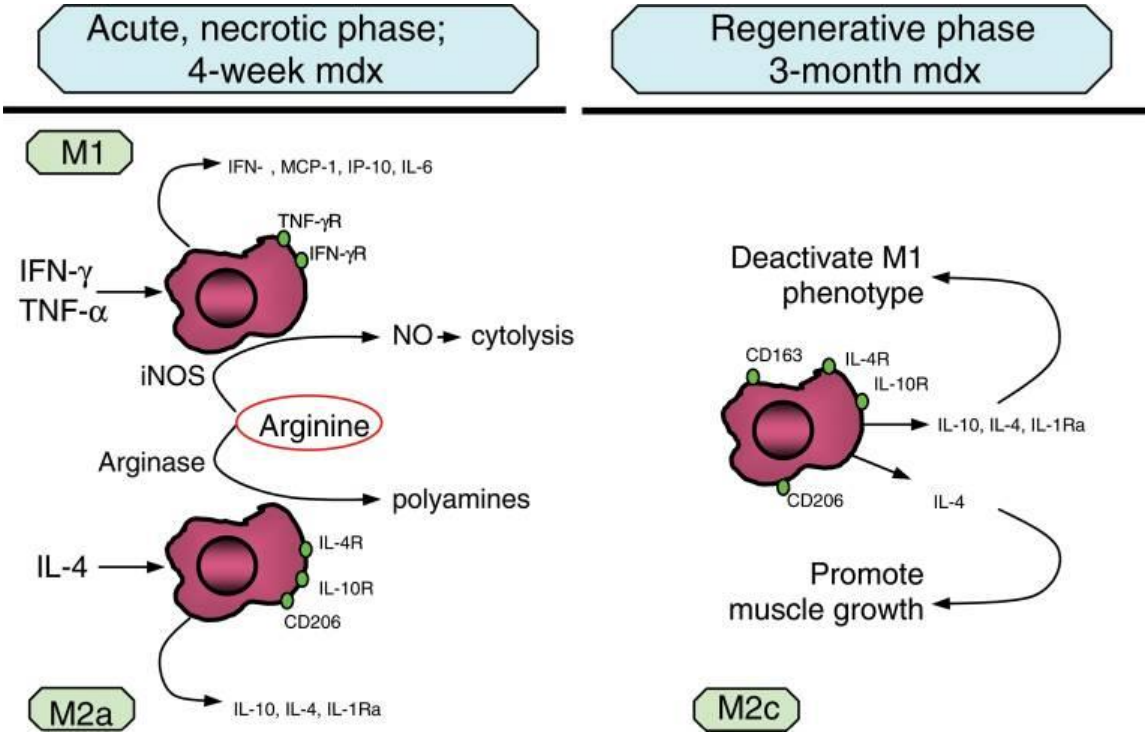

Figure 11: Diagram representing the macrophage phenotypes that dominate the peak phase of necrosis and the regenerative phase (S. A. Villalta et al., 2009)

2.3 The role of Oxidative Stress in DMD

Reactive oxygen species (ROS) are highly reactive molecules due to the presence of one or more unpaired electrons. They are produced under physiological conditions due to the partial reduction of molecular oxygen and are involved in many cellular processes including cell growth, immunity, necrosis, apoptosis, protease activity and gene expression.

As it is a highly metabolic tissue, the skeletal muscle constantly produces moderate levels of ROS and reactive nitrogen species (RNS). To maintain the levels of free radicals within hormetic ranges, the skeletal muscle is equipped with a sophisticated antioxidant system. The endogenous antioxidant defense system can work directly by scavenging ROS through the expression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), or indirectly by inducing the cytoprotective (phase II) response, i.e. HO-1, NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), γ-glutamylcysteine synthetase (GCS) (Kozakowska et al., 2015).

However, if ROS formation exceeds the physiological buffering capacity either due to ROS overproduction or failure to remove them by antioxidant defenses, this results in Oxidative Stress. This condition can lead to cell dysfunction and death due to DNA damage, protein oxidation, and lipid peroxidation; and is tightly correlated with myofiber necrosis and inflammation in DMD (Grounds et al., 2020). As oxidative stress is an important mediator of DMD, patients present an evident increase of oxidation (Tidball & Wehling-Henricks, 2007). Dystrophic muscles may even be more susceptible to oxidative damage according to the

increased cell death response of *mdx* myotubes to free radical-induced injury but not for other forms of metabolic stress (Rando et al., 1998).

Proposed sources of oxidative stress in DMD include inflammatory cells, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), decoupling of NOS, impaired mitochondrial function along with insufficient stress response. Myeloperoxidase is mainly secreted by neutrophils and to a lesser extent by monocytes and macrophages in the area of inflammation. *Mdx* muscles exhibit an increased activity of this enzyme. This heme protein oxidizes chloride in the presence of hydrogen peroxide to form the highly reactive oxidant hypochlorous acid, capable of causing tissue damage (Terrill et al., 2016).

Pro-inflammatory cytokines such as IL-1β, TNF-α and Oxidative Stress activate the transcription factor NF-κB through the subsequent phosphorylation, polyubiquitination and proteasomal degradation of the inhibitor protein IκB (I kappa B). NF-κB then translocates to the nucleus where it binds with DNA and amplifies the generation of pro-inflammatory mediators and ROS notably by regulating NOX activity (Gauss et al., 2007; Gius et al., 1999; Wong & Tergaonkar, 2009). Indeed, increased levels of IL-1β, TNF-α and NF-κB are reported in DMD and *mdx* mice (Lawler, 2011).

NADPH oxidase is an enzyme complex utilizing NADPH as a substrate to convert molecular oxygen to ROS, generally superoxide or hydrogen peroxide. Although NOX is highly expressed in inflammatory cells, including neutrophils and macrophages, three isoforms of NOX are present in the skeletal muscle: NOX1, NOX2 and NOX4. Increased levels of NOX2 subunits are found in muscles of *mdx*, even prior to the necrotic state, thus before evidence of muscle damage or inflammation, and also in cultured primary myotubes from *mdx* mice. Moreover, also in pre-necrotic *mdx* mice, there is an increase in the expression of endogenous antioxidants, such as SOD and catalase, and in the levels of lipid peroxidation and oxidized GSH, which are used as markers of oxidative stress (Disatnik et al., 1998; L. F. Ferreira & Laitano, 2016). These findings put forward the causative role of oxidative stress in the pathology independently of inflammation. The consequential loss of nNOS from the DGC has been reported to increase NOX activity, inflammation, and protein degradation, and to alter satellite cell activation (Lawler, 2011).

The Nrf2 signaling pathway plays a pivotal role in oxidative stress and inflammation. Under normal conditions, Nrf2 is kept inactive by being bound to its cytoplasmic endogenous inhibitor Keap1, which promotes its ubiquitination and degradation. Upon exposure to stress signals such as free radicals, the conformational change of Keap1 induces its dissociation from Nrf2 which in turn translocates to the nucleus. There, Nrf2 binds to antioxidant-related elements (ARE) and modulates the expression of a myriad of defensive genes, including those encoding for SOD, CAT, NQO1 and HO-1. HO-1 is responsible for the synthesis of biliverdin and bilirubin, which are non-enzymatic antioxidants present in the skeletal muscle.

Nrf2 targets inflammation by downregulating NF-κB, and binding in the vicinity of inflammatory cytokine genes such as IL-1β and IL-6, leading to their transcriptional inhibition (Kobayashi et al., 2016). HO-1 is at the core of Nrf2-mediated NF-κB inhibition by catalyzing the degradation of heme into carbon monoxide (CO), Fe^{2+} and biliverdin which is consequently reduced to the antioxidant bilirubin. The Nrf2 pathway is activated in dystrophic patients to counteract oxidative stress, however low levels of HO-1 coupled with high IL-6 concentrations disrupt the compensatory mechanism and exacerbate inflammation (Petrillo et al., 2017).

Moreover, decreased expression of HO-1 worsen muscular dystrophy while pharmacological induction of HO-1 improves the phenotype of *mdx* mice (Chan et al., 2016).

Nonetheless, with its association in the regulation of over 600 target genes, the Nrf2 signaling pathway is also involved in prolonging satellite cell proliferation by upregulating MyoD and downregulating Myogenin, calcium handling, mithormesis, autophagy and heat shock proteins. With its ability to tackle simultaneously numerous cellular processes, Nrf2 is a particularly interesting therapeutical target for the multisystemic disorder DMD (Kourakis, Timpani, de Haan, et al., 2021; Pietraszek-Gremplewicz et al., 2018) (*Figure 12*).

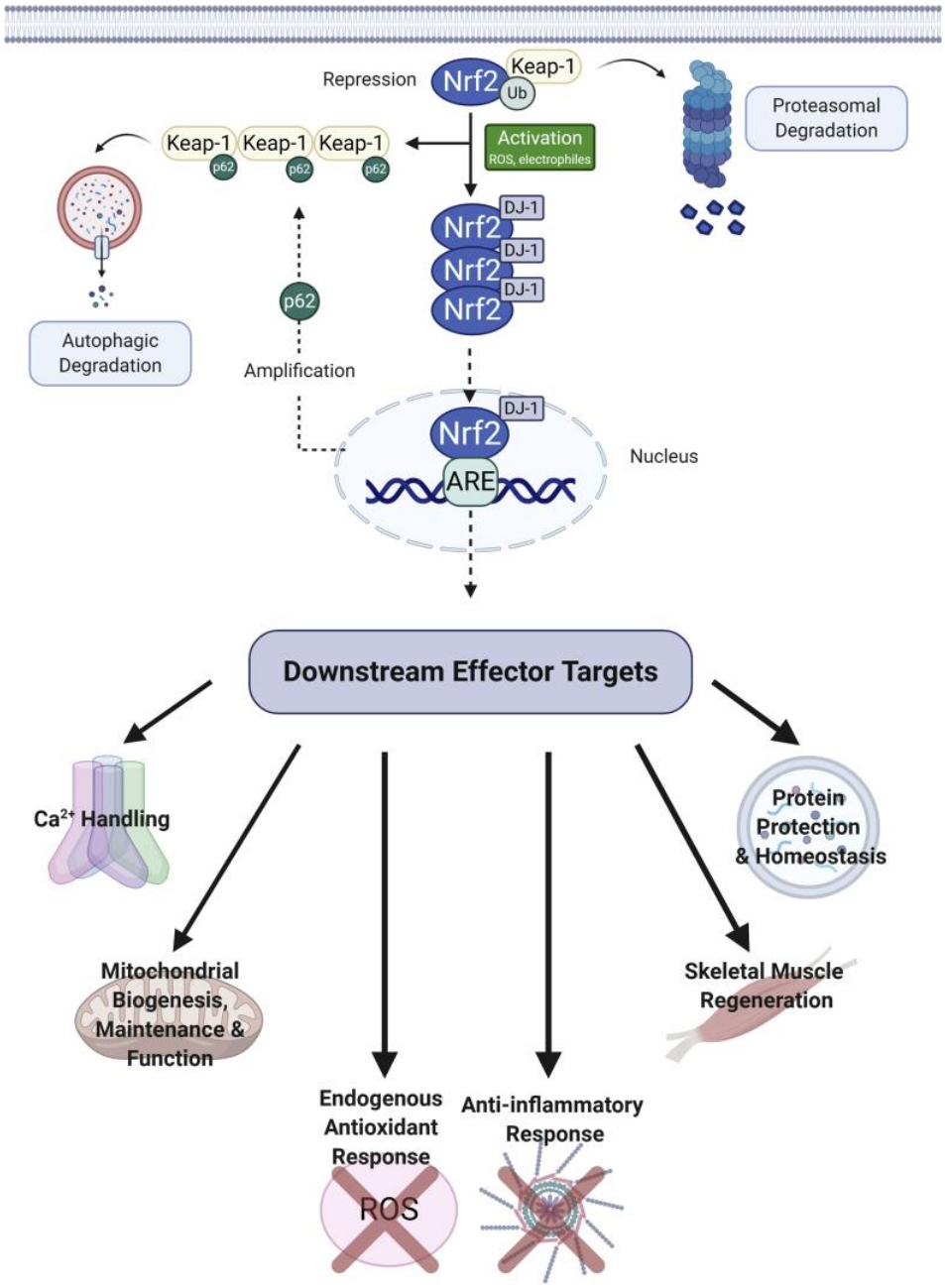

Figure 12: Nuclear erythroid 2-related factor (Nrf2) signaling pathway and its downstream target effects (Kourakis, Timpani, de Haan, et al., 2021)

2.4 Current therapeutical approaches to DMD

Among the 325 studies on DMD registered in the database of the U.S. National Library of Medicine there are currently 26 ongoing clinical trials with diverse interventions (https://clinicaltrials.gov/ct2/results/details?cond=Duchenne+Muscular+Dystrophy, ClinicalTrials.gov, 2021).

Since the core of DMD is a genetic mutation causing the absence of functional dystrophin protein, restoring its function or expression with gene-directed techniques is the primary approach, and has been extensively studied (*Table 1, Figure 13*). These efforts even lead to commercially available gene-based approaches targeting specific DMD mutations, thus providing help only to a small subgroup of patients.

Despite the exponential progress in this field, finding the ultimate cure for DMD will be extremely challenging. The first obstacle resides in the targeted tissue: the skeletal muscle, which is highly abundant, postmitotic and wrapped in layers of connective tissue which hamper the delivery of expression vectors such as viral particles or stem cells. Even if we achieve to restore the expression of dystrophin, the loss of skeletal muscle starts very early and cannot be restored with the technologies currently available. There is thus a pressing need for a rapid diagnosis and intervention.

Another major obstacle is the host immune response, which can be triggered by the vector but also the delivered protein, so the combination with another agent able to manage the immune system is an attractive therapeutic strategy. Therefore, there is also an important focus in targeting other pathways involved in the pathology of this multisystemic disease, that, even if not curative, can improve the quality of life and slow down the progression of the disease (*Table 2, Figure 13*). Considering that inflammation and oxidative stress are detrimental in many instances, pharmacological approaches targeting these processes could potentially synergize with gene-based approaches and other pathologies (Mercuri et al., 2019; Sun, Shen, et al., 2020; Yao et al., 2021).

Therapeutic strategies	Mechanism of action	Chemistry	Drug route	Current stage
Exon skipping				
Golodirsen	Exon 53 skipping	Antisense oligonucleotides	Intravenous	Approved (FDA)
Eteplirsen	Exon 51 skipping	Antisense oligonucleotides	Intravenous	Approved (FDA)
Viltolarsen	Exon 53 skipping	Antisense oligonucleotides	Intravenous	Approved (FDA)
Casimersen	Exon 45 skipping	Phosphorodiamidate morpholino oligomer	Intravenous	Phase II/III
SRP-5051	Exon 51 skipping	Peptide-conjugated phosphorodiamidate morpholino oligomer	Intravenous	Phase II
DS-5141b	Exon 45 skipping	2 ENA antisense	Intravenous	Phase I/II
Stop codon readthrough				
Ataluren	Readthrough strategy of nonsense mutations	Small molecule	Oral	Approved (EMA), confirmatory Phase III,
NPC14(Arbekacin Sulfate)	Readthrough strategy of nonsense mutations	Small molecule	Intravenous	Phase II
Gene addition				
PF-06939926	AAV9 gene therapy	Recombinant adeno-associated virus and codon-optimized human micro-dystrophin	Intravenous	Phase III
rAAVrh74.MHCK7	AAV9 gene therapy	Recombinant adeno-associated virus and codon-optimized human micro-dystrophin	Intravenous	Phase I/II
SGT-001	AAV9 gene therapy	Recombinant adeno-associated virus and codon-optimized human micro-dystrophin	Intravenous	Phase I/II
Genome editing				
CRISPR-Cas9	Removes DNA encoding a specific target exon	AAVs- CRISPR-Cas9 system	–	Pre-clinical
Protein replacement				
C1100 (Ezutromid)	Upregulation of utrophin	Small molecule	Oral	Phase II
rAAVrh74.MCK.GALGT2	Upregulation of utrophin	Recombinant adeno-associated virus and GALGT2 gene	Intravenous	Phase I/II
Myoblast transplantation				
Donor-derived myoblasts	Fuse with host muscle fibers	Myoblasts grown	Intravenous	Phase I/II

Table 1: The current therapeutic strategies targeting the primary cause of DMD and their status (Yao et al., 2021)

Therapeutic strategies	Mechanism of action	Chemistry	Drug route	Current stage
Anti-fibrotic				
Pamrevlumab	Monoclonal anti-CTGF antibody	Antibody	Intravenous	Phase II
Losartan	Angiotensin II type 1 receptor blocker	Small molecule	Oral	Phase II
Halofuginone	Inhibitor of collagen a1 and MMP2	Small molecule	Oral	Phase I/II (Suspended)
Infliximab	Antibody to human TNF-α	Antibody	–	Pre-clinical
Suramin	Inhibits TGF-β	Small molecule	–	Pre-clinical
Imatinib Mesylate (Gleevec)	Inhibits TGF-β	Small molecule	–	Pre-clinical
Anti-inflammatory				
Corticosteroids (Prednisone, Prednisolone, and Deflazacort)	NF-κB inhibition	Small molecule	Oral	Phase III
Edasalonexent	NF-kB inhibition	Small molecule	Oral	Phase III
Vamorolone (VBP-15)	NF-kB inhibition	Small molecule	Oral	Phase II
Increlex (R)	Recombinant IGF-1	Fusion protein	Subcutaneous	Phase II
TAS-205	Hematopoietic prostaglandin D synthase inhibitor	Small molecule	Oral	Phase II a
Flavocoxid	NF-kB inhibition	Small molecule	Oral	Phase I
Givinostat	Histone deacetylase (HDAC) inhibitor	Small molecule	Oral	Phase I/II
Tamoxifen	Estrogen receptor modulator	Small molecule	Oral	Phase III
Reduction of muscle damage				
Ca^{2+} dysregulation				
Rimeporide	Sodium–hydrogen exchanger 1 inhibitor	Small molecule	Oral	Phase Ib
AT-300	Blocks mechanosensitive Ca^{2+} channels	Small molecule	–	Pre-clinical
Recombinant Mitsugumin 53	Facilitates membrane repair at sites of injury	Recombinant proteins	–	Pre-clinical
BGP-15	Hsp72 inducer	Small molecule	–	Pre-clinical
Streptomycin	Nonspecific Ca^{2+} channel blocker	Small molecule	–	Pre-clinical
Oxidative stress				
Coenzyme Q10	Electron acceptor for NADH and succinate dehydrogenase	Small molecule	Oral	Phase III
Idebenone	Antioxidant	Small molecule	Oral	Phase III
N-acetylcysteine	Endogenous antioxidant	Small molecule	–	Pre-clinical
Muscle ischemia				
Sildenafil	PDE5 inhibitor	Small molecule	Oral	Phase I/ II
L-Arginine	Metabolic support	Recombinant proteins	Oral	Phase I
Tadalafil	PDE5 inhibitor	Small molecule	Oral	Phase III (Terminated)
Muscle atrophy				
GLPG0492	β2-Agonist, increases cAMP	Small molecule	Oral	Phase I
Urocortin	Increases cAMP	Ca^{2+}-independent phospholipase A2 type β	–	Pre-clinical
rAAV1.CMV.huFollistatin344	Delivery of follistatin using adeno-associated virus	AAV1-Follistatin	Intramuscular	Phase I/II
Bone homeostasis				
Zoledronic acid	Inhibits bone resorption	Small molecule	Intravenous	Phase III
Alendronate (ALN)	Improves bone mineral density	Small molecule	–	Pre-clinical

Table 2: The current therapeutic strategies targeting the secondary pathology of DMD and their status (Yao et al., 2021)

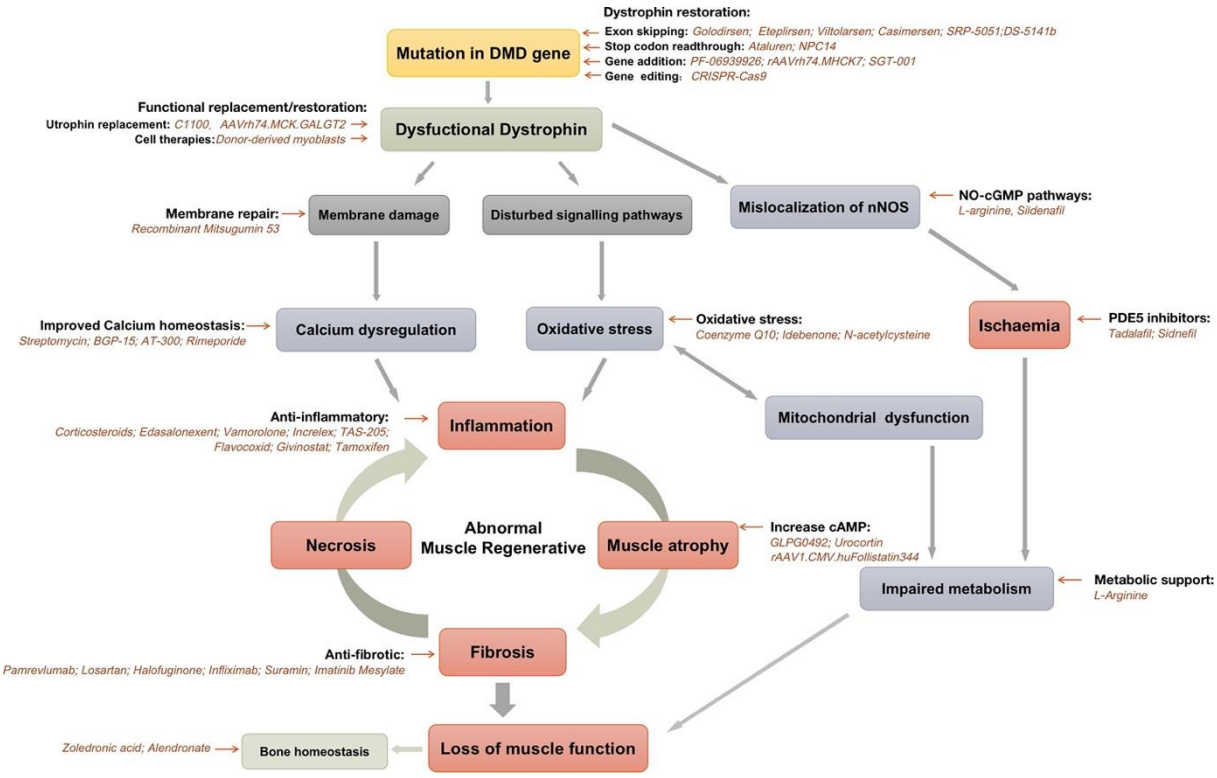

Figure 13: Schematic representation of the current pharmacological approaches for DMD (Yao et al., 2021)

Corticosteroids

In the absence of a cure, the glucocorticosteroids with prednisone/prednisolone and deflazacort represent the gold standard treatment of DMD. Their implementation in the 1990s has helped to prolong ambulation and life expectancy, nonetheless their mechanisms of action still remain to be fully elucidated. The beneficial outcome of corticosteroids to treat a primary muscle disease is even paradoxical considering they are also known to trigger muscle atrophy (Szabo et al., 2021).

In muscular dystrophy, corticosteroids are believed to act as immunosuppressants as they result in a significant reduction of the inflammatory infiltrate in the muscle. However, their range of effects is broad as they have been reported to affect myogenesis and metabolism, reduce proteolysis and myonuclear apoptosis as well as increase the expression of utrophin, a dystrophin homolog. They can improve muscle performance via the Krüppel-like factor 15 and sarcolemmal repair by direct upregulation of Annexin 1 and 6.

Glucocorticoids diffuse through the cell membrane and form a receptor-ligand complex by binding to the cytoplasmic nuclear hormone receptor, glucocorticoid receptor (GR), to then translocate to the nucleus. Here, glucocorticosteroids exert their anti-inflammatory action through the process of trans-repression or trans-activation by binding to the NF-κB binding element or glucocorticoid response element (GRE), respectively. However, if the complex binds to negative GRE, this cis-repression triggers the mediation of adverse effects (Kourakis, Timpani, Campelj, et al., 2021).

Unfortunately, the chronic treatment with high dosage of corticosteroids comes along with tremendous side effects including important weight gain, cushingoid appearance, stunted growth, osteoporosis, and behavioral changes. Recent findings indicate that intermittent steroid dosing as opposed to daily administration provides the benefits of glucocorticoids with a lower profile of adverse effects. There is no clear evidence whether prednisone or deflazacort gives a better outcome (Quattrocelli et al., 2017).

Currently, Vamolorone, a first-in-class dissociative steroid, is under investigation in a phase II trial (Vamolorone: NCT05185622) and is very promising. Since it induces primarily the trans-repression effect, it bypasses most adverse effects of the other corticosteroids (Verhaart & Aartsma-Rus, 2019).

Histone deacetylase inhibitors

Histone deacetylases (HDAC) are enzymes that lead to compacted and transcriptionally repressed chromatin by removing acetyl groups from histone. Conversely, Histone deacetylase inhibitors (HDACi) promote genetic transcription and represent the first generation of epigenetic drugs (Delcuve et al., 2012). Dystrophin deficient mice display a deregulated HDAC activity, such as HDAC2, which perturbs the transcriptional outcome of the myofibers and can be restored by HDACi treatment. HDACi promote myogenesis *in vitro* and *in vivo* and have a positive outcome in *mdx* mice (Consalvi et al., 2014). They specifically target FAPs by blocking their adipogenic potential and promoting satellite cell differentiation in young dystrophic mice. However, once mice are old their resistance to respond to HDACi is detrimental (Mozzetta et al., 2013).

The HDACi have been reported to induce the expression of follistatin, increase the myofiber size and reduce fibrosis and fatty tissue. Moreover, the progressive alteration of mitochondrial biogenesis associated to the increased deacetylation of peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) promoter in *mdx* mice is sensitive to Givinostat (Consalvi et al., 2011, 2014; Giovarelli et al., 2021). A phase III study of Givinostat in ambulatory DMD patients is currently ongoing (Givinostat: NCT03373968).

Exon Skipping

Based on the idea that most patients with DMD could in theory produce a Becker muscular dystrophy (BMD)-like dystrophin (in-frame deleted protein), if the reading frame would be corrected, the exon-skipping strategy aim to do so by hiding an exon from the RNA splicing machinery of the cell (Aartsma-Rus et al., 2017; Echevarría et al., 2018). This approach uses antisense oligonucleotides (AONs), which are small pieces of 20-30bp of modified RNA or DNA, to bind a specific sequence in the targeted exon in the pre-mRNA dystrophin transcript. By masking splicing signals on the RNA, AONs hybridization leads to the exclusion of both the intron and its adjacent exon. An in-frame RNA is generated by skipping the targeted exon and consequently a truncated but still partially functional protein can be translated. An important obstacle to the AON approach is that it is mutation-specific, as different mutations require the skipping of the appropriate exons to correct the reading frame. Thus, a particular

exon skipping provides treatment to only a small group of patients (Aartsma-Rus & van Ommen, 2007).

The FDA approved in 2016 Eteplirsen, a phosphorodiamidate morpholino oligomer (PMO) AON skipping exon 51. Afterwards, the FDA approved provisionally Golodirsen (FDA 2019) and Vilotarsen (FDA 2020), both drugs targeted for DMD exon 53, and casimersen (FDA 2021), targeted for exon 45.

If Eteplirsen was able to show its safety and functional benefits, even in the long-term, its approval remains controversial due to its minor production of dystrophin. Indeed, challenges of unmodified PMOs include the pharmacokinetics with the limited cellular uptake, especially insufficient in the heart (Q. L. Lu et al., 2011), the fast clearance from systemic circulation and the short-term efficacy (Sun, Shen, et al., 2020). As such, repetitive high doses are required for clinical use (Moulton & Moulton, 2010). To overcome these issues, Eteplirsen has been conjugated with a proprietary cell-penetrating peptide, SRP-5051 (Tsoumpra et al., 2019). This peptide-conjugated Eteplirsen is currently in phase II study (Eteplirsen: NCT03985878), however, toxicity concerns are growing (Sheikh & Yokota, 2021).

Stop Codon Readthrough

Nonsense mutations represent 10% of total mutations analyzed in DMD patients (Bladen et al., 2015). This point mutation changes an amino acid codon into a stop codon, causing the premature halting of translation, which leads to the production of non-functional dystrophin. The concept of read-through therapies is to use a small molecule to interact with the ribosome, which forces the insertion of an alternative amino acid at the point of the premature stop codon to allow the translational read through, leading to the generation of relatively functional dystrophin.

If the aminoglycoside antibiotic gentamicin was shown to have readthrough abilities, its overall effectiveness is unclear and its chronic use has been prohibited due to its toxicity (Bertoni & Namgoong, 2016; Vitiello et al., 2019). To reduce the side effects of gentamicin, another antibiotic negamycin and its synthetic analogues were developed, such as TCP-1109 (13x) in 2019, showing a promising potential (Hamada et al., 2019).

A small oxadiazole derivative administered orally, Ataluren, has been granted conditional approval by the EMA but has been rejected by the FDA. If Ataluren seems well tolerated, its efficacy has been inconclusive as although it showed functional improvement, there are no reports demonstrating dystrophin restoration (Berger et al., 2020; Mercuri et al., 2020). An ongoing phase II study estimated to be completed in 2022 aims to assess the safety and pharmacokinetics of Ataluren (Ataluren: NCT04336826).

Gene-addition therapy

The transfer of a functional dystrophin gene in dystrophic muscles as a potential treatment has been suggested as soon as the genetic cause of the disease was identified. However, this approach presents many challenges. Most viral vectors do not efficiently infect skeletal muscle apart from the adeno-associated virus (AAV). AAV happens to be very small and with a limited transgene capacity while the dystrophin gene is enormous. A potential

solution is inspired by BMD, which is generally caused by large in-frame deletions in the dystrophin gene. This generates small dystrophins which are partly functional, as they still contain critical binding domains resulting in a much milder phenotype compared to DMD. Accordingly, micro-dystrophin has been engineered to fit in the AAV vector and is currently under clinical trials, but lacking the C-terminal domain, it cannot fully restore the function of WT dystrophin. The mini-dystrophin gene comprises all functional domains and has a better bioactivity than micro-dystrophin but exceeds the packing capacity of the AAV vector. The trans-splicing AAV system allowing to double or triple the AAV packing size has been developed. This method consists in independent AAV vectors containing sequential parts of the dystrophin gene which can associate to generate the full-length protein (Koo et al., 2014).

Although progress has been achieved since this extraordinary idea was first conceived, there are still pending challenges including: the limited transfection efficiency and longevity, the widespread distribution of the targeted tissue and the management of the immune system. The immune responses triggered by both the viral capsid and the delivered proteins are major safety concerns, especially in high dose delivery. Using different AAV serotypes and miniaturized utrophin (μUtro) are interesting tracks to overcome immunogenic issues (Y. Song et al., 2019; Sun, Shen, et al., 2020; Yao et al., 2021).

Gene Editing

CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 (CRISPR associated protein) system consists of an endonuclease Cas9 and a single-guide RNA (sgRNA) and represents a powerful tool to rectify genetic abnormalities in DMD. The CRISPR/Cas9 technology induces double-stranded DNA breaks at specific places of the editing sequence targeted by sgRNA to activate DNA repair system. Subsequent nonhomologous end-joining (NHEJ) leads to exon-skipping to restore the reading frame, while homology-directed repair (HDR) could replace DMD mutations with correct sequences and produce normal dystrophin (Babačić et al., 2019). However, if NHEJ-mediated exon deletion has a higher error rate than HDR-mediated accurate genome correction, the latter is usually less efficient than its counterpart. To tackle this issue, two small molecules, Brefeldin A and L755507, have been found to improve HDR genomic correction efficiency (Yu et al., 2015). Moreover, sgRNA may locally match to other nontargeted sequences and lead to off-target effects due to the extreme complexity of the genome. Aiming to limit the off-target exposure, scientists have adjusted the length and improved the stability of guide RNA. Different types of Cas9 endonucleases derived from diverse sources have been reported to enhance targeting specificity (Cho et al., 2014; Fu et al., 2014; C. M. Lee et al., 2016; Müller et al., 2016; Ran et al., 2015). Furthermore, with the assistance of the more specific endonuclease FokI to construct a fused endonuclease complex with Cas9, gene editing was achieved with low off-target effects (Terao et al., 2016).

60% of DMD patients could potentially benefit from CRISPR/Cas9 gene-editing delivered by AAV vectors, however, its efficacy is highly dependent on the dose of AAV posing the obstacle of the excessive immune response (Y. Zhang et al., 2020). More research is needed to elucidate the mechanism and frequency of off-target effects as well as to generate more specific Cas9 enzymes (Hollinger & Chamberlain, 2015; Zheng et al., 2018).

Stem cell therapy

The idea of transplanting stem cells to treat muscular dystrophies emerged even before the genetic causes were identified. The rationale of this technique is to inject normal muscle precursor cells in dystrophic muscles to replenish the satellite cell pool with dystrophin competent cells. (Sun, Serra, et al., 2020). The optimal cell source can dispense a copy of the normal DMD gene and act on both skeletal and cardiac muscles. Other than satellite cells, different types of adult stem cells such as $CD133^+$ cells, bone marrow-derived side population cells, mesoangioblasts, and adipose-derived stem cells, were reported to participate in the regeneration of dystrophic muscle (He et al., 2020; Yao et al., 2021). The transplantation of satellite cells from muscle biopsies was found to increase the expression of dystrophin in *mdx* mice (X. Xu et al., 2015). If the multipotent stem cells derived from normal human skeletal muscles, and the $CD133^+$ cells were found to have myogenic potential (Meng et al., 2014), both, however, showed no obvious functional benefits in DMD patients (Meng et al., 2018; Torrente et al., 2007). Human-induced pluripotent stem cell (hiPSC) is a promising cell source for reconstructing dystrophic muscles (Danisovic et al., 2018). The hiPSCs are retrieved from the dystrophic patients and can be genetically corrected with CRISPR/Cas9 technique to restore intact dystrophin expression (Hochheiser et al., 2018).

Despite the apparent engraftment of these cell-based therapies in *mdx* mice (Young et al., 2016), because of the insufficient capacities of these stem cells to survive and migrate along with the additional tumorigenic risk and immune rejection, more research is needed before the possible clinical application (Y. Jin et al., 2020). An ongoing phase I/II clinical trial, estimated to be completed in 2024, is investigating whether the intramuscular transplantation of normal myoblast is safe and can enhance the strength of the injected muscle (Myoblast Transplantation: NCT02196467).

Chapter 3: Antioxidants as potential pharmacological strategy for DMD

As Oxidative stress is a hallmark of DMD, various antioxidant therapies have been investigated to counteract the elevated reactive oxygen and nitrogen species (RONS) generation associated with myonecrosis and inflammation (Allen et al., 2016; Datta & Ghosh, 2020; Lawler, 2011). Antioxidant treatment of *mdx* mice showed benefits in maintaining muscle mass and function, while reducing oxidative stress and inflammation (https://doi.org/10.1016/j.redox.2020.101803). Most of the compounds tested showed a direct ROS-scavenging activity, as well as the ability to regulate molecules involved in the generation of RONS.

Some of them have even reached the clinical phase of drug development. Coenzyme Q10 (CoQ10) binds the inner mitochondrial membrane and functions as an electron acceptor of the respiratory chain and as a modulator of the mitochondrial transition pore, reducing calcium accumulation. CoQ10 added to prednisone treatment led to an increase in muscle strength in DMD patients (Papucci et al., 2003; Spurney et al., 2011). Epigallocatechin gallate, the predominant polyphenol found in green tea and Epicatechin, a cacao flavonoid are both ROS scavengers and potent inducers of mitochondrial biogenesis (Nakae et al., 2012; Ramirez-Sanchez et al., 2014) which have just completed phase II/III and I/II study, respectively (Epicatechin: NCT02964377; Epigallocatechin-Gallate: NCT01183767).

3.1 N-Acetylcysteine

N-Acetylcysteine (NAC) is a widely used dietary antioxidant mostly due to its role as a precursor of L-cysteine that is necessary for the synthesis of glutathione (GSH) rather than as a direct ROS scavenger through its thiol group (Ezeriņa et al., 2018). Pre-clinical studies on *mdx* mice have shown that NAC administration can alleviate the dystrophic pathology in both skeletal and cardiac muscle. Taken together, NAC was found to improve muscle strength, notably for the diaphragm in which it was also able to increase Nrf2 expression and reduce lipid peroxidation, TNF-α, IL-1β and fibrosis. NAC was able to protect from exercise-induced damage and myonecrosis, improve calcium handling and reduce NF-κB activation. NAC administration also increased the sarcolemmal expression of β-dystroglycan and utrophin, a close homologue of dystrophin. This not only shows the efficacy of this amino acid but also reinforces the role of oxidative stress in the pathology by dysregulating dystrophin-associated proteins (Burns et al., 2019; de Senzi Moraes Pinto et al., 2013; Fauconnier et al., 2010; Terrill et al., 2012; Whitehead et al., 2008).

NAC has yet to be tested in DMD patients despite the impressive pre-clinical data. However, a recent study put forward an unexpected significant suppression of body mass gain (Pinniger et al., 2017). In this study, *mdx* mice received the dose of 2% NAC in drinking water, still without being accompanied by an elevation in cysteine levels which would be an indication of toxicity. Although a reduced body mass gain could be considered an advantage in lessening

muscle stress in dystrophy, it should be investigated with extreme caution (O'Halloran et al., 2018). Moreover, if NAC supplementation is not efficient in all models to prevent skeletal muscle dysfunction (Farid et al., 2005).

3.2 Quercetin

Quercetin is a safe polyphenolic flavonoid abundantly present in fruits and vegetables. This compound has potent antioxidant, anti-inflammatory, and PGC-1α pathway activating properties and has already been shown to help in the prevention of various diseases such as cancer, cardiovascular diseases, and osteoporosis (Boots et al., 2008; Hsu et al., 2021).
Quercetin exerts its antioxidant activity mostly by scavenging free radicals, regulating glutathione levels, enzymatic activity, signal transduction pathways and chelating transition metal ions. This compound prohibits key enzymes associated with oxidative stress including NOX (NOX1, NOX2 and NOX4) and Xanthine oxidase while upregulating levels of phase II antioxidant enzymes such as SOD, catalase, GSH peroxidase, HO-1 and NQO1.
Quercetin has been reported to improve the Nrf2-ARE signaling pathway and to inhibit p38MAPK/iNOS and NF-κB signaling pathway (Liu & Guo, 2015; Mohammadtaghvaei et al., 2021; Tanigawa et al., 2007; D. Xu et al., 2019).

In *mdx* mice, chronic Quercetin supplementation improved endogenous antioxidant protein and mitochondrial content by elevating levels of SOD2, GPX, and PGC-1α in the heart. Quercetin administration provided cardioprotection against pathological inflammation, notably by lowering macrophage infiltration and lowering NF-κB and COX2 content, and facilitated DGC assembly although no benefits were observed regarding cardiac function (Ballmann et al., 2017). Quercetin alleviated disease-related muscle injury in *mdx* diaphragm by enhancing the ratio myofiber/area along with fewer centrally nucleated fibers and infiltrating immune cells, however, muscle function was not examined in this work (Hollinger et al., 2015). In a different study, quercetin enrichment enhanced skeletal muscle function and spontaneous physical activity despite similar degrees of muscle damage (Spaulding et al., 2016).

3.3 Plumbagin

Plumbagin is a bioactive naphthoquinone mainly isolated from the root of *Plumbago zeylanica* used in traditional Asian medicine in various cases including infections, and hepatic disorders. Besides being a strong antioxidant, Plumbagin also has anti-inflammatory, antitumor and antibacterial activities (Panichayupakaranant & Ahmad, 2016; Tilak et al., 2004).

Plumbagin protects from oxidative stress by inducing the Nrf-2 pathway along with the downstream activation of glutathione and phase II antioxidant enzymes such as catalase, SOD, GSH peroxidase and S-transferase, NQO1, and HO-1 (Kumar et al., 2013; K. H. Wang & Li, 2018). Concomitantly, Plumbagin prevents the formation of free radicals, scavenges them, and inhibits lipid peroxidation. Plumbagin can also dampen NOX4 activity in macrophages *in vivo* and in *vitro* (Z. Zhang et al., 2016).

Regarding its anti-inflammatory effect, Plumbagin inhibits NF-κB, cytokines including TNF-α, IL-1β and IL-6 as well as pro-inflammatory mediators including iNOS and COX2 (Luo et al., 2010).

Collectively, these properties of Plumbagin have been demonstrated *in vivo* on spinal cord injury-induced oxidative stress and inflammation in rats (W. Zhang et al., 2014), rat paw edema induced by carrageenan (Luo et al., 2010), mice infected with malaria (Gupta et al., 2018), in rat mitochondria from various tissues (Tilak et al., 2001); and *in vitro* on various cell models including macrophages (RAW 264.7) (T. Wang et al., 2014), primary cultures of chondrocytes and nucleus pulposus (Chu et al., 2016; Guo et al., 2017), Human embryonic kidney 293, brain tumor LN229 cells (Ding et al., 2010), and neuronal cells (PC12) (K. H. Wang & Li, 2018). However, the therapeutic potential of Plumbagin for muscular dystrophies has not yet been investigated.

Chapter 4: Intrinsic role of ASMase in Inflammation and Oxidative Stress

4.1 Sphingolipids in Cell Signaling

Sphingolipids constitute one of the major classes of eukaryotic lipids. If initially sphingolipids were considered solely as structural components of the plasma membrane, they have recently emerged as bioactive lipids with a crucial role in a plethora of physiological and pathophysiological processes including cell migration, survival, proliferation, differentiation, apoptosis, inflammation, and tumorigenesis. Sphingolipids are amphipathic lipids consisting of a sphingoid backbone that is N-acetylated with various fatty acid chains. The complexity and interconnection of the sphingolipid metabolism rely on its regulation by various enzymes along with fluxes of different metabolites and explain the diverse and sometimes opposing effects of these bioactive lipids in different cell types (Hannun & Obeid, 2008; Maceyka & Spiegel, 2014) (*Figure 14*).

The sphingolipid metabolism consists of four key pathways: i. the *de novo* biosynthesis of ceramide which begins in the endoplasmic reticulum with the condensation of serine and palmitate ii. the hydrolysis of sphingomyelin present in the cell membrane by sphingomyelinase which produces ceramide iii. the conversion of ceramide into sphingomyelin or complex sphingolipids such as sphingomyelin and glycosphingolipids iv. the conversion of ceramide into bioactive molecules such as ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P) (Pralhada Rao et al., 2013).

Ceramide and sphingosine are generally implicated in inflammation, proapoptotic signaling, and growth arrest whereas S1P promotes cell growth and survival, inhibiting ceramide-induced apoptosis. This homeostatic system is referred to as the ceramide/S1P rheostat, with sphingosine kinase playing a prominent regulatory role by phosphorylating sphingosine to form S1P (Newton et al., 2015).

Ceramides are considered to be the central hub of the sphingolipid metabolism as they participate in multiple physiological and pathological activities. Ceramides are mainly generated by sphingomyelinases in the cell membrane. Due to their structural and chemical properties, ceramides have the tendency to self-associate to form ceramide-enriched microdomains or larger platforms, especially when they are the product of sphingomyelinase in the outer leaflet. These platforms reorganize the cell membrane and amplify cell signaling by clustering receptors such as CD95, CD40, CD14 and signaling molecules in or underneath the platforms upon stimulation (Grassmé et al., 2001, 2002; Pfeiffer et al., 2001). The clustering of these molecules may modify the structure of the receptors, activate the downstream signaling targets, and exclude suppressive proteins. Ceramides can also act as secondary messengers, as their domains can interact and activate various enzymes such as cathepsin D and kinase suppressor of Ras (Heinrich et al., 2004; Y. Zhang & Yao, 1997).

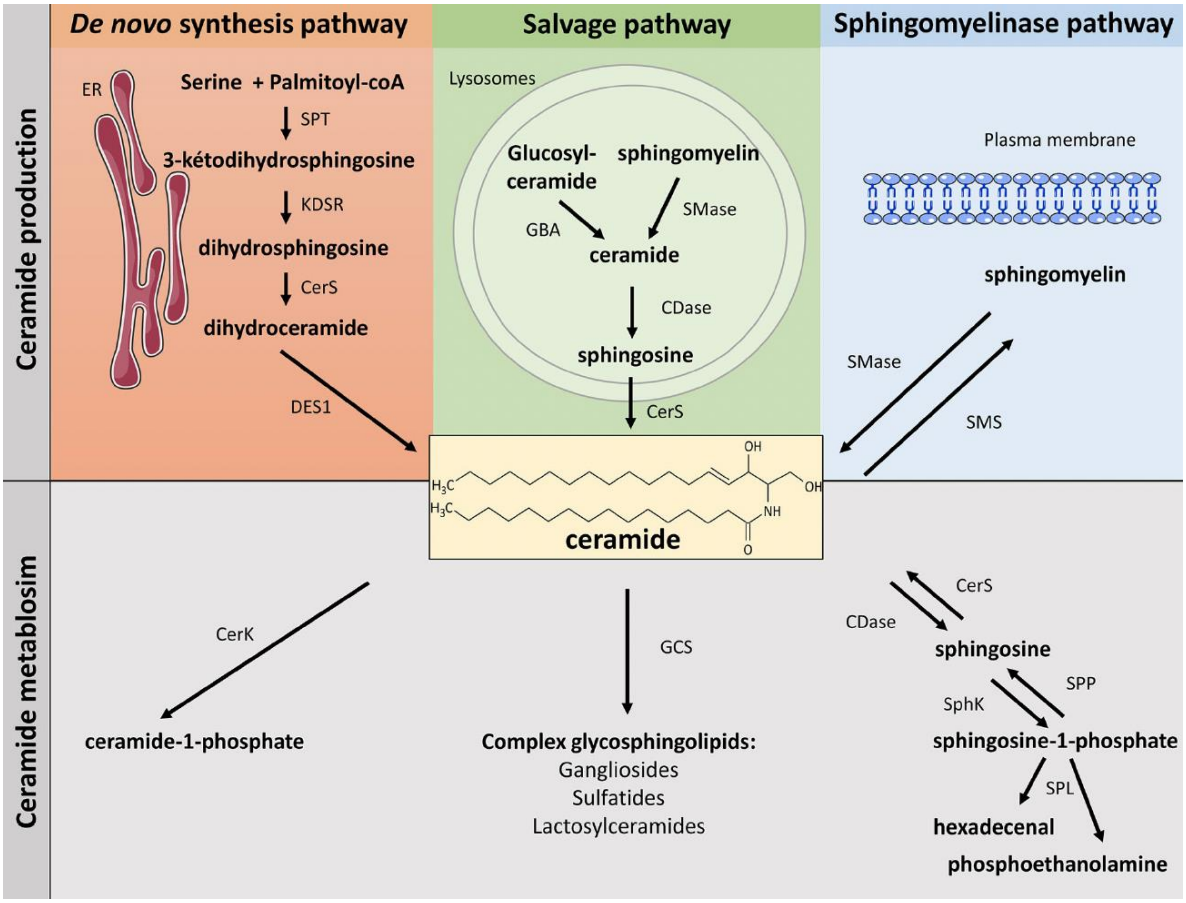

Figure 14: The major pathways governing the metabolism of sphingolipids (Tan-Chen et al., 2020)

4.2 ASMase and FIASMA

Sphingomyelinase is a phosphodiesterase that hydrolyzes sphingomyelin to produce phosphorylcholine and ceramide (*Figure 15*). According to their optimal pH for enzyme activity, sphingomyelinases can be classified into three groups (acid, neutral and alkaline) and are further divided according to their primary structure, localization, and cation dependence (Goñi & Alonso, 2002). Acidic and neutral sphingomyelinases are ubiquitously expressed in most tissues and are considered as the primary contributors in ceramide generation whereas alkaline sphingomyelinase is exclusively expressed in the intestine and liver and is involved in the digestion of dietary sphingomyelin (Duan et al., 2003). Acid sphingomyelinase (ASMase) is encoded by the SMDP1 (Sphingomyelin Phosphodiesterase 1) gene. Due to post-translational modification, two forms of ASMase exist: lysosomal (L-ASMase) and secreted (S-ASMase). During its processing in lysosomes, where it resides, L-ASMase interacts with zinc but its activation is independent of it. On the contrary, S-ASMase activation relies on zinc and is released outside the cell by the secretory pathway. When cells are stimulated, L-ASMase and S-ASM have a coregulatory effect, typically showing that S-ASMase increases while L-ASMase present a compensatory decrease (Xiang et al., 2021) (*Figure 16*).

The range of receptor-and non-receptor-mediated stimuli activating ASMase exceeds those reported for any other enzyme. ASMase agonists include inflammatory cytokines (TNF-α, IL-1β), oxidative stress (H_2O_2), chemotherapeutic agents, radiation (UV light) and pathogens (Zeidan & Hannun, 2010).

Studies on apoptosis have unraveled the mechanism of ASMase activation. Upon CD95 stimulation, ASMase is activated through the translocation from intracellular compartments to the plasma membrane in an exocytic pathway requiring Syntaxin 4, a t-SNARE protein (Perrotta et al., 2010).

As altered ASMase activity is implicated in numerous diseases, it is both an attractive therapeutic target and a promising clinical biomarker (Kornhuber et al., 2015). Therefore, in recent years researchers have focused their attention on identifying drugs that can inhibit the activity of the enzyme, calling them Functional Inhibitors of ASMase (FIASMAs) (Kornhuber et al., 2015).

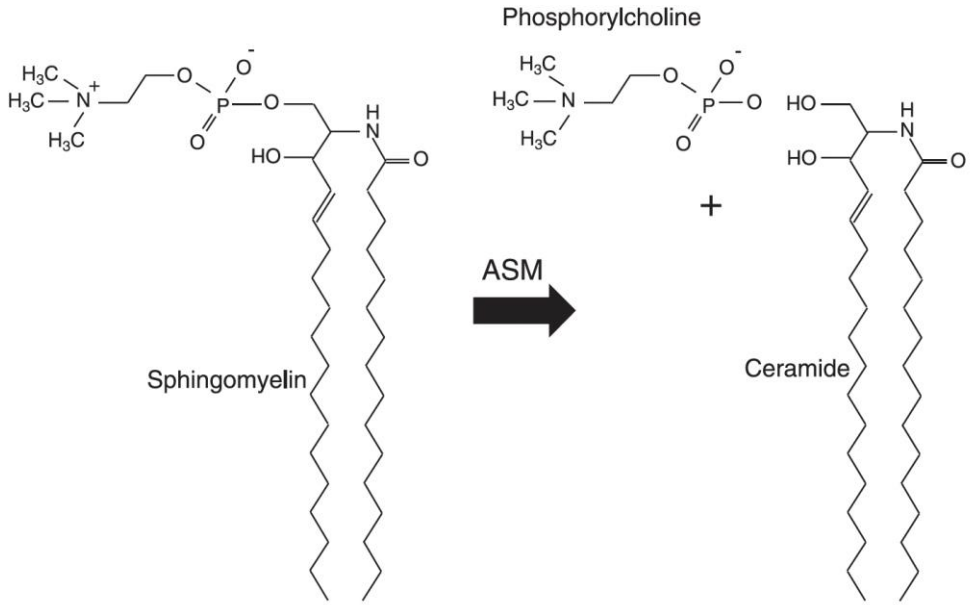

Figure 15: Acid Sphingomyelinase (ASMase) catalyzes the hydrolysis of Sphingomyelin to form Ceramide (Smith & Schuchman, 2008)

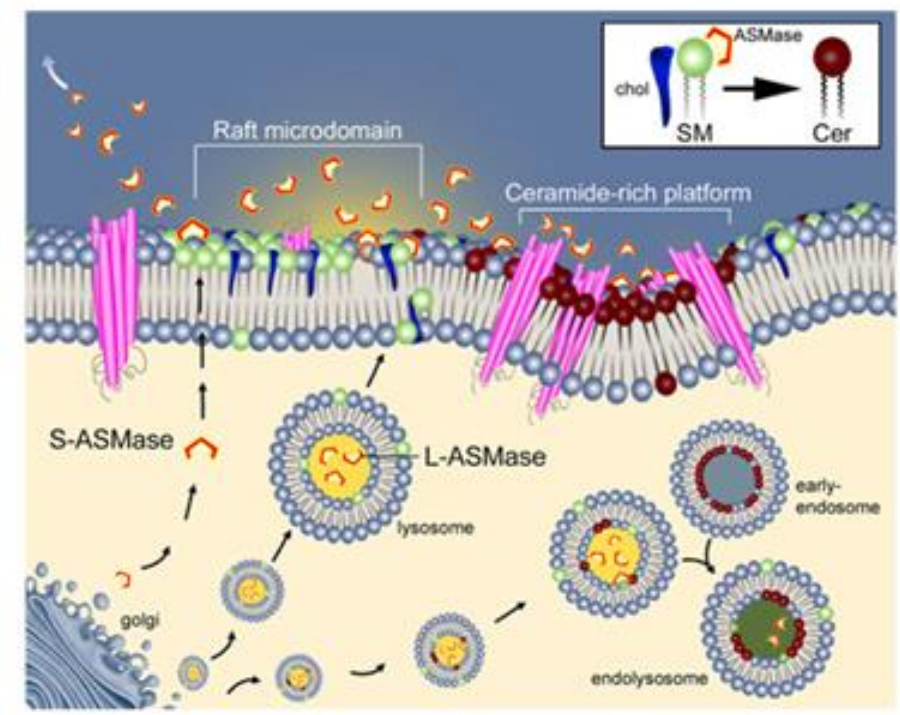

Figure 16: The role of ASMase depending upon its localization (Truman et al., 2011)

Due to their potential anti-apoptotic, neuroprotective and anti-inflammatory effects there are broad clinical applications for FIASMA including: stroke, Alzheimer's and Parkinson's disease and sepsis.

FIASMAs do not directly inhibit ASMase but result in its functional inhibition. FIASMAs are weak bases that accumulate in acidic compartments like lysosomes because they become protonated at the acidic pH and can no longer cross the membrane (acidic trapping). The attachment of ASMase to the inner lysosomal membrane protects it from proteolytic degradation and relies on electrostatic force. However, the high concentrations of the protonated FIASMA alter the electrostatic properties of the lysosomal membrane, subsequently leading ASMase detachment, inactivity, and proteolytic degradation (*Figure 17*).

Several novel FIASMAs have been identified, such as Fluoxetine, Sertraline and Nortriptyline. Most of the currently known FIASMAs are already licensed for medical use in humans, most likely to be minimally toxic and potentially rapidly available. Increasing FIASMAs concentrations leads to a decrease in ASMase activity following sigmoidal concentration-effects curves. Several FIASMAs efficiently inhibit ASMase in a concentration range commonly used in human therapy. Importantly, FIASMAs do not lead to total degradation of ASMase but instead leave a residual basal activity. This is the reason why patients treated with FIASMAs do not show symptoms of Niemann-Pick-disease, a lysosomal storage disease caused by ASMase deficiency. Even after prolonged administration of FIASMAs, ASMase activity remains low, there is no habituation effect, nor a rebound effect (Beckmann et al., 2014; Kornhuber et al., 2010).

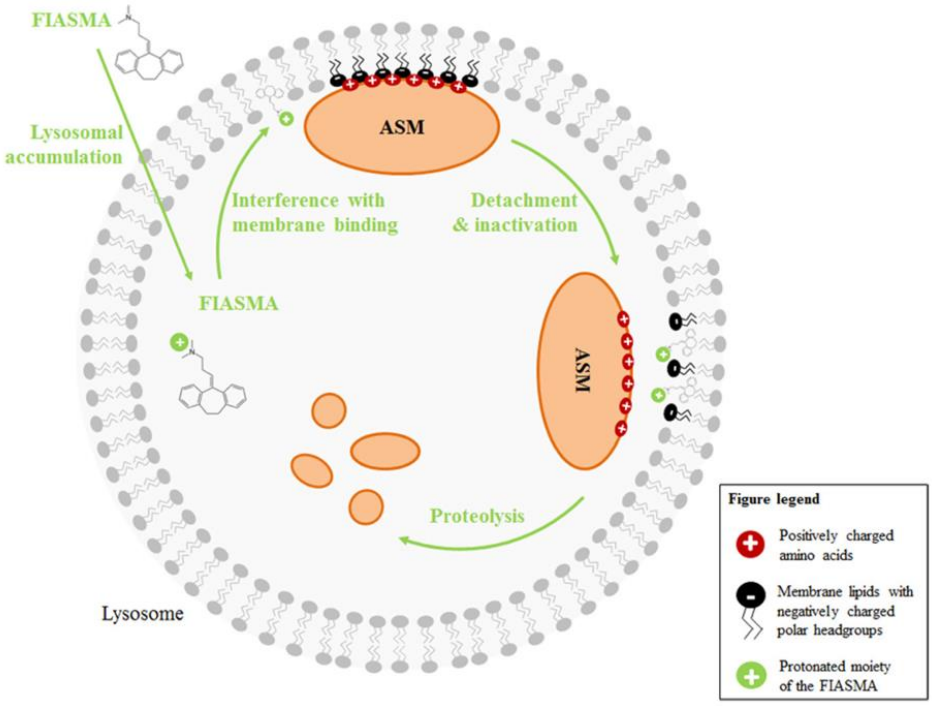

Figure 17: Mechanism of action of Functional Inhibitors of ASMase (FIASMAs) (Beckmann et al., 2014)

4.3 ASMase in Oxidative Stress

Enzymes mediating ROS secretion such as NOX aggregate in the ceramide-enriched platforms (Y. Zhang et al., 2008). It has been shown that lipid rafts contribute to the efficient release of ROS and that NOX4 subunits clustered in lipid rafts form complexes resulting in an increased production of superoxide anion. Considering that, ROS may be a secondary product of ASMase activation (Dumitru et al., 2007; P. L. Li et al., 2007; Vieceli Dalla Sega et al., 2014).

On the other hand, it seems that ROS such as H_2O_2 are not only a trigger of ASMase (X. Li et al., 2012; Managò et al., 2015) but can be considered as bioregulators of ASMase as there is increasing evidence showing that they are required for ASMase to perform its function (Zeidan & Hannun, 2010). Indeed, ASMase activation can be blocked by ROS scavenging and NADPH inhibition (Boini et al., 2010; Peng et al., 2015) and activated by direct oxidation of C-terminal cysteine (Qiu et al., 2003).

It has even been demonstrated that ASMase dampens muscle force in an oxidant-dependent manner with NOX and mitochondria as candidate sources of oxidant (L. Ferreira et al., 2010; L. F. Ferreira et al., 2012; Won & Singh, 2006; D. X. Zhang et al., 2003). A recent study revealed that ASMase needs the NADPH $p47^{phox}$ subunit, which is required for the function of NOX2, to weaken the diaphragm (Bost et al., 2015).

Moreover, it has been observed that decreasing ASMase activity in BEAS-2B epithelial cells results in an increase of Nrf2 (MacFadden-Murphy et al., 2017), which is a master player

of the antioxidant and anti-inflammatory response and is essential for the initiation of healing (Shela et al., 2016; Süntar et al., 2021).

This feed-forward mechanism in which both ROS and ASMase rely on each other is encouraging to target oxidative stress with FIASMAs and antioxidants with a potential synergistic action.

4.4 ASMase in Immunity and Inflammation

ASMase plays an irreplaceable role in the regulation of immune cell function including macrophages, Natural killer and T cells (Bai & Guo, 2017; Xiang et al., 2021).

ASMase bioactivity is crucial for macrophage biology from the beginning as this enzyme is necessary for monocyte differentiation to macrophage (Langmann et al., 1999). ASMase is also responsible for promoting inflammatory signals and cytokine production which can be triggered by inflammatory stimuli such as LPS, IL-1β or TNF-α thus participating in the exacerbation of the inflammatory response (J. Jin et al., 2013; Sakata et al., 2007; Truman et al., 2012). The importance of ASMase in the inflammatory response is reinforced by its requirement for NF-κB activation. ASMase is also crucial for efficient phago-lysosomal fusion (Schramm et al., 2008). The assembly and activation of the TLR4 receptor following LPS binding to CD14 require ceramides produced by ASMase (Cuschieri et al., 2007). This is particularly interesting considering that ablation of TLR4 in *mdx* mice ameliorated multiple aspects of the dystropathology by alleviating inflammation and shifting macrophages towards a more anti-inflammatory ($iNOS^{neg}CD206^{pos}$) profile (Giordano et al., 2015).

Regarding T-cells, ASMase mediates CD3 and CD28 signals, and determine T-cell activation/proliferation. ASMase regulates cytotoxic activity of $CD8^+$ T cells, as ASMase-deficient cells show decreased production of cytokine including IFN-γ (Herz et al., 2009). ASMase dominates Th1 responses, but negatively regulates Treg function. Thus, inhibition of ASMase lead to an increase of the proportion of Treg cell among $CD4^+$ T cells (Bai & Guo, 2017; Schneider-Schaulies & Beyersdorf, 2018). Interestingly, this immunosuppressive lymphocyte Treg possesses the functional capacity to suppress muscle damage in DMD by restraining the development of type 1 inflammatory response (A. S. Villalta et al., 2014).

Adding to its inflammatory role, ASMase regulates the activation of inflammasomes, such as NLPR3, reported to be involved in the pathogenesis of DMD (Boursereau et al., 2018), which mediates the activation of caspase-1 and the production of the pro-inflammatory cytokines IL-18 and IL-1β (Grassmé et al., 2014; C. Li et al., 2020; Martinon et al., 2002).

ASMase is also involved in the increase of CCL5 levels in cells. Interestingly, this chemokine has been suggested to be associated with myofiber necrosis in DMD patients (de Paepe et al., 2012; Jenkins et al., 2011).

The direct and specific implication of ASMase in the inflammation of exercise-induced skeletal muscle damage has been recently reported. Following acute strenuous exercise, IL-6 and creatine kinase (CK) levels were increased concomitantly with ASMase, unlike neutral sphingomyelinase and serine palmitoyltransferase-1, an enzyme involved in the *de novo*

biosynthesis of ceramides. Remarkably, the treatment with the FIASMA Imipramine reduced both IL-6 and CK elevations along with cleaved caspase-3 levels (Y.-I. Lee & Leem, 2019).

In the light of this evidence, we decided to investigate the involvement of ASMase in DMD pathology. To do so, we assessed ASMase activity in tibialis anterior (TA) and diaphragm (DIA) muscles of both WT and *mdx* mice at the stage of peak muscle necrosis and at the regenerating phase, meaning 1 and 3 months of age respectively (Turk et al., 2005) (*Figure 18*). Our preliminary results demonstrated the increased activity of the enzyme in *mdx* muscles and suggest a strong connection between DMD pathology and ASMase activation.

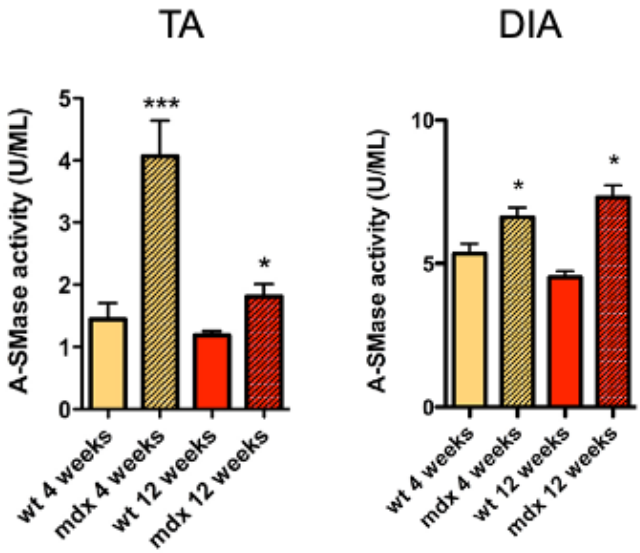

Figure 18: ASMase activity (U/ML) measured in muscle lysates by a specific fluorimetric assay. The detection was carried out in triplicate (n=10 animals for each time point). Values are expressed as ± SEM; **p<0.05; ***p<0.0001* vs WT.

AIM OF THE STUDY

The main goal of this thesis is to define a novel alternative therapeutic strategy for DMD by targeting the ASMase pathway and testing antioxidants that may synergize with ASMase inhibition. ASMase has been implicated in various inflammatory- and oxidative stress-associated disorders, which are two hallmarks of DMD (Maceyka & Spiegel, 2014; Tidball et al., 2018; Truman et al., 2011; Xiang et al., 2021). We demonstrated lately its significant increase in *mdx* mice at 1 and 3 months of age, the stages of peak necrosis and regeneration, respectively. To elucidate whether hampering ASMase is worth considering to treat DMD, the experimental strategy is organized in two main parts:

AIM 1: Pharmacological inhibition of ASMase as a therapeutic strategy for DMD. The first aim of this study is to achieve pharmacological inhibition of ASMase in DMD. The main drugs known to be functional inhibitors of ASMase (FIASMAs) are antidepressant drugs such as tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) (Kornhuber et al., 2010). As a proof-of-concept, we first started with Amitriptyline, as there are previous reports on its benefits on inflammation and muscle function in *mdx* mice (Carre-Pierrat et al., 2011; Manning et al., 2014). Considering the side effects of TCAs, we then tested the efficacy of the SSRIs Fluoxetine and Sertraline in *mdx* mice to validate the possibility to target ASMase in DMD therapy.

AIM 2: Antioxidant treatment in DMD: the role of ASMase and its therapeutic potential. ASMase is tightly related to oxidative stress, a process that also plays an important role in the progression of DMD. Thus, we evaluated the therapeutic value of the antioxidants: Plumbagin, Quercetin and N-acetylcysteine (NAC) with particular attention to their effect on ASMase activity. NAC is perceived as a control as it has already been shown to improve the pathology of *mdx* mice.

The results obtained by the experiments of AIM 1 and 2 have helped us to identify the most efficient FIASMA and antioxidant in DMD treatment to combine them, by evaluating their effects on muscle function, inflammation, and oxidative stress. The future perspective is to examine if the increased nucleophilic tone by the selected antioxidant may enhance the effect of the FIASMA. This strategy holds the potential of improving muscle integrity and alleviating the depressive symptoms often experienced by DMD patients. Polytherapy is expected to be more effective than monotherapy in general, as it targets multiple pathogenic mechanisms.

MATERIALS AND METHODS

Mdx Mice and treatments

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. All procedures were carried out in strict accordance with the Italian law on animal care (D.L. 26/2014, implementation of the 2010/63/UE) and approved by the University of Milan Animal Welfare Body and by the Italian Minister of Health (n°924/2018-PR; n°19/2021-PR). All efforts were made to reduce both animal suffering and the number of animals used.

To study DMD, the experiments were performed on the *mdx* mice C57BL/10ScSn-Dmd^{mdx}/J. WT mice with the same genetical background were used as a control in some experiments. The C57BL/10ScSn-Dmd^{mdx}/J mice belong to the most commonly used model for decades, they carry a spontaneous point mutation (C-to-T transition) within exon 23 of the dystrophin gene introducing in a premature stop codon (Sicinski et al., 1989). This nonsense mutation leads to the absence of full-length dystrophin and accounts for approximately one third of the mutations reported in DMD patients (Willmann et al., 2009).

For the preliminary experiment with the FIASMA Amitriptyline, starting from 1 month of age, mice (n = 6-10 mice per group) were treated for 5 weeks. Amitriptyline (10mg/kg/day, Sigma Aldrich) was delivered intraperitoneally.

For the long-term experiment, one month old mice (n = 6-10 mice per group) were treated for 3 months with either a FIASMA: Fluoxetine, Sertraline, or an antioxidant molecule: Plumbagin, Quercetin or N-acetylcysteine (NAC). As NAC is an effective endogenous antioxidant currently in pre-clinical studies for DMD, we used it as a reference for our antioxidants (Yao et al., 2021). Fluoxetine (5mg/kg/day), Sertraline (5mg/kg/day) and NAC (1,5g/kg/day) were administered in the drinking water. The concentrations of the drug in the water were calculated weekly according to the weight of the animals and their water consumption to guarantee accurate dosage. To ensure drug stability, the water was changed three times a week and was given in dark water feeding bottles. Plumbagin (30mg/kg/day) and Quercetin (50mg/kg/day) were administered in the diet, prepared by Mucedola srl at the concentration of 250mg/Kg and 400mg/Kg, respectively. The concentration of the compounds was calculated based on the daily food intake we measured in these animals. The vehicle controls received the same water and diet without any drug added.

ASMase-KO mice and acute muscle damage

To study the role of Acid Sphingomyelinase (ASMase) physiologically and following acute muscle injury, we took advantage of the ASMase-KO mice (C57BL/6N strain). They were generated crossing heterozygous mice and were always compared to wild-type (WT) littermates used as controls. Genotypes were determined by PCR (Horinouchi et al., 1995).

The analysis of skeletal muscles (tibialis anterior, gastrocnemius, quadriceps and diaphragm) was done in males and females of 1, 2 and 3months of age.

Acute muscle damage was induced by injecting CTX from *Naja pallida* (50 µL, 10 µM, L8102, Latoxan, Portes-lès-Valence, France), in the tibialis anterior (TA) of 2.5 months old, mice anaesthetized with 4% isoflurane (Rigamonti et al., 2013). Mice were sacrificed at 1, 3, 5, 7, 14, and 21 days after injury. Damaged muscles were collected and snap-frozen in liquid nitrogen for RNA analyses. For histology, muscles were collected and directly frozen in liquid nitrogen-cool isopentane and stored at −80 ◦C until processed.

Exhaustion Treadmill Test

Mice were made to run on the standard treadmill machine Exer 3/6 Treadmill (Colombus Instruments, Columbus, OH, USA) horizontally to assess their resistance to fatigue following the TREAT-NMD SOP (http://www.treatnmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.003.pdf). The exhaustion treadmill test was performed after an appropriate acclimatization period consisting of three sessions at 8cm/sec for 5 min. The assay consisted of 5 min at 8cm/sec, then the speed was increased by 2cm/sec every minute until reaching either 50cm/sec or exhaustion. The criterion for exhaustion was determined as the inability of the mouse to go back to run within 10 sec after direct contact with the electric stimulus grid. Running time, speed and distance are provided by the software.

Whole Body Tension Test

The Whole Body Tension (WBT) test was used to determine the ability of mice to exert tension in a forward pulling maneuver that is elicited by stroking the tail of the mice in accordance with the SOP of TREAT-NMD (http://www.treatnmd.eu/downloads/file/sops/dmd/MDX/dmd_m.2.2.006.pdf).

The tails were connected to an MP150 System transducer (BIOPAC Systems, Goleta, CA 931117, USA) with a metal thread (one end of the thread being tied to the tail using adhesive tape and the other end to the transducer). Mice were placed into a small tube lined with an aluminium mesh. Forward pulling movements were elicited by a stroke of the tail with serrated forceps and the corresponding tensions were recorded using the AcqKnowledge software recording system (BIOPAC Systems). Between 20 and 30 pulling tensions were recorded during each session. The WBT was determined by dividing the average of the top five or top ten forward pulling tensions by the body weight and represent the maximum phasic tension that can be developed over several attempts.

ASMase activity assay

The activity of ASMase was assessed on both skeletal muscle and macrophage homogenates obtained by using Ultra-Turrax® homogenator (T 10, IKA, Wilgminton, NC, USA) followed by 3 freeze-thaw cycles, and sonication for 10 s at 55% power (Sonopuls Ultrasonic homogenizer HD 2070, Bandelin, Berlin, Germany) in water as this assay is not compatible with common lysis buffers. Homogenates (30 µg per sample) were assayed for

ASMase activity using the ASMase assay kit (Echelon Biosciences, Salt Lake City, UT, USA) following the manufacturer's protocol. Fluorescence analysis was performed using a GloMax-Multi detection system plate reader (Promega, Madison, WI, USA; excitation: 365 nm, emission 410–460 nm).

RNA Extraction and RT-qPCR

The mRNA expression evaluation was performed as previously described (Perrotta et al., 2016, 2018). Total RNA from muscles, satellite cells and macrophages was isolated by phase separation in PureZOL reagent (Bio-Rad) according to the manufacturer's protocol. After solubilization in RNase-free water, total RNA was quantified by Nanodrop 2000 spectrophotometer (ThermoFisher). Total RNA (800–1000 ug) was retro-transcribed using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). RPL32, RPL38, 36B4 and GADPH have been used as housekeeping genes for normalization by using the $2^{-\Delta\Delta CT}$. The primers pairs used for RT-qPCR analysis are detailed in the table below.

Name	Primer Sequences
IL-1β	F: 5’-CCCTGCAGCTGGAGAGTGTGGA-3’
	R: 5’-TGTGCTCTGCTTGTGAGGTGCTG-3’
TNF-α	F: 5’-CCCACGTCGTAGCAAACCACC-3’
	R: 5’-TCGGGGCAGCCTTGTCCCTT-3’
CD80	F: 5'- AGTTTCTCTTTTTCAGGTTGTGAA-3'
	R: 5'-CACCCGGCAGATGCTAAAGA-3'
NOS2	F: 5'-GTTCTCAGCCCAACAATACAAGA-3'
	R: 5'-GTGGACGGGTCGATGTCAC-3'
IL-6	F: 5'-TAGTCCTTCCTACCCCAATTTCC-3'
	R: 5'-TTGGTCCTTAGCCACTCCTTC-3'
ARG1	F: 5'-CTCCAAGCCAAAGTCCTTAGA-3'
	R: 5'-AGGAGCTGTCATTAGGGACAT-3'
CD163	F: 5'-CTCCTGTGGACTCTGAAGCG-3'
	R: 5'-CTCTGAATGACCCCCGAGGA-3'
CD206	F: 5'-ATGGATTGCCCTGAACAGCA-3'
	R: 5'-TGTACCGCACCCTCCATCTA-3'
IL-10	F: 5'-GCTCTTACTGACTGGCATGAG-3'
	R: 5'-CGCAGCTCTAGGAGCATGTG-3'
F4/80	F: 5'-TGACTCACCTTGTGGTCCTAA-3'
	R: 5'-CTTCCCAGAATCCAGTCTTTCC-3'
ASMase	F:5'-TGGGACTCCTTTGGATGGG-3'
	R:5'-CGGCGCTATGGCACTGAAT-3'
Nrf2	F: 5'-CATTCCCGAATTACAGTGTC-3'
	R: 5'-GGAGATCGATGAGTAAAAATGG-3'
HO-1	F: 5'-CATGAAGAACTTTCAGAAGGG-3'
	R: 5'-TAGATATGGTACAAGGAAGCC-3'
SOD3	F: 5'-AGAGAGAGTATTTGGGAACC-3'
	R: 5'-AAACTAAGCTGCAAAGTCTC-3'
GPX1	F: 5'-GGAGAATGGCAAGAATGAAG-3'
	R: 5'-TTCGCACTTCTCAAACAATG-3'
NOX4	F: 5'-TTTCTCAGGTGTGCATGTAGC-3'
	R: 5'-GCGTAGGTAGAAGCTGTAACCA-3'
IGF-1	F: 5'-GTGTGGACCGAGGGGCTTTTACTTC-3'
	R: 5'-GCTTCAGTGGGGCACAGTACATCTC-3'
MyoD	F: 5'-CTGGCGCCGCTGCCTTCTAC-3'
	R: 5'-GGCCGCTGTAATCCATCATGCCA-3'
Myogenin	F: 5'-GACCCTACAGACGCCCACAATC-3'
	R: 5'-ACACCCAGCCTGACAGACAATC-3'
Myf6	F: 5'-GACAGCAGTGGAGAGGAAC-3'
	R: 5'-ACAGTCCGACGCTTCAGG-3'
DMD	F: 5’-GGAAGAAGTAGAGGACTGTTATG-3’
	R: 5’-AGGTCTAGGAGGCGTTTTCC-3’
MyHCII	F: 5’-AAGCGAAGAGTAAGGCTGTC-3’
	R: 5’-GTGATTGCTTGCAAAGGAAC-3’
MyHCIV	F: 5’-ACAAGCTGCGGGTGAAGAGC-3’
	R: 5'-CAGGACAGTGACAAAGAACG-3’
RPL32	F: 5'-TTAAGCGAAACTGGCGGAAAC-3'
	R: 5'-TTGTTGCTCCCATAACCGATG-3'
RPL38	F: 5'-GAAGGATGCCAAGTCTGTCAA-3'
	R: 5'-GAGGGCTGGTTCATTTCAGA-3'
36B4	F: 5'-AGGATATGGGATTCGGTCTCTTC-3'
	R: 5'-TCATCCTGCTTAAGTGAACAAACT-3'
GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3'
	R:5'-TCCACCACCCTGTTGCTGTA-3'

Histology and Immunofluorescence of skeletal muscle

Muscles were dissected and directly frozen to allow the preparation of 7 μm thick sections cut with a cryostat for both morphological and immunofluorescence analysis. Hematoxylin-eosin (H&E, Bio Optica, Milan, Italy), Sirius Red (Direct Red 80: 365548, Sigma-Aldrich) and Immunofluorescence were performed as previously described (Burdi et al., 2009; Rittié, 2017; Sciorati et al., 2009).

Briefly for immunofluorescence, sections were first fixed for 10 min with 4% paraformaldehyde (PFA) in PBS and then blocked with PBS enriched with 10% normal goat serum (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 1h. The primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Sections were then incubated with the appropriate fluorescent-labelled secondary antibodies diluted in blocking solution for 1h at room temperature. Nuclei were counterstained with DAPI (PureBlu, Bio-Rab, Hercules, CA, USA) before mounting the slides with the Fluoroshield Histology Mounting Medium (Sigma-Aldrich) (Amato et al., 2018). To expose the proliferating satellite cells in the muscle, the sections were boiled in sodium citrate (C9999, Sigma-Aldrich) for 15 min before blocking them with 10% normal goat serum and 0.5% Triton in PBS (Cazzato et al., 2013; Zecchini et al., 2019).

To measure the cross-sectional area (CSA) of myofibers, muscle sections were stained with an anti-laminin antibody (Sigma-Aldrich). The ImageJ software was used to determine the CSA of 1000 to 3000 individual myofibres from at least three different fields for every muscle section. For each muscle, at least four to nine sections were analyzed (Catalani et al., 2016; Guardiola et al., 2012).

For cultured satellite cell staining, image analysis was performed by using ImageJ software. Fusion index, diameter of myotubes, number of nuclei/myotubes and myotubes > 5 nuclei were counted from five to ten microscopic fields randomly chosen. Fusion index was calculated as the percentage of number of nuclei within myotubes over the total number of nuclei. All the antibodies used are listed in the two tables below.

Images were acquired using a DMI4000 B fluorescence microscope Leica automated inverted microscope equipped with a DCF310 digital camera (Leica Microsystems, Wetzlar, Germany) or the ZOE^{TM} Fluorescent Cell imager (Bio-Rad) and the Leica TCS SP8 System equipped with Leica DMi8 inverted microscope, for confocal imaging.

Primary Antibodies					
Protein	**Marker**	**Specie**	**Conjugate**	**Dilution**	**Company/Code**
Laminin A	ECM	Rabbit	Unconjugated	D1:250	Sigma/L93932
CD45	Immune cell	Rat	FITC	D1:50	Miltenyi Biotec/130-116-500
CD80	M1	Armenian Hamster	Alexa Fluor 488	D1:10	BioLegend/104716
CD206	M2	Rat	Alexa Fluor 647	D1:20	BioLegend/141712
MyoD	Satellite cell	Mouse	Unconjugated	D1:20	Dako/M3512
Ki67	Proliferation	Rabbit	Unconjugated	D1:200	Abcam/ab16667
MF20	Differentiation	Mouse	Unconjugated	D1:2	Developmental Studies Hybridoma Bank
MyHC-Emb	Regeneration	Mouse	Unconjugated	D1:50	Santa Cruz Biotechnology/sc-53091
Collagen I	Collagen	Rabbit	Unconjugated	D1:100	Abcam/ab34710

Secondary Antibodies				
Target Species	**Host**	**Conjugate**	**Dilution**	**Company/Code**
Rabbit	Goat	Alexa Fluor 647	D1:500	Invitrogen/A21244
Rat	Goat	Alexa Fluor 647	D1:500	Invitrogen/A21247
Mouse	Goat	Alexa Fluor 546	D1:500	Invitrogen/A11003
Mouse	Goat	Alexa Fluor 488	D1:500	Invitrogen/A11001
Rabbit	Goat	Alexa Fluor 488	D1:500	Invitrogen/A11008
Rabbit	Goat	Alexa Fluor 546	D1:500	Invitrogen/A11010

Creatine Kinase Evaluation

We obtained blood samples from the orbital sinus of mice anaesthetized with 4% isoflurane. The blood was centrifuged at 3000 rpm for 15min at 4°C and the supernatant was used to measure the Creatine Kinase (CK) activity (units per liter U/L) in the serum using an indirect colorimetric assay (Randox Laboratories, Crumlin, Northern Ireland, UK).

Flow Cytometry

Skeletal muscles were harvested, minced and digested with in a freshly prepared solution of Dispase and type II collagenase diluted in DMEM (Sigma-Aldrich) at 37°C for 40 min. Disaggregation was stopped with 20% FBS and cells were filtered through a 70 µm cell strainer (Miltenyi Biotec). The collected cells were washed with PBS and incubated with primary conjugated antibodies for 15 min at room temperature.

They were analyzed 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, Vancouver, BC, Canada) and CD34-Alexa Fluor 647 (BD Pharmingen™, San Diego, CA, USA) double-positive cells and CD45-PE-Cy7, CD31-PE-Cy7, CD80-FITC, CD86-FITC, CD14-FITC (eBioscience, San Diego, CA, USA) and Sca1-FITC (BD Pharmingen™) negative cells (Pasut et al., 2013). M1 macrophages were identified as CD45-PE-Cy7 (eBioscience), F4/80-PE (Miltenyi Biotec), CD80-FITC (eBioscience), MHCII-Brilliant Violet (Biolegend) positive cells and CD206-AF647 (Sony Biotechnology) negative; M2 macrophages as CD45-PE-Cy7 (eBioscience), F4/80-PE (Miltenyi Biotec), CD206-AF647 (Sony Biotechnology) positive cells and CD80-FITC (eBioscience), MHCII-BV (Biolegend) negative cells (Assi et al., 2015; Perrotta et al., 2018).

Drug quantification in skeletal muscle

Different HPLC/MS/MS methods and instruments (according to the dimension and the characteristics of the molecules) have been set up and validated for the quantification of the administrated drugs in the *mdx* muscles by using the respective internal standard (IS) (Fluoxetine-d5-hydrochloride, Sertraline-d3-Hydrochloride, Quercetin-d3-Hydrochloride).

At the end of treatment, when animals were sacrificed gastrocnemius samples were removed, immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. The

muscles were then homogenized in water (10% w/v) and 200 µl of the obtained mixture were purified, extracted and analyzed. Briefly, for Fluoxetine and Sertraline quantification, an aliquot of tissue homogenate, spiked with IS, were diluted with 2 ml of Buffer pH 9 and extracted by liquid-liquid extraction with 4 ml of chloroform/heptane/isopropyl alcohol (55/33/17). For Quercetin quantification the dilution was performed with 2 ml of Buffer pH 4 followed by the extraction. After organic phase evaporation residual was dissolved in 50 µL of methanol and immediately injected for the analysis.

Primary Cell Isolation and Culture

Bone marrow precursor cells (MONO) were harvested by flushing femurs and tibiae of 2–3 months old WT and ASMase-KO mice and cultured for 5 days in α-MEM (37 ◦C, 5% CO2 in a humidified atmosphere) containing 10% FBS and of 100 ng/mL macrophage-specific colony-stimulating factor (M-CSF) (Miltenyi Biotec) to generate macrophages. Cells were cultured for 2 additional days in the presence of 10 ng/mL of M-CSF and 50 ng/mL IFN-γ (Miltenyi Biotec) to generate M1 cells or for 4 additional days with 10 ng/mL M-CSF and 10 ng/mL IL-4 (Miltenyi Biotec) to generate M2a cells (Cervia et al., 2016; Perrotta et al., 2014, 2018). To assess the effect of pharmacological inhibition of ASMase on macrophage differentiation/polarization, Amitriptyline hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) (Cervia et al., 2016) concentration of were added to WT cells to reach the final concentration of 5 µM on the days 0, 3 and 5 of culture.

Satellite cells from 1 month old WT and ASMase-KO mice were obtained from muscles of hindlimbs and forelimbs by using the tissue dissociation protocol of gentleMACS™ Octo Dissociator with Heaters (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by magnetic depletion of lineage PECAM1-, PTPRC-, ITGAM-, and LY6A/Sca-1-positive cells using the Satellite Cell Isolation Kit (Miltenyi Biotec), following the manufacturer's instructions (Zecchini et al., 2019).

Satellite cells were cultured in DMEM (EuroClone, Milan, Italy) supplemented with 20% fetal bovine serum (FBS) (EuroClone,), 3% chick embryo extract (United States Biological, Salem, MA, USA), 10 ng/mL basic fibroblast growth factor (FGFb) (PeproTech, Cranbury, NJ, USA) and 1% penicillin-streptomycin (EuroClone) on Matrigel-coated (Corning, New York, NY, USA) plates at 37 ◦C with 5% CO2 for 4 days. To assess proliferation, satellite cells were plated at a confluence of 1.5×10^4 cell/cm^2 in growth medium and cultured for 24 h. For the differentiation experiment, cells were plated at a confluence 5×10^4 cell/cm^2 in medium containing 2% Horse Serum (EuroClone) instead of FBS and cultured for 48 h (De Palma et al., 2014).

Statistical analyses

The fewest animals possible were used to obtain valid scientific results. 5-8 animals were used for each experimental group and analytical method. Statistical significance of raw data between the groups was evaluated using unpaired (independent samples) or paired (matched observations) Student's t-test or one-way ANOVA followed by Bonferroni or Tukey post-tests (multiple comparisons) or two-way ANOVA with correction for multiple

comparisons using the Sidak test (grouped analysis). When data are not normally distributed, the Mann-Whitney test was used. The results are expressed as means ± SEM of the indicated n values. p values ≤ 0.05 were considered statistically significant. The analysis was carried out by using GraphPad Prism software package (GraphPad Software, San Diego, CA, USA).

RESULTS

Proof-of-concept of the potential of targeting ASMase by treating *mdx* mice with the FIASMA Amitriptyline

To test the therapeutic potential of FIASMAs in *mdx* mice, we performed a short-term treatment with Amitriptyline. This tricyclic antidepressant (TCA) has already been reported to be beneficial in *mdx* mice by alleviating skeletal muscle inflammation as well as symptoms of anxiety and depression, but its role as a FIASMA has not been investigated in this study (Manning et al., 2014). Therefore, we treated *mdx* mice with the same dose of Amitriptyline (10 mg/kg/day) for 5 weeks, starting at 1 month of age, while the controls received only the vehicle.

At the end of the treatment, we found a significant decrease in ASMase activity in the muscle lysates of the tibialis anterior (TA) from the treated group (*Figure 1A*). Histological analysis revealed a decreased inflammatory infiltrate in sections of the TA from the treated animals by Hematoxylin and Eosin (H&E) staining (*Figure 1B*).

These data were confirmed by the decrease of necrotic fibers in the treated mice as evidenced by the immunofluorescence targeting the immunoglobulin G (mIgG) uptake by myofibers of the TA (*Figure 1C*).

Furthermore, the liquid chromatography/tandem mass spectrometry assay performed on muscle lysates evidenced the presence of the drug in the targeted tissue (0.16 ± 0.05pg/mg). Treated animals did not manifest any side effects such as distress or anorexia.

These findings corroborate the strong connection between increased levels of ASMase and the chronic muscle damage of DMD and bring to light the pharmacological therapeutic potential of targeting ASMase.

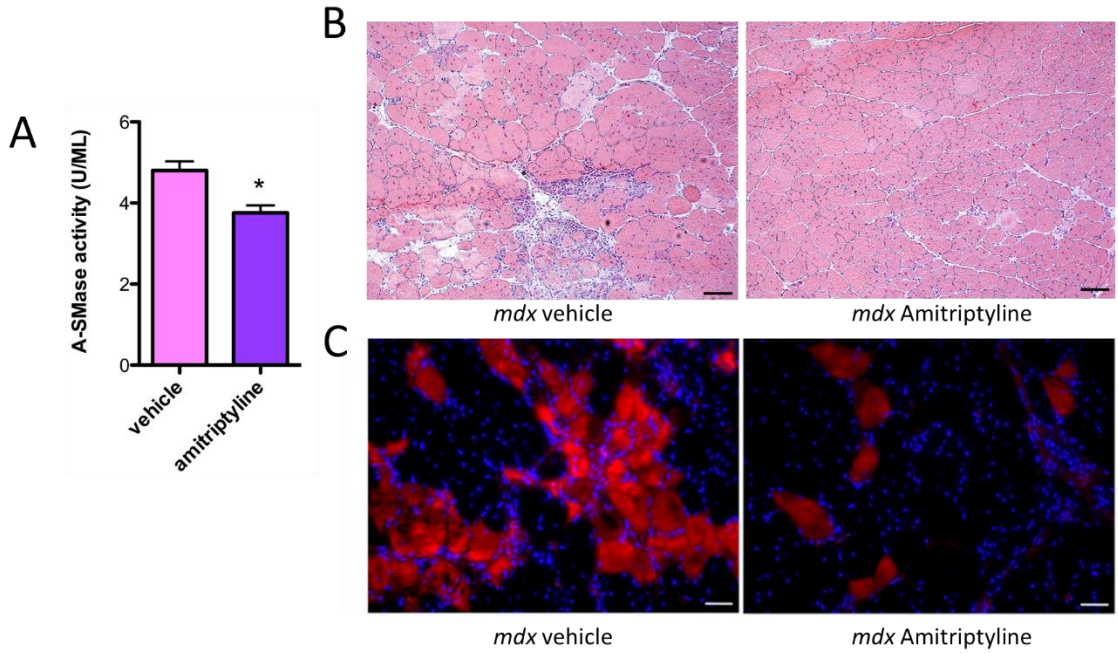

Figure 1. The FIASMA Amitriptyline ameliorates the condition of *mdx* mice
Mdx mice were administered Amitriptyline (10 mg/kg/day) or only its vehicle. TA were collected and analyzed after 5 weeks of treatment.
A) ASMase activity (U/mL) of the TA.
B) Hematoxylin & Eosin staining (scale bar = 100 μm) of the TA.
C) mIgG staining of necrotic fibers (scale bar = 50 μm) of the TA.
Values are expressed as mean ± SEM; (n = 5 mice per group) *$p<0.005$, **$p<0.001$, ***$p<0.0001$ vs. *mdx* vehicle.

Health status evaluation of the treated animals

After confirming with the FIASMA Amitriptyline that inhibiting ASMase is an attractive therapeutical strategy for DMD, we decided to proceed with an alternative category of FIASMAs: SSRIs, i.e. Fluoxetine and Sertraline, as they have a better safety profile than TCAs, for a long-term treatment as DMD is a chronic disorder. Moreover, we tested the effects of the antioxidant molecules: Plumbagin, Quercetin, and NAC.

Starting from 1 month of age, mice were treated for 3 months with either a FIASMA, or an antioxidant. During the first 10 days of treatment, animals were monitored daily to assess their general health condition and evaluate their water and food intake. For the rest of the treatment, mice were examined 3 times a week.

None of the treatments affected water nor food consumption and consequently the weight of the mice (*Table 1 and Figure 2*). Importantly, no signs of distress (i.e. anxiety) or pathology attributable to drug adverse reactions were observed during the period of treatment.

	Mdx Vehicle	*Mdx* Fluoxetine	*Mdx* Sertraline	*Mdx* NAC	*Mdx* Plumbagin	*Mdx* Quercetin
Daily food intake (g; mean ± SD)	4.087 ± 0.2589	4.194 ± 0.2718	4.254 ± 0.1771	4.255 ± 0.2727	3.945 ± 0.2831	3.984 ± 0.3050
Daily water intake (ml; mean ± SD)	4.205 ± 0.5559	4.086 ± 0.5775	4.162 ± 0.5979	4.838 ± 0.4777	3.947 ± 0.3964	4.182 ± 0.8959

Table 1. Mean daily food and water intake per animal during the period of treatment (n = 6-10 mice per group).

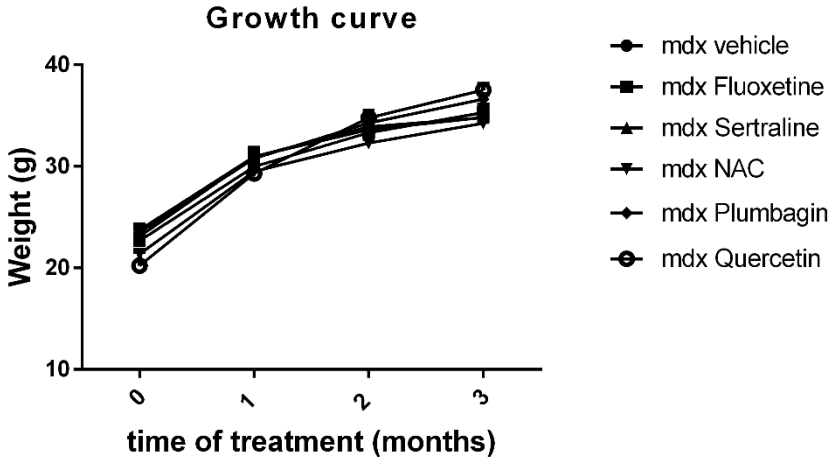

Figure 2. Health status evaluation of the treated animals
Growth curves of *mdx* mice during the period of treatment, i.e. 3 months from 1 to 4 months of age (n = 6-10 mice per group).

FIASMAs and Antioxidants improve the phenotype of *mdx* mice

As the ultimate goal of a treatment for DMD is to improve muscular function, we performed the exhaustion treadmill test at the end of our therapy to evaluate its efficacy. This test allows the assessment of the dystrophic state *in vivo* by measuring both the distance run and the time to exhaustion in accordance with the standardized operating procedures (SOPs) from www.treat-nmd.eu (https://treat-nmd.org/wp-content/uploads/2016/08/MDX-DMD_M.2.1.003-34.pdf).

Both Fluoxetine and Sertraline did not improve the muscular performance assessed by the running distance and time of *mdx* mice (*Figure 3A*). However, as they are antidepressants, we also administered them to WT mice to exclude any cognitive bias. We noticed that both SSRIs significantly reduced their running distance and time until exhaustion compared to WT vehicle mice (*Figure 3A*). This impairment can cover a possible beneficial action of FIASMAs on skeletal muscles.

On the other hand, all the tested antioxidants were able to improve muscle function assessed by their performance on the treadmill (*Figure 3B*) and the recovery score versus WT values (*Table 2*), which is an impartial way to rate the effectiveness of a treatment on a given parameter.

To investigate further the efficacy of our treatment we performed most of our experiments, including imaging analyses, on the diaphragm (DIA), as it is the muscle reproducing best the dystropathology reported in DMD patients (Stedman et al., 1991).

As expected, the muscles obtained from untreated *mdx* mice (*mdx* vehicle) revealed a progressive degeneration, with the appearance of necrotic and calcified fibers (*Figure 4A*) and Sirius Red staining revealed frequent foci of fibrosis (*Figure 4B and Table 3*). All the treatments resulted in an improved morphology of the muscle, as shown by the H&E staining (*Figure 4A*) with reduced myonecrosis and calcification. Of interest, we observed a nearly normal muscle architecture following Sertraline, Plumbagin, and Quercetin administration. Moreover, all the treated mice, apart from the ones of the Fluoxetine group, showed a reduced fibrosis (*Figure 4B and Table 3*). Muscle integrity, characterized by a decrease of areas of myonecrosis evidenced by mIgG staining (Bencze et al., 2019), was remarkably restored after each treatment, especially with Plumbagin and Quercetin (*Figure 4C and Table 3*).

This decrease in muscle degeneration paralleled with the situation during the treatment. Indeed, we found a considerable decrease in serum Creatine Kinase (CK) activity, a marker of muscle damage, in all the treated groups 1 month after the beginning of the experiment (*Figure 4D).*

All the treatments lead to increased muscle regeneration measured by positive staining for embryonic myosin (MyHC-emb) (*Figure 5A and Table 3*) and by the elevated mRNA expression in both DIA and TA of Myogenin and Myf6 (MRF4) (*Figure 5B*), which are fundamental transcription factors for muscle differentiation.

These data on muscle function and structure show that both FIASMAs and antioxidants improve the phenotype of *mdx* mice, which is particularly promising regarding Plumbagin as it is the first time this molecule is tested in dystrophic mice.

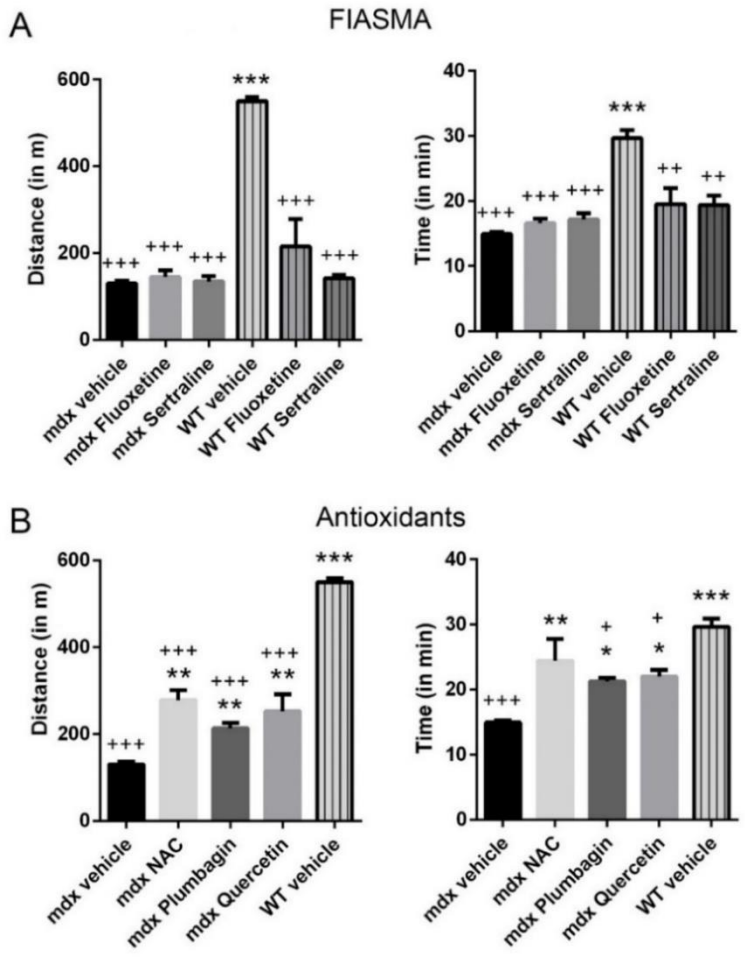

Figure 3. FIASMAs and antioxidants improve the muscle function of *mdx* mice

A-B) Muscular performances after treatment assessed by exhaustion treadmill running test, measuring both distance run (meters-m; A-B left panel) and time to exhaustion (minutes-min; A-B right panel). **A)** Mice (*mdx* and WT) treated or not (vehicle) with FIASMA. **B)** Mice (*mdx* and WT) treated or not (vehicle) with antioxidants and WT as control.

n = 5-8 mice; **p<0.05, **p<0.001, ***p<0.0001* vs. *mdx* vehicle*; +p<0.05, ++p<0.001, +++p<0.0001* vs. WT vehicle.

	Recovery score - Distance	**Recovery score - Time**
***Mdx* NAC**	35%	64%
***Mdx* Plumbagin**	20%	43%
***Mdx* Quercetin**	29%	48%

Table 2. Recovery scores vs WT of *mdx* mice (n = 5-8 mice) treated with NAC, Plumbagin and Quercetin.

The formula used: [*mdx* treated] − [*mdx* untreated] / [wild type] − [*mdx* untreated] × 100 is in accordance with the SOPs described by TREAT-NMD (https://treat-nmd.org/wp-content/uploads/2016/08/MDX-DMD_M.1.1_001-21.pdf).

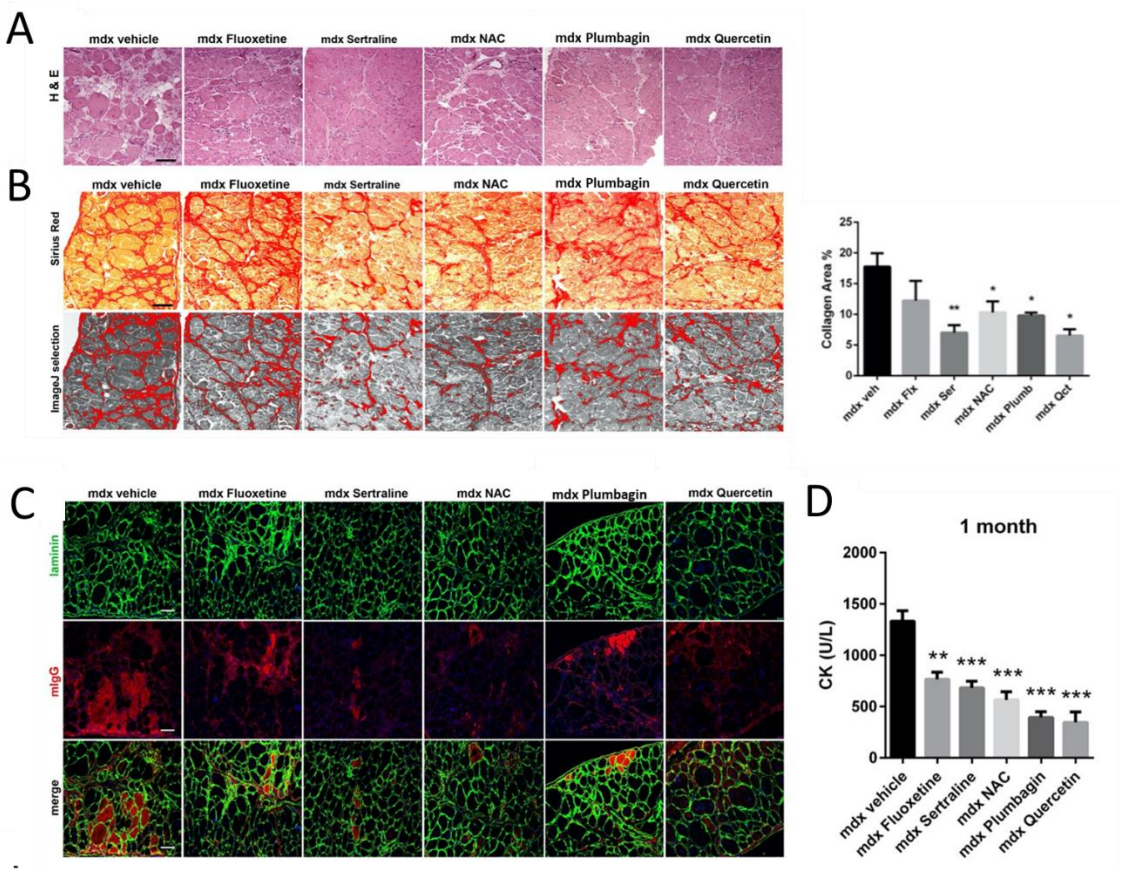

Figure 4. FIASMAs and antioxidants improve the phenotype of *mdx* mice

A-B) Histological images of the DIA (**A**: H&E staining; **B**: Sirius Red staining) obtained after 3 months of treatment, representative of 10 images per mouse (n = 3). Scale bar = 20 µm.

B) The graph displays the quantification of Sirius red staining (collagen deposition).

* *p<0.05*, ** *p<0.001*, vs. *mdx* vehicle. Values are expressed as mean ± SEM.

C-D) Evaluation of muscle damage assessed as Myonecrosis (**C**) in the DIA and serum CK (**D**).

C) Mouse IgG (mIgG) (red) staining labels damaged myofibers.

D) Serum CK levels measured in *mdx* mice treated or not with a FIASMA or an antioxidant for 1 month.

n = 5-8 mice; * *p<0.05*, ** *p<0.001*, *** *p<0.0001* vs. *mdx* vehicle.

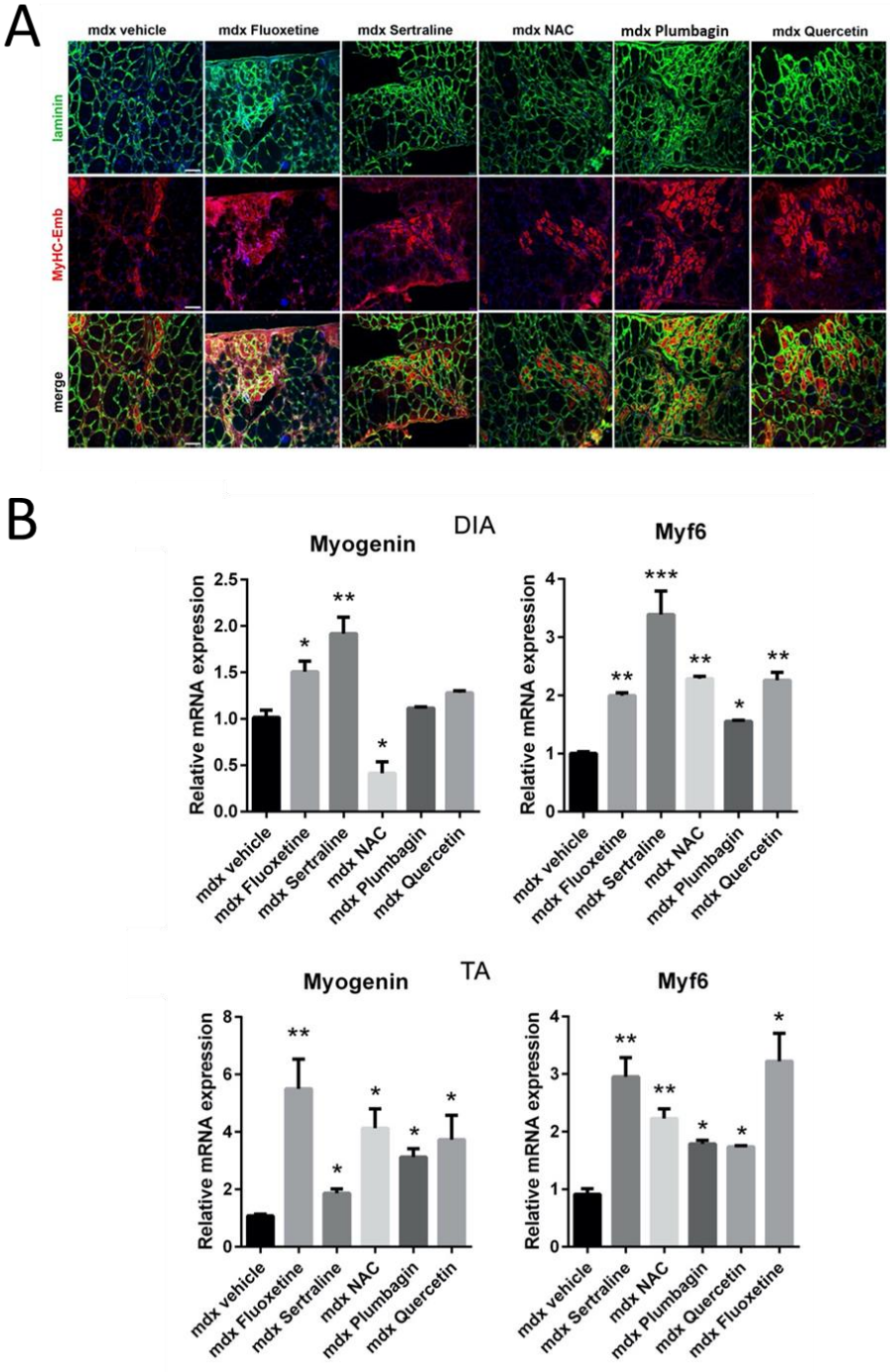

Figure 5. FIASMAs and antioxidants increase the muscle regeneration of *mdx* mice

A) MyHC-Emb (red) staining labels regenerating fibers. Laminin (green) is used as sarcolemma marker to identify fiber area. Nuclei were counterstained with DAPI. The figure shows representative images (10 images per animal; n = 3 mice). Scale bar = 20 µm.

B) RT-qPCR analysis of the myogenic regulatory factors Myogenin and Myf6 (MRF4) in DIA (upper panels) and TA (bottom panels) of *mdx* mice (treated or not).

Values are expressed as mean ± SEM (n ≥ 3 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *mdx* vehicle.

	Fibrosis (Collagen)	Myonecrosis (mIgG)	Regeneration (MyHC-Emb)
***mdx* Vehicle**	+++	+++	+
***mdx* Fluoxetine**	++	++	++
***mdx* Sertraline**	+	+	++
***mdx* NAC**	+	+	++
***mdx* Plumbagin**	+	+/-	+++
***mdx* Quercetin**	+	+/-	++

Table 3. Histopathological evaluation of muscle fibrosis, necrosis and regeneration
Analysis performed on 10 images of the DIA per animal (n = 3); (legend of the table: +/- = almost negative; + = weakly positive; ++ = positive; +++ = strongly positive).

FIASMAs and Plumbagin decrease ASMase activity in the skeletal muscle of *mdx* mice

Then we measured the activity of ASMase in the DIA, the muscle recapitulating best the human pathology (Stedman et al., 1991).

The FIASMAs decreased ASMase activity in the DIA as expected. However, the antioxidant Plumbagin also achieved to inhibit the enzyme, while Quercetin did not (*Figure 6*).

Additionally, to confirm that our treatments reached the targeted tissue, in collaboration with the laboratory of Medical Chemistry of DIBIC "L. Sacco" we quantified by Mass Spectrometry the administered compounds in lysates of gastrocnemius muscle (*Table 4*).

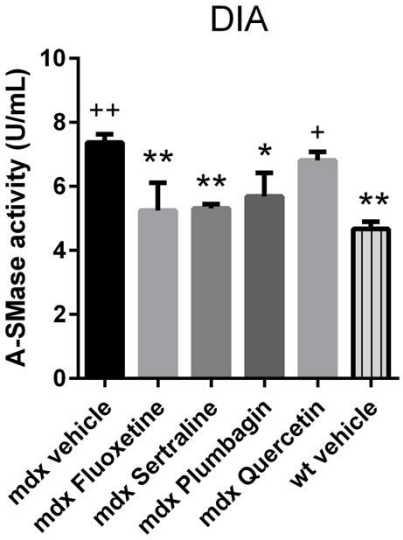

Figure 6. FIASMAs and Plumbagin decrease ASMase activity in the skeletal muscle of *mdx* mice
ASMase activity (U/mL), measured in DIA homogenates (n = 5-8 mice); ***p<0.001* vs. *mdx* vehicle; *+p<0.05, ++p<0.001* vs. WT vehicle.

Molecule	Concentration (ng/g ± SD)
Fluoxetine	514.5 ± 184.5 (n = 4)
Sertraline	78.60 ± 15.84 (n = 4)
Quercetin	0.3450 ± 0.1630 (n = 4)

Table 4. Drug concentrations in the muscles of *mdx* mice

FIASMAs and Antioxidants attenuate Oxidative Stress in *mdx* mice

As Oxidative Stress is a hallmark feature of DMD, we explored the effects exerted by the chosen FIASMAs, which are already known to have antioxidative properties (Behr et al., 2012) and antioxidants on the expression of several enzymes involved in the antioxidant defense system, thanks to our collaboration with the Biochemistry Unit of the University of Bologna. Both Plumbagin and Quercetin are known to elicit their antioxidant properties by activating the Nrf2 pathway, and antioxidant enzymes which also count as its downstream targets, for example glutathione, SOD, CAT, HO-1, and NQO1, and as direct ROS scavenger (Ding et al., 2010; Guo et al., 2017; Kumar et al., 2013; Lin et al., 2004; Mohammadtaghvaei et al., 2021; D. Xu et al., 2019).

Nrf2 expression was increased by Fluoxetine and Plumbagin (*Figure 7*), corroborating with previous findings (Mendez-David et al., 2015). However, the effect on ASMase expression was not significant.

All the treatments were able to induce the expression of HO-1, a rate-limiting enzyme in the catabolism of heme, and SOD3, a direct superoxide scavenger, while only Fluoxetine was able to promote GPX1, a direct scavenger of peroxide (*Figure 7*).

Unexpectedly, the expression of an enzyme primarily producing H_2O_2: NOX4, was increased in muscles from Plumbagin and Quercetin treated mice (*Figure 7*).

Altogether, these findings show that FIASMAs and antioxidants improve the antioxidant defense system of *mdx* mice, especially with Fluoxetine and Plumbagin which effectively activated Nrf2.

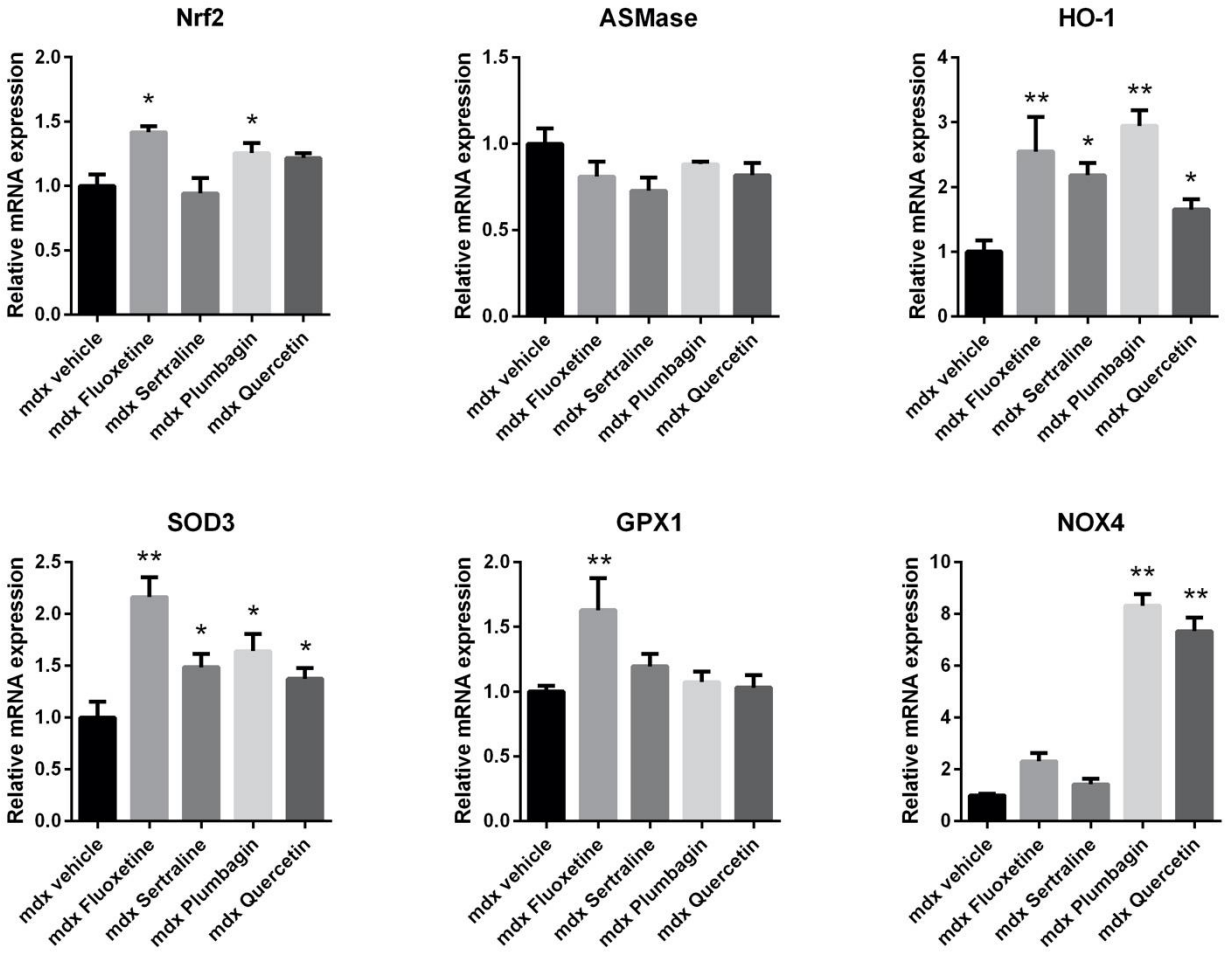

Figure 7. FIASMAs and antioxidants attenuate Oxidative Stress in *mdx* mice

RT-qPCR analysis of the oxidative stress markers Nrf2, HO-1, SOD3, GPX1, NOX4, and ASMase (SMPD1) in muscles of *mdx* mice (treated or not).

Values are expressed as mean ± SEM (n ≥ 3 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *mdx* vehicle.

FIASMAs and Antioxidants alleviate and modulate inflammation towards an anti-inflammatory state in *mdx* mice

The chronic pro-inflammatory state plays a key role in muscular dystrophy. As macrophages are the main actors of this process, with the pro-inflammatory M1 macrophages worsening muscle injury and the anti-inflammatory M2 macrophages promoting tissue repair, regulating the balance between M1 and M2 phenotypes has been considered an attractive therapeutic strategy (Rosenberg et al., 2015; S. A. Villalta, Deng, et al., 2011).

We found that all our treatments were able to reduce the overall immune response, as shown by the positive staining for CD45 in sections of DIA muscles (*Figure 8 and Table 5*).

To explore this lowered inflammation more in detail we evaluated the M1/M2 ratio in muscles by flow cytometry (*Figure 9A*) as well as the expression of M1 and M2 markers in DIA and TA by RT-qPCR (*Figure 9B-E*). We observed a promising decrease of the M1/M2 ratio for Sertraline and Plumbagin (*Figure 9A*) and noticed that the reduced positivity for CD45 in the DIA paralleled with the lowered expression of IL-1β for all the treatments, as well as for TNF-α, except for Sertraline (*Figure 9B*). Interestingly, in the TA we observed a significant anti-inflammatory action for all the antioxidants administered, while no modifications were found for the FIASMA Fluoxetine (*Figure 9C*).

On the other hand, regarding the anti-inflammatory effect assessed by the increase of the M2 markers IL-10, CD163 and ARG1, the best results were obtained with Fluoxetine in both DIA and TA (*Figure 9 D-E*), while Plumbagin was able to increase the expression of ARG1 in both muscles. All the drugs achieved to increase CD163 expression in the TA (*Figure 9E*).

Importantly, this anti-inflammatory effect exerted by FIASMAs and antioxidants paralleled with the improved muscle function and regeneration we observed in *mdx* mice.

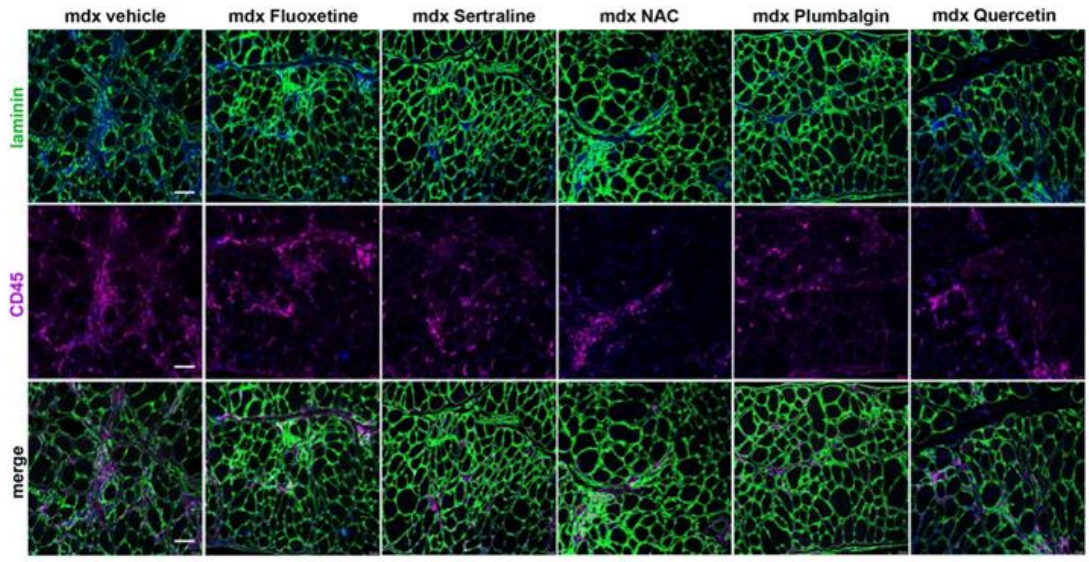

Figure 8. FIASMAs and antioxidants alleviate inflammation in mdx mice
Evaluation of the inflammatory infiltrate. CD45 (purple) staining labels immune cells infiltrated in the muscle. Laminin (green) is used as sarcolemma marker to identify fiber area. Nuclei were counterstained with DAPI.
The figure shows representative images (10 images per animal; n = 3 mice). Scale bar = 20 µm.

	Inflammation ($CD45^+$)
***mdx* Vehicle**	+++
***mdx* Fluoxetine**	++
***mdx* Sertraline**	+
***mdx* NAC**	+
***mdx* Plumbagin**	+
***mdx* Quercetin**	++

Table 5. Histopathological evaluation of muscle inflammation
Analysis performed on 10 images of the DIA per animal (n = 3); (legend of the table: +/- = almost negative; + = weakly positive; ++ = positive; +++ = strongly positive).

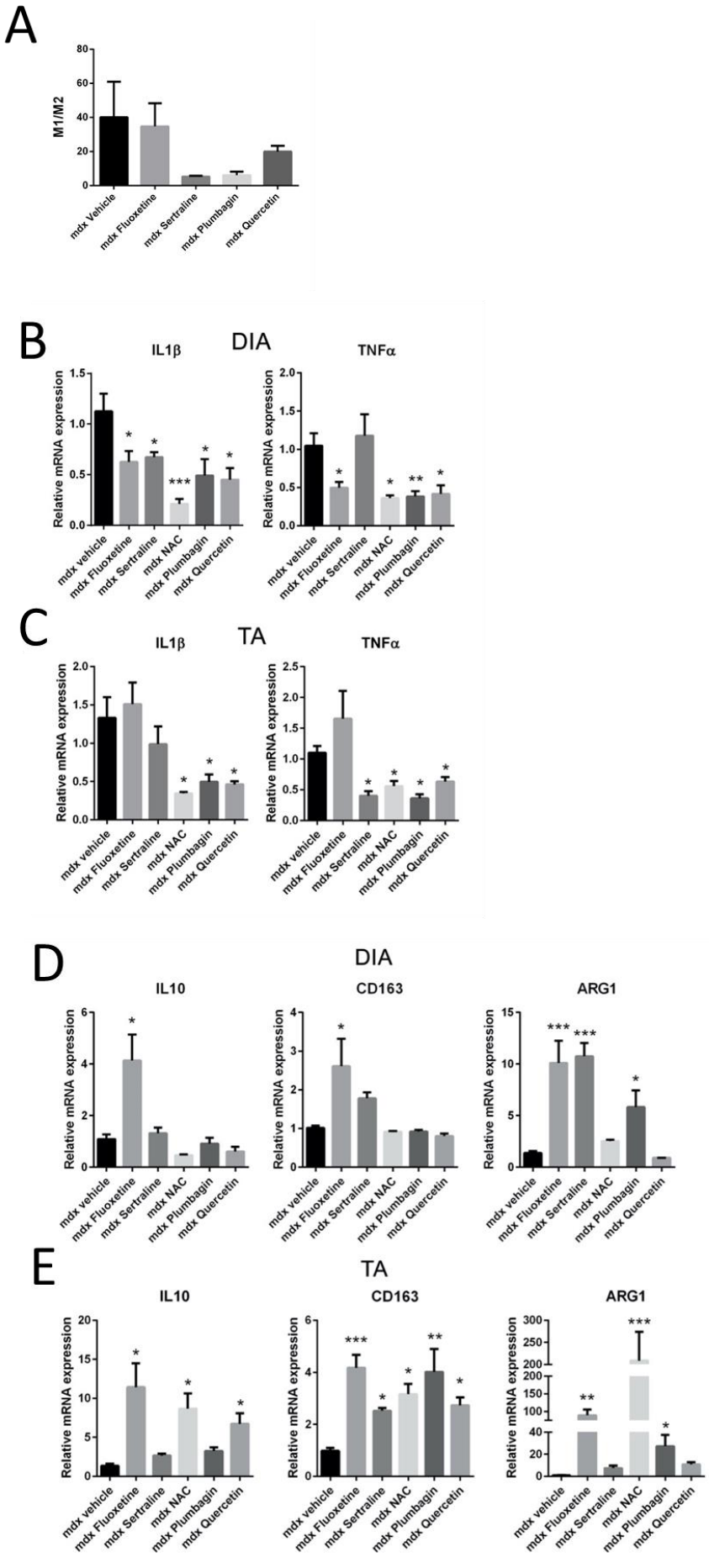
A
M1/M2
mdx Vehicle
mdx Fluoxetine
mdx Sertraline
mdx Plumbagin
mdx Quercetin
B
DIA
IL1β
TNFα
Relative mRNA expression
mdx vehicle
mdx Fluoxetine
mdx Sertraline
mdx NAC
mdx Plumbagin
mdx Quercetin
C
TA
IL1β
TNFα
D
DIA
IL10
CD163
ARG1
E
TA
IL10
CD163
ARG1

Figure 9. FIASMAs and antioxidants modulate inflammation towards an anti-inflammatory state in mdx mice

A) Evaluation of the M1/M2 ratio by Flow cytometry in muscle lysates, with M1 macrophages labeled as $CD45^{+}F4/80^{+}CD206^{-}CD80^{+}MHCII^{+}$ and M2 macrophages as $CD45^{+}F4/80^{+}CD206^{+}CD80^{-}MHCII^{-}$.

B-C) RT-qPCR analysis of the M1 markers/cytokines IL-1β, TNF-α in DIA (**B**) and TA (**C**) of *mdx* mice (treated or not).

D-E) RT-qPCR analysis of the M2 markers/cytokines IL-10, CD163 and ARG1 in DIA (**D**) and TA (**E**) of *mdx* mice (treated or not).

All values are expressed as mean ± SEM (n ≥ 3 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *mdx* vehicle.

ASMase ablation shifts macrophage polarization towards an M2 phenotype

The first clue that ASMase is involved in M1 macrophage biology is that its activity is increased in differentiated macrophages (Mp) and M1 but not M2 macrophages derived from WT mice with respect to monocytes *in vitro* (*Figure 10A*), which is in line with the literature stating that ASMase is activated by stress stimuli including lipopolysaccharide, TNF-α and IL-1β.

In order to corroborate our hypothesis on the role of ASMase in inflammation and muscle damage/regeneration, we took advantage of ASMase-KO mice, starting from the analysis of macrophages (Roux-Biejat et al., 2021).

We found that the absence of ASMase results in a significantly reduced expression of IL-1β, TNF-α, CD80 and NOS2 in comparison to the WT counterparts, particularly in macrophages cultured in M1-polarizing conditions, but also to some extent for M2 (*Figure 10B*).

Similarly, we noticed a reduction of IL-1β, TNF-α and CD80 when treating WT M1 macrophages with the FIASMA Amitriptyline (*Figure 10C-D*).

While we observed no differences for CD206 and CD163 in ASMase-KO macrophages cultured in M2-polarizing conditions, we detected a surge in the expression of these M2 markers in M1 macrophages of ASMase-KO mice (*Figure 10E*).

Moreover, these data paralleled with the increase of Nrf2 expression in M1 macrophages lacking ASMase (*Figure 10F*), thus indicating a possible involvement of this transcription factor in the molecular machinery triggered by ASMase.

Noteworthy, the similar expression of the marker F4/80 in both WT and ASMase-KO revealed that ASMase-KO derived cells differentiate normally, therefore suggesting that ASMase does not affect macrophage differentiation (*Figure 10G*).

Altogether these results show that ASMase has a crucial role in M1 polarization and that its absence shifts macrophages towards an M2 phenotype.

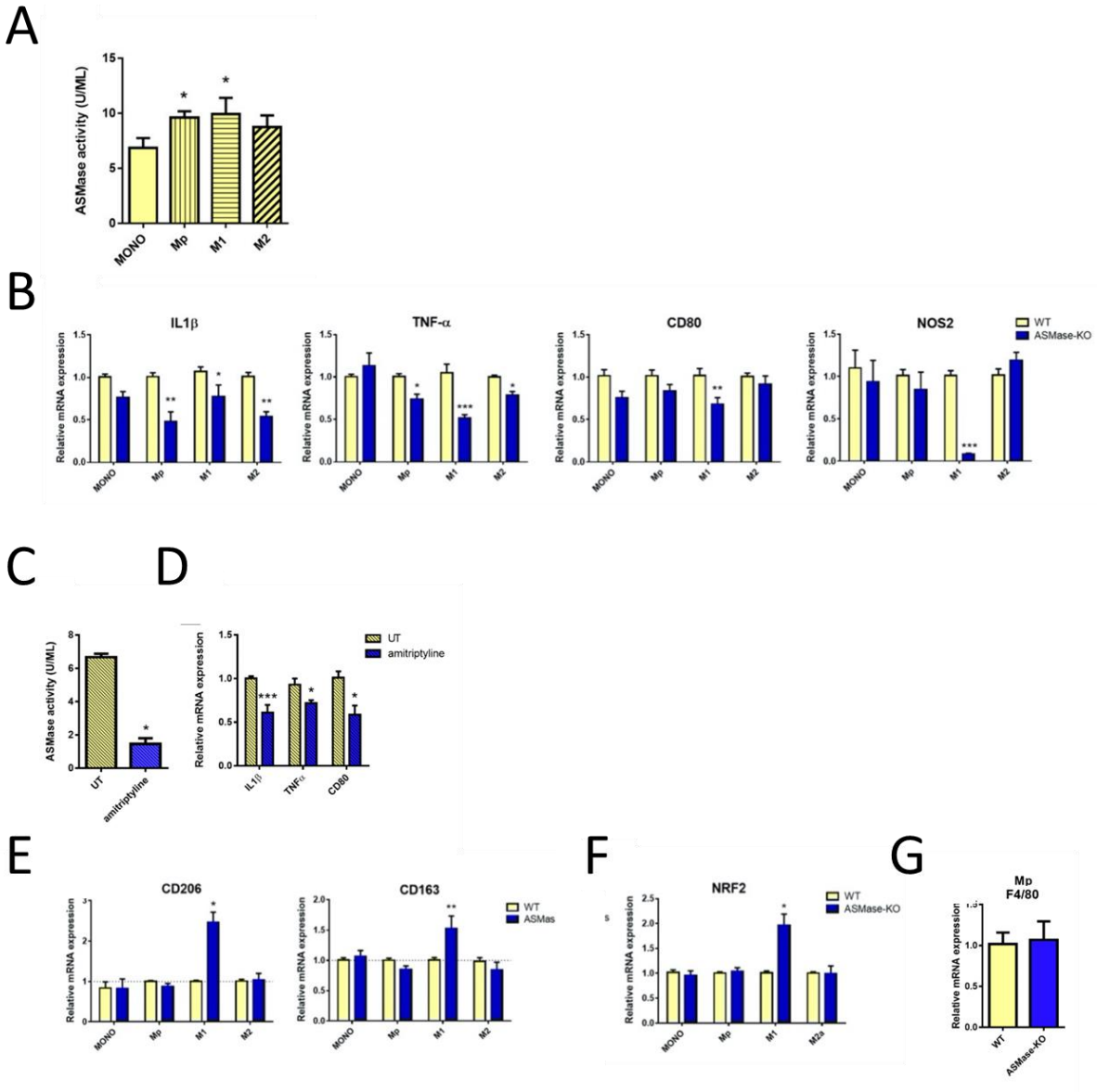

Figure 10. ASMase ablation shifts macrophage polarization towards an M2 phenotype

A) ASMase activity measured at different stages of macrophages differentiation and polarization, i.e., monocytes (MONO), differentiated macrophages (Mp), M1-polarized macrophages (M1) and M2-polarized macrophages (M2) from WT mice.

Values are expressed as mean ± SEM macrophages (Mp). Values are expressed as mean ± SEM ($n \geq 3$ mice). * $p < 0.05$ vs. MONO.

B) RT-qPCR analysis of the M1 markers IL-1β, TNF-α, CD80 and NOS2 (iNOS) in MONO, Mp, M1 and M2 from WT and ASMase-KO mice.

Values are expressed as mean ± SEM ($n \geq 3$ mice) normalized vs the untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the respective WT.

C) ASMase activity measured in macrophages from WT mice treated with the FIASMA Amitriptyline (5 μM) during the differentiation.

Values are expressed as mean ± SEM (n = 4 mice). * $p < 0.05$ vs the untreated control.

D) RT-qPCR analysis of the M1 markers IL-1β, TNF-α, CD80 in Amitriptyline (5 μM) treated macrophages.
Values are expressed as mean ± SEM (n ≥ 3 mice). * $p < 0.05$, *** $p < 0.001$ vs. the respective untreated control.
E-F) RT-qPCR analysis of the M2 markers CD206, and CD163 (**E**) and Nrf2 (**F**) in MONO, Mp, M1 and M2 from WT and ASMase-KO mice.
Values are expressed as mean ± SEM (n ≥ 3 mice) normalized vs the untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the respective WT.
G) RT-qPCR analysis of the F4/80 marker in differentiated macrophages (Mp) isolated from WT and ASMase-KO mice muscles (n = 3).
Values are expressed as mean ± SEM.

ASMase ablation reduces inflammation favoring anti-inflammatory mediators after acute muscle injury

As a well-orchestrated inflammation is crucial for muscle regeneration, we explored the potential of inhibiting ASMase to modulate the immune response after acute muscle injury. To do so, we injected cardiotoxin (CTX) in the TA of WT and ASMase-KO mice and retrieved it at different time points of healing.

The increase of ASMase activity we observed starting from 3 days after the injury peaking at 5 and 7 days (*Figure 11A*), suggests an involvement of this enzyme in muscle regeneration *in vivo*.

Compared to their WT littermates, we found in ASMase-KO mice a reduced leukocyte infiltration, assessed by counting $CD45^+$ cells at the site of injury (*Figure 11B-C*). Importantly, the pro-inflammatory state was inhibited in ASMase-KO animals as demonstrated by the lower expression of IL-1β and IL-6 seen 3 and 5 days after the CTX injection (*Figure 11D*). This was reinforced by the reduced positivity to immunolabeled CD80 with no difference concerning CD206 (*Figure 11E*), and accordingly, the decreased M1/M2 ratio assessed by flow cytometry (*Figure 11F*).

These findings show that the absence of ASMase can modify the course of inflammation *in vivo*. The concomitant elevation of Insulin-like growth factor 1 (IGF-1) (*Figure 11D*), a potent enhancer of tissue regeneration, indicates that such changes in the niche of satellite cells are beneficial (Tidball & Welc, 2015).

Moreover, the anti-inflammatory effect in the absence of ASMase, following tissue damage was confirmed by the enhanced expression of Nrf2 (*Figure 11G*), along with its downstream genes involved in the antioxidant response: HO-1, GPX1 and SOD3 (*Figure 11H*).

Taken together, these data show that the absence of ASMase alleviates inflammation and oxidative stress, by effectively activating Nrf2 and shifting macrophage polarization towards the M2 phenotype associated with tissue repair *in vivo*. This scenario following acute muscle injury in ASMase-KO mice parallel with our results obtained with FIASMAs and antioxidants in *mdx* mice in the context of chronic injury.

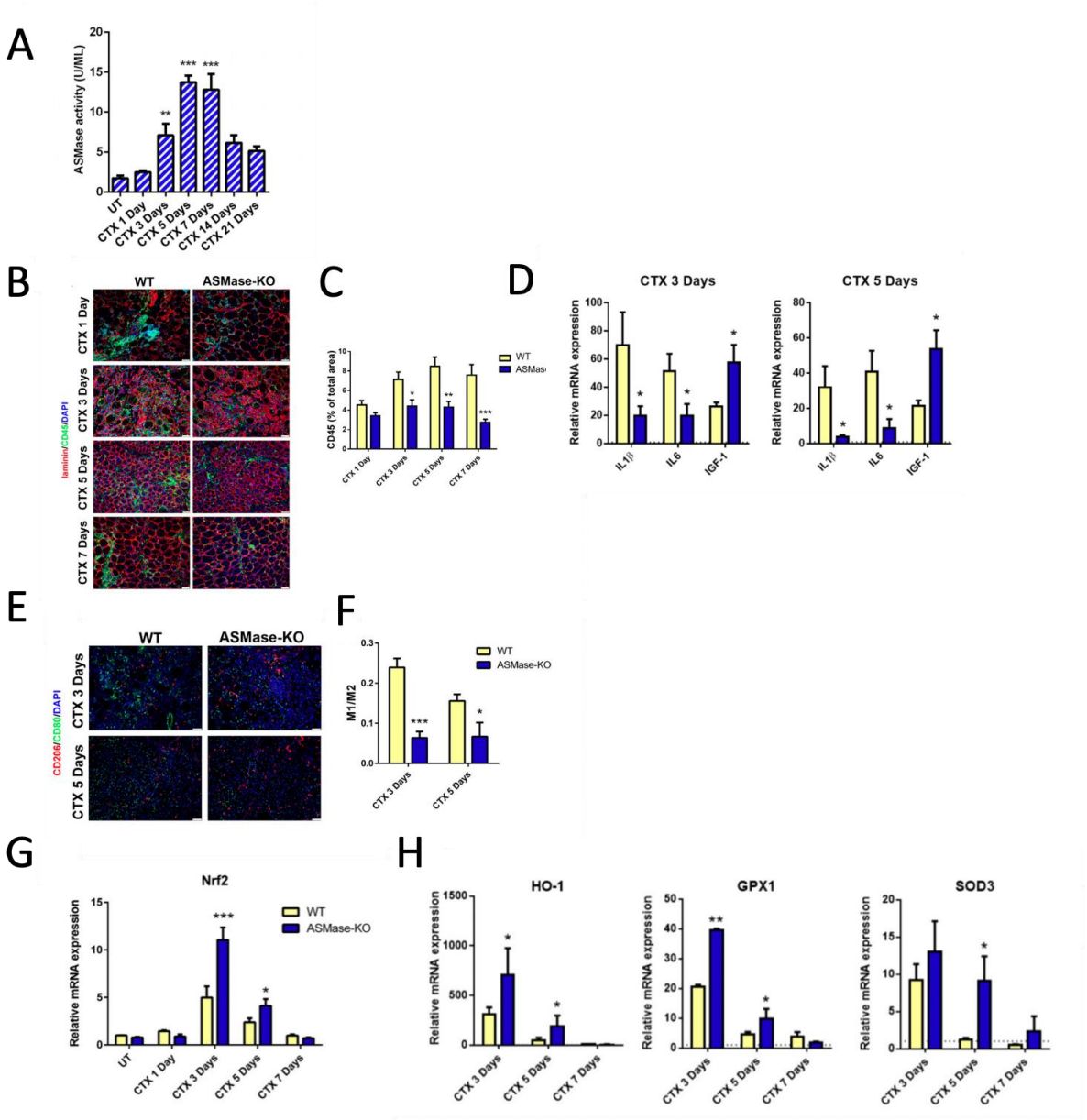

Figure 11. ASMase ablation reduces inflammation favoring anti-inflammatory mediators after acute muscle injury

A) Acid sphingomyelinase activity measured at different time points after CTX injection in tibialis anterior of WT mice ($n \geq 4$ mice).

Values are expressed as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$ vs. the untreated control (UT).

B) Representative images of CD45 immunostaining and DAPI nuclear counterstaining (blue) of transverse sections from WT and ASMase-KO TA muscles at different time points after CTX injection.

C) CD45 positive ($CD45^+$) quantification. Values are expressed as mean ± SEM ($n = 3$ mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the respective WT.

D) RT-qPCR analysis of inflammatory markers IL-1β, IL-6 and IGF-1 at 3 (left graph) and 5 (right graph) days after CTX injection. Values are expressed as mean ± SEM ($n \geq 4$ mice) normalized vs. the untreated controls (dashed line).

E) Representative images of CD206 (red) and CD80 (green) immunostaining and DAPI nuclear counterstaining (blue) of transverse sections from WT and ASMase-KO TA muscles at 3 and 5 days after CTX injection.
F) M1/M2 ratio quantification by flow cytometry in TA muscles at 3 and 5 days after CTX injection in WT and ASMase-KO mice ($n \geq 3$ mice for each group).
Values are expressed as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ vs. the respective WT.
G) RT-qPCR analysis of Nrf2 at different time points after CTX injection.
Values are expressed as mean ± SEM ($n \geq 3$ mice) normalized vs the untreated control. * $p < 0.05$, *** $p < 0.001$ vs. the respective WT control. UT: untreated.
H) RT-qPCR analysis of HO-1, GPX1 and SOD3 at different time points after CTX injection.
Values are expressed as mean ± SEM ($n \geq 3$ mice) normalized vs. the untreated controls (dashed line). * $p < 0.05$, ** $p < 0.01$ vs. the respective WT.

ASMase ablation improves skeletal muscle regeneration after acute damage

To test the hypothesis that ablation of ASMase is beneficial in the context of muscle damage we compared muscle regeneration of ASMase-KO and WT mice after cardiotoxin injection.

We evaluated the CSA of regenerating centrally nucleated fibers and the expression of embryonic myosin (MyHC-emb) as a proxy for muscle regeneration at 5 and 7 days after injury (*Figure 12A-B and 12D-E*) as these time points correspond to the highest activity of ASMase (*Figure 11A*), and regenerative myogenesis and tissue remodeling are ongoing.

Although the number of regenerating myofibers was the same in WT and ASMase-KO TA (*Figure 12G*), their increased size along with the decreased expression of MyHC-emb in ASMase-KO mice compared to WT in both time points (*Figure 12A-B and 12D-E*) indicate an enhanced growth of the new fibers resulting from an accelerated myogenesis.

Contemporarily, we found an increase in mRNA expression of dystrophin (DMD) (*Figure* 12*C-F*) which is an indicator of myofiber maturity (Yoshimoto et al., 2020).

The increased number of satellite cells in ASMase-KO mice at 3 and 5 days post-injury in comparison to WT mice, assessed by flow cytometry and immunofluorescence (*Figure 12H-I*), further supported these findings.

As no differences were found in the later stages of muscle regeneration (14 days after muscle injury) (*Figure 12J*), we concluded that the effect was transient.

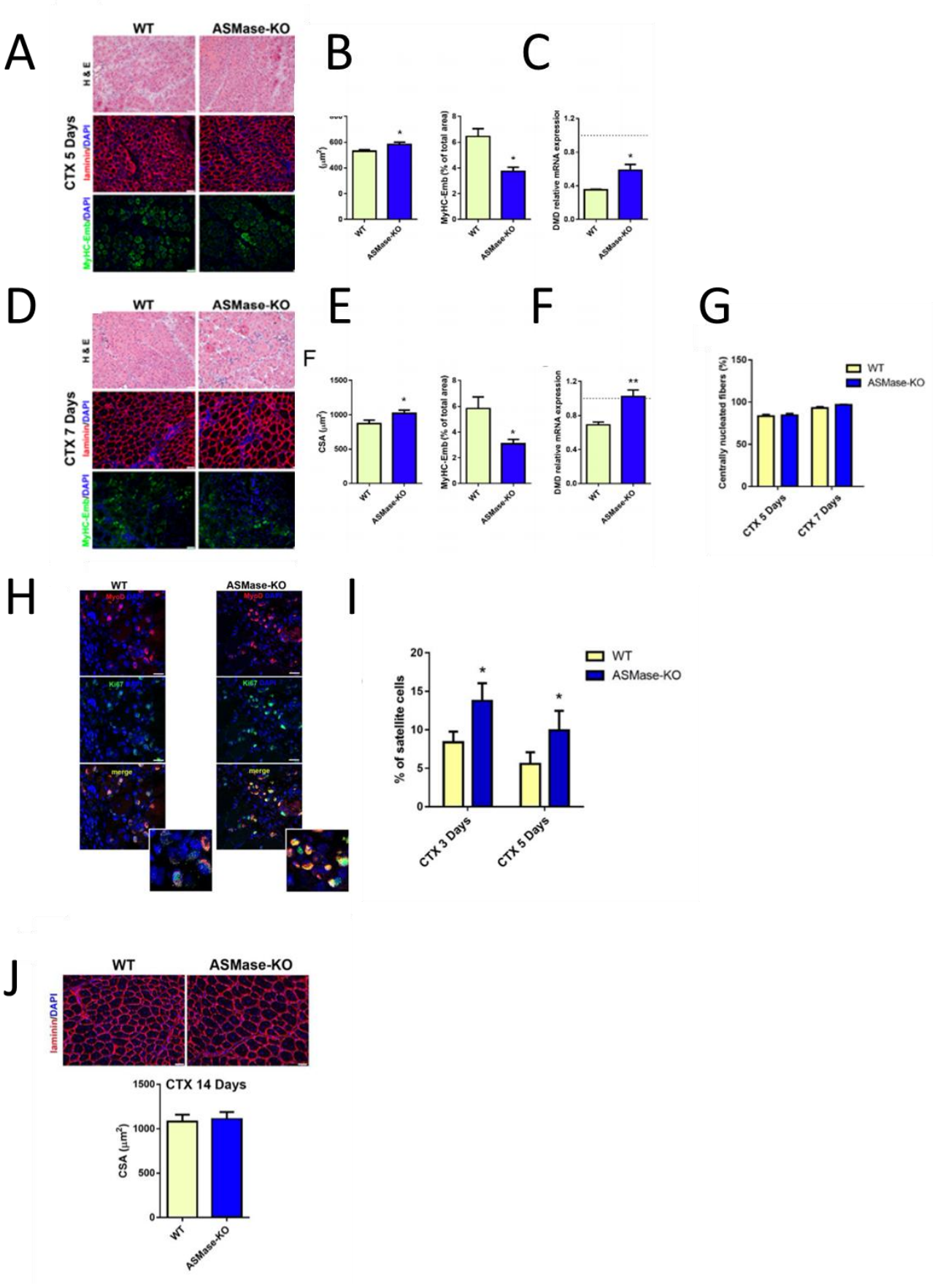
A
WT
ASMase-KO
H&E
CTX 5 Days
laminin/DAPI
MyHC-Emb/DAPI
B
C
D
CTX 7 Days
E
F
G
Centrally nucleated fibers (%)
CTX 5 Days
CTX 7 Days
H
Ki67
merge
I
% of satellite cells
CTX 3 Days
CTX 5 Days
J
CTX 14 Days
CSA (μm²)

Figure 12. ASMase ablation improves skeletal muscle regeneration after acute damage
A-G) Analysis of TA muscles at 5 and 7 days after CTX injection in WT and ASMase-KO mice.
A) Representative images at 5 days after CTX injection of H&E staining (top panels), laminin (middle panels) and MyHC-Emb (bottom panels) immunostaining of transverse sections of TA muscles. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm.
B) Mean CSA quantification (left graph) of regenerating fibers (centrally nucleated) measured on laminin staining (n = 3 mice) and MyHC-Emb quantification (right graph) (n = 4 mice) of TA muscles at 5 days after CTX injection.
Values are expressed as mean ± SEM. * $p < 0.05$ vs. the WT.
C) RT-qPCR analysis of DMD at 5 days after CTX injection. Values are expressed as mean ± SEM (n = 3 mice) normalized vs. the untreated controls (dashed line). * $p < 0.05$, ** $p < 0.01$ vs. the WT.
D) Representative images at 7 days after CTX injection of H&E staining (top panels), laminin (middle panels) and MyHC-Emb (bottom panels) immunostaining of transverse sections of TA muscles. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm.
E) Mean CSA quantification (left graph) of regenerating fibers (centrally nucleated) measured on laminin staining (n = 3 mice) and MyHC-Emb quantification (right graph) (n = 4 mice) of TA muscles at 7 days after CTX injection.
Values are expressed as mean ± SEM. * $p < 0.05$ vs. the WT.
F) RT-qPCR analysis of DMD at 5 days after CTX injection. Values are expressed as mean ± SEM (n = 3 mice) normalized vs. the untreated controls (dashed line). * $p < 0.05$, ** $p < 0.01$ vs. the WT.
G) Quantification of centrally nucleated fibers (%) measured in H&E-stained transversal sections of TA muscles from WT and ASMase-KO mice at 5 and 7 days after CTX injection.
Values are expressed as mean ± SEM.
H) Representative images of proliferating Satellite Cells, *i.e.* MyoD (Red) and Ki67 (green) positive cells, in TA muscles of WT and ASMase-KO mice at 5 days after CTX injection. Nuclei were counterstained with DAPI (blue). Scale Bar = 20 μm.
I) Satellite Cell quantification by flow cytometry in TA muscles of WT and ASMase-KO mice at 3 and 5 days after CTX injection ($n \geq 4$ mice).
Values are expressed as mean ± SEM. * $p < 0.05$ vs the respective WT control.
J) Representative images of laminin immunostaining of transverse sections of TA muscles at 14 days after CTX injection. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm. The graph shows the mean CSA quantification measured on laminin staining (n = 3 mice). Values are expressed as mean ± SEM.

ASMase ablation does not affect the phenotype/function of Skeletal Muscle *in vivo* and *in vitro*

Finally, to clearly demonstrate that ASMase controls muscle regeneration by modulating the immune response, we investigated ASMase-KO mice muscles and compared them to their WT counterparts, during their growth.

Muscle weights and gross morphology assessed by histology and measurement of the fiber size (CSA) from both genotypes were indistinguishable, as showed our analysis of TA, gastrocnemius (GA), quadriceps (QUAD), and DIA at 1, 2 and 3 months of age to evaluate muscle growth (*Figure 13A-E*).

In agreement with a normal muscle phenotype, ablation of ASMase did not alter the strength of the mice as they had the same performance at the Whole Body Tension Test (WBT) (*Figure 13F*).

Considering the fundamental role of Satellite Cells in muscle regeneration, we wondered whether the absence of ASMase would affect their capabilities to proliferate and differentiate. Thus, we established primary muscle progenitor cell culture from ASMase-KO and WT mice. After 24h of culture, to analyze their proliferation, we compared by immunostaining the percentage of cells positive for the cell cycle marker Ki67 (F*igure 14A-B*). After 48h, we analyzed their differentiation by confronting their fusion index, diameter, number of nuclei per myotube, and myotubes with more than 5 nuclei (*Figure 14C-D*). We also analyzed the expression of the myogenic factors: MyoD, Myogenin, MyHCII, MyHCIV for both time points (*Figure 14E*).

As we found no difference *in vitro* in between the two genotypes, we concluded that ASMase ablation does not affect the proliferation and differentiation of satellite cells.

Altogether, these results indicate that early muscle regeneration is accelerated in ASMase-KO mice by modulating the microenvironment of the injured muscle, without affecting satellite cell myogenic properties directly.

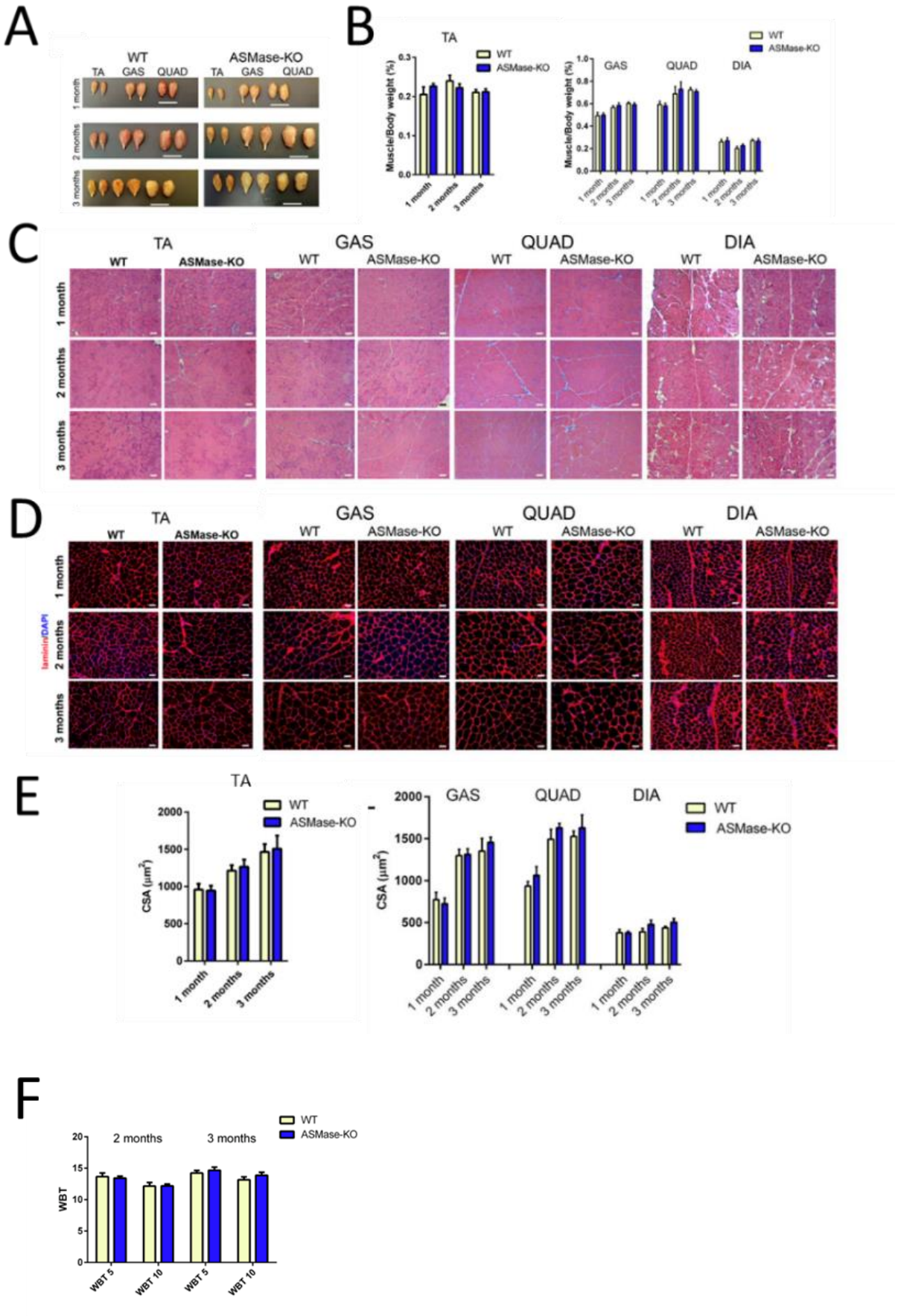
A
WT
ASMase-KO
TA
GAS
QUAD
1 month
2 months
3 months
B
TA
WT
ASMase-KO
Muscle/Body weight (%)
GAS
QUAD
DIA
C
TA
GAS
QUAD
DIA
D
laminin/DAPI
E
CSA (μm²)
F
2 months
3 months
WBT
WBT 5
WBT 10

Figure 13. Characterization of the skeletal muscle phenotype in ASMase-KO mice *in vivo*
A) Representative images of Tibialis anterior (TA), Gastrocnemius (GAS) and Quadriceps (QUAD) muscles of 1 month, 2 months and 3 months old WT and ASMase-KO mice. Scale Bar = 10 mm.
B) Muscle weight, normalized to body weight, of TA, GAS, QUAD and DIA muscles of 1, 2, and 3 months old WT and ASMase-KO mice ($n \geq 4$ mice).
C-D) H&E staining (**C**) and Laminin (red) and DAPI (Blue) (**D**) immunostaining of cryosections of muscles of TA, GAS, QUAD and DIA of 1, 2, and 3 months old WT and ASMase-KO mice. Scale Bar = 50 μm.
E) Quantification of mean CSA of TA, GAS, QUAD and DIA of 1, 2, and 3 months old WT and ASMase-KO mice ($n \geq 4$ mice) measured using ImageJ based on laminin staining.
F) WBT measurements determined by dividing the average of the top ten or top five forward pulling tensions, respectively, by the body weight in 1, 2, and 3 months old WT and ASMase-KO mice (n= 10).

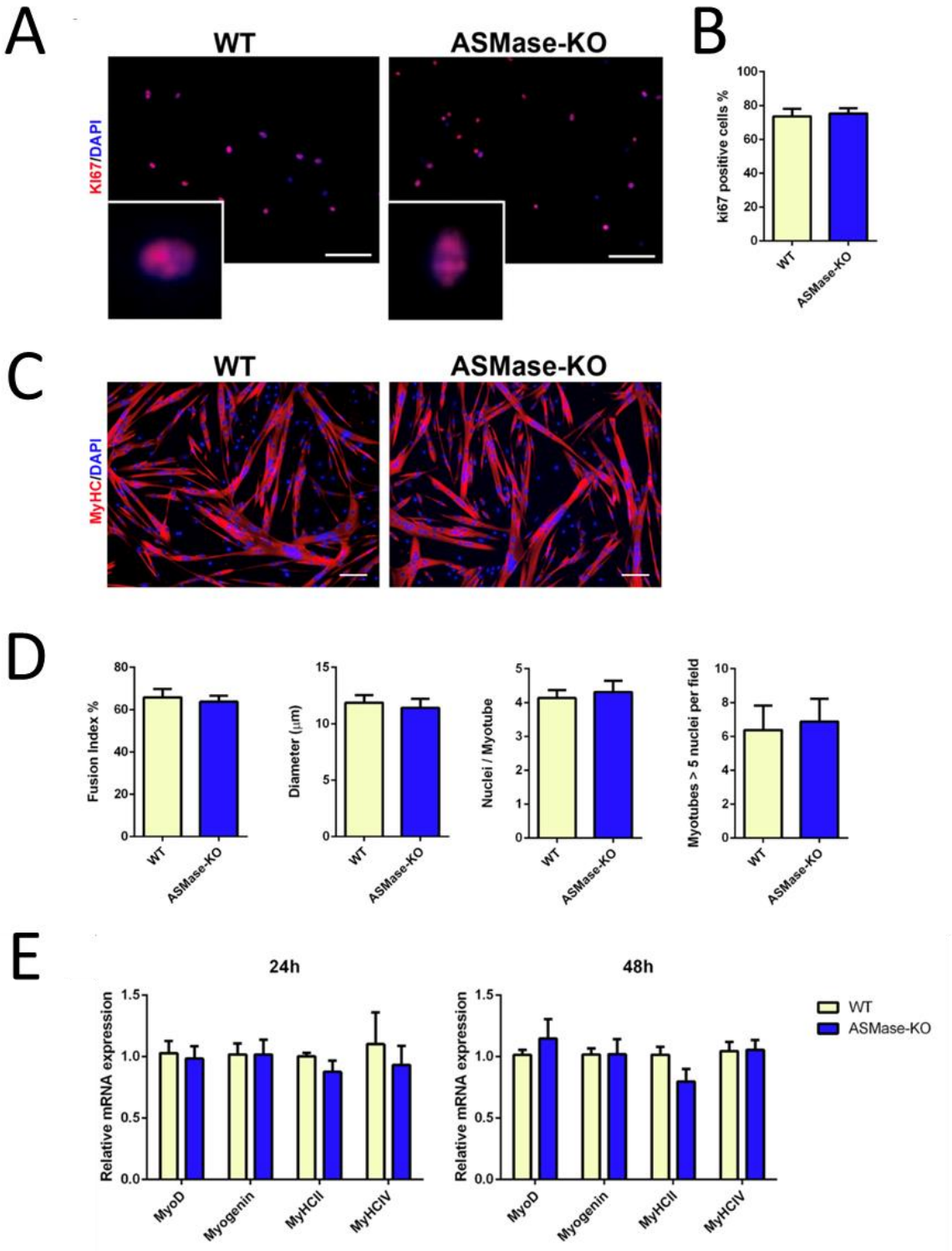
A
WT
ASMase-KO
Ki67/DAPI
B
ki67 positive cells %
100
80
60
40
20
0
WT
ASMase-KO
C
WT
ASMase-KO
MyHC/DAPI
D
Fusion Index %
80
60
40
20
0
WT
ASMase-KO
Diameter (μm)
15
10
5
0
WT
ASMase-KO
Nuclei / Myotube
5
4
3
2
1
0
WT
ASMase-KO
Myotubes > 5 nuclei per field
10
8
6
4
2
0
WT
ASMase-KO
E
24h
48h
Relative mRNA expression
1.5
1.0
0.5
0.0
MyoD
Myogenin
MyHCII
MyHCIV
WT
ASMase-KO

Figure 14. Characterization of the skeletal muscle phenotype in ASMase-KO mice *in vitro*
A) Representative Ki67 immunostaining (red) and DAPI nuclear counterstaining (blue) of satellite cells isolated from WT and ASMase-KO mice and cultured for 24 h (Proliferation). Scale bar = 100 µm.
B) Percentage of Ki67 positive satellite cells on total DAPI staining. Values are expressed as mean ± SEM (n = 5 mice).
C) Representative MyHC immunostaining (red) and DAPI nuclear counterstaining (blue) of satellite cells isolated from WT and ASMase-KO mice and cultured for 48 h (Differentiation). Scale bar = 100 µm.
D) Fusion index, mean myotubes diameter, mean number of myonuclei/myotube and percentage of myotubes with more than 5 nuclei of satellite cells from WT and ASMase-KO mice (n = 9 mice).
E) RT-qPCR analysis of myogenic markers Myod, Myog, and MyHCII and MyHCIV during proliferation (24h) and differentiation (48h) of satellite cells ($n \geq 4$ mice).

DISCUSSION

The enzyme ASMase plays a major role in the metabolism of sphingolipids by generating ceramide, a lipid second messenger involved in a wide range of physiological and pathological mechanisms (Zeidan & Hannun, 2010). ASMase is activated by multiple stress stimuli and in turn, induces the production of key mediators of Inflammation and Oxidative Stress. As both of these processes are hallmarks of DMD (Petrillo et al., 2017; Tidball et al., 2018) and we recently demonstrated the role of ASMase in the progression of this muscular dystrophy; here, we explored whether targeting this enzyme by FIASMAs (Kornhuber et al., 2010) and/or antioxidant molecules, would be a pertinent therapeutic strategy. We provide evidence that acting on the ASMase pathway induces a protective effect from the development of the pathology with improvements of both morphological and functional muscle features.

FIASMAs and Antioxidants improve mdx mice pathophysiology

The first proof-of-concept evidence of ASMase modulation in DMD has been obtained in a short-term experiment by administering the FIASMA and TCA Amitriptyline to *mdx* mice. As mouse model for the experiments of this work we decided to use C57BL/10ScSn-Dmd^{mdx}/J, the one recommended by TREAT-NMD (https://treat-nmd.org/resources-support/research-overview/preclinical-research/animal-model-choice-for-dmd/) to evaluate drug efficacy for DMD. Despite their milder phenotype with respect to the human pathology, they remain the most extensively used model as the recently developed ones, aiming to increase the severity of the condition, usually rely on additive effects of either structural or myogenic gene knockouts that do not occur in the human disease (Willmann et al., 2009; Yucel et al., 2018). Our results demonstrated the ability of the drug to decrease ASMase in skeletal muscle along with the reduction of inflammation. This is in line with previous data on the beneficial effect of Amitriptyline in improving *mdx* mice phenotype by modulating inflammation and supports our hypothesis of the anti-inflammatory effect of ASMase inhibition (Manning et al., 2014).

As DMD is a chronic disease, a long-term treatment of *mdx* mice has been carried out, using other FIASMAs renowned to be safer: Fluoxetine and Sertraline, belonging to the class of SSRIs. Furthermore, we tested the antioxidant molecules Plumbagin, Quercetin and NAC. The latter was used as a control for the efficacy of our protocol as NAC is currently in pre-clinical studies for DMD (Yao et al., 2021).

In the exhaustion treadmill running test, used to determine the improvement of muscle function in mice under treatment, *mdx* mice treated with Plumbagin or Quercetin performed significantly better than the dystrophic controls, reaching a recovery score similar to NAC, while both FIASMAs did not appear to improve muscle function. Indeed, *mdx* mice treated with Fluoxetine or Sertraline ran the same distance and time as the *mdx* vehicle. However, a limit to this test may be the involvement of behavior. As the treated WT performed poorly compared to the WT vehicle, we suggest that a cognitive impairment following the treatment can cover a possible beneficial action of FIASMAs on skeletal muscle. In recent studies, Fluoxetine treatment in mice (18mg/kg/day for 6 weeks) increased endurance abilities and more

significantly grip strength (Tutakhail et al., 2019). The possible reasons for which we did not obtain the same outcome on the treadmill could be related to differences in the treadmill protocol, the dosage of Fluoxetine which could modify its activating or sedating effect, the duration of the treatment as well as the strain of mice (Beasley et al., 1992). Nonetheless, these results along with the increased muscle mass in the Fluoxetine group are particularly encouraging and considering the more striking difference observed with the grip strength test, this prompts us to take advantage of other tests to assess muscle strength.

All the treatments tested improved muscle morphology, reaching a nearly normal architecture with Sertraline, Plumbagin and Quercetin administration. Fibrosis was also greatly reduced in all the treatments, apart from Fluoxetine. Importantly, treated *mdx* mice showed a significant decrease in muscle damage, assessed by evaluation of myonecrosis, measurement of serum Creatine Kinase during treatment (Klein et al., 2017) and inflammation. In parallel, we also noticed an enhanced regeneration, with higher expression of key regulators of myogenesis: Myogenin, which controls the transition from proliferating myoblasts to differentiating myotubes and, particularly, Myf6 which is involved in myofiber maturation and is the predominant myogenic factor in mature differentiated muscle (Ganassi et al., 2020; Moretti et al., 2016; Zammit, 2017). Noteworthy, the proportion of satellite cells expressing Myogenin entering the differentiation program is peculiarly low in DMD muscles (Kottlors & Kirschner, 2010).

The increase of regeneration in the context of muscular dystrophy, with its continuous rounds of degeneration, may be considered as an indicator of muscle damage (Guiraud et al., 2019). However, it is also known that this perpetuating vicious cycle of degeneration and regeneration in DMD depletes the pool of satellite cells (A. Lu et al., 2014). Furthermore, as dystrophin is normally expressed in satellite cells, its absence distorts the polarity of these stem cells, causing a deficit in asymmetric divisions resulting in a reduced rate of myogenic progenitor cell generation (Dumont, Wang, et al., 2015; Y. X. Wang et al., 2019).

Altogether these data account for a general improvement of the dystrophic phenotype following the treatments, which can generate a more favorable environment for Satellite Cells known to be particularly influenced by macrophages: to live, proliferate and differentiate (Boldrin et al., 2015; Madaro et al., 2019; Schertzer et al., 2007).

The similarities between inhibition of ASMase in acute and chronic muscle damage

FIASMAs achieved to decrease ASMase activity in the diaphragm, the muscle recapitulating best the human pathology and thus the muscle we used for most of the experiments (Stedman et al., 1991), therefore corroborating the role of this enzyme in the pathophysiology of the disease. The antioxidant Plumbagin was also able to decrease the enzyme activity, while Quercetin failed to modify it. However, since no difference was found in the expression of ASMase, we can hypothesize that the protective effect of the compound relies on the modulation of the enzyme activity rather than its expression as for FIASMAs which induce the lysosomal degradation of ASMase (Kornhuber et al., 2010). This decrease in Plumbagin-treated muscles could be solely related to its antioxidant and anti-inflammatory properties already demonstrated (Wang & Li, 2018), although it seems worth investigating whether this molecule does not have more direct control over ASMase.

All the treatments attenuated Oxidative Stress by increasing the expression of members of the antioxidant system: HO-1 and SOD3 while only Fluoxetine was able to promote GPX1. This antioxidative property of the FIASMA is in line with previous studies (Behr et al., 2012; Tutakhail et al., 2019). Unexpectedly, we observed a surge of NOX4 gene expression in Plumbagin and Quercetin-treated muscles. This could be ascribed to a compensation mechanism after inhibition of its activity by antioxidants. However, NOX4 upregulation, effective or not, is clearly counteracted by the induction of the antioxidant system defense as evidenced by histopathological and functional evaluation of muscles. Nonetheless, this deserves more research in order to be confirmed, for example *in vitro* on macrophages and satellite cells to verify that the direct antioxidant effect due to inhibition of the activity of NOX4 counterbalances the induction of its expression, resulting from a presumed compensation mechanism.

This induction of HO-1 by the FIASMAs confirms previous reports (Shin et al., 2009). This is encouraging considering the role of HO-1 in the antioxidative and anti-inflammatory response, as well as cell survival and cell cycle regulation. Its importance has even been reinforced recently by the worsening of the disease in dystrophin-deficient animals lacking the Hmox1 gene (Hmox1$^{-/-}$*mdx*) (Pietraszek-Gremplewicz et al., 2018).

Only Fluoxetine and Plumbagin were able to induce Nrf2 expression, which corroborates with previous findings (Guo et al., 2017; Mendez-David et al., 2015) and is particularly promising considering the potential of this transcription factor to simultaneously target separate pathological features of DMD including inflammation and oxidative stress (Kourakis, Timpani, de Haan, et al., 2021).

Both FIASMAs and antioxidants alleviated inflammation and promoted its shift towards an anti-inflammatory state. All the treatments reduced the expression of the M1 markers: IL-1β and TNF-α mostly in both diaphragm and tibialis anterior or only one of them for Sertraline and Fluoxetine. These pro-inflammatory cytokines are elevated in *mdx* mice and their inhibition has been found beneficial (Evans et al., 2009; Hodgetts et al., 2006; Huang et al., 2009). Regarding the anti-inflammatory M2 markers known to drive tissue repair (S. A. Villalta, Deng, et al., 2011) assessed by the evaluation of IL-10, CD163 and ARG1, the best results were obtained with Fluoxetine which increased all of them in both muscles as well as with Plumbagin that modulated the expression of ARG1. This surge of IL-10 expression induced by Fluoxetine is a positive sign as it has been demonstrated that IL-10 reduces the pathology of muscular dystrophy (S. A. Villalta, Rinaldi, et al., 2011). Moreover, IL-10 is a strong inducer of CD163, which promotes muscle repair by shifting macrophages towards the M2c phenotype and its expression was increased with all the treatments in the tibialis anterior (S. A. Villalta et al., 2009). Additionally, supporting this switch in macrophage phenotype, Plumbagin and Sertraline decreased the M1/M2 ratio.

Altogether, these antioxidative and anti-inflammatory effects exerted by both FIASMAs and antioxidants are consistent with the improvement in muscle function and regeneration.

Similar results were obtained in an acute muscle injury model by using ASMase-KO mice (Roux-Biejat et al., 2021), thus suggesting that the events observed in treated *mdx* mice are related to the decrease of ASMase activity. We first found *in vitro* that ASMase plays a crucial role in M1 polarization and that its absence shifts macrophages towards an M2 phenotype as attested the decrease of IL-1β, TNF-α, CD80 and NOS2 concomitantly with the

increase of CD163, CD206 and Nrf2 in ASMase-KO macrophages cultured in M1 conditions compared to the WT counterparts. Noteworthy, this switch was also observed when administering the FIASMA Amitriptyline to WT M1 macrophages. The molecular/cellular pathways involved in macrophage phenotype polarization during muscle injury/regeneration are still under investigation. Our results pointed out an involvement of ASMase, likely through Nrf2 and are in line with the data of Kobayashi and colleagues that have recently clarified that Nrf2 activation inhibits the expression of inflammatory cytokines in M1 macrophages, thus blunting the inflammatory response (Kobayashi et al., 2016).

To explore the relevancy of the modulation of the immune system by ASMase, we evaluated *in vivo* the regenerative capacities of ASMase-KO and WT mice, who physiologically show no difference in their skeletal muscle system. Following CTX injection, ASMase-KO muscles displayed a decrease in inflammation along with a shift towards the M2 phenotype with a decrease in IL-1β and IL-6 expression and in the M1/M2 ratio. ASMase ablation also alleviated Oxidative Stress by inducing the antioxidant defense system with HO-1, GPX1 and SOD3. In the absence of ASMase, there is a peak in Nrf2 expression, as we observed *in vitro* in M1 macrophages of ASMase-KO mice. These data further confirm that the crosstalk between the ASMase/ceramide pathway and inflammation is tight and multi-faceted. Indeed, if it is clearly demonstrated that the activation of ASMase by inflammatory cytokines is often mandatory for their intracellular signaling to be effective, it is also established that ASMase may, in turn, stimulate cytokine expression and release (Falcone et al., 2004; Hofmeister et al., 1997; Perry et al., 2014).

In parallel with the modulation of inflammation, muscle regeneration was improved as attested the increased size of regenerating fibers along with the increased expression of DMD, a reflector of myofiber maturity (Yoshimoto et al., 2020), and IGF-1 a hormone important in muscle growth and maintenance also recognized to improve regeneration by directly expanding the pool of satellite cells and concomitantly driving macrophages towards the M2 phenotype (Y. H. Song et al., 2013; Tidball & Welc, 2015). Consistently, the number of satellite cells was also increased after the damage, although in normal conditions we found no difference between the two genotypes. These data corroborate our observations on damage/regeneration in *mdx* mice after treatment with FIASMAs.

Nevertheless, we are aware that further investigations are necessary to clearly dissect the molecular pathway underlying ASMase inhibition, as for instance, investigating *in vitro* on primary cultures of macrophages and satellite cell and *in vivo* in *mdx* mice the effects of ARC39, a recently discovered molecule that is a direct and specific inhibitor of ASMase, and has even been found safe when administered intraperitoneally in mice (Naser et al., 2020).

Potential and Future Perspectives of FIASMAs and Antioxidants in the therapy of DMD

The results obtained so far are particularly encouraging regarding the safety of the long-term treatment with FIASMAs and antioxidants. Indeed, the closely monitored mice showed no signs of distress (i.e. anxiety) or pathology attributable to drug adverse events. Our major concern was related to the FIASMA administration, as although we chose the safest among the available ones, *i.e.* Fluoxetine and Sertraline, they belong to the selective serotonin reuptake inhibitor class of antidepressant drugs. The role of serotonin (5-hydroxytryptamine) in satiety

control is well recognized and these serotonergic drugs are known to reduce appetite and consequently weight in humans and rodents (Halford et al., 2011). On the other hand, they have also been associated with weight gain (Shin et al., 2009). However, the weight loss was observed in rodents at higher doses of Fluoxetine starting from 10mg/kg/day (Aggarwal et al., 2016). As this is the first time these SSRIs are used for their FIASMA properties in *mdx* mice we set for the lowest possible dose we estimated sufficient to elicit a response: 5mg/kg/day, considering that up until 18mg/kg/day Fluoxetine administered chronically to mice, the plasma concentrations of Fluoxetine are in the range of patients taking 20-80mg Fluoxetine (i.e. Prozac) per day (Dulawa et al., 2004). Regarding Sertraline, as the human equivalent dose (HED) formula used to extrapolate the drug dosage from one animal species to another is: HED (mg/kg) = Animal dose (mg/kg) x (Animal K_m/Human K_m) where human K_m=37 and mouse K_m =3 (Reagan-Shaw et al., 2008), the HED of the 5mg/kg/day administered to our treated *mdx* mice is 0,41mg/kg. Considering that the maximum human dose of Sertraline as an antidepressant drug is 3 mg/kg (Romanelli et al., 2019), this is more than 7 times above the dosage we chose.

Studies in mice also reported that both Fluoxetine and Sertraline at 10 and 20 mg/kg/day respectively attenuate postischemic brain injury (Shin et al., 2009). Additionally, it was shown that 2,5 and 5 mg/kg/day Fluoxetine have a stronger neuroprotective effect than 10 mg/kg/day, with the lowest dose resulting in the most remarkable effect (Zhu et al., 2012). Regarding Sertraline, a dose of 1 mg/kg/day was found sufficient to decrease serum IL-6 in mice (Romanelli et al., 2019).

Regarding Plumbagin and Quercetin, these antioxidants are natural dietary supplements with growing evidence showing their safety (Badwaik et al., 2019; Harwood et al., 2007; Panichayupakaranant & Ahmad, 2016; Patel et al., 2018; Rauf et al., 2018; Sumsakul et al., 2016). In our experiments, Plumbagin provided similar benefits to NAC, which has already given promising results in *mdx* mice (de Senzi Moraes Pinto et al., 2013; Whitehead et al., 2008). However, there is a concern regarding the safety of NAC that has been posed not only in studies on dystrophic mice (Pinniger et al., 2017) but also by the FDA (https://www.fda.gov/food/cfsan-constituent-updates/fda-requests-information-relevant-use-nac-dietary-supplement) which is currently requesting for more information for its use a food supplement.

These findings support our idea to test the efficacy of a lower dosage of the FIASMAs to be used in combination with an antioxidant.

Conclusion

The goal of this thesis was to determine the most effective FIASMA and antioxidant molecule, which appear to be Fluoxetine and Plumbagin respectively, with the future perspective of combining them. This is encouraging for Plumbagin as it is the first time it is tested in muscular dystrophy. The rationale of this combination is to improve treatment efficacy by regulating the ASMase pathway at different levels so that the two molecules together could benefit from each other in an additive/synergistic way. As antioxidants are available on the market as dietary supplements and present none or few side effects, their addition, allowing to diminish the dose of FIASMA would, in turn, further reduce adverse events.

There is a pressing need for an alternative therapeutic strategy for DMD, as in the absence of a definite cure, corticosteroids are part of care recommendations, but they delay the progression of DMD at the cost of severe side effects. Moreover, an obstacle to the advances in gene therapy is the host immune response, thus a combination with another agent able to modulate it could be a solution. FIASMAs coupled with an antioxidant could potentially help in these cases, but also in alleviating the depressive symptoms often displayed by the patients as these FIASMAs are antidepressants. Furthermore, drug repurposing allows time- and cost-savings as the drugs are already commercially available with a known safety profile.

REPORT

Duchenne Muscular Dystrophy (DMD) is the most common and severe muscular dystrophy, leading to death by the third decade of life. It is caused by loss-of-function mutations in the dystrophin gene. However, two other mechanisms overlapping each other play an important role in the pathophysiology:

- **Inflammation**, which is adapted to acute, infrequent muscle damage but misapplied in the context of chronic injury. This has been demonstrated by removing inflammatory cell types in the *mdx* mice, the murine model of DMD, as well as through the inhibition of key mediators of inflammation (Brunelli & Rovere-Querini, 2008; Grounds et al., 2020; Madaro & Bouché, 2014).
- **Oxidative stress**, as this process can lead to cell death and dystrophic muscles exhibit an increase in producing free radicals and compensatory antioxidant enzymes already at the pre-necrotic stage (Disatnik et al., 1998; Rando et al., 2002).

Acid Sphingomyelinase (ASMase) is an enzyme catalyzing the hydrolysis of sphingomyelins to ceramides, which are key players in cellular stress response. Sphingomyelinases are stimulated during inflammation and in response to oxidative stress (Nikolova-Karakashian & Reid, 2011; Won & Singh, 2006). Their importance in inflammatory-associated disorders along with their therapeutic potential is rising interest (Maceyka & Spiegel, 2014). Functional inhibitors of ASMase (FIASMAs) (Kornhuber et al., 2010), including mostly antidepressants, work by inducing the lysosomal degradation of ASMase, and are known to have a good safety profile.

Importantly, the involvement of ASMase in the progression of DMD, was previously demonstrated in the laboratory of Pharmacology of DIBIC in experiments on the tibialis anterior (TA) and diaphragm (DA) muscles of *mdx* mice, compared to wild-type (WT) mice.

Therefore, the focus of this PhD study was to investigate whether targeting this enzyme by using FIASMAs, and/or antioxidants molecules, would be a pertinent therapeutic strategy for DMD.

First, as a proof-of-concept we performed a short-term experiment with Amitriptyline, a tricyclic antidepressant and a FIASMA which has already been proven to be beneficial in *mdx* mice, although its effect on ASMase in dystrophic muscles has not been yet analyzed (Manning et al., 2014). We administered Amitriptyline to *mdx* mice (10mg/kg, for 6 weeks), while the control group received the vehicle. By histopathology, we noticed a reduced inflammation in the treated group, and a reduction of myonecrosis, thus supporting previous findings. This improvement was paralleled by reduced ASMase activity assayed in the muscle, and the presence of the drug in the targeted tissue by mass spectrometry.

Therefore, we then treated chronically *mdx* mice with a FIASMA i.e. Fluoxetine and Sertraline at 5mg/kg or an antioxidant molecule i.e. Plumbagin (30mg/kg), Quercetin (50mg/kg) and N-acetylcysteine (NAC) (1.5g/kg) for 3 months. As the tested FIASMAs belong to selective serotonin reuptake inhibitors (SSRIs), they have fewer side effects than Amitriptyline. NAC was used as a control as its efficacy in *mdx* mice was reported in numerous studies (de Senzi Moraes Pinto et al., 2013; Whitehead et al., 2008).

Both Plumbagin and Quercetin improved the muscle function evaluated by the exhaustion treadmill test compared to *mdx* vehicle and reached a recovery score versus WT similar to NAC. However, the potential improvement of muscle function was possibly hidden in FIASMA-treated *mdx* mice due to the behavioral effect of these SSRIs (Beasley et al., 1992), as suggested by the poor performance of WT mice treated with the same drugs compared to the WT vehicle.

All the treatments however improved muscle morphology compared to *mdx* vehicle with a reduced inflammatory infiltrate and fibrosis as showed by the Hematoxylin and Eosin and Sirius Red stainings and significantly decreased muscle damage evidenced by mIgG staining and serum Creatine Kinase activity. In parallel, the muscle regeneration was enhanced as attested by the increased positivity for embryonic myosin and expression of key regulators of myogenesis, Myogenin and Myf6.

We found that both FIASMAs tested and Plumbagin were able to decrease ASMase activity in the diaphragm, the muscle recapitulating best the human pathology (Stedman et al., 1991) and thus used in most of our experiments; however, Quercetin failed to modify enzyme activity.

Both FIASMAs and antioxidants reduced and modulated inflammation towards an anti-inflammatory state as evidenced by the decreased positivity for CD45 and expression of the M1 macrophage markers, worsening muscle damage: IL-1β and TNF-α, in parallel with the increase in M2 macrophage markers promoting tissue repair IL-10, CD163 and ARG1 (Madaro et al., 2019; S. A. Villalta et al., 2009). We also observed a promising decrease of the M1/M2 ratio for Plumbagin and Sertraline by flow cytometry.

All the treatment also attenuated oxidative stress by increasing the expression of several enzymes involved in the antioxidant defense system: HO-1, SOD3 and GPX1, which was modulated only by Fluoxetine.

Only Fluoxetine and Plumbagin induced Nrf2 expression, which plays a pivotal role in oxidative stress and inflammation holding great potential in DMD therapy as this transcription factor can simultaneously target separate pathological features of the disease (Kourakis, Timpani, de Haan, et al., 2021).

In order to clearly understand the role of ASMase in the process of regeneration after injury we investigated this issue in ASMase-KO mice (Roux-Biejat et al., 2021). Of note, although ASMase ablation did not affect the phenotype/function of skeletal muscle and satellite cells per se, it shifted macrophage polarization towards an M2, pro-regenerative, phenotype. The absence of ASMase led to a significantly lower expression of IL-1β, TNF-α, CD80 and NOS2 in comparison to the WT counterparts *in vitro*, particularly in macrophages cultured in M1-polarizing conditions. Similarly, we noticed a reduction of IL-1β, TNF-α and CD80 when treating WT M1 macrophages with the FIASMA Amitriptyline. Importantly, while we observed no differences for CD206 and CD163 in ASMase-KO macrophages cultured in M2-polarizing conditions, we detected a surge in the expression of these M2 markers in M1 macrophages of ASMase-KO mice. Moreover, these data paralleled with the increase of Nrf2 expression in M1 macrophages lacking ASMase, thus indicating a possible involvement of this transcription factor in the molecular machinery triggered by ASMase. These results put forward the crucial role of ASMase in M1 polarization.

To explore the potential of inhibiting ASMase to affect muscle regeneration by modulating the immune response *in vivo*, we injected cardiotoxin (CTX) in the TA of both genotypes and retrieved it at different time points of muscle regeneration. Compared to their WT littermates, we found in ASMase-KO mice a reduced leukocyte infiltration at the site of injury along with a lower pro-inflammatory state as demonstrated the lower expression of IL-1β and IL-6. This was reinforced by the decreased M1/M2 macrophage ratio.

Moreover, the anti-inflammatory effect in the absence of ASMase following tissue damage was confirmed by the enhanced expression of Nrf2, along with its downstream genes involved in the antioxidant response: HO-1, GPX1 and SOD3.

The concomitant elevation of Insulin-like growth factor 1 (IGF-1), a potent enhancer of tissue regeneration indicates that such changes in the niche of satellite cells are beneficial for muscle regeneration (Tidball & Welc, 2015). As a consequence, we found an increased size of the regenerating myofibres coupled with a decreased expression of embryonic myosin in the injured TA of ASMase-KO versus WT. This indicates an enhanced growth of the new fibers resulting from an accelerated myogenesis. Contemporarily, we found in ASMase-KO mice an increase in mRNA expression of dystrophin (DMD) which is an indicator of myofiber maturity (Yoshimoto et al., 2020).

Taken together, these data show that the absence of ASMase alleviates inflammation and oxidative stress, by effectively activating Nrf2 and shifting macrophage polarization towards the M2 phenotype associated with tissue repair *in vivo*. This scenario following acute muscle injury in ASMase-KO mice parallel with our results obtained with FIASMAs and antioxidants in *mdx* mice in the context of chronic injury; therefore, suggesting that the events observed in treated *mdx* mice are related to the decrease of ASMase activity.

We provide evidence that acting on the ASMase pathway induces a protective effect from the progression of the pathology with improvements of both functional and morphological muscle features. These results are particularly encouraging to provide an alternative therapeutic strategy to the incurable DMD as corticosteroids are part of care recommendations to slow the development of the disease, but they are paired with severe side effects. As these FIASMAs are antidepressants, they could alleviate the depressive symptoms often displayed by the patient. Modulating ASMase could also help gene-therapy in reaching the therapeutic efficacy as one important challenge is the host immune response. Moreover, drug repurposing allows time- and cost-saving as the drugs are already commercially available with a known safety profile. Finally, the efficacy could potentially be improved in a synergistic/additive manner by combining a FIASMA with an antioxidant, our results showing that the most efficient ones in their class are Fluoxetine and Plumbagin. Noteworthy, this is the first time Plumbagin is tested in the context of muscular dystrophy.

PUBLICATIONS

- ***Acid Sphingomyelinase Controls Early Phases of Skeletal Muscle Regeneration by Shaping the Macrophage Phenotype***
  Paulina Roux-Biejat, Marco Coazzoli, Pasquale Marrazzo, Silvia Zecchini, Ilaria Di Renzo, Cecilia Prata, Alessandra Napoli, Claudia Moscheni, Matteo Giovarelli, Maria Cristina Barbalace, Elisabetta Catalani, Maria Teresa Bassi, Clara De Palma, Davide Cervia, Marco Malaguti, Silvana Hrelia, Emilio Clementi, Cristiana Perrotta.
  Cells, 2021 Nov 5;10(11):3028.
  doi: 10.3390/cells10113028

- ***Givinostat as metabolic enhancer reverting mitochondrial biogenesis deficit in Duchenne Muscular Dystrophy***
  Matteo Giovarelli, Silvia Zecchini, Giorgia Catarinella, Claudia Moscheni, Patrizia Sartori, Cecilia Barbieri, Paulina Roux-Biejat, Alessandra Napoli, Chiara Vantaggiato, Davide Cervia, Cristiana Perrotta, Emilio Clementi, Lucia Latella, Clara De Palma.
  Pharmacological Research,Volume 170, 2021, 105751,ISSN 1043-6618,
  doi: 10.1016/j.phrs.2021.105751

- ***Acid Sphingomyelinase Downregulation Enhances Mitochondrial Fusion and Promotes Oxidative Metabolism in a Mouse Model of Melanoma.***
  Marco Coazzoli[#], Alessandra Napoli[#], Paulina Roux-Biejat[#], Clara De Palma, Claudia Moscheni, Elisabetta Catalani, Silvia Zecchini, Vincenzo Conte, Matteo Giovarelli, Sonia Caccia, Patrizia Procacci, Davide Cervia, Emilio Clementi, Cristiana Perrotta.
  Cells, 2020; 9(4):848.
  doi: 10.3390/cells9040848.
  # equal contribution.

- ***Drp1 overexpression induces desmin disassembling and drives kinesin-1 activation promoting mitochondrial trafficking in skeletal muscle.***
  Matteo Giovarelli, Silvia Zecchini, Emanuele Martini, Massimiliano Garrè, Sara Barozzi, Michela Ripolone, Laura Napoli, Marco Coazzoli, Chiara Vantaggiato, Paulina Roux-Biejat, Davide Cervia, Claudia Moscheni, Cristiana Perrotta, Dario Parazzoli, Emilio Clementi, Clara De Palma,
  Cell Death and Differentiation. 2020, 27(8):2383-2401.
  doi: 10.1038/s41418-020-0510-7.

CONGRESSES AND SEMINARS

XVIII IIM Meeting – Online, 2021
Poster Presentation: *"The Acid Sphingomyelinase Pathway as a promising target of inflammation and oxidative stress in Duchenne Muscular Dystrophy"*

Annual PhD Student School 2021- PhD in Pharmacological Biomolecular, Experimental and Clinical Sciences, Università degli Studi di Milano – Gargano, Italy, 2021
Oral Presentation: "*The Acid Sphingomyelinase Pathway as a promising target of Inflammation and Oxidative Stress in Duchenne Muscular Dystrophy*"

IV Edition of Spring School - PhD in Pharmacological Biomolecular, Experimental and Clinical Sciences, Università degli Studi di Milano – Chiesa in Valmalenco, Italy, 2020
Oral Presentation: *"Targeting Inflammation and Oxidative Stress with the Acid Sphingomyelinase Pathway in Duchenne Muscular Dystrophy"*

XVI IIM Meeting – Assisi, Italy, 2019
Oral Presentation: *"Inhibition of Acid Sphingomyelinase as Novel Alternative Therapy for Duchenne Muscular Dystrophy"*

III Edition of Spring School - PhD in Pharmacological Biomolecular, Experimental and Clinical Sciences, Università degli Studi di Milano – Chiesa in Valmalenco, Italy, 2019
Oral Presentation: *"Inhibition of ASMase and mitochondria reactivation as the novel alternative strategies for Duchenne Muscular Dystrophy treatment"*

BIBLIOGRAHY

Aartsma-Rus, A., Ginjaar, I. B., & Bushby, K. (2016). The importance of genetic diagnosis for Duchenne muscular dystrophy. *J Med Genet. 2016 Mar;53(3):145-51. Doi: 10.1136/Jmedgenet-2015-103387. PMID: 26754139; PMCID: PMC4789806.*

Aartsma-Rus, A., Straub, V., Hemmings, R., Haas, M., Schlosser-Weber, G., Stoyanova-Beninska, V., Mercuri, E., Muntoni, F., Sepodes, B., Vroom, E., & Balabanov, P. (2017). Development of Exon Skipping Therapies for Duchenne Muscular Dystrophy: A Critical Review and a Perspective on the Outstanding Issues. In *Nucleic Acid Therapeutics* (Vol. 27, Issue 5, pp. 251–259). Mary Ann Liebert Inc. https://doi.org/10.1089/nat.2017.0682

Aartsma-Rus, A., & van Ommen, G. J. B. (2007). Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. In *RNA* (Vol. 13, Issue 10, pp. 1609–1624). https://doi.org/10.1261/rna.653607

Aggarwal, A., Jethani, S. L., Rohatgi, R. K., & Kalra, J. (2016). Selective serotonin re-uptake inhibitors (SSRIs) induced weight changes: A dose and duration dependent study on albino rats. *Journal of Clinical and Diagnostic Research*, *10*(3), AF01–AF03. https://doi.org/10.7860/JCDR/2016/16482.7376

Alderton, J. M., & Steinhardt, R. A. (2000). Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *Journal of Biological Chemistry*, *275*(13), 9452–9460. https://doi.org/10.1074/jbc.275.13.9452

Allen, D. G., Whitehead, N. P., & Froehner, S. C. (2016). Absence of dystrophin disrupts skeletal muscle signaling: roles of Ca^{2+} , reactive oxygen species, and nitric oxide in the development of muscular dystrophy Allen DG, Whitehead NP, Froehner SC. Absence of Dystrophin Disrupts Skeletal Muscle Signaling: Roles of Ca 2 , Reactive Oxygen Species, and Nitric Oxide in the Development of Muscular Dystrophy. *Physiol Rev*, *96*, 253–305. https://doi.org/10.1152/physrev.00007.2015.-Dystrophin

Amato, R., Catalani, E., Dal Monte, M., Cammalleri, M., di Renzo, I., Perrotta, C., Cervia, D., & Casini, G. (2018). Autophagy-mediated neuroprotection induced by octreotide in an ex vivo model of early diabetic retinopathy. *Pharmacological Research*, *128*, 167–178. https://doi.org/10.1016/j.phrs.2017.09.022

Angelini, C., di Mauro, S., & Margreth, A. (1968). Relationship of serum enzyme changes to muscle damage in vitamin E deficiency of the rabbit. *Lo Sperimentale; Archivio Di Biologia Normale e Patologica*, *118*(5), 349–369.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R. K., & Chazaud, B. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *Journal of Experimental Medicine*, *204*(5), 1057–1069. https://doi.org/10.1084/jem.20070075

Assi, E., Cervia, D., Bizzozero, L., Capobianco, A., Pambianco, S., Morisi, F., De Palma, C., Moscheni, C., Pellegrino, P., Clementi, E., & Perrotta, C. (2015). Modulation of Acid Sphingomyelinase in Melanoma Reprogrammes the Tumour Immune Microenvironment. *Mediators of Inflammation*, *2015*. https://doi.org/10.1155/2015/370482

Ataluren: NCT04336826. https://clinicaltrials.gov/ct2/show/NCT04336826
A Study to Evaluate the Safety and Pharmacokinetics of Ataluren in Participants From ≥6 Months to <2 Years of Age With Nonsense Mutation Duchenne Muscular Dystrophy (nmDMD)
Estimated Study Completion Date: 2022

Babačić, H., Mehta, A., Merkel, O., & Schoser, B. (2019). CRISPR-cas gene-editing as plausible treatment of neuromuscular and nucleotide-repeat-expansion diseases: A

systematic review. In *PLoS ONE* (Vol. 14, Issue 2). Public Library of Science. https://doi.org/10.1371/journal.pone.0212198

Badwaik, H. R., Kumari, L., Nakhate, K., Verma, V. S., & Sakure, K. (2019). Phytoconstituent plumbagin: Chemical, biotechnological and pharmaceutical aspects. In *Studies in Natural Products Chemistry* (Vol. 63, pp. 415–460). Elsevier B.V. https://doi.org/10.1016/B978-0-12-817901-7.00013-7

Bai, A., & Guo, Y. (2017). Acid sphingomyelinase mediates human CD4+ T-cell signaling: potential roles in T-cell responses and diseases. *Cell Death & Disease*, *8*(7), e2963. https://doi.org/10.1038/cddis.2017.360

Ballmann, C., Denney, T., Beyers, R. J., Quindry, T., Romero, M., Selsby, J. T., & Quindry, J. C. (2017). Long-term dietary quercetin enrichment as a cardioprotective countermeasure in mdx mice. *Experimental Physiology*, *102*(6), 635–649. https://doi.org/10.1113/EP086091

Beasley, C. M., Sayler, M. E., Weiss, A. M., & Potvin, J. H. (1992). Fluoxetine Activating and Sedating Effects at Multiple Fixed Doses. *International Clinical Psychopharmacology, 8(4), 271–275.*

Beckmann, N., Sharma, D., Gulbins, E., Becker, K. A., & Edelmann, B. (2014). Inhibition of acid sphingomyelinase by tricyclic antidepressants and analogons. *Frontiers in Physiology*, *5 AUG*(September), 1–14. https://doi.org/10.3389/fphys.2014.00331

Behr, G. A., Moreira, J. C. F., & Frey, B. N. (2012). Preclinical and clinical evidence of antioxidant effects of antidepressant agents: Implications for the pathophysiology of major depressive disorder. In *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2012/609421

Belcastro, A. N., Shewchuk, L. D., & Raj, D. A. (1998). Exercise-induced muscle injury: A calpain hypothesis. *Molecular and Cellular Biochemistry*, *179*(1–2), 135–145. https://doi.org/10.1023/A:1006816123601

Bencze, M., Periou, B., Baba-Amer, Y., & Authier, F. J. (2019). Immunolabelling myofiber degeneration in muscle biopsies. *Journal of Visualized Experiments*, *2019*(154). https://doi.org/10.3791/59754

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology*, *4*(2). https://doi.org/10.1101/cshperspect.a008342

Berger, J., Li, M., Berger, S., Meilak, M., Rientjes, J., & Currie, P. D. (2020). Effect of Ataluren on dystrophin mutations. *Cell Mol Med. 2020 Jun;24(12):6680-6689. Doi: 10.1111/Jcmm.15319.. PMID: 32343037; PMCID: PMC7299694*.

Bertoni, C., & Namgoong, J. (2016). Clinical potential of ataluren in the treatment of Duchenne muscular dystrophy. *Degenerative Neurological and Neuromuscular Disease*, 37. https://doi.org/10.2147/dnnd.s71808

Biressi, S., Molinaro, M., & Cossu, G. (2007). Cellular heterogeneity during vertebrate skeletal muscle development. *Developmental Biology*, *308*(2), 281–293. https://doi.org/10.1016/j.ydbio.2007.06.006

Bladen, C. L., Salgado, D., Monges, S., Foncuberta, M. E., Kekou, K., Kosma, K., Dawkins, H., Lamont, L., Roy, A. J., Chamova, T., Guergueltcheva, V., Chan, S., Korngut, L., Campbell, C., Dai, Y., Wang, J., Barišić, N., Brabec, P., Lahdetie, J., … Lochmüller, H. (2015). The TREAT-NMD DMD global database: Analysis of more than 7,000 duchenne muscular dystrophy mutations. *Human Mutation*, *36*(4), 395–402. https://doi.org/10.1002/humu.22758

Boini, K. M., Zhang, C., Xia, M., Han, W. Q., Brimson, C., Poklis, J. L., & Li, P. L. (2010). Visfatin-induced lipid raft redox signaling platforms and dysfunction in glomerular

endothelial cells. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1801*(12), 1294–1304. https://doi.org/10.1016/j.bbalip.2010.09.001

Boldrin, L., Zammit, P. S., & Morgan, J. E. (2015). Satellite cells from dystrophic muscle retain regenerative capacity. *Stem Cell Research*, *14*(1), 20–29. https://doi.org/10.1016/j.scr.2014.10.007

Boots, A. W., Haenen, G. R. M. M., & Bast, A. (2008). Health effects of quercetin: From antioxidant to nutraceutical. In *European Journal of Pharmacology* (Vol. 585, Issues 2–3, pp. 325–337). https://doi.org/10.1016/j.ejphar.2008.03.008

Bost, E. R., Frye, G. S., Ahn, B., & Ferreira, L. F. (2015). Diaphragm dysfunction caused by sphingomyelinase requires the p47phox subunit of NADPH oxidase. *Respiratory Physiology and Neurobiology*, *205*, 47–52. https://doi.org/10.1016/j.resp.2014.10.011

Boursereau, R., Abou-Samra, M., Lecompte, S., Noel, L., & Brichard, S. M. (2018). Downregulation of the NLRP3 inflammasome by adiponectin rescues Duchenne muscular dystrophy. *BMC Biology*, *16*(1). https://doi.org/10.1186/s12915-018-0501-z

Brunelli, S., & Rovere-Querini, P. (2008). The immune system and the repair of skeletal muscle. In *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2008.06.008

Burdi, R., Rolland, J. F., Fraysse, B., Litvinova, K., Cozzoli, A., Giannuzzi, V., Liantonio, A., Camerino, G. M., Sblendorio, V., Capogrosso, R. F., Palmieri, B., Andreetta, F., Confalonieri, P., de Benedictis, L., Montagnani, M., & de Luca, A. (2009). Multiple pathological events in exercised dystrophic mdx mice are targeted by pentoxifylline: Outcome of a large array of in vivo and ex vivo tests. *Journal of Applied Physiology*, *106*(4), 1311–1324. https://doi.org/10.1152/japplphysiol.90985.2008

Burns, D. P., Drummond, S. E., Bolger, D., Coiscaud, A., Murphy, K. H., Edge, D., & O'halloran, K. D. (2019). N-acetylcysteine decreases fibrosis and increases force-generating capacity of mdx diaphragm. *Antioxidants*, *8*(12). https://doi.org/10.3390/antiox8120581

Cappellari, O., Mantuano, P., & de Luca, A. (2020). "The Social Network" and Muscular Dystrophies: The Lesson Learnt about the Niche Environment as a Target for Therapeutic Strategies. *Cells*, *9*(7). https://doi.org/10.3390/cells9071659

Carre-Pierrat, M., Lafoux, A., Tanniou, G., Chambonnier, L., Divet, A., Fougerousse, F., Huchet-Cadiou, C., & Ségalat, L. (2011). Pre-clinical study of 21 approved drugs in the mdx mouse. *Neuromuscular Disorders*, *21*(5), 313–327. https://doi.org/10.1016/j.nmd.2011.01.005

Carter, J. C., Sheehan, D. W., Prochoroff, A., & Birnkrant, D. J. (2018). Muscular Dystrophies. *Clinics in Chest Medicine*, *39*(2), 377–389. https://doi.org/10.1016/j.ccm.2018.01.004

Catalani, E., Proietti Serafini, F., Zecchini, S., Picchietti, S., Fausto, A. M., Marcantoni, E., Buonanno, F., Ortenzi, C., Perrotta, C., & Cervia, D. (2016). Natural products from aquatic eukaryotic microorganisms for cancer therapy: Perspectives on anti-tumour properties of ciliate bioactive molecules. In *Pharmacological Research* (Vol. 113, pp. 409–420). Academic Press. https://doi.org/10.1016/j.phrs.2016.09.018

Cazzato, D., Assi, E., Moscheni, C., Brunelli, S., De Palma, C., Cervia, D., Perrotta, C., & Clementi, E. (2013). Nitric oxide drives embryonic myogenesis in chicken through the upregulation of myogenic differentiation factors. *Exp Cell Res. 2014 Jan 15;320(2):269-80. Doi: 10.1016/j.Yexcr.2013.11.006. . PMID: 24240125.*

Cervia, D., Assi, E., De Palma, C., Giovarelli, M., Bizzozero, L., Pambianco, S., Renzo, I. di, Zecchini, S., Moscheni, C., Vantaggiato, C., Procacci, P., Clementi, E., & Perrotta, C. (2016). *Essential role for acid sphingomyelinase-inhibited autophagy in melanoma response to cisplatin* (Vol. 7, Issue 18). www.impactjournals.com/oncotarget

Chan, M. C., Ziegler, O., Liu, L., Rowe, G. C., Das, S., Otterbein, L. E., & Arany, Z. (2016). Heme oxygenase and carbon monoxide protect from muscle dystrophy. *Skeletal Muscle*, *6*(1). https://doi.org/10.1186/s13395-016-0114-6

Chazaud, B., Brigitte, M., Yacoub-Youssef, H., Arnold, L., Gherardi, R., Sonnet, C., Lafuste, P., & Chretien, F. (2009). Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exercise and Sport Sciences Reviews*, *37*(1), 18–22. https://doi.org/10.1097/JES.0b013e318190ebdb

Chinoy, H., & Cooper, R. G. (2018). *Muscular dystrophies and other genetic myopathies Myositis (Oxford Rheumatology Library) Muscular dystrophies and other genetic myopathies*. 1–15. https://doi.org/10.1093/med/9780198754121.001.0001

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J. S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research*, *24*(1), 132–141. https://doi.org/10.1101/gr.162339.113

Chu, H., Yu, H., Ren, D., Zhu, K., & Huang, H. (2016). Plumbagin exerts protective effects in nucleus pulposus cells by attenuating hydrogen peroxide-induced oxidative stress, inflammation and apoptosis through NF-B and Nrf-2. *International Journal of Molecular Medicine*, *37*(6), 1669–1676. https://doi.org/10.3892/ijmm.2016.2564

Consalvi, S., Saccone, V., Giordani, L., Minetti, G., Mozzetta, C., & Puri, P. L. (2011). Histone deacetylase inhibitors in the treatment of muscular dystrophies: Epigenetic drugs for genetic diseases. *Molecular Medicine*, *17*(5–6), 457–465. https://doi.org/10.2119/molmed.2011.00049

Consalvi, S., Saccone, V., & Mozzetta, C. (2014). Histone deacetylase inhibitors: A potential epigenetic treatment for Duchenne muscular dystrophy. In *Epigenomics* (Vol. 6, Issue 5, pp. 547–560). Future Medicine Ltd. https://doi.org/10.2217/epi.14.36

Cossu, G., de Angelis, L., Borello, U., Berarducci, B., Buffa, V., Sonnino, C., Coletta, M., Vivarelli, E., Bouche', M., Lattanzi, L., Tosoni, D., di Donna, S., Berghella, L., Salvatori, G., Murphy, P., Cusella-De Angelis, M. G., & Molinaro, M. (2000). Determination, diversification and multipotency of mammalian myogenic cells. *International Journal of Developmental Biology*, *44*(6), 699–706. https://doi.org/10.1387/ijdb.11061434

Cuschieri, J., Bulger, E., Billgrin, J., Garcia, I., & Maier, R. v. (2007). Acid sphingomyelinase is required for lipid raft TLR4 complex formation. *Surgical Infections*, *8*(1), 91–105. https://doi.org/10.1089/sur.2006.050

Danisovic, L., Culenova, M., & Csobonyeiova, M. (2018). Induced pluripotent stem cells for duchenne muscular dystrophy modeling and therapy. In *Cells* (Vol. 7, Issue 12). MDPI. https://doi.org/10.3390/cells7120253

Datta, N., & Ghosh, P. S. (2020). Update on Muscular Dystrophies with Focus on Novel Treatments and Biomarkers. *Current Neurology and Neuroscience Reports*, *20*(6). https://doi.org/10.1007/s11910-020-01034-6

De Paepe, B., Creus, K. K., Martin, J. J., & de Bleecker, J. L. (2012). Upregulation of chemokines and their receptors in duchenne muscular dystrophy: Potential for attenuation of myofiber necrosis. *Muscle and Nerve*, *46*(6), 914–916. https://doi.org/10.1002/mus.23481

De Palma, C., & Clementi, E. (2012). Nitric oxide in myogenesis and therapeutic muscle repair. *Molecular Neurobiology*, *46*(3), 682–692. https://doi.org/10.1007/s12035-012-8311-8

De Palma, C., Morisi, F., Pambianco, S., Assi, E., Touvier, T., Russo, S., Perrotta, C., Romanello, V., Carnio, S., Cappello, V., Pellegrino, P., Moscheni, C., Bassi, M. T., Sandri, M., Cervia, D., & Clementi, E. (2014). Deficient nitric oxide signalling impairs skeletal muscle growth and performance: Involvement of mitochondrial dysregulation. *Skeletal Muscle*, *4*(1). https://doi.org/10.1186/s13395-014-0022-6

de Senzi Moraes Pinto, R., Ferretti, R., Moraes, L. H. R., Neto, H. S., Marques, M. J., & Minatel, E. (2013). N-Acetylcysteine treatment reduces TNF-α levels and myonecrosis in diaphragm muscle of mdx mice. *Clinical Nutrition*, *32*(3), 472–475. https://doi.org/10.1016/j.clnu.2012.06.001

Delcuve, G. P., Khan, D. H., & Davie, J. R. (2012). Roles of histone deacetylases in epigenetic regulation: Emerging paradigms from studies with inhibitors. In *Clinical Epigenetics* (Vol. 4, Issue 1). https://doi.org/10.1186/1868-7083-4-5

Ding, Y., Chen, Z.-J., Liu, S., Che, D., Vetter, M., & Chang, C.-H. (2010). Inhibition of Nox-4 activity by plumbagin, a plant-derived bioactive naphthoquinone. *Journal of Pharmacy and Pharmacology*, *57*(1), 111–116. https://doi.org/10.1211/0022357055119

Disatnik, M. H., Dhawan, J., Yu, Y., Beal, M. F., Whirl, M. M., Franco, A. A., & Rando, T. A. (1998). Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic state. *Journal of the Neurological Sciences*, *161*(1), 77–84. https://doi.org/10.1016/s0022-510x(98)00258-5

Dort, J., Fabre, P., Molina, T., & Dumont, N. A. (2019). Macrophages Are Key Regulators of Stem Cells during Skeletal Muscle Regeneration and Diseases. *Stem Cells International*, *2019*(Dmd). https://doi.org/10.1155/2019/4761427

Duan, R. D., Cheng, Y., Hansen, G., Hertervig, E., Liu, J. J., Syk, I., Sjöström, H., & Nilsson, Å. (2003). Purification, localization, and expression of human intestinal alkaline sphingomyelinase. *Journal of Lipid Research*, *44*(6), 1241–1250. https://doi.org/10.1194/jlr.M300037-JLR200

Duchenneandyou.com. https://duchenneandyou.com/caring-for-a-child-with-duchenne/
Figure adapted from Birnkrant 2018 Part 3; Bushby 2010; Goemans 2014; Sussman 2002

Dulawa, S. C., Holick, K. A., Gundersen, B., & Hen, R. (2004). Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology*, *29*(7), 1321–1330. https://doi.org/10.1038/sj.npp.1300433

Dumitru, C. A., Zhang, Y., Li, X., & Gulbins, E. (2007). Ceramide: A novel player in reactive oxygen species-induced signaling? In *Antioxidants and Redox Signaling* (Vol. 9, Issue 9, pp. 1535–1540). https://doi.org/10.1089/ars.2007.1692

Dumont, N. A., Bentzinger, C. F., Sincennes, M. C., & Rudnicki, M. A. (2015). Satellite cells and skeletal muscle regeneration. *Comprehensive Physiology*, *5*(3), 1027–1059. https://doi.org/10.1002/cphy.c140068

Dumont, N. A., Wang, Y. X., von Maltzahn, J., Pasut, A., Bentzinger, C. F., Brun, C. E., & Rudnicki, M. A. (2015). Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. *Nature Medicine*, *21*(12), 1455–1463. https://doi.org/10.1038/nm.3990

Duque, G. A., & Descoteaux, A. (2014). Macrophage cytokines: Involvement in immunity and infectious diseases. *Frontiers in Immunology*, *5*(OCT), 1–12. https://doi.org/10.3389/fimmu.2014.00491

Duxson, M. J., Usson, Y., & Harris, A. J. (1989). The origin of secondary myotubes in mammalian skeletal muscles: Ultrastructural studies. *Development*, *107*(4), 743–750. https://doi.org/10.1242/dev.107.4.743

Echevarría, L., Aupy, P., & Goyenvalle, A. (2018). Exon-skipping advances for Duchenne muscular dystrophy. In *Human Molecular Genetics* (Vol. 27, Issue R2, pp. R163–R172). Oxford University Press. https://doi.org/10.1093/hmg/ddy171

Epicatechin: NCT02964377. https://clinicaltrials.gov/ct2/show/NCT02964377
Plus Epicatechin Duchenne Muscular Dystrophy in Non-ambulatory Adolescents
Study Completion Date: 2018

Epigallocatechin-Gallate: NCT01183767. https://clinicaltrials.gov/ct2/show/NCT01183767

Sunphenon Epigallocatechin-Gallate (EGCg) in Duchenne Muscular Dystrophy (SUNIMUD)
Study Completion Date: 2018

Eteplirsen: NCT03985878. https://clinicaltrials.gov/ct2/show/NCT03985878
A Study to Evaluate Safety, Tolerability, and Efficacy of Eteplirsen in Patients With Duchenne Muscular Dystrophy
Estimated Study Completion Date: 2027

Evans, N. P., Misyak, S. A., Robertson, J. L., Bassaganya-Riera, J., & Grange, R. W. (2009). Immune-Mediated Mechanisms Potentially Regulate the Disease Time-Course of Duchenne Muscular Dystrophy and Provide Targets for Therapeutic Intervention. In *PM and R* (Vol. 1, Issue 8, pp. 755–768). https://doi.org/10.1016/j.pmrj.2009.04.010

Ezeriņa, D., Takano, Y., Hanaoka, K., Urano, Y., & Dick, T. P. (2018). N-Acetyl Cysteine Functions as a Fast-Acting Antioxidant by Triggering Intracellular H 2 S and Sulfane Sulfur Production. *Cell Chemical Biology*, *25*(4), 447-459.e4. https://doi.org/10.1016/j.chembiol.2018.01.011

Fahy, N., de Vries-van Melle, M. L., Lehmann, J., Wei, W., Grotenhuis, N., Farrell, E., van der Kraan, P. M., Murphy, J. M., Bastiaansen-Jenniskens, Y. M., & van Osch, G. J. V. M. (2014). Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. *Osteoarthritis and Cartilage*, *22*(8), 1167–1175. https://doi.org/10.1016/j.joca.2014.05.021

Falcone, S., Perrotta, C., De Palma, C., Pisconti, A., Sciorati, C., Capobianco, A., Rovere-Querini, P., Manfredi, A. A., & Clementi, E. (2004). Activation of Acid Sphingomyelinase and Its Inhibition by the Nitric Oxide/Cyclic Guanosine 3′,5′-Monophosphate Pathway: Key Events in Escherichia coli- Elicited Apoptosis of Dendritic Cells . *The Journal of Immunology*, *173*(7), 4452–4463. https://doi.org/10.4049/jimmunol.173.7.4452

Farid, M., Reid, M. B., Li, Y. P., Gerken, E., & Durham, W. J. (2005). Effects of dietary curcumin or N-acetylcysteine on NF-κB activity and contractile performance in ambulatory and unloaded murine soleus. *Nutrition and Metabolism*, *2*. https://doi.org/10.1186/1743-7075-2-20

Fauconnier, J., Thireau, J., Reiken, S., Cassan, C., Richard, S., Matecki, S., Marks, A. R., & Lacampagne, A. (2010). Leaky RyR2 trigger ventricular arrhythmias in Duchenne muscular dystrophy. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1559–1564. https://doi.org/10.1073/pnas.0908540107

Ferreira, L. F., & Laitano, O. (2016). Regulation of NADPH oxidases in skeletal muscle. *Free Radical Biology and Medicine*, *98*, 18–28. https://doi.org/10.1016/j.freeradbiomed.2016.05.011

Ferreira, L. F., Moylan, J. S., Stasko, S., Smith, J. D., Campbell, K. S., Reid, M. B., & Reid, M. B. (2012). Sphingomyelinase depresses force and calcium sensitivity of the contractile apparatus in mouse diaphragm muscle fibers. *J Appl Physiol*, *112*, 1538–1545. https://doi.org/10.1152/japplphysiol.01269.2011.-Diseases

Ferreira, L., Moylan, J., Gilliam, L., Smith, J., Nikolova-Karakashian, M., & Reid, M. (2010). Sphingomyelinase stimulates oxidant signaling to weaken skeletal muscle and promote fatigue. *Am J Physiol Cell Physiol.;299(3):C552-60. Doi: 10.1152/Ajpcell.00065.2010. Epub 2010 Jun 2. PMID: 20519448; PMCID: PMC2944322.*

Flanigan, K. M. (2014). Duchenne and becker muscular dystrophies. *Neurologic Clinics*, *32*(3), 671–688. https://doi.org/10.1016/j.ncl.2014.05.002

Forcina, L., Cosentino, M., & Musarò, A. (2020). Mechanisms Regulating Muscle Regeneration: Insights into the Interrelated and Time-Dependent Phases of Tissue Healing. *Cells*, *9*(5). https://doi.org/10.3390/cells9051297

Frenette, J., Cai, B., & Tidball, J. G. (2000). Complement activation promotes muscle inflammation during modified muscle use. *American Journal of Pathology*, *156*(6), 2103–2110. https://doi.org/10.1016/S0002-9440(10)65081-X

Frontera, W. R., & Ochala, J. (2015). Skeletal Muscle: A Brief Review of Structure and Function. *Behavior Genetics*, *45*(2), 183–195. https://doi.org/10.1007/s00223-014-9915-y

Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature Biotechnology*, *32*(3), 279–284. https://doi.org/10.1038/nbt.2808

Ganassi, M., Badodi, S., Wanders, K., & Zammit, P. S. (2020). Myogenin is an essential regulator of adult myofibre growth and muscle stem cell homeostasis. *ELife*, *9*, 1–23. https://doi.org/10.7554/eLife.60445

Gao, Q., & McNally, E. (2015). The Dystrophin Complex: structure, function and implications for therapy. *Compr Physiol*, *5*(3), 1223–1239. https://doi.org/10.1016/j.physbeh.2017.03.040

Garg, K., Corona, B. T., & Walters, T. J. (2015). Therapeutic strategies for preventing skeletal muscle fibrosis after injury. *Frontiers in Pharmacology*, *6*(APR). https://doi.org/10.3389/fphar.2015.00087

Gauss, K. A., Nelson-Overton, L. K., Siemsen, D. W., Gao, Y., DeLeo, F. R., & Quinn, M. T. (2007). Role of NF-κB in transcriptional regulation of the phagocyte NADPH oxidase by tumor necrosis factor-α. *Journal of Leukocyte Biology*, *82*(3), 729–741. https://doi.org/10.1189/jlb.1206735

Giordano, C., Mojumdar, K., Liang, F., Lemaire, C., Li, T., Richardson, J., Divangahi, M., Qureshi, S., & Petrof, B. J. (2015). Toll-like receptor 4 ablation in mdx mice reveals innate immunity as a therapeutic target in Duchenne muscular dystrophy. *Human Molecular Genetics*, *24*(8), 2147–2162. https://doi.org/10.1093/hmg/ddu735

Giovarelli, M., Zecchini, S., Catarinella, G., Moscheni, C., Sartori, P., Barbieri, C., Roux-Biejat, P., Napoli, A., Vantaggiato, C., Cervia, D., Perrotta, C., Clementi, E., Latella, L., & De Palma, C. (2021). Givinostat as metabolic enhancer reverting mitochondrial biogenesis deficit in Duchenne Muscular Dystrophy. *Pharmacological Research*, *170*. https://doi.org/10.1016/j.phrs.2021.105751

Gius, D., Botero, A., Shah, S., & Curry, H. A. (1999). Intracellular oxidation/reduction status in the regulation of transcription factors NF-kB and AP-1. In *Toxicology Letters* (Vol. 106).

Givinostat: NCT03373968. https://clinicaltrials.gov/ct2/show/NCT03373968
Givinostat in Duchenne's Muscular Dystrophy Long-term Safety and Tolerability Study
Estimated Study Completion Date: 2023

Goñi, F., & Alonso, A. (2002). Sphingomyelinases: enzymology and membrane activity. *FEBS Lett. ;531(1):38-46. Doi: 10.1016/S0014-5793(02)03482-8. PMID: 12401200.*

Grassmé, H., Carpinteiro, A., Edwards, M. J., Gulbins, E., & Becker, K. A. (2014). Regulation of the inflammasome by ceramide in cystic fibrosis lungs. *Cellular Physiology and Biochemistry*, *34*(1), 45–55. https://doi.org/10.1159/000362983

Grassmé, H., Jekle, A., Riehle, A., Schwarz, H., Berger, J., Sandhoff, K., Kolesnick, R., & Gulbins, E. (2001). CD95 Signaling via Ceramide-rich Membrane Rafts. *Journal of Biological Chemistry*, *276*(23), 20589–20596. https://doi.org/10.1074/jbc.M101207200

Grassmé, H., Jendrossek, V., Bock, J., Riehle, A., & Gulbins, E. (2002). Ceramide-Rich Membrane Rafts Mediate CD40 Clustering. *The Journal of Immunology*, *168*(1), 298–307. https://doi.org/10.4049/jimmunol.168.1.298

Grounds, M. D., Terrill, J. R., Al-Mshhdani, B. A., Duong, M. N., Radley-Crabb, H. G., & Arthur, P. G. (2020). Biomarkers for Duchenne muscular dystrophy: Myonecrosis,

inflammation and oxidative stress. *DMM Disease Models and Mechanisms*, *13*(2), 1–12. https://doi.org/10.1242/DMM.043638

Guardiola, O., Lafuste, P., Brunelli, S., Iaconis, S., Touvier, T., Mourikis, P., de Bock, K., Lonardo, E., Andolfi, G., Bouché, A., Liguori, G. L., Shen, M. M., Tajbakhsh, S., Cossu, G., Carmeliet, P., & Minchiotti, G. (2012). Cripto regulates skeletal muscle regeneration and modulates satellite cell determination by antagonizing myostatin. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(47). https://doi.org/10.1073/pnas.1204017109

Guiraud, S., Edwards, B., Squire, S. E., Moir, L., Berg, A., Babbs, A., Ramadan, N., Wood, M. J., & Davies, K. E. (2019). Embryonic myosin is a regeneration marker to monitor utrophin-based therapies for DMD. *Human Molecular Genetics*, *28*(2), 307–319. https://doi.org/10.1093/hmg/ddy353

Guo, Y. X., Liu, L., Yan, D. Z., & Guo, J. P. (2017). Plumbagin prevents osteoarthritis in human chondrocytes through Nrf-2 activation. *Molecular Medicine Reports*, *15*(4), 2333–2338. https://doi.org/10.3892/mmr.2017.6234

Gupta, A. C., Mohanty, S., Saxena, A., Maurya, A. K., & Bawankule, D. U. (2018). Plumbagin, a vitamin K3 analogue ameliorate malaria pathogenesis by inhibiting oxidative stress and inflammation. *Inflammopharmacology*, *26*(4), 983–991. https://doi.org/10.1007/s10787-018-0465-1

Halford, J. C. G., Boyland, E. J., Lawton, C. L., Blundell, J. E., & Harrold, J. A. (2011). Serotonergic Anti-Obesity Agents Past Experience and Future Prospects. In *Drugs* (Vol. 71, Issue 17).

Hamada, K., Omura, N., Taguchi, A., Baradaran-Heravi, A., Kotake, M., Arai, M., Takayama, K., Taniguchi, A., Roberge, M., & Hayashi, Y. (2019). New Negamycin-Based Potent Readthrough Derivative Effective against TGA-Type Nonsense Mutations. *ACS Medicinal Chemistry Letters*, *10*(10), 1450–1456. https://doi.org/10.1021/acsmedchemlett.9b00273

Hannun, Y. A., & Obeid, L. M. (2008). Principles of bioactive lipid signalling: Lessons from sphingolipids. *Nature Reviews Molecular Cell Biology*, *9*(2), 139–150. https://doi.org/10.1038/nrm2329

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., & Lines, T. C. (2007). A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*, *45*(11), 2179–2205. https://doi.org/10.1016/j.fct.2007.05.015

He, R., Li, H., Wang, L., Li, Y., Zhang, Y., Chen, M., Zhu, Y., & Zhang, C. (2020). Engraftment of human induced pluripotent stem cell-derived myogenic progenitors restores dystrophin in mice with duchenne muscular dystrophy. *Biological Research*, *53*(1). https://doi.org/10.1186/s40659-020-00288-1

Heinrich, M., Neumeyer, J., Jakob, M., Hallas, C., Tchikov, V., Winoto-Morbach, S., Wickel, M., Scheider-Brachert, W., Trauzold, A., Hethke, A., & Schütze, S. (2004). Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. In *Cell Death and Differentiation* (Vol. 11, Issue 5, pp. 550–563). https://doi.org/10.1038/sj.cdd.4401382

Herz, J., Pardo, J., Kashkar, H., Schramm, M., Kuzmenkina, E., Bos, E., Wiegmann, K., Wallich, R., Peters, P. J., Herzig, S., Schmelzer, E., Krönke, M., Simon, M. M., & Utermöhlen, O. (2009). Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes. *Nature Immunology*, *10*(7), 761–768. https://doi.org/10.1038/ni.1757

Hochheiser, K., Kueh, A. J., Gebhardt, T., & Herold, M. J. (2018). CRISPR/Cas9: A tool for immunological research. In *European Journal of Immunology* (Vol. 48, Issue 4, pp. 576–583). Wiley-VCH Verlag. https://doi.org/10.1002/eji.201747131

Hodgetts, S., Radley, H., Davies, M., & Grounds, M. D. (2006). Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice. *Neuromuscular Disorders*, *16*(9–10), 591–602. https://doi.org/10.1016/j.nmd.2006.06.011

Hofmeister, R., Wiegmann, K., Korherr, C., Bernardo, K., Krönke, M., & Falk, W. (1997). Activation of acid sphingomyelinase by interleukin-1 (IL-1) requires the IL-1 receptor accessory protein. *Journal of Biological Chemistry*, *272*(44), 27730–27736. https://doi.org/10.1074/jbc.272.44.27730

Hollinger, K., & Chamberlain, J. S. (2015). Viral vector-mediated gene therapies. In *Current Opinion in Neurology* (Vol. 28, Issue 5, pp. 522–527). Lippincott Williams and Wilkins. https://doi.org/10.1097/WCO.0000000000000241

Hollinger, K., Shanely, R. A., Quindry, J. C., & Selsby, J. T. (2015). Long-term quercetin dietary enrichment decreases muscle injury in mdx mice. *Clinical Nutrition*, *34*(3), 515–522. https://doi.org/10.1016/j.clnu.2014.06.008

Horinouchi, K., Erlich, S., Perl, D. P., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Desnick, R. J., Stewart, C. L., & Schuchman, E. H. (1995). Acid sphingomyelinase deficient mice: A model of types A and B Niemann–Pick disease. *Nature Genetics*, *10*(3), 288–293. https://doi.org/10.1038/ng0795-288

Hsu, M. Y., Hsiao, Y. P., Lin, Y. T., Chen, C., Lee, C. M., Liao, W. C., Tsou, S. C., Lin, H. W., & Chang, Y. Y. (2021). Quercetin alleviates the accumulation of superoxide in sodium iodate-induced retinal autophagy by regulating mitochondrial reactive oxygen species homeostasis through enhanced deacetyl-SOD2 via the Nrf2-PGC-1α-Sirt1 pathway. *Antioxidants*, *10*(7). https://doi.org/10.3390/antiox10071125

Huang, P., Zhao, X. S., Fields, M., Ransohoff, R. M., & Zhou, L. (2009). Imatinib attenuates skeletal muscle dystrophy in mdx mice . *The FASEB Journal*, *23*(8), 2539–2548. https://doi.org/10.1096/fj.09-129833

Jenkins, R. W., Clarke, C. J., Canals, D., Snider, A. J., Gault, C. R., Heffernan-Stroud, L., Wu, B. X., Simbari, F., Roddy, P., Kitatani, K., Obeid, L. M., & Hannun, Y. A. (2011). Regulation of CC ligand 5/rantes by acid sphingomyelinase and acid ceramidase. *Journal of Biological Chemistry*, *286*(15), 13292–13303. https://doi.org/10.1074/jbc.M110.163378

Jin, J., Zhang, X., Lu, Z., Perry, D. M., Li, Y., Russo, S. B., Cowart, L. A., Hannun, Y. A., & Huang, Y. (2013). Acid sphingomyelinase plays a key role in palmitic acid-amplified inflammatory signaling triggered by lipopolysaccharide at low concentrations in macrophages. *American Journal of Physiology - Endocrinology and Metabolism*, *305*(7). https://doi.org/10.1152/ajpendo.00251.2013

Jin, Y., Shen, Y., Su, X., Weintraub, N. L., & Tang, Y. (2020). Effective restoration of dystrophin expression in iPSC Mdx-derived muscle progenitor cells using the CRISPR/Cas9 system and homology-directed repair technology. *Computational and Structural Biotechnology Journal*, *18*, 765–773. https://doi.org/10.1016/j.csbj.2020.03.012

Kang, P. B., & Griggs, R. C. (2015). Advances in muscular dystrophies. *JAMA Neurology*, *72*(7), 741–742. https://doi.org/10.1001/jamaneurol.2014.4621

Kelly, A. M., & Zacks, S. I. (1969). The histogenesis of rat intercostal muscle. *The Journal of Cell Biology*, *42*(1), 135–153. https://doi.org/10.1083/jcb.42.1.135

Klein, S. M., Prantl, L., Geis, S., Felthaus, O., Dolderer, J., Anker, A. M., Zeitler, K., Alt, E., & Vykoukal, J. (2017). Circulating serum CK level vs. muscle impairment for in situ

monitoring burden of disease in Mdx-mice. *Clinical Hemorheology and Microcirculation*, *65*(4), 327–334. https://doi.org/10.3233/CH-16195

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., Tanaka, N., Moriguchi, T., Motohashi, H., Nakayama, K., & Yamamoto, M. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms11624

Koo, T., Popplewell, L., Athanasopoulos, T., & Dickson, G. (2014). Triple trans-splicing adeno-associated virus vectors capable of transferring the coding sequence for full-length dystrophin protein into dystrophic mice. *Human Gene Therapy*, *25*(2), 98–108. https://doi.org/10.1089/hum.2013.164

Kornhuber, J., Rhein, C., Müller, C. P., & Mühle, C. (2015). Secretory sphingomyelinase in health and disease. In *Biological Chemistry* (Vol. 396, Issues 6–7, pp. 707–736). Walter de Gruyter GmbH. https://doi.org/10.1515/hsz-2015-0109

Kornhuber, J., Tripal, P., Reichel, M., Mühle, C., Rhein, C., Muehlbacher, M., Groemer, T. W., & Gulbins, E. (2010). Functional Inhibitors of Acid Sphingomyelinase (FIASMAs): A Novel Pharmacological Group of Drugs with Broad Clinical Applications. *Cell Physiol Biochem 2010;26:09-20*. https://doi.org/10.1007/978-3-7091-1368-4-9

Kottlors, M., & Kirschner, J. (2010). Elevated satellite cell number in Duchenne muscular dystrophy. *Cell and Tissue Research*, *340*(3), 541–548. https://doi.org/10.1007/s00441-010-0976-6

Kourakis, S., Timpani, C. A., Campelj, D. G., Hafner, P., Gueven, N., Fischer, D., & Rybalka, E. (2021). Standard of care versus new-wave corticosteroids in the treatment of Duchenne muscular dystrophy: Can we do better? *Orphanet Journal of Rare Diseases*, *16*(1). https://doi.org/10.1186/s13023-021-01758-9

Kourakis, S., Timpani, C. A., de Haan, J. B., Gueven, N., Fischer, D., & Rybalka, E. (2021). Targeting Nrf2 for the treatment of Duchenne Muscular Dystrophy. *Redox Biology*, *38*(August 2020), 101803. https://doi.org/10.1016/j.redox.2020.101803

Kozakowska, M., Pietraszek-Gremplewicz, K., Jozkowicz, A., & Dulak, J. (2015). The role of oxidative stress in skeletal muscle injury and regeneration: focus on antioxidant enzymes. *Journal of Muscle Research and Cell Motility*, *36*(6), 377–393. https://doi.org/10.1007/s10974-015-9438-9

Kumar, S., Gautam, S., & Sharma, A. (2013). Antimutagenic and antioxidant properties of plumbagin and other naphthoquinones. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *755*(1), 30–41. https://doi.org/10.1016/j.mrgentox.2013.05.007

Langmann, T., Buechler, C., Ries, S., Schaeffler, A., Aslanidis, C., Schuierer, M., Weiler, M., Sandhoff, K., de Jong, P. J., & Schmitz, G. (1999). Transcription factors Sp1 and AP-2 mediate induction of acid sphingomyelinase during monocytic differentiation. *Journal of Lipid Research*, *40*(5), 870–880. https://doi.org/10.1016/s0022-2275(20)32122-2

Laterza, O. F., Lim, L., Garrett-Engele, P. W., Vlasakova, K., Muniappa, N., Tanaka, W. K., Johnson, J. M., Sina, J. F., Fare, T. L., Sistare, F. D., & Glaab, W. E. (2009). Plasma microRNAs as sensitive and specific biomarkers of tissue injury. *Clinical Chemistry*, *55*(11), 1977–1983. https://doi.org/10.1373/clinchem.2009.131797

Lawler, J. M. (2011). Exacerbation of pathology by oxidative stress in respiratory and locomotor muscles with Duchenne muscular dystrophy. *Journal of Physiology*, *589*(9), 2161–2170. https://doi.org/10.1113/jphysiol.2011.207456

Lee, C. M., Cradick, T. J., & Bao, G. (2016). The neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Molecular Therapy*, *24*(3), 645–654. https://doi.org/10.1038/mt.2016.8

Lee, Y.-I., & Leem, Y.-H. (2019). Acid sphingomyelinase inhibition alleviates muscle damage in gastrocnemius after acute strenuous exercise. *J Exerc Nutrition Biochem. 23(2):1-6. Doi:10.20463/Jenb.2019.0009*. https://doi.org/10.20463/jenb.2018.0010

Lemos, D. R., Babaeijandaghi, F., Low, M., Chang, C. K., Lee, S. T., Fiore, D., Zhang, R. H., Natarajan, A., Nedospasov, S. A., & Rossi, F. M. V. (2015). Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors. *Nature Medicine*, *21*(7), 786–794. https://doi.org/10.1038/nm.3869

Li, C., Guo, S., Pang, W., & Zhao, Z. (2020). Crosstalk Between Acid Sphingomyelinase and Inflammasome Signaling and Their Emerging Roles in Tissue Injury and Fibrosis. In *Frontiers in Cell and Developmental Biology* (Vol. 7). Frontiers Media S.A. https://doi.org/10.3389/fcell.2019.00378

Li, P. L., Zhang, Y., & Yi, F. (2007). Lipid raft redox signaling platforms in endothelial dysfunction. In *Antioxidants and Redox Signaling* (Vol. 9, Issue 9, pp. 1457–1470). https://doi.org/10.1089/ars.2007.1667

Li, X., Gulbins, E., & Zhang, Y. (2012). Oxidative stress triggers Ca 2+ -dependent lysosome trafficking and activation of acid sphingomyelinase. *Cellular Physiology and Biochemistry*, *30*(4), 815–826. https://doi.org/10.1159/000341460

Lin, H. C., Cheng, T. H., Chen, Y. C., & Juan, S. H. (2004). Mechanism of heme oxygenase-1 gene induction by quercetin in rat aortic smooth muscle cells. *Pharmacology*, *71*(2), 107–112. https://doi.org/10.1159/000076947

Liu, Y., & Guo, M. (2015). Studies on transition metal-quercetin complexes using electrospray ionization tandem mass spectrometry. *Molecules*, *20*(5), 8583–8594. https://doi.org/10.3390/molecules20058583

Lovering, R. M., Porter, N. C., & Block, R. J. (2005). The muscular dystrophies: From genes to therapies. *Physical Therapy*, *85*(12), 1372–1388. https://doi.org/10.1093/ptj/85.12.1372

Lu, A., Poddar, M., Tang, Y., Proto, J. D., Sohn, J., Mu, X., Oyster, N., Wang, B., & Huard, J. (2014). Rapid depletion of muscle progenitor cells in dystrophic mdx/utrophin-/- mice. *Human Molecular Genetics*, *23*(18), 4786–4800. https://doi.org/10.1093/hmg/ddu194

Lu, Q. L., Yokota, T., Takeda, S., Garcia, L., Muntoni, F., & Partridge, T. (2011). The status of exon skipping as a therapeutic approach to duchenne muscular dystrophy. In *Molecular Therapy* (Vol. 19, Issue 1, pp. 9–15). Nature Publishing Group. https://doi.org/10.1038/mt.2010.219

Luce, L., Carcione, M., Mazzanti, C., Buonfiglio, P. I., Dalamón, V., Mesa, L., Dubrovsky, A., Corderí, J., & Giliberto, F. (2021). Theragnosis for Duchenne Muscular Dystrophy. *Frontiers in Pharmacology*, *12*(June), 1–14. https://doi.org/10.3389/fphar.2021.648390

Luo, P., Wong, Y. F., Ge, L., Zhang, Z. F., Liu, Y., Liu, L., & Zhou, H. (2010). Anti-inflammatory and analgesic effect of plumbagin through inhibition of nuclear factor-κB activation. *Journal of Pharmacology and Experimental Therapeutics*, *335*(3), 735–742. https://doi.org/10.1124/jpet.110.170852

Maceyka, M., & Spiegel, S. (2014). Sphingolipid metabolites in inflammatory disease. In *Nature* (Vol. 510, Issue 7503, pp. 58–67). Nature Publishing Group. https://doi.org/10.1038/nature13475

MacFadden-Murphy, E., Roussel, L., Martel, G., Bérubé, J., & Rousseau, S. (2017). Decreasing SMPD1 activity in BEAS-2B bronchial airway epithelial cells results in increased NRF2 activity, cytokine synthesis and neutrophil recruitment. *Biochemical and Biophysical Research Communications*, *482*(4), 645–650. https://doi.org/10.1016/j.bbrc.2016.11.087

Madaro, L., & Bouché, M. (2014). From innate to adaptive immune response in muscular dystrophies and skeletal muscle regeneration: The role of lymphocytes. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/438675

Madaro, L., Torcinaro, A., de Bardi, M., Contino, F. F., Pelizzola, M., Diaferia, G. R., Imeneo, G., Bouchè, M., Puri, P. L., & de Santa, F. (2019). Macrophages fine tune satellite cell fate in dystrophic skeletal muscle of mdx mice. *PLoS Genetics*, *15*(10). https://doi.org/10.1371/journal.pgen.1008408

Mah, J. (2016). Current and emerging treatment strategies for Duchenne muscular dystrophy. *Neuropsychiatric Disease and Treatment*, *Volume 12*, 1795–1807. https://doi.org/10.2147/NDT.S93873

Managò, A., Becker, K. A., Carpinteiro, A., Wilker, B., Soddemann, M., Seitz, A. P., Edwards, M. J., Grassmé, H., Szabò, I., & Gulbins, E. (2015). Pseudomonas aeruginosa Pyocyanin Induces Neutrophil Death via Mitochondrial Reactive Oxygen Species and Mitochondrial Acid Sphingomyelinase. *Antioxidants and Redox Signaling*, *22*(13), 1097–1110. https://doi.org/10.1089/ars.2014.5979

Manning, J., Kulbida, R., Rai, P., Jensen, L., Bouma, J., Singh, S. P., O'Malley, D., & Yilmazer-Hanke, D. M. (2014). Amitriptyline is efficacious in ameliorating muscle inflammation and depressive symptoms in the mdx mouse model of Duchenne muscular dystrophy. *Experimental Physiology*, *99*(10), 1370–1386. https://doi.org/10.1113/expphysiol.2014.079475

Mantovani, A., Garlanda, C., & Locati, M. (2009). Macrophage diversity and polarization in atherosclerosis: A question of balance. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *29*(10), 1419–1423. https://doi.org/10.1161/ATVBAHA.108.180497

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*, *25*(12), 677–686. https://doi.org/10.1016/j.it.2004.09.015

Martinon, F., Burns, K., & Rg Tschopp, J. (2002). The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-that they possess several distinct protein/protein inter-action domains which are used to assemble large multi-component complexes. Apaf-1, for example, contains an N-terminal CARD followed by a NBS/self-oligomer-ization domain and a C-terminal WD-40 repeat (Jaro. In *Molecular Cell* (Vol. 10).

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of Biophysical and Biochemical Cytology*, *9*, 493–495. https://doi.org/10.1083/jcb.9.2.493

Mendell, J. R., Shilling, C., Leslie, N. D., Flanigan, K. M., Al-Dahhak, R., Gastier-Foster, J., Kneile, K., Dunn, D. M., Duval, B., Aoyagi, A., Hamil, C., Mahmoud, M., Roush, K., Bird, L., Rankin, C., Lilly, H., Street, N., Chandrasekar, R., & Weiss, R. B. (2012). Evidence-based path to newborn screening for duchenne muscular dystrophy. *Annals of Neurology*, *71*(3), 304–313. https://doi.org/10.1002/ana.23528

Mendez-David, I., Tritschler, L., el Ali, Z., Damiens, M. H., Pallardy, M., David, D. J., Kerdine-Römer, S., & Gardier, A. M. (2015). Nrf2-signaling and BDNF: A new target for the antidepressant-like activity of chronic fluoxetine treatment in a mouse model of anxiety/depression. *Neuroscience Letters*, *597*, 121–126. https://doi.org/10.1016/j.neulet.2015.04.036

Meng, J., Chun, S., Asfahani, R., Lochmüller, H., Muntoni, F., & Morgan, J. (2014). Human skeletal muscle-derived CD133+ Cells form functional satellite cells after intramuscular transplantation in immunodeficient host mice. *Molecular Therapy*, *22*(5), 1008–1017. https://doi.org/10.1038/mt.2014.26

Meng, J., Muntoni, F., & Morgan, J. (2018). CD133+ cells derived from skeletal muscles of Duchenne muscular dystrophy patients have a compromised myogenic and muscle regenerative capability. *Stem Cell Research*, *30*, 43–52. https://doi.org/10.1016/j.scr.2018.05.004

Mercuri, E., Bönnemann, C. G., & Muntoni, F. (2019). Muscular dystrophies. *The Lancet*, *394*(10213), 2025–2038. https://doi.org/10.1016/S0140-6736(19)32910-1

Mercuri, E., Muntoni, F., Osorio, A. N., Tulinius, M., Buccella, F., Morgenroth, L. P., Gordish-Dressman, H., Jiang, J., Trifillis, P., Zhu, J., Kristensen, A., Santos, C. L., Henricson, E. K., McDonald, C. M., & Desguerre, I. (2020). Safety and effectiveness of ataluren: Comparison of results from the STRIDE Registry and CINRG DMD Natural History Study. *Journal of Comparative Effectiveness Research*, *9*(5), 341–360. https://doi.org/10.2217/cer-2019-0171

Mikita, J., Dubourdieu-Cassagno, N., Deloire, M. S., Vekris, A., Biran, M., Raffard, G., Brochet, B., Canron, M.-H., Franconi, J.-M., Boiziau, C., & Petry, K. G. (2011). Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. In *Mult Scler. 2011 Jan;17(1):2-15. doi: 10.1177/1352458510379243. PMID: 20813772.*

Mohammadtaghvaei, N., Afarin, R., Mavalizadeh, F., Shakerian, E., Bavarsad, S. S., & Mohammadzadeh, G. (2021). Effect of quercetin on the expression of noxs and p-smad3c in tgf-b-activated hepatic stellate cell line lx-2. *Hepatitis Monthly*, *21*(6). https://doi.org/10.5812/hepatmon.116875

Moretti, I., Ciciliot, S., Dyar, K. A., Abraham, R., Murgia, M., Agatea, L., Akimoto, T., Bicciato, S., Forcato, M., Pierre, P., Uhlenhaut, N. H., Rigby, P. W. J., Carvajal, J. J., Blaauw, B., Calabria, E., & Schiaffino, S. (2016). MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms12397

Moulton, H. M., & Moulton, J. D. (2010). Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy. In *Biochimica et Biophysica Acta - Biomembranes* (Vol. 1798, Issue 12, pp. 2296–2303). https://doi.org/10.1016/j.bbamem.2010.02.012

Mozzetta C., Consalvi S., Saccone V., Tierney M., Diamantini A., Mitchell K.J., Marazzi G., Borsellino G., Battistini L., Sassoon D., Sacco A., Puri P.L.. Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice. EMBO Mol Med. 2013 Apr;5(4) 626-639. https://doi.org/10.1002/emmm.201202096

Müller, M., Lee, C. M., Gasiunas, G., Davis, T. H., Cradick, T. J., Siksnys, V., Bao, G., Cathomen, T., & Mussolino, C. (2016). Streptococcus thermophilus CRISPR-Cas9 systems enable specific editing of the human genome. *Molecular Therapy*, *24*(3), 636–644. https://doi.org/10.1038/mt.2015.218

Muntoni, F., Torelli, S., & Ferlini, A. (2003). Dystrophin and mutations: One gene, several proteins, multiple phenotypes. *Lancet Neurology*, *2*(12), 731–740. https://doi.org/10.1016/S1474-4422(03)00585-4

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J. L., Mosser, D. M., Natoli, G., Saeij, J. P., Schultze, J. L., Shirey, K. A., … Wynn, T. A. (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*, *41*(1), 14–20. https://doi.org/10.1016/j.immuni.2014.06.008

Musarò, A. (2014). The Basis of Muscle Regeneration. *Advances in Biology*, *2014*(Table 1), 1–16. https://doi.org/10.1155/2014/612471

Myoblast Transplantation: NCT02196467. https://clinicaltrials.gov/ct2/show/NCT02196467 Transplantation of Myoblasts to Duchenne Muscular Dystrophy (DMD) Patients Estimated Study Completion Date: 2024

Nakae, Y., Dorchies, O. M., Stoward, P. J., Zimmermann, B. F., Ritter, C., & Ruegg, U. T. (2012). Quantitative evaluation of the beneficial effects in the mdx mouse of epigallocatechin gallate, an antioxidant polyphenol from green tea. *Histochemistry and Cell Biology*, *137*(6), 811–827. https://doi.org/10.1007/s00418-012-0926-3

Naser, E., Kadow, S., Schumacher, F., Mohamed, Z. H., Kappe, C., Hessler, G., Pollmeier, B., Kleuser, B., Arenz, C., Becker, K. A., Gulbins, E., & Carpinteiro, A. (2020). Characterization of the small molecule ARC39, a direct and specific inhibitor of acid sphingomyelinase in vitro. *Journal of Lipid Research*, *61*(6), 896–910. https://doi.org/10.1194/jlr.ra120000682

Newton, J., Lima, S., Maceyka, M., & Spiegel, S. (2015). Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. In *Experimental Cell Research* (Vol. 333, Issue 2, pp. 195–200). Academic Press Inc. https://doi.org/10.1016/j.yexcr.2015.02.025

Nikolova-Karakashian, M. N., & Reid, M. B. (2011). Sphingolipid metabolism, oxidant signaling, and contractile function of skeletal muscle. *Antioxidants and Redox Signaling*, *15*(9), 2501–2517. https://doi.org/10.1089/ars.2011.3940

Nowak, K. J., & Davies, K. E. (2004). Duchenne muscular dystrophy and dystrophin: Pathogenesis and opportunities for treatment: Third in molecular medicine review series. In *EMBO Reports* (Vol. 5, Issue 9, pp. 872–876). https://doi.org/10.1038/sj.embor.7400221

O'Halloran, K. D., Murphy, K. H., & Burns, D. P. (2018). Antioxidant therapy for muscular dystrophy: caveat lector! In *Journal of Physiology* (Vol. 596, Issue 4, pp. 737–738). Blackwell Publishing Ltd. https://doi.org/10.1113/JP275598

Orimo, S., Hiyamuta, E., Arahata, K., & Sugita, H. (1991). Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. *Muscle & Nerve*, *14*(6), 515–520. https://doi.org/10.1002/mus.880140605

Panichayupakaranant, P., & Ahmad, M. I. (2016). Plumbagin and its role in chronic diseases. In *Advances in Experimental Medicine and Biology* (Vol. 929, pp. 229–246). Springer New York LLC. https://doi.org/10.1007/978-3-319-41342-6_10

Papucci, L., Schiavone, N., Witort, E., Donnini, M., Lapucci, A., Tempestini, A., Formigli, L., Zecchi-Orlandini, S., Orlandini, G., Carella, G., Brancato, R., & Capaccioli, S. (2003). Coenzyme Q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *Journal of Biological Chemistry*, *278*(30), 28220–28228. https://doi.org/10.1074/jbc.M302297200

Pasut, A., Jones, A. E., & Rudnicki, M. A. (2013). Isolation and culture of individual myofibers and their satellite cells from adult skeletal muscle. *Journal of Visualized Experiments*, *73*. https://doi.org/10.3791/50074

Patel, R. v., Mistry, B. M., Shinde, S. K., Syed, R., Singh, V., & Shin, H. S. (2018). Therapeutic potential of quercetin as a cardiovascular agent. In *European Journal of Medicinal Chemistry* (Vol. 155, pp. 889–904). Elsevier Masson SAS. https://doi.org/10.1016/j.ejmech.2018.06.053

Pedersen, B. K. (2013). Muscle as a secretory organ. *Comprehensive Physiology*, *3*(3), 1337–1362. https://doi.org/10.1002/cphy.c120033

Peng, H., Li, C., Kadow, S., Henry, B. D., Steinmann, J., Becker, K. A., Riehle, A., Beckmann, N., Wilker, B., Li, P. L., Pritts, T., Edwards, M. J., Zhang, Y., Gulbins, E., & Grassmé, H. (2015). Acid sphingomyelinase inhibition protects mice from lung edema and lethal Staphylococcus aureus sepsis. *Journal of Molecular Medicine*, *93*(6), 675–689. https://doi.org/10.1007/s00109-014-1246-y

Perrotta, C., Bizzozero, L., Cazzato, D., Morlacchi, S., Assi, E., Simbari, F., Zhang, Y., Gulbins, E., Bassi, M. T., Rosa, P., & Clementi, E. (2010). Syntaxin 4 is required for acid

sphingomyelinase activity and apoptotic function. *Journal of Biological Chemistry*, *285*(51), 40240–40251. https://doi.org/10.1074/jbc.M110.139287

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. *American Journal of Pathology*, *184*(1), 230–247. https://doi.org/10.1016/j.ajpath.2013.10.006

Perrotta, C., Buonanno, F., Zecchini, S., Giavazzi, A., Proietti Serafini, F., Catalani, E., Guerra, L., Belardinelli, M. C., Picchietti, S., Fausto, A. M., Giorgi, S., Marcantoni, E., Clementi, E., Ortenzi, C., & Cervia, D. (2016). Climacostol reduces tumour progression in a mouse model of melanoma via the p53-dependent intrinsic apoptotic programme. *Scientific Reports*, *6*. https://doi.org/10.1038/srep27281

Perrotta, C., Cervia, D., di Renzo, I., Moscheni, C., Bassi, M. T., Campana, L., Martelli, C., Catalani, E., Giovarelli, M., Zecchini, S., Coazzoli, M., Capobianco, A., Ottobrini, L., Lucignani, G., Rosa, P., Rovere-Querini, P., De Palma, C., & Clementi, E. (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*(MAY). https://doi.org/10.3389/fimmu.2018.01186

Perry, D. M., Newcomb, B., Adada, M., Wu, B. X., Roddy, P., Kitatani, K., Siskind, L., Obeid, L. M., & Hannun, Y. A. (2014). Defining a role for acid sphingomyelinase in the p38/interleukin-6 pathway. *Journal of Biological Chemistry*, *289*(32), 22401–22412. https://doi.org/10.1074/jbc.M114.589648

Petrillo, S., Pelosi, L., Piemonte, F., Travaglini, L., Forcina, L., Catteruccia, M., Petrini, S., Verardo, M., D'Amico, A., Musaró, A., & Bertini, E. (2017). Oxidative stress in Duchenne muscular dystrophy: Focus on the NRF2 redox pathway. *Human Molecular Genetics*, *26*(14), 2781–2790. https://doi.org/10.1093/hmg/ddx173

Pfeiffer, A., Böttcher, A., Orsó, E., Orsó, O., Kapinsky, M., Nagy, P., Bodnárbodn´bodnár, A., Spreitzer, I., Liebisch, G., Drobnik, W., Gempel, K., Horn, M., Holmer, S., Hartung, T., Multhoff, G., Schütz, G., Schindler, H., Ulmer, A. J., Heine, H., … Schmitz, G. (2001). Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Lipopolysaccharide and Ceramide Docking to CD14 Provokes Ligand-Specific Receptor Clustering in Rafts. Eur. J. Immunol., 31: 3153-3164. Https://Doi.Org/10.1002/1521-4141(200111)31:11<3153::AID-IMMU3153>3.0.CO;2-0*.

Pietraszek-Gremplewicz, K., Kozakowska, M., Bronisz-Budzynska, I., Ciesla, M., Mucha, O., Podkalicka, P., Madej, M., Glowniak, U., Szade, K., Stepniewski, J., Jez, M., Andrysiak, K., Bukowska-Strakova, K., Kaminska, A., Kostera-Pruszczyk, A., Jozkowicz, A., Loboda, A., & Dulak, J. (2018). Heme Oxygenase-1 Influences Satellite Cells and Progression of Duchenne Muscular Dystrophy in Mice. *Antioxidants and Redox Signaling*, *29*(2), 128–148. https://doi.org/10.1089/ars.2017.7435

Pinniger, G. J., Terrill, J. R., Assan, E. B., Grounds, M. D., & Arthur, P. G. (2017). Pre-clinical evaluation of N-acetylcysteine reveals side effects in the mdx mouse model of Duchenne muscular dystrophy. *Journal of Physiology*, *595*(23), 7093–7107. https://doi.org/10.1113/JP274229

Pownall, M. E., Gustafsson, M. K., & Emerson, C. P. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annual Review of Cell and Developmental Biology*, *18*(February 2002), 747–783. https://doi.org/10.1146/annurev.cellbio.18.012502.105758

Pralhada Rao, R., Vaidyanathan, N., Rengasamy, M., Mammen Oommen, A., Somaiya, N., & Jagannath, M. R. (2013). Sphingolipid Metabolic Pathway: An Overview of Major Roles

Played in Human Diseases. *Journal of Lipids*, *2013*, 1–12. https://doi.org/10.1155/2013/178910

Qiu, H., Edmunds, T., Baker-Malcolm, J., Karey, K. P., Estes, S., Schwarz, C., Hughes, H., & van Patten, S. M. (2003). Activation of human acid sphingomyelinase through modification or deletion of C-terminal cysteine. *Journal of Biological Chemistry*, *278*(35), 32744–32752. https://doi.org/10.1074/jbc.M303022200

Quattrocelli, M., Barefield, D. Y., Warner, J. L., Vo, A. H., Hadhazy, M., Earley, J. U., Demonbreun, A. R., & McNally, E. M. (2017). Intermittent glucocorticoid steroid dosing enhances muscle repair without eliciting muscle atrophy. *Journal of Clinical Investigation*, *127*(6), 2418–2432. https://doi.org/10.1172/JCI91445

Ramirez-Sanchez, I., de Los Santos, S., Gonzalez-Basurto, S., Canto, P., Mendoza-Lorenzo, P., Palma-Flores, C., Ceballos-Reyes, G., Villarreal, F., Zentella-Dehesa, A., & Coral-Vazquez, R. (2014). (-)-Epicatechin improves mitochondrial-related protein levels and ameliorates oxidative stress in dystrophic δ-sarcoglycan null mouse striated muscle. *FEBS Journal*, *281*(24), 5567–5580. https://doi.org/10.1111/febs.13098

Ran, F. A., Cong, L., Yan, W. X., Scott, D. A., Gootenberg, J. S., Kriz, A. J., Zetsche, B., Shalem, O., Wu, X., Makarova, K. S., Koonin, E. v., Sharp, P. A., & Zhang, F. (2015). In vivo genome editing using Staphylococcus aureus Cas9. *Nature*, *520*(7546), 186–191. https://doi.org/10.1038/nature14299

Rando, T. A., Alto, P., Diseases, N., Diego, S., & Program, E. B. (2002). *Oxidative stress and the pathogenesis of muscular dystrophies*. *November*, 175–186. https://doi.org/10.1097/01.PHM.0000029774.56528.A6

Rando, T. A., Disatnik, M. H., Yu, Y., & Franco, A. (1998). Muscle cells from mdx mice have an increased susceptibility to oxidative stress. *Neuromuscular Disorders*, *8*(1), 14–21. https://doi.org/10.1016/S0960-8966(97)00124-7

Rauf, A., Imran, M., Khan, I. A., ur-Rehman, M., Gilani, S. A., Mehmood, Z., & Mubarak, M. S. (2018). Anticancer potential of quercetin: A comprehensive review. In *Phytotherapy Research* (Vol. 32, Issue 11, pp. 2109–2130). John Wiley and Sons Ltd. https://doi.org/10.1002/ptr.6155

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *The FASEB Journal*, *22*(3), 659–661. https://doi.org/10.1096/fj.07-9574lsf

Relaix, F., Bencze, M., Borok, M. J., der Vartanian, A., Gattazzo, F., Mademtzoglou, D., Perez-Diaz, S., Prola, A., Reyes-Fernandez, P. C., Rotini, A., & Taglietti. (2021). Perspectives on skeletal muscle stem cells. *Nature Communications*, *12*(1), 1–11. https://doi.org/10.1038/s41467-020-20760-6

Relaix, F., & Zammit, P. S. (2012). Satellite cells are essential for skeletal muscle regeneration: The cell on the edge returns centre stage. *Development (Cambridge)*, *139*(16), 2845–2856. https://doi.org/10.1242/dev.069088

Rigamonti, E., Touvier, T., Clementi, E., Manfredi, A. A., Brunelli, S., & Rovere-Querini, P. (2013). Requirement of Inducible Nitric Oxide Synthase for Skeletal Muscle Regeneration after Acute Damage. *The Journal of Immunology*, *190*(4), 1767–1777. https://doi.org/10.4049/jimmunol.1202903

Rittié, L. (2017). Method for picrosirius red-polarization detection of collagen fibers in tissue sections. In *Methods in Molecular Biology* (Vol. 1627, pp. 395–407). Humana Press Inc. https://doi.org/10.1007/978-1-4939-7113-8_26

Roh, J. S., & Sohn, D. H. (2018). Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Network*, *18*(4), 1–14. https://doi.org/10.4110/in.2018.18.e27

Romanelli, M. M., da Costa-Silva, T. A., Cunha-Junior, E., Dias Ferreira, D., Guerra, J. M., Galisteo, A. J., Pinto, E. G., Barbosa, L. R. S., Torres-Santos, E. C., & Tempone, A. G.

(2019). Sertraline Delivered in Phosphatidylserine Liposomes Is Effective in an Experimental Model of Visceral Leishmaniasis. *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00353

Rosenberg, A. S., Puig, M., Nagaraju, K., Hoffman, E. P., Villalta, S. A., Rao, V. A., Wakefield, L. M., & Woodcock, J. (2015). Immune-mediated pathology in Duchenne muscular dystrophy. In *Science Translational Medicine* (Vol. 7, Issue 299). American Association for the Advancement of Science. https://doi.org/10.1126/scitranslmed.aaa7322

Rőszer, T. (2015). Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm. 2015:816460. Doi: 10.1155/2015/816460. Epub 2015 May 18. PMID: 26089604; PMCID: PMC4452191.* https://doi.org/10.32388/n35phw

Roux-Biejat, P., Coazzoli, M., Marrazzo, P., Zecchini, S., di Renzo, I., Prata, C., Napoli, A., Moscheni, C., Giovarelli, M., Barbalace, M. C., Catalani, E., Bassi, M. T., De Palma, C., Cervia, D., Malaguti, M., Hrelia, S., Clementi, E., & Perrotta, C. (2021). Acid Sphingomyelinase Controls Early Phases of Skeletal Muscle Regeneration by Shaping the Macrophage Phenotype. *Cells*, *10*(11), 3028. https://doi.org/10.3390/cells10113028

Sakata, A., Ochiai, T., Shimeno, H., Hikishima, S., Yokomatsu, T., Shibuya, S., Toda, A., Eyanagi, R., & Soeda, S. (2007). Acid sphingomyelinase inhibition suppresses lipopolysaccharide-mediated release of inflammatory cytokines from macrophages and protects against disease pathology in dextran sulphate sodium-induced colitis in mice. *Immunology*, *122*(1), 54–64. https://doi.org/10.1111/j.1365-2567.2007.02612.x

Scharner, J., & Zammit, P. S. (2011). The muscle satellite cell at 50: The formative years. *Skeletal Muscle*, *1*(1), 1–13. https://doi.org/10.1186/2044-5040-1-28

Schertzer, J. D., Gehrig, S. M., Ryall, J. G., & Lynch, G. S. (2007). Modulation of Insulin-like Growth Factor (IGF)-I and IGF-binding protein interactions enhances skeletal muscle regeneration and ameliorates the dystrophic pathology in mdx mice. *American Journal of Pathology*, *171*(4), 1180–1188. https://doi.org/10.2353/ajpath.2007.070292

Schmalbruch, H., & Lewis, D. M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve. (4):617-26. Doi: 10.1002/(Sici)1097-4598(200004)23:4<617::Aid-Mus22>3.0.Co;2-y. PMID: 10716774.*

Schneider-Schaulies, J., & Beyersdorf, N. (2018). CD4+ Foxp3+ regulatory T cell-mediated immunomodulation by anti-depressants inhibiting acid sphingomyelinase. *Biol Chem.;399(10):1175-1182. Doi: 10.1515/Hsz-2018-0159. PMID: 29908119.* https://doi.org/10.1515/hsz-2011-xxxx

Schramm, M., Herz, J., Haas, A., Krönke, M., & Utermöhlen, O. (2008). Acid sphingomyelinase is required for efficient phago-lysosomal fusion. *Cellular Microbiology*, *10*(9), 1839–1853. https://doi.org/10.1111/j.1462-5822.2008.01169.x

Sciorati, C., Touvier, T., Buono, R., Pessina, P., François, S., Perrotta, C., Meneveri, R., Clementi, E., & Brunelli, S. (2009). Necdin is expressed in cachectic skeletal muscle to protect fibers from tumor-induced wasting. *Journal of Cell Science*, *122*(8), 1119–1125. https://doi.org/10.1242/jcs.041665

Severinsen, M. C. K., & Pedersen, B. K. (2020). Muscle–Organ Crosstalk: The Emerging Roles of Myokines. *Endocrine Reviews*, *41*(4), 594–609. https://doi.org/10.1210/ENDREV/BNAA016

Shechter, R., London, A., Varol, C., Raposo, C., Cusimano, M., Yovel, G., Rolls, A., Mack, M., Pluchino, S., Martino, G., Jung, S., & Schwartz, M. (2009). Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS Medicine*, *6*(7). https://doi.org/10.1371/journal.pmed.1000113

Shechter, R., Miller, O., Yovel, G., Rosenzweig, N., London, A., Ruckh, J., Kim, K. W., Klein, E., Kalchenko, V., Bendel, P., Lira, S. A., Jung, S., & Schwartz, M. (2013). Recruitment

of Beneficial M2 Macrophages to Injured Spinal Cord Is Orchestrated by Remote Brain Choroid Plexus. *Immunity*, *38*(3), 555–569. https://doi.org/10.1016/j.immuni.2013.02.012

Sheikh, O., & Yokota, T. (2021). Pharmacology and toxicology of eteplirsen and SRP-5051 for DMD exon 51 skipping: an update. In *Archives of Toxicology*. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00204-021-03184-z

Shela, S. B., Narasimhan, M., Shanmugam, G., Litovsky, S. H., Gounder, S. S., Karan, G., Arulvasu, C., Kensler, T. W., Hoidal, J. R., Darley-Usmar, V. M., & Rajasekaran, N. S. (2016). Disruption of nuclear factor (erythroid-derived-2)-like 2 antioxidant signaling: A mechanism for impaired activation of stem cells and delayed regeneration of skeletal muscle. *FASEB Journal*, *30*(5), 1865–1879. https://doi.org/10.1096/fj.201500153

Shin, T. K., Kang, M. S., Lee, H. Y., Seo, M. S., Kim, S. G., Kim, C. D., & Lee, W. S. (2009). Fluoxetine and sertraline attenuate postischemic brain injury in mice. *The Korean Journal of Physiology & Pharmacology: Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *13(3), 257–263.*

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., & Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: A point mutation. *Science*, *244*(4912). https://doi.org/10.1126/science.2662404

Smith, E. L., & Schuchman, E. H. (2008). The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *The FASEB Journal*, *22*(10), 3419–3431. https://doi.org/10.1096/fj.08-108043

Song, Y. H., Song, J. L., Delafontaine, P., & Godard, M. P. (2013). The therapeutic potential of IGF-I in skeletal muscle repair. In *Trends in Endocrinology and Metabolism* (Vol. 24, Issue 6, pp. 310–319). https://doi.org/10.1016/j.tem.2013.03.004

Song, Y., Morales, L., Malik, A. S., Mead, A. F., Greer, C. D., Mitchell, M. A., Petrov, M. T., Su, L. T., Choi, M. E., Rosenblum, S. T., Lu, X., VanBelzen, D. J., Krishnankutty, R. K., Balzer, F. J., Loro, E., French, R., Propert, K. J., Zhou, S., Kozyak, B. W., … Stedman, H. H. (2019). Non-immunogenic utrophin gene therapy for the treatment of muscular dystrophy animal models. *Nature Medicine*, *25*(10), 1505–1511. https://doi.org/10.1038/s41591-019-0594-0

Spaulding, H. R., Ballmann, C. G., Quindry, J. C., & Selsby, J. T. (2016). Long-Term quercetin dietary enrichment partially protects dystrophic skeletal muscle. *PLoS ONE*, *11*(12). https://doi.org/10.1371/journal.pone.0168293

Spurney, C. F., Rocha, C. T., Henricson, E., Florence, J., Mayhew, J., Gorni, K., Pasquali, L., Pestronk, A., Martin, G. R., Hu, F., Nie, L., Connolly, A. M., & Escolar, D. M. (2011). CINRG pilot trial of coenzyme Q10 in steroid-treated Duchenne muscular dystrophy. *Muscle Nerve. 2011 Aug;44(2):174-8. Doi: 10.1002/Mus.22047. Epub. PMID: 21698649; PMCID: PMC3136634.*

Stedman, H. H., Sweeney, H. L., Shrager, J. B., Maguire, H. C., Panettieri, R. A., Petrof, B., Narusawa, M., Leferovich, J. M., Sladky, J. T., & Kelly, A. M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature ;352(6335):536-9. Doi: 10.1038/352536a0. PMID: 1865908.*

Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., & Suttles, J. (2005). Macrophages Sequentially Change Their Functional Phenotype in Response to Changes in Microenvironmental Influences. *The Journal of Immunology*, *175*(1), 342–349. https://doi.org/10.4049/jimmunol.175.1.342

Sumsakul, W., Plengsuriyakarn, T., & Na-Bangchang, K. (2016). Pharmacokinetics, toxicity, and cytochrome P450 modulatory activity of plumbagin. *BMC Pharmacology and Toxicology*, *17*(1). https://doi.org/10.1186/s40360-016-0094-5

Sun, C., Serra, C., Lee, G., & Wagner, K. R. (2020). Stem cell-based therapies for Duchenne muscular dystrophy. In *Experimental Neurology* (Vol. 323). Academic Press Inc. https://doi.org/10.1016/j.expneurol.2019.113086

Sun, C., Shen, L., Zhang, Z., & Xie, X. (2020). Therapeutic strategies for duchenne muscular dystrophy: An update. *Genes*, *11*(8), 1–25. https://doi.org/10.3390/genes11080837

Süntar, I., Çetinkaya, S., Panieri, E., Saha, S., Buttari, B., Profumo, E., & Saso, L. (2021). Regulatory role of nrf2 signaling pathway in wound healing process. In *Molecules* (Vol. 26, Issue 9). MDPI AG. https://doi.org/10.3390/molecules26092424

Szabo, S. M., Salhany, R. M., Deighton, A., Harwood, M., Mah, J., & Gooch, K. L. (2021). The clinical course of Duchenne muscular dystrophy in the corticosteroid treatment era: a systematic literature review. In *Orphanet Journal of Rare Diseases* (Vol. 16, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13023-021-01862-w

Tan-Chen, S., Guitton, J., Bourron, O., le Stunff, H., & Hajduch, E. (2020). Sphingolipid Metabolism and Signaling in Skeletal Muscle: From Physiology to Physiopathology. In *Frontiers in Endocrinology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fendo.2020.00491

Tanigawa, S., Fujii, M., & Hou, D. X. (2007). Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radical Biology and Medicine*, *42*(11), 1690–1703. https://doi.org/10.1016/j.freeradbiomed.2007.02.017

Terao, M., Tamano, M., Hara, S., Kato, T., Kinoshita, M., & Takada, S. (2016). Utilization of the CRISPR/Cas9 system for the efficient production of mutant mice using crRNA/tracrRNA with Cas9 nickase and FokI-dCas9. *Exp Anim. 2016 Jul 29;65(3):275-83. Doi: 10.1538/Expanim.15-0116. PMID: 26972821; PMCID: PMC4976241.*

Terrill, J. R., Pinniger, G. J., Graves, J. A., Grounds, M. D., & Arthur, P. G. (2016). Increasing taurine intake and taurine synthesis improves skeletal muscle function in the mdx mouse model for Duchenne muscular dystrophy. *Journal of Physiology*, *594*(11), 3095–3110. https://doi.org/10.1113/JP271418

Terrill, J. R., Radley-Crabb, H. G., Grounds, M. D., & Arthur, P. G. (2012). N-Acetylcysteine treatment of dystrophic mdx mice results in protein thiol modifications and inhibition of exercise induced myofibre necrosis. *Neuromuscular Disorders*, *22*(5), 427–434. https://doi.org/10.1016/j.nmd.2011.11.007

Tidball, J. G. (2005). Inflammatory processes in muscle injury and repair. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *288*(2 57-2), 345–353. https://doi.org/10.1152/ajpregu.00454.2004

Tidball, J. G., & Wehling-Henricks, M. (2007). The role of free radicals in the pathophysiology of muscular dystrophy. *Journal of Applied Physiology*, *102*(4), 1677–1686. https://doi.org/10.1152/japplphysiol.01145.2006

Tidball, J. G., & Wehling-Henricks, M. (2014). Nitric oxide synthase deficiency and the pathophysiology of muscular dystrophy. *Journal of Physiology*, *592*(21), 4627–4638. https://doi.org/10.1113/jphysiol.2014.274878

Tidball, J. G., & Welc, S. S. (2015). Macrophage-derived IGF-1 is a potent coordinator of myogenesis and inflammation in regenerating muscle. In *Molecular Therapy* (Vol. 23, Issue 7, pp. 1134–1135). Nature Publishing Group. https://doi.org/10.1038/mt.2015.97

Tidball, J. G., Welc, S. S., & Wehling-Henricks, M. (2018). Immunobiology of Inherited Muscular Dystrophies. *Comprehensive Physiology*, *8*(4), 1313–1356. https://doi.org/10.1002/cphy.c170052

Tierney, M. T., & Sacco, A. (2016). Satellite Cell Heterogeneity in Skeletal Muscle Homeostasis. *Trends Cell Biol. 2016 Jun;26(6):434-444. Doi: 10.1016/j.Tcb.2016.02.004. PMID: 26948993; PMCID: PMC4877266.* https://doi.org/10.1016/j.tcb.2016.02.004.Satellite

Tilak, J. C., Devasagayam, T. P. A., Banerjee, M., Adhikari, S., Chintalwar, G. J., & Chattopadhyay, S. (2001). *Differential Antioxidant Effects of Plumbagin in Rat Tissues*.

Torrente, Y., Belicchi, M., Marchesi, C., D'antona, G., Cogiamanian, F., Pisati, F., Gavina, M., Giordano, R., Tonlorenzi, R., Fagiolari, G., Lamperti, C., Porretti, L., Lopa, R., Sampaolesi, M., Vicentini, L., Grimoldi, ¶ N, Tiberio, F., Songa, V., Baratta, # P, … Bresolin, N. (2007). Autologous Transplantation of Muscle-Derived CD133 + Stem Cells in Duchenne Muscle Patients. *Cell Transplantation*, *16*, 563–577. www.cognizantcommunication.com

Trovato, F. M., Imbesi, R., Conway, N., & Castrogiovanni, P. (2016). Morphological and functional aspects of human skeletal muscle. *Journal of Functional Morphology and Kinesiology*, *1*(3), 289–302. https://doi.org/10.3390/jfmk1030289

Truman, J. P., al Gadban, M. M., Smith, K. J., & Hammad, S. M. (2011). Acid sphingomyelinase in macrophage biology. In *Cellular and Molecular Life Sciences* (Vol. 68, Issue 20, pp. 3293–3305). https://doi.org/10.1007/s00018-011-0686-6

Truman, J. P., al Gadban, M. M., Smith, K. J., Jenkins, R. W., Mayroo, N., Virella, G., Lopes-Virella, M. F., Bielawska, A., Hannun, Y. A., & Hammad, S. M. (2012). Differential regulation of acid sphingomyelinase in macrophages stimulated with oxidized low-density lipoprotein (LDL) and oxidized LDL immune complexes: Role in phagocytosis and cytokine release. *Immunology*, *136*(1), 30–45. https://doi.org/10.1111/j.1365-2567.2012.03552.x

Tsoumpra, M. K., Fukumoto, S., Matsumoto, T., Takeda, S., Wood, M. J. A., & Aoki, Y. (2019). Peptide-conjugate antisense based splice-correction for Duchenne muscular dystrophy and other neuromuscular diseases. In *EBioMedicine* (Vol. 45, pp. 630–645). Elsevier B.V. https://doi.org/10.1016/j.ebiom.2019.06.036

Turk, R., Sterrenburg, E., de Meijer, E. J., van Ommen, G. J. B., den Dunnen, J. T., & 't Hoen, P. A. C. (2005). Muscle regeneration in dystrophin-deficient mdx mice studied by gene expression profiling. *BMC Genomics*, *6*. https://doi.org/10.1186/1471-2164-6-98

Tutakhail, A., Nazari, Q. A., Khabil, S., Gardier, A., & Coudore, F. (2019). Muscular and mitochondrial effects of long-term fluoxetine treatment in mice, combined with physical endurance exercise on treadmill. *Life Sciences*, *232*. https://doi.org/10.1016/j.lfs.2019.05.064

Vamolorone: NCT05185622. https://clinicaltrials.gov/ct2/show/NCT05185622
A Study to Assess Vamorolone in Boys Ages 2 to <4 Years and 7 to <18 Years With Duchenne Muscular Dystrophy (DMD)
Estimated Study Start Date: 2022

Verhaart, I. E. C., & Aartsma-Rus, A. (2019). Therapeutic developments for Duchenne muscular dystrophy. *Nature Reviews Neurology*, *15*(7), 373–386. https://doi.org/10.1038/s41582-019-0203-3

Vieceli Dalla Sega, F., Zambonin, L., Fiorentini, D., Rizzo, B., Caliceti, C., Landi, L., Hrelia, S., & Prata, C. (2014). Specific aquaporins facilitate Nox-produced hydrogen peroxide transport through plasma membrane in leukaemia cells. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1843*(4), 806–814. https://doi.org/10.1016/j.bbamcr.2014.01.011

Villalta, A. S., Rosenthal, W., Martinez, L., Kaur, A., Sparwasser, T., Tidball, J. G., Margeta, M., Spencer, M. J., & Bluestone, J. A. (2014). Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med.;6(258):258ra142. Doi:10.1126/Scitranslmed.3009925*.
https://doi.org/10.1126/scitranslmed.3009925.Regulatory

Villalta, S. A., Deng, B., Rinaldi, C., Wehling-Henricks, M., & Tidball, J. G. (2011). IFN-γ Promotes Muscle Damage in the mdx Mouse Model of Duchenne Muscular Dystrophy by

Suppressing M2 Macrophage Activation and Inhibiting Muscle Cell Proliferation . *The Journal of Immunology*, *187*(10), 5419–5428. https://doi.org/10.4049/jimmunol.1101267

Villalta, S. A., Nguyen, H. X., Deng, B., Gotoh, T., & Tidbal, J. G. (2009). Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Human Molecular Genetics*, *18*(3), 482–496. https://doi.org/10.1093/hmg/ddn376

Villalta, S. A., Rinaldi, C., Deng, B., Liu, G., Fedor, B., & Tidball, J. G. (2011). Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype. *Human Molecular Genetics*, *20*(4), 790–805. https://doi.org/10.1093/hmg/ddq523

Vitiello, L., Tibaudo, L., Pegoraro, E., Bello, L., & Canton, M. (2019). Teaching an old molecule new tricks: Drug repositioning for duchenne muscular dystrophy. In *International Journal of Molecular Sciences* (Vol. 20, Issue 23). MDPI AG. https://doi.org/10.3390/ijms20236053

Wang, K. H., & Li, B. Z. (2018). Plumbagin protects against hydrogen peroxide-induced neurotoxicity by modulating NF-κB and Nrf-2. *Archives of Medical Science*, *14*(5), 1112–1118. https://doi.org/10.5114/aoms.2016.64768

Wang, T., Wu, F., Jin, Z., Zhai, Z., Wang, Y., Tu, B., Yan, W., & Tang, T. (2014). Plumbagin inhibits LPS-induced inflammation through the inactivation of the nuclear factor-kappa B and mitogen activated protein kinase signaling pathways in RAW 264.7 cells. *Food and Chemical Toxicology*, *64*, 177–183. https://doi.org/10.1016/j.fct.2013.11.027

Wang, Y. X., Feige, P., Brun, C. E., Hekmatnejad, B., Dumont, N. A., Renaud, J. M., Faulkes, S., Guindon, D. E., & Rudnicki, M. A. (2019). EGFR-Aurka Signaling Rescues Polarity and Regeneration Defects in Dystrophin-Deficient Muscle Stem Cells by Increasing Asymmetric Divisions. *Cell Stem Cell*, *24*(3), 419-432.e6. https://doi.org/10.1016/j.stem.2019.01.002

Wehling, M., Spencer, M. J., & Tidball, J. G. (2001). A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *Journal of Cell Biology*, *155*(1), 123–131. https://doi.org/10.1083/jcb.200105110

Whitehead, N. P., Pham, C., Gervasio, O. L., & Allen, D. G. (2008). N-Acetylcysteine ameliorates skeletal muscle pathophysiology in mdx mice. *Journal of Physiology*, *586*(7), 2003–2014. https://doi.org/10.1113/jphysiol.2007.148338

Willmann, R., Possekel, S., Dubach-Powell, J., Meier, T., & Ruegg, M. A. (2009). Mammalian animal models for Duchenne muscular dystrophy. In *Neuromuscular Disorders* (Vol. 19, Issue 4, pp. 241–249). https://doi.org/10.1016/j.nmd.2008.11.015

Wink, D. A., Hanbauer, I., Laval, F., Cook, J. A., Krishna, M. C., & Mitchell, J. B. (1994). Nitric Oxide Protects against the Cytotoxic Effects of Reactive Oxygen Species. In *Annals of the New York Academy of Sciences* (Vol. 738, Issue 1, pp. 265–278). https://doi.org/10.1111/j.1749-6632.1994.tb21812.x

Wolfe, R. R. (2006). The underappreciated role of muscle in health and disease. *American Journal of Clinical Nutrition*, *84*(3), 475–482. https://doi.org/10.1093/ajcn/84.3.475

Won, J. S., & Singh, I. (2006). Sphingolipid signaling and redox regulation. *Free Radical Biology and Medicine*, *40*(11), 1875–1888. https://doi.org/10.1016/j.freeradbiomed.2006.01.035

Wong, E. T., & Tergaonkar, V. (2009). Roles of NF-κB in health and disease: Mechanisms and therapeutic potential. In *Clinical Science* (Vol. 116, Issue 6, pp. 451–465). https://doi.org/10.1042/CS20080502

Xiang, H., Jin, S., Tan, F., Xu, Y., Lu, Y., & Wu, T. (2021). Physiological functions and therapeutic applications of neutral sphingomyelinase and acid sphingomyelinase. In

Biomedicine and Pharmacotherapy (Vol. 139). Elsevier Masson s.r.l. https://doi.org/10.1016/j.biopha.2021.111610

Xu, D., Hu, M. J., Wang, Y. Q., & Cui, Y. L. (2019). Antioxidant activities of quercetin and its complexes for medicinal application. In *Molecules* (Vol. 24, Issue 6). MDPI AG. https://doi.org/10.3390/molecules24061123

Xu, X., Wilschut, K. J., Kouklis, G., Tian, H., Hesse, R., Garland, C., Sbitany, H., Hansen, S., Seth, R., Knott, P. D., Hoffman, W. Y., & Pomerantz, J. H. (2015). Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles. *Stem Cell Reports*, *5*(3), 419–434. https://doi.org/10.1016/j.stemcr.2015.07.016

Yang, W., & Hu, P. (2018). Skeletal muscle regeneration is modulated by inflammation. *Journal of Orthopaedic Translation*, *13*, 25–32. https://doi.org/10.1016/j.jot.2018.01.002

Yao, S., Chen, Z., Yu, Y., Zhang, N., Jiang, H., Zhang, G., Zhang, Z., & Zhang, B. (2021). Current Pharmacological Strategies for Duchenne Muscular Dystrophy. In *Frontiers in Cell and Developmental Biology* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fcell.2021.689533

Yin, H., Price, F., & Rudnicki, M. A. (2013). Satellite cells and the muscle stem cell niche. *Physiological Reviews*, *93*(1), 23–67. https://doi.org/10.1152/physrev.00043.2011

Yiu, E. M., & Kornberg, A. J. (2015). Duchenne muscular dystrophy. *Journal of Paediatrics and Child Health*, *51*(8), 759–764. https://doi.org/10.1111/jpc.12868

Yoshimoto, Y., Ikemoto-Uezumi, M., Hitachi, K., Fukada, S. I., & Uezumi, A. (2020). Methods for Accurate Assessment of Myofiber Maturity During Skeletal Muscle Regeneration. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.00267

Yoshioka, Y., Yamamuro, A., & Maeda, S. (2003). Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway. In *British Journal of Pharmacology* (Vol. 139, Issue 1, pp. 28–34). https://doi.org/10.1038/sj.bjp.0705206

Young, C. S., Hicks, M. R., Ermolova, N. v., Nakano, H., Jan, M., Younesi, S., Karumbayaram, S., Kumagai-Cresse, C., Wang, D., Zack, J. A., Kohn, D. B., Nakano, A., Nelson, S. F., Miceli, M. C., Spencer, M. J., & Pyle, A. D. (2016). A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells. *Cell Stem Cell*. https://doi.org/10.1016/j.stem.2016.01.021

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., la Russa, M., Xie, M., Ding, S., & Qi, L. S. (2015). Small molecules enhance crispr genome editing in pluripotent stem cells. *Cell Stem Cell*, *16*(2), 142–147. https://doi.org/10.1016/j.stem.2015.01.003

Yucel, N., Chang, A. C., Day, J. W., Rosenthal, N., & Blau, H. M. (2018). Humanizing the mdx mouse model of DMD: the long and the short of it. NPJ Regenerative medicine, 3, 4. https://doi.org/10.1038/s41536-018-0045-4

Zammit, P. S. (2017). Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. In *Seminars in Cell and Developmental Biology* (Vol. 72, pp. 19–32). Elsevier Ltd. https://doi.org/10.1016/j.semcdb.2017.11.011

Zecchini, S., Giovarelli, M., Perrotta, C., Morisi, F., Touvier, T., di Renzo, I., Moscheni, C., Bassi, M. T., Cervia, D., Sandri, M., Clementi, E., & De Palma, C. (2019). Autophagy controls neonatal myogenesis by regulating the GH-IGF1 system through a NFE2L2- and DDIT3-mediated mechanism. *Autophagy*, *15*(1), 58–77. https://doi.org/10.1080/15548627.2018.1507439

Zeidan, Y. H., & Hannun, Y. A. (2010). The Acid Sphingomyelinase/Ceramide Pathway: Biomedical Significance and Mechanisms of Regulation. In *Current Molecular Medicine* (Vol. 10).

Zhang, D. X., Zou, A. ping, & Li, P. lan. (2003). Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *American Journal of Physiology - Heart and Circulatory Physiology*, *284*(2 53-2). https://doi.org/10.1152/ajpheart.00697.2002

Zhang, W., Cheng, L., Hou, Y., Si, M., Zhao, Y. P., & Nie, L. (2014). Plumbagin Protects Against Spinal Cord Injury-induced Oxidative Stress and Inflammation in Wistar Rats through Nrf-2 Upregulation. *Drug Research*, *65*(9), 495–499. https://doi.org/10.1055/s-0034-1389950

Zhang, Y., Li, H., Min, Y.-L., Sanchez-Ortiz, E., Huang, J., Mireault, A. A., Shelton, J. M., Kim, J., Mammen, P. A., Bassel-Duby, R., & Olson, E. N. (2020). Enhanced CRISPR-Cas9 correction of Duchenne muscular dystrophy in mice by a self-complementary AAV delivery system. In *Sci. Adv* (Vol. 6). http://advances.sciencemag.org/

Zhang, Y., Li, X., Carpinteiro, A., & Gulbins, E. (2008). Acid Sphingomyelinase Amplifies Redox Signaling in Pseudomonas aeruginosa -Induced Macrophage Apoptosis . *The Journal of Immunology*, *181*(6), 4247–4254. https://doi.org/10.4049/jimmunol.181.6.4247

Zhang, Y., & Yao, B. (1997). Kinase Suppressor of Ras Is Ceramide-Activated Protein Kinase. In *Cell* (Vol. 89).

Zhang, Z., Deng, W., Kang, R., Xie, M., Billiar, T., Wang, H., Cao, L., & Tang, D. (2016). Plumbagin protects mice from lethal sepsis by modulating immunometabolism upstream of PKM2. *Molecular Medicine*, *22*, 162–172. https://doi.org/10.2119/molmed.2015.00250

Zhao, P., & Hoffman, E. P. (2004). Embryonic Myogenesis Pathways in Muscle Regeneration. *Developmental Dynamics*, *229*(2), 380–392. https://doi.org/10.1002/dvdy.10457

Zheng, L., Shi, J., & Mu, Y. (2018). Dynamics changes of CRISPR-Cas9 systems induced by high fidelity mutations. *Physical Chemistry Chemical Physics*, *20*(43), 27439–27448. https://doi.org/10.1039/c8cp04226h

Zhu, B. G., Sun, Y., Sun, Z. Q., Yang, G., Zhou, C. H., & Zhu, R. S. (2012). Optimal Dosages of Fluoxetine in the Treatment of Hypoxic Brain Injury Induced by 3-Nitropropionic Acid: Implications for the Adjunctive Treatment of Patients after Acute Ischemic Stroke. *CNS Neuroscience and Therapeutics*, *18*(7), 530–535. https://doi.org/10.1111/j.1755-5949.2012.00315.x