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PERSISTENT INHIBITION OF MITOCHONDRIAL BIOGENESIS IN DYSTROPHIC MICE: IDENTIFICATION OF NITRIC OXIDE-DEPENDENT SALVAGE PATHWAY

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SUMMARY

Muscular dystrophies are a group of genetic diseases showing muscle degeneration characterized by progressive skeletal muscle weakness, defects in muscle proteins fiber necrosis, and progressive substitution of fibers with connective and adipose tissue.

The therapeutic protocols currently in use, based on corticosteroid administration, provide some delay in the progression of the disease, but they are associated with severe side effects. The genetic approaches (exon skipping and antisense oligonucleotides) currently being investigated show some degree of success, however they are directed to specific subsets of population and cannot restore fully the damage already caused by the disease to the muscle.

Several studies had demonstrate that the pathophysiology of muscular dystrophies correlates with an altered synthesis of nitric oxide (NO), in fact neuronal nitric oxide synthase (nNOS) is absent from the sarcolemma and relocated to the cytosol, with total muscle NOS activity being thus reduced.

During the years, nitric oxide donors were identified as good candidate molecules for Duchenne Muscular dystrophy therapy and recently, our group found that a NO donor, molsidomine, is able to slow disease progression and to restore the functional capacity of damaged muscle, significantly enhancing spontaneous and forced motor activities[1]. Nitric oxide regulates some mitochondrial functions, such as morphology and complexes activity; moreover mitochondrial dysfunction has long been suspected to be an important pathogenetic feature in muscular dystrophies even if their role is not fully understood.

The aim of this project is to analyze the mitochondrial profile of alpha-Sarcoglican (a-SG) null mice, a mouse model for Limb Girdle muscular dystrophy 2D (LGMD 2D) and to evaluate a possible effect of molsidomine on mitochondrial function. Our long term aim is to define approaches that limit muscle wasting, with a dual finality, on the one hand to ameliorate the dystrophic symptoms per se, on the other to increase the efficiency of cell/gene therapies, in a combined therapy for the disease. To this end elucidation of novel possible targets is necessary and this is the final goal of this project.

We find out a severe reduction in mitochondrial content in both *tibialis anterior* and diaphragm accounting for a lower OxPhos capacity of these muscles. The respiratory rates relative to mitochondrial DNA suggest that mitochondrial content is the major

determinant of the lower oxidative capacity of a-SG null muscles. The low mitochondrial content in dystophyc mice is due to a persistent inhibition of the mitochondrial biogenesis pathway. Unexpectedly, the treatment with the NO-donor molsidomine is not able to restore mitochondrial content in a-SG$^{-/-}$ mice, but it is able to improve significantly their oxidative capacity, triggering a therapeutic fiber switch and stimulating fatty acid oxidation rather than improving mitochondrial function per se. Molsidomine promotes in fact an important deacetylation and activation of peroxisome proliferator-activated receptor γ coactivator 1-gene α (PGC-1α), the principal trascriptional co-activator involved in muscle fiber type determination. Deacetylation of PGC-1α occurs through a nitric oxide-dependent AMP activated protein kinase (APMK) activation leading to an increase expression and activity of the deacetylase Sirt1.

Altogether these results highlight for the first time a defective mitochondrial biogenesis in LGMD 2D impairing mitochondrial metabolism and define the increase in OxPhos capacity associated with fiber switch as rescue mechanism with a mechanism independent on mitochondrial biogenesis but focused on lipid metabolism.

INTRODUCTION

Chapter 1: Muscular dystrophies

Muscular dystrophies are a clinically, genetically, and biochemically heterogeneous group of genetic disorders affecting primarily the skeletal muscle [2]. They are characterized by progressive muscle weakness that affects limb, axial, and facial muscles with different severity. In some specific forms, also other muscles can be affected such as respiratory muscles, cardiac smooth muscles, and swallowing muscles. In rare variants, the disorder is associated with the damage of other organs or tissues, including brain, eyes, inner ear and skin. There is a high degree of variability between different forms of muscular dystrophy, in terms of severity, age of onset, rate of progression and prognosis.

In the past two decades a better knowledge of the mechanisms underlying muscular dystrophies, the important improvements in standards of care and new therapeutic approaches have changed both the natural history and long-term perspectives of these disorders. The progression of genetic analyses has also resulted in an unexpected expansion of the clinical range of mutations associated with the disease, including allelic disorders that share no features with the first muscular dystrophy described [3-4]. The availability of clinical guidelines based on expert consensus ameliorated the standards of cares, the prevention and the management of complications, and definitely the clinical course and the survival of patients. Finally, better knowledge about the molecular basis of these disorders led to the development of new treatment approaches, several of which are already in clinical trials [5].

The most common and extensively studied muscular dystrophies are those involving mutations of the dystrophin-dystroglycan-laminin network [6], (Table 1). The organization of the dystrophin complex at the sarcolemmal level of muscle fibers is shown in Figure 1 and demonstrates the pivotal role of dystrophin for muscle stability, since it provides a mechanical link between the internal cytoskeleton and the extracellular matrix. The dystrophin-associated proteins include dystroglycan, sarcoglycan, and cytoplasmic (syntrophin) subcomplexes. The dystroglycan complex is formed by of α-dystroglycan, which associates with the basal-lamina protein merosin in the extracellular matrix, and β-dystroglycan, a transsarcolemmal protein that binds α-dystroglycan and dystrophin. The

SG complex includes four trans-sarcolemmal proteins (α-, β-, γ and δ-SG) that associate each other and function as a single unit, with δ-SG serving as the link to β-dystroglycan of the dystroglycan complex. Within the cytoplasmic subcomplex, the COOH-terminal region of dystrophin is bound to syntrophin, whereas the amino-terminus of dystrophin binds F-actin.

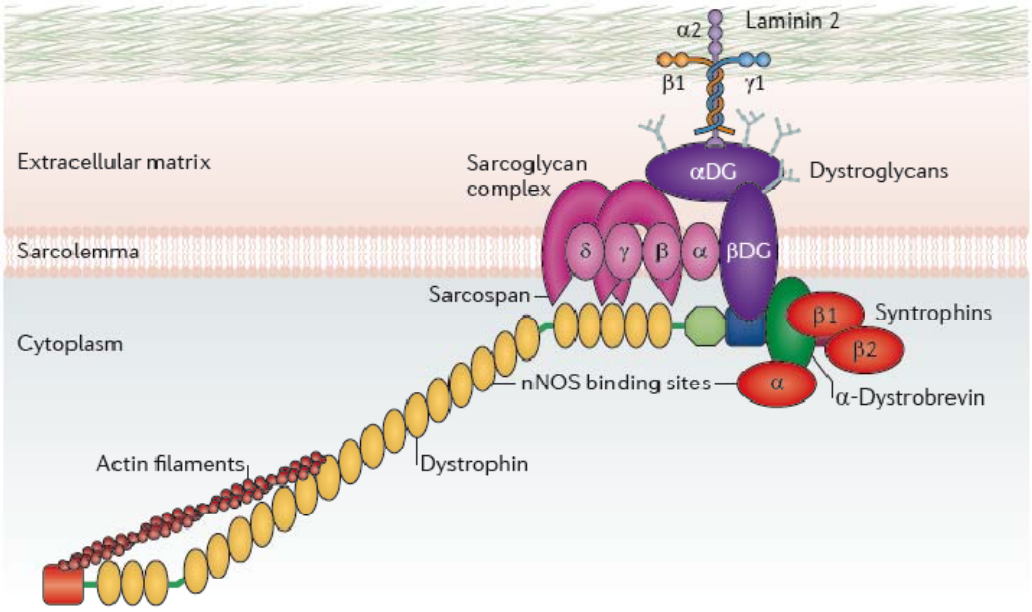

Figure 1: The dystrophin-associated protein complex

Mutations of dystrophin gene are the most common cause of muscular dystrophy accounting for Duchenne and Becker muscular dystrophies, both X-linked, recessively inherited disorders. In terms of incidence, together with Duchenne Muscular Dystrophy (DMD), particularly relevant is also the Limb girdle Muscular Dystrophy 2D (LGMD-2D) characterized by the lack of alpha sarcoglycan (a-SG) in the dystrophin-sarcoglycan complex which also leads to muscle degeneration. These two forms share some similarities in how skeletal muscle undergoes degeneration.

Skeletal muscle of dystrophic patients is more susceptible to eccentric contraction-induced injury than the healthy one. In response to this type of injury, damaged muscle fibers undergo repeated cycles of necrosis and regeneration, during which satellite cells, the primary myogenic precursor cells, become activated to regenerate muscle fibers. However, the regenerative process is largely inefficient and results in fibrotic changes and

fatty deposition in skeletal muscle and the most severely affected skeletal muscle is the diaphragm.

At present there is no satisfactory therapy for this pathology, apart for the treatment with corticosteroids that, however, provides only temporary improvements and is associated with severe side effects. The genetic approaches (exon skipping and antisense oligonucleotides) currently being investigated show some degree of success, although they are directed to specific subsets of population and cannot restore fully the damage already caused by the disease to the muscle.

Gene/Protein Product	Cellular/Localization Function	Disease	Ref.
Ememin	Nuclear envelope	X-linked recessive Emery-Dreifus muscular dystrophy	8
Lamin A/C	Nuclear envelope	Autosomal dominant Emery-Dreifus muscular dystrophy	8
Poly(A) binding protein 2	mRNA metabolism	Oculopharyngeal muscular dystrophy	87
Expanded trinucleotide repeats in 3′ UTR of cAMP-dependent protein kinase	mRNA metabolism	Congential myotonic dystrophy	55
Expanded trinucleotide repeats in 3′ UTR of cAMP-dependent protein kinase	mRNA metabolism	Myotonic dystrophy	55
Dysferlin	Myofibril plasma membrane maintenance	Miyoshi/LGMD-2B	59
Calpain III	Myofibril plasma membrane maintenance	LGMD-2A	81
Caveolin	Myofibril plasma membrane maintenance	LGMD-1C	65
α-2 Laminin	Membrane cytoskeleton structural support	Congenital muscular dystrophy	31, 74
Dystrophin	Membrane cytoskeleton structural support	Duchenne muscular dystrophy, Becker muscular dystrophy	46, 48, 54
Sarcoglycan complex	Membrane cytoskeleton structural support	LGMD-2C, -2D, -2E, -2F	4

LGMD, limb-girdle muscular dystrophy.

Table 1: Genes associate with selected muscular dystrophies [6]

1.1 Classification and epidemiology

For many years, muscular dystrophies have been classified in accordance to the main clinical manifestations and age of onset (limb girdle muscular dystrophies, Emery-Dreifuss muscular dystrophy, and congenital muscular dystrophies). Furthermore, the heterogeneous groups such as limb girdle muscular dystrophies or congenital muscular dystrophies were sub-classified according to their inheritance and the genetic defect responsible for the individual forms (*LGMD1A*, *LGMD1B*, *LGMD2A*, and *LGMD2B*), where the number 1 indicated the dominant inheritance whereas the number 2 the

recessively inherited disorders. The letters A, B, and C indentified the different genes involved. The improved understanding of the mechanisms underlying these forms provided new clues about their classification that cannot be based only on the previous assumption that every clinical phenotype is related to a distinct genetic defect. The old classification, based on the clinical findings, is now always accompanied by information about the primary protein defect and their localization and function as shown in Table 2 [5].

	Inheritance	OMIM number	Locus	Genesymbol	Protein	Main localisation
Duchenne or Becker muscular dystrophy	X-R	310200 (Duchenne); 300376 (Becker)	Xq21·2	*DMD*	Dystrophin	Sarcolemma-associated protein
Limb girdle muscular dystrophy						
Type 1A	AD	159000	5q31	*MYOT*	Myotilin	Sarcomere-associated protein (Z disc)
Type 1B	AD	159001	1q21·2	*LMNA*	Lamin A/C	Nuclear lamina-associated protein
Type 1C	AD	607780	3p25	*CAV3*	Caveolin-3	Sarcolemma-associated protein
Type 1D	AD	603511	7q	*DNAJB6*	Co-chaperone DNAJB6	Sarcomere-associated protein (Z disc)
Type 1E	AD	602067	6q23	*DES*	Desmin	Intermediate filament protein
Type 1F	AD	608423	7q32	Unknown	Unknown	Unknown
Type 1G	AD	609115	4p21	Unknown	Unknown	Unknown
Type 1H	AD	613530	3p23–p25	Unknown	Unknown	Unknown
Type 2A	AR	253600	15q15·1	*CAPN3*	Calpain-3	Myofibril-associated proteins
Type 2B	AR	253601	2p13	*DYSF*	Dysferlin	Sarcolemma-associated protein
Type 2C	AR	253700	13q12	*SGCG*	γ-sarcoglycan	Sarcolemma-associated protein
Type 2D	AR	608099	17q12–q21·33	*SGCA*	α-sarcoglycan	Sarcolemma-associated protein
Type 2E	AR	604286	4q12	*SGCB*	β-sarcoglycan	Sarcolemma-associated protein
Type 2F	AR	601287	5q33	*SGCD*	δ-sarcoglycan	Sarcolemma-associated protein
Type 2G	AR	601954	17q12	*TCAP*	Titin cap (telethonin)	Sarcomere-associated protein (Z disc)
Type 2H	AR	254110	9q31–q34	*TRIM32*	Tripartite motif-containing 32 (ubiquitin ligase)	Sarcomeric-associated protein (Z disc)
Type 2I	AR	607155	19q13·3	*FKRP*	Fukutin-related protein	Putative glycosyltransferase enzymes
Type 2J	AR	608807	2q31	*TTN*	Titin	Sarcomeric protein
Type 2K	AR	609308	9q34	*POMT1*	Protein-1-O-mannosyl-transferase 1	Glycosyltransferase enzymes
Type 2L	AR	611307	11p14·3	*ANO5*	Anoctamin 5	Transmembrane protein, possible sarcoplasmic reticulum
Type 2M	AR	611588	9q31	*FKTN*	Fukutin	Putative glycosyltransferase enzymes
Type 2N	AR	613158	14q24	*POMT2*	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes
Type 2O	AR	613157	1p34	*POMGNT1*	Protein-O-linked mannose β 1,2-N-aminyltransferase 1	Glycosyltransferase enzymes
Type 2P	AR	613818	3p21	*DAG1*	Dystrophin-associated glycoprotein 1	Sarcomeric-associated protein
Type 2Q	AR	613723	8q24	*PLEC1*	Plectin 1	Sarcolemma-associated protein (Z disc)
Facioscapulohumeral muscular dystrophy						
Type 1	AD	158900	4q35	Unknown	DUX4 and chromatin rearrangement	Nuclear
Type 2	AD	158901	18	Unknown	SMCHD1	Structural maintenance of chromosomes flexible hinge domain containing 1
Emery Dreifuss muscular dystrophy						
X-linked type 1	X-R	310300	Xq28	*EMD*	Emerin	Nuclear membrane protein
X-linked type 2	X-R	300696	Xq27·2	*FHL1*	Four and a half LIM domain 1	Sarcomere and sarcolemma
Autosomal dominant	AD	2181350	1q21·2	*LMNA*	Lamin A/C	Nuclear membrane protein
Autosomal recessive	AR	604929	1q21·2	*LMNA*	Lamin A/C	Nuclear membrane protein
With nesprin-1 defect	AD	612998	6q25	*SYNE1*	Spectrin repeat containing, nuclear envelope 1 (nesprin-1)	Nuclear membrane protein
With nesprin-2 defect	AD	5612999	4q23	*SYNE2*	Spectrin repeat containing, nuclear envelope 2 (nesprin-2)	Nuclear membrane protein
Congenital muscular dystrophy with merosin deficiency (MDC1A)	AR	607855	6q2	*LAMA2*	Laminin α2 chain of merosin	Extracellular matrix proteins
Congenital muscular dystrophy	AR	604801	1q42	Unknown	Unknown	Unknown
Congenital muscular dystrophy and abnormal glycosylation of dystroglycan (MDC1C)	AR	606612	19q13	*FKRP*	Fukutin-related protein	Putative glycosyltransferase enzymes
Congenital muscular dystrophy and abnormal glycosylation of dystroglycan (MDC1D)	AR	608840	22q12	*LARGE*	Like-glycosyl transferase	Putative glycosyltransferase enzymes
Fukuyama congenital muscular dystrophy	AR	253800	9q31–q33	*FCMD*	Fukutin	Putative glycosyltransferase enzymes

(Continues on next page)

	Inheritance	OMIM number	Locus	Gene symbol	Protein	Main localisation
(Continued from previous page)						
Walker-Warburg syndrome						
With fukutin defect	AR	236670	9q31–q33	*FCMD*	Fukutin	Putative glycosyltransferase enzymes
With protein-O-mannosyl-transferase 1 defect	AR	236670	9q34	*POMT1*	Protein-1-O-mannosyl-transferase 1	Glycosyltransferase enzymes
With protein-O-mannosyl-transferase 2 defect	AR	236670	14q24	*POMT2*	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes
With protein-O-linked mannose β1,2-N-aminyltransferase 1 defect	AR	236670	1p34	*POMGNT1*	Protein-O-linked mannose β1,2-N-aminyltransferase 1	Glycosyltransferase enzymes
With fukutin related protein defect	AR	236670	19q13	*FKRP*	Fukutin related protein	Putative glycosyltransferase enzymes
Muscle-eye-brain disease						
With protein-O-linked mannose β1,2-N-aminyltransferase 1 defect	AR	253280	1p34	*POMGNT1*	Protein-O-linked mannose β1,2-N-aminyltransferase 1	Glycosyltransferase enzymes
With fukutin-related protein defect	AR	253280	19q13	*FKRP*	Fukutin-related protein	Putative glycosyltransferase enzymes
With protein-O-mannosyl-transferase 2 defect	AR	253280	14q24	*POMT2*	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes
Congenital muscular dystrophy due to glycosylation disorder	AR	NA	9q34.1	*DPM2*	Dolichyl-phosphate mannosyltransferase polypeptide 2	Glycosyltransferase enzymes
Congenital muscular dystrophy due to glycosylation disorder	AR	NA	1q21-3	*DPM3*	Dolichyl-phosphate mannosyltransferase polypeptide 3	Glycosyltransferase enzymes
Congenital muscular dystrophy with mitochondrial structural abnormalities	mtDNA	602541	22q13	*CHKB*	Choline kinase	Sarcolemmal and mitochondrial membrane
Congenital muscular dystrophy with rigid spine syndrome	AR	602771	1p36	*SEPN1*	Selenoprotein N1	Endoplasmic reticulum protein
Ullrich syndrome						
With collagen type VI subunit α1 defect	AR	254090	21q22-3	*COL6A1*	Collagen type VI, subunit α1	Extracellular matrix proteins
With collagen type VI subunit α2 defect	AR	254090	21q22-3	*COL6A2*	Collagen type VI, subunit α2	Extracellular matrix proteins
With collagen type VI subunit α3 defect	AR	254090	2q37	*COL6A3*	Collagen type VI, subunit α3	Extracellular matrix proteins
Congenital muscular dystrophy with integrin α7 defect	AR	613204	12q13	*ITGA7*	Integrin α7	External sarcolemmal protein
Congenital muscular dystrophy with integrin α9 defect	AR	NA	3p21-3	*ITGA9*	Integrin α9	External sarcolemmal protein
Muscular dystrophy with generalised lipodystrophy	AR	NA	17q21–q23	*PTRF*	Polymerase I and transcript release factor (cavin-1)	Ttubules and sarcolemma
Oculopharyngeal muscular dystrophy	AD or AR	164300	14q11-2	PABPN1	Polyadenylate binding protein nuclear 1	Unknown

X-R=X-linked recessive. OMIM=Online Mendelian Inheritance in Man. AD=autosomal dominant. AR=autosomal recessive. NA=not assigned.

Table 1: Classification of muscular dystrophies

Table 2: Classification of muscular dystrophies [5]

Duchenne muscular dystrophy is the most common inherited muscle disease of childhood, with an estimated prevalence in northern England of 8.29 per 100 000 boys; a milder allelic variant, Becker muscular dystrophy, has a slightly lower prevalence of 7.29 per 100 000 boys. Myotonic dystrophy is the most common form in adults, with an estimated prevalence of 10.6 per 100 000 men, followed by facioscapulohumeral muscular dystrophy (FSH), with an estimated prevalence of three per 100 000 men [7]. Between limb girdle muscular dystrophies, the recessive forms are more common than the dominant ones and limb girdle muscular dystrophy 2A seems to be more prevalent in southern Europe, whereas limb girdle muscular dystrophy 2I is more diffuse in northern Europe, followed by limb girdle muscular dystrophy 2B [8-9].

Geographic regions seem to play an important role in terms of frequencies also in some particular forms of congenital muscular dystrophy such as Fukuyama congenital muscular dystrophy, that is the most common form of congenital muscular dystrophy in Japan and is caused by a founder recessive mutation [10].

1.2 Clinical manifestations

The molecular heterogeneity of muscular dystrophies is accompanied by great variability in terms of clinical manifestation and age of onset that can change from birth or childhood to adulthood.

In general, congenital muscular dystrophies have evident clinical signs at birth or in the first few months of life, however other dystophies, such as Duchenne muscular dystrophy or some forms of the limb girdle muscular dystrophies, show clinical sign during early or late childhood when independent ambulation has been achieved. Finally, other limb girdle muscular myotonic dystrophy and facioscapulohumeral muscular dystrophies do not appear until adulthood. The common feature of these different forms of pathologies is definitely the marked skeletal muscle weakness with a different distribution that can be a useful tool to distinguish between different variants. (Figure 2) [2].

In several forms, the peculiar distribution of weakness allows the disorder to be identified rapidly, as is the case of FSH and oculopharyngeal muscular dystrophy. Moreover often muscle weakness is associated with either muscle atrophy or relative hypertrophy, as seen in Duchenne muscular dystrophy, Becker muscular dystrophy, and several limb girdle muscular dystrophies. Myotonic dystrophy is unique because it is associated with stiffness of various muscles (and difficulties in relaxation of grip), a phenomenon known as myotonia. Progressive rigidity of the elbow, Achilles tendon, and spine almost invariably occurs in Emery-Dreifuss muscular dystrophy and in Ullrich congenital muscular dystrophy, but is less common in limb girdle muscular dystrophy 2A and is rare in other variants [5].

In general the progression of the disease is very variable and is mainly controlled by the severity of the individual mutation affecting each gene. In most patients affected by congenital muscular dystrophy variants, ambulation is never achieved; in DMD and LGMD ambulation is achieved but it will be lost with the progression of the diseases, indeed children become progressively weaker by the end of the first decade, and they lose the ambulation about in the early or middle teenage years and they are oblige to use whellchair. In the other forms of limb girdle muscular dystrophies and in most cases of facioscapulohumeral muscular dystrophy, ambulation can be maintained and wheelchair assistance is needed only later in life. Clinical severity of myotonic dystrophy is extremely variable, ranging from fatal outcome to minimally affected adults with only

cataracts and grip myotonia. Respiratory impairment is frequent and its severity is not always related to the degree of motor impairment. In most muscular dystrophies in which this complication occurs, respiratory insufficiency happens only after loss of ambulation as a result of generalized weakness of inspiratory and expiratory muscles. In other forms, this manifestation is the result of selective diaphragmatic weakness.

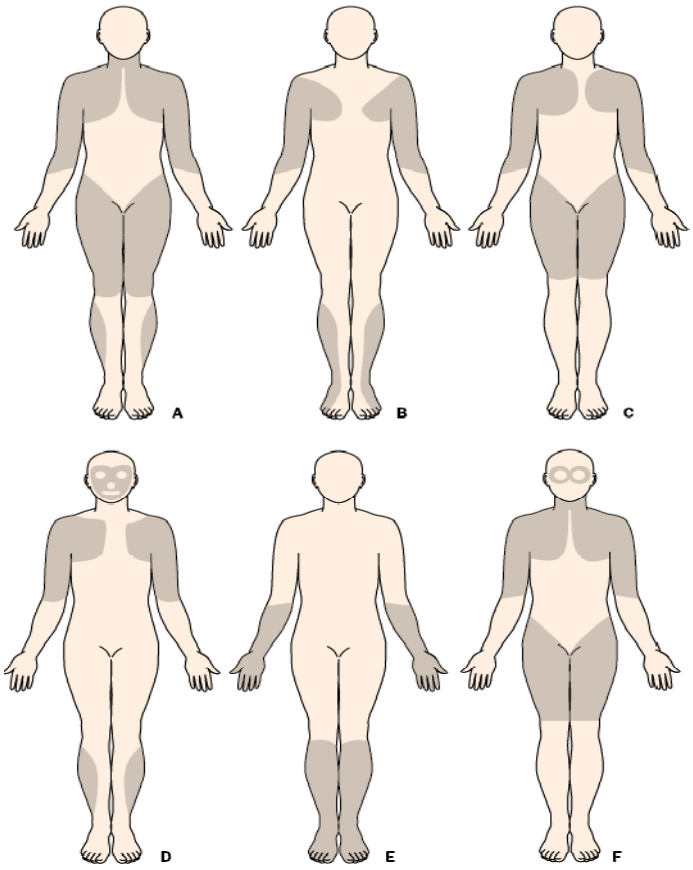

Figure 2: Distribution of predominant muscle weakness in different types of dystrophy.
A, Duchenne-type and Becker-type; B, Emery-Dreifuss; C, limb-girdle; D, facioscapulohumeral; E, distal, F, oculopharyngeal. [2].

Respiratory insufficiency typically starts at night, resulting in disturbed sleep, morning drowsiness and headaches, loss of appetite, and frequent chest infections.

Cardiac involvement is common in many muscular dystrophies, but is not a consistent finding [11-12]. Age of onset, progression, and type of cardiac involvement are variable. Although in DMD and some LGMD dilated cardiomyopathy is the main presenting cardiac concern, in others, such as Emery-Dreifuss muscular dystrophy, conduction defects are a severe and consistent feature [13].

In some congenital muscular dystrophies and rarely in some LGMD, functional or structural brain damage occurs. Example of functional deficiency are present in DMD and in myotonic dystrophy [14], in particular in Duchenne muscular dystrophy, a third of boys have non-progressive mental retardation and behavioral or psychiatric comorbidities (eg, attention deficit disorder or autism) [15]. Structural brain defects have also been recorded in the advanced stages of DMD and of myotonic dystrophy.

1.3 Limb–Girdle Muscular Dystrophies

Limb-girdle muscular dystrophy describes a heterogeneous group of muscle disorders characterized by the predominantly proximal distribution of limb–girdle weakness. For decades, the LGMD diagnosis was an exclusionary one: when Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy, FSH, myotonic dystrophy, metabolic myopathies, and other syndromic disorders were ruled out, the patient was assigned an LGMD diagnosis. Their current classification based on inheritance pattern, comes from the discovery of genetically distinct subtypes of LGMD and the most common forms are the autosomal recessive disorders, called LGMD2 (2A–2I), whereas less diffuse are the autosomal dominant forms called LGMD1 with subtypes (1A-1E) [16].

The autosomal-recessive LGMDs are a heterogeneous group of muscular diseases characterized by progressive muscle weakness and, in severe cases, death in the second or third decade of life [17]. The more severe phenotypes are often caused by mutations in the SG genes α (LGMD-2D), β (LGMD-2E), γ (LGMD-2F), and δ (LGMD-2C). The sarcoglycan complex stabilizes the association between dystrophin, dystroglycan and the extracellular matrix maintaining the mechanical stability of the plasma membrane during muscle contraction. Sarcoglycanopathies mimic dystrophinopathies, often with an early onset and with the development of muscle hypertrophy, although cognitive deficit is rare.

A primary deficiency of any single SG leads to partial or complete absence of all other sarcoglycans on the sarcolemma, suggesting that sarcoglycans are mutually dependent on each other [18]. Phenotypically, the SG deficiencies are indistinguishable from the dystrophin deficiencies [19], but if dystrophinopathies show secondary deficiencies of all SG proteins, sarcoglycanopathies usually show normal levels of dystrophin.

Accordingly, LGMDs represent an important group of muscular dystrophies, widely used to investigate the dystrophic muscular damage and for this reason different mouse models with mutations in sarcoglycans genes have been generated. In particular α-SG-deficient null mutant transgenic mice have been generated targeting the disruption of the α-SG gene [20]. These mice show the complete absence of α-SG transcript and protein, a complete loss of the SG complex and sarcospan, and develop a progressive muscular dystrophy similar to its human homologue, LGMD-2D.

In contrast to *mdx* mouse, the murine model of DMD, a-SG null mice show persistent muscle necrosis progressive with age, which is an important hallmark of the human disease. Moreover these mice demonstrate significant muscle hypertrophy and muscle-specific changes in contractile properties. Taken together this mouse model is suitable for elucidating the pathogenesis of limb-girdle muscular dystrophies and for the development of therapeutic strategies for this disease.

Chapter 2: Therapeutic approaches to muscular dystrophy

2.1 Current treatments

The lack or alteration of structural proteins, such as dystophyn or sarcoglycan proteins, into the musculoskeletal system results in a chronic influx of calcium into the myofibers, causing cellular death and inflammatory responses. For this reason corticosteroids have been proposed as a pharmacological therapy for dystrophinopathies, in order to counteract muscle necrosis, inflammation and to reduce the muscle membrane susceptibility to damage. At the moment, although there are no specific cures for the different forms of muscular dystrophy, corticosteroid administration represents the therapeutic protocol currently in use for DMD and other types of muscular dystrophy improving quality of life and extending lifespan [21].

After preclinical studies, the unequivocal proof of efficacy of glucocorticoids was established in a randomized, double-blind controlled trial in DMD patients [22]. At six months prednisone treatment compared to placebo improved muscle strength and pulmonary functions, as well as the time needed to rise from supine to standing, to walk 9 m, and to climb four stairs ($p < 0.001$ for all comparisons). Some years later, similar results were documented using deflazacort, an alternative, sodium-sparing glucocorticoid that was shown to prolong ambulation ($p < 0.005$) [23]. Furthermore, a recent blind placebo-controlled study demonstrated that a high-dose administered by a weekend regimen of prednisone (10 mg/kg/wk divided over 2 days) produced fewer side effects compared to standard daily dosing while retaining the benefits that were observed with daily prednisone for DMD [24].

In general the corticosteirod treatments provide some delay in the progression of the disease, but they are associated with severe side effects, such as weight gain with a cushingoid appearance. DMD boys on steroids are also at risk of hypertension, cataract formation, loss of bone density, vertebral compression fractures, and long bone fractures. Long-term administration may be limited in some cases by steroid-induced behavioral problems frequently observed with this class of drugs [25].

Also for the large majority of LGMD patients, there are no decisive therapies and treatments remain palliative and supportive. The management of the disease involves physiotherapy to prevent joint deformities and promote walking, further passive

stretching should be instituted early, soon after diagnosis both in LGMD and DMD. With regard to lower limb surgery, which has been studied more in the DMD population, bilateral hip and knee replacement, aponeurectomy of the iliotibial band, or Achilles tendon lengthening may prolong ambulation, but these procedures remain controversial. Surgery may prolong assisted standing ability and, in turn, prevent long-term spine deformities and back pain. Considering cardiomyopathy or conduction defects often developed by patient, heart monitoring is very important. Although specific guidelines for each type of LGMD are still lacking, repeat electrocardiography (ECG)/Holter monitoring and echocardiography every 2 to 5 years are recommened, even for asymptomatic patients. Angiotensin-converting enzyme inhibitors and β-blockers, well described to have a beneficial effect on DMD, are recommended in the presence of left ventricular dysfunction [16].

Since inflammation is an early and important pathophysiological event, the pharmacologic approach with steroids has been reported to be beneficial also in some types of LGMD, including LGMD2I, LGMD 2L and LGMD 2D, as already demonstrated for DMD [26-28].

At present, there is no effective therapy to stop the lethal progression of these diseases, although several promising experimental strategies are currently under investigation. These include gene therapy aimed at reintroducing a functional recombinant version of the dystrophin gene using adeno-associated, lentiviral or adenoviral vectors, as well as modification of the dystrophin pre-mRNA, commonly referred as exon skipping. Both strategies held great promises with several clinical trials ongoing, but cell-based therapies and pharmacological approaches such as the up-regulation of utrophin or read-through strategies for nonsense mutations have also been investigated as summarized in figure 3.

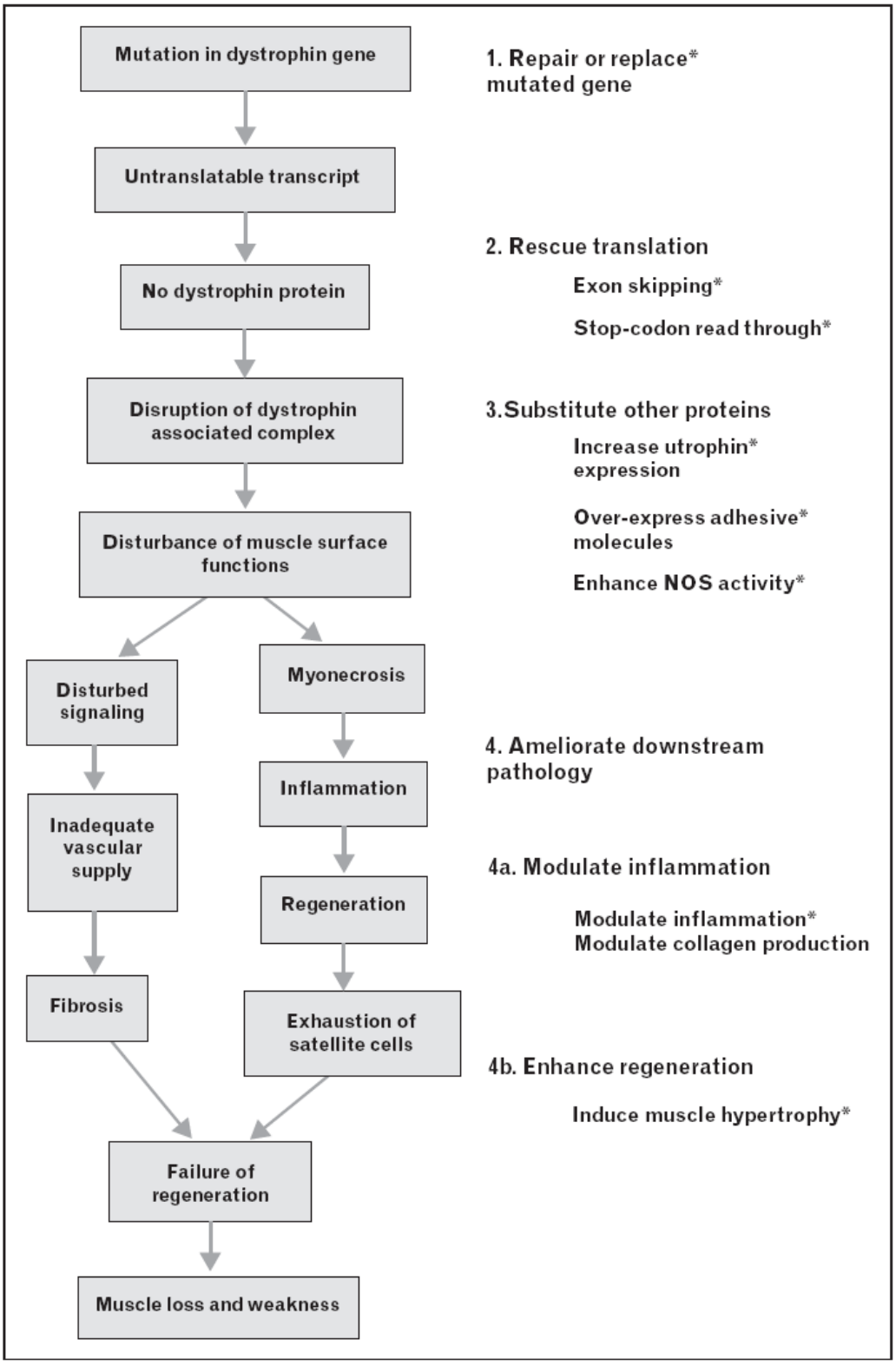

Figure 3: The main stages in the pathological cascade arising from a null-mutation of the dystrophin gene, to the left, together with the potential therapeutic actions to combat each of these stages, to the right [29].

2.2 Gene therapy for DMD and LGMD

The muscular dystrophies, including DMD and LGMD, caused by single gene mutation are attractive candidates for gene therapy.

Since the discovery of the molecular basis of DMD in the 1980s, the introduction of a functional dystrophyn gene into the muscle cells has been deeply investigated, but numerous challenges delay the clinical implementation of this approach beyond initial expectations.

DMD arises from null mutations in the *dmd* gene that result in the near complete absence of dystrophin protein, or in rare cases from mutations that lead to production of a non-functional dystrophin, such as ones lacking critical domains near the carboxy-terminus of the protein. The allelic form, Becker muscular dystrophy (BMD), also arises from mutations in the *dmd* gene, however these mutations produce reduced amounts or truncated forms of dystrophin [30]

The size of the gene is a very big challenge since dystrophin is a 427 kDa cytoskeletal protein that is translated by the largest gene in the human genome (>2.4 MBP) with an mRNA of 14 kilobases (kb) (11 kb coding region of dystrophin from start codon to stop codon). Moreover, the muscular tissue makes up greater than 40% of the body mass, and a gene therapy approach for DMD requires the delivery of a new dystrophin gene to all muscles of the body, including the diaphragm and the heart. Since the disease requires the treatment of such a large mass of tissue, it is believed that the optimal strategy of delivery may be *via* the vasculature. Nevertheless, it is important to underlie that the therapeutic effect depends on the amount of protein delivered and the state of the disease at the time of treatment. In fact, in older patients, who show profound muscle lost as well as marked fibrotic changes and fatty deposition, dystrophin delivery to muscle cells might have a limited therapeutic efficacy [31]. However, not all the systems for gene delivery are suitable for systemic use, due to different abilities to cross blood and tissue barriers together with immunogenic problems. [32]. To overcome this problem, several groups are now investigating the potential of delivering utrophin, a dystrophin homologue, which is expected to be less immunogenic [33-34].

The most promising viral vectors under investigation for gene therapy of DMD are adeno-associated virus (AAV) given its known persistence in healthy muscle and absence of pathogenicity. However, its cloning capacity of ≤ 5 kb is a potentially limiting obstacle

for successful DMD gene transfer. Therefore scientists paid attention to develop and characterize internally deleted, mini or micro-dystrophin constructs. Dystrophin is composed of 4 major structural domains: an N-terminal actin-binding domain; a central rod domain (a portion of which also binds actin) composed of 24 spectrin-like repeats and 4 hinge domains (the fourth carries a WW domain important for binding to part of the DGC); a cysteine-rich domain; followed by a distal C-terminal domain that interacts with members of the DGC at the sarcolemmal level (Figure 4A) [35]. The idea that functional mini dystrophin constructs may be useful came from the finding that BMD patients with very mild dystrophy can carry large deletions in the *dmd* gene [36]. Consequently, mini-dystrophin and highly miniaturized micro-dystrophin constructs are now being tested using both viral and non-viral models of delivery to determine their potential use as therapeutic proteins (Figure 4 B).

A phase I gene therapy trial using AAV delivery of a mini-dystrophin gene supported the safety and tolerability of intramuscular delivery; however, dystrophin expression was limited [25,37]. Since this first gene therapy trial in DMD, advances have been made in vectors design, muscle specific promoters, and mini-dystrophin constructs. [38].

Others strategies of gene replacement are those that involve non-integrating gene delivery tools for a non viral gene therapy. Thus, one of the many advantages of this system over conventional viral vectors is to avoid any risk of immune response owing to viral capsids or other viral proteins and insertional mutagenesis [39]. Among the non-integrating vectors, the most commonly used are plasmids, human artificial chromosomes (HACs) and transposons. Interestingly, almost all of them have a large cloning capacity, but there are still problems indeed plasmids are less efficient than the systemic injection of AAV vectors and can only be used for certain muscles [40].

All the strategies mentioned above propose the replacement of the mutated gene with a new functional one; others promising approaches are related to the possibility to repair, instead of replace, the genetic defect in a mutation specific manner.

Exon skipping for example is an alternative gene therapy approach for DMD, aimed at repairing the patient's own gene, targeting the defect at the pre-mRNA level allowing one or more exons to be omitted to restore the dystrophin reading frame. This strategy is possible because approximately 70% of mutations responsible for DMD lead to a disrupted reading frame, resulting in a truncated non functional dystrophin protein [41-42].

This result is obtained with different oligonucleotides, typically 20–30 nucleotides in length and complementary to regions of the pre-mRNA transcript. The antisense oligonucleotides (AON) target specific exons, hiding them from the splicing machinery and causing their skipping during the splicing process. Exon skipping is the only approach to show efficacy in a large animal (dog) model [43] and it has been considered the most promising strategy for DMD.

Pre-clinical efficacy has been demonstrated in the *mdx*, dystrophin/utrophin knock-out mouse, and the dystrophin-deficient dog using two different AON, a 2'O-methyl-ribo-oligonucleosidephosphorothioate (2'OMe) and a phosphorodiamidate morpholino (PMO) [44-45].

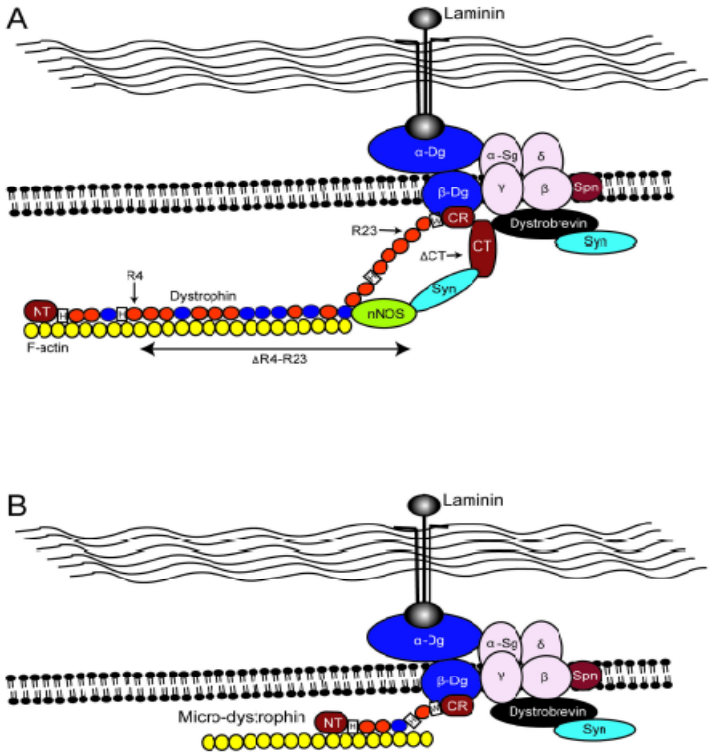

Figure 4: Micro-dystophin constructs
(**A**) Dystrophin is crucial to link the cytosolic actin skeleton of the muscle fiber to the extracellular matrix. (**B**) Micro-dystrophin constructs that lack a large portion of the rod domain (ΔR4–R23), and the CT domain are currently being developed and tested to treat DMD. These micro-dystrophins are highly functional and are capable of restoring the DGC as shown. The only differences in the DGC that associates with micro-dystrophin are a lack of nNOS binding and fewer associated syntrophins [31].

Also clinical trials have been performed with PMO and 2'OMe delivered directly to muscle and targeting exon 51 (Figure 5). Both oligomers demonstrated early evidence of efficacy [46-47], but their use was limited by the fact that they could not be used for a significant number of DMD patients, in particular those with large deletions or with mutations in regulatory or N-/C-terminal regions of dystrophin [42] were excluded.

Gene targeting and endonucleases is another important tool for gene modification in muscular dystrophy and is based on the use of endonucleases engineered to induce double-strand breaks in specific DNA sequences that subsequently will be spontaneously repaired [48].

Gene transfer in LGMD2D, is an approach that shows a more satisfactory outcome compared to DMD. First of all, the predominant mutation in LGMD2D is a missense mutation in the α-sarcoglycan gene, providing the basis for gene expression with lower immunogenicity compared to dystrophin. Moreover the full length α-SG cDNA is <2 kb and is well within the packaging capacity of AAV. In a double blind, randomized controlled study, was observed a persistent and sustainable gene expression of α-sarcoglycan following gene transfer into extensor *digitorum brevis* muscle, mediated by AAV with a muscle-specific tMCK promoter, not previously used in a clinical trial [49].

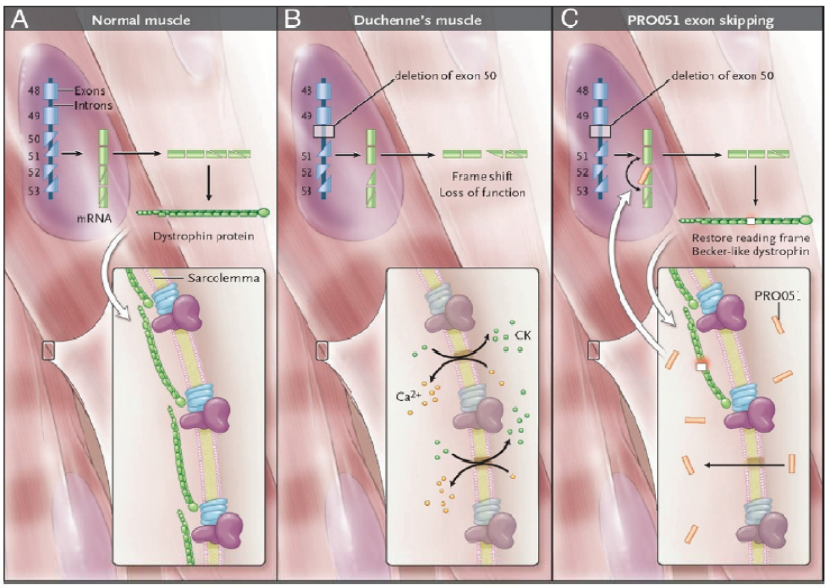

Figure 5: Mechanism of action of AO exon-skipping drugs.
A) Dystrophin gene splicing in healthy muscle, in which all 79 exons are precisely spliced together to maintain the protein translational reading frame. **B)** A patient with DMD with a deletion of exon 50. The remaining exons are spliced together, but there is a disruption of the reading frame. **C)** The mechanism of action of PRO051, an AO drug targeting exon 51. The exon 51 sequence (adjacent to the missing exon 50 sequence) is skipped, so that the mRNA splices exon 49 to 52. The new deletion is able to be translated into semifunctional Becker-like dystrophin, resulting in partial repair of the myofiber plasma membrane [50].

2.3 Cellular therapy for DMD and LGMD

In the last years, cell therapies, in which cells bearing a functional dystrophin gene are transplanted to treat muscular dystrophy, acquired increased attention. Potentially therapeutic cells can be obtained either from patients, in which cells are corrected *ex vivo* and re-implanted (autologous transfer), or from healthy donors and injected into a dystrophic patient (allogeneic transfer). Ideally, transplanted cells should be able to migrate from blood to muscle and should form myotubes further they could also increase satellite cell into the niche and self-renew process to sustain a long-lasting treatment.

Satellite cells are quiescent unipotent stem cells, located underneath the basal lamina of adult skeletal muscle fibers [51]. In response to injury, satellite cells become activated and differentiate into myoblasts, which proliferate and fuse to repair or replace the damaged fibers. A fraction of activated satellite cells returns to quiescence to maintain the pool of progenitor cells. Given their natural commitment, it has been easy to consider satellite cells as the leading candidate for muscle regeneration in dystrophic mice, in fact, experiments carried out in the late 1980s by Partridge *et al.* showed that intramuscular injections of normal myoblasts into the *mdx* mouse resulted in cell fusion and expression of dystrophin in *mdx* myofibers [52]. Unfortunately subsequent clinical trials failed to deliver significant levels of dystrophin, probably as a result of poor survival, limited migration and immune rejection of transplanted cells [53-54].

In addition to satellite cells, several other stem cell types possess the ability to differentiate into myofibers. These include total bone marrow-derived stem cells, blood- and marrow derived side population and CD133+ cells, mesenchymal cells, and mesoangioblasts. In contrast to myoblasts and satellite cells, all of these have been shown to be compatible with systemic delivery through the circulatory system [31].

Mesoangioblasts, for example, are vessel-associated stem/progenitor cells isolated from the skeletal muscle vasculature of different species and are able to differentiate into skeletal myofibres and to cross the vessel barrier, allowing them to be delivered through the bloodstream [55-56]. Intra-arterial transplantation of these cells was able to ameliorate the dystrophic phenotype of different pre-clinical models (mice and dogs) of muscular dystrophy [56-58]. Moreover mesoangioblasts modulated immune responses [59] and these results supported a phase I/II clinical trial based upon the intra-arterial allogeneic transplantation of these cells.

Also mesenchymal stem cells (MSCs) isolated from various sources have been shown to undergo some skeletal myogenesis [60-61], but work from independent groups demonstrated that, although they were able to engraft skeletal muscle, MSCs did not ameliorate the phenotype of mdx mice [62-63].

In the last few years a growing interest about the therapeutic prospective offered by pluripotent stem cells is emerging. This involve the use of embryonic stem cells (ESC) and pluripotent stem cells (iPS). Both these cell types could be an extraordinary potential clinical tool useful for the treatment of any skeletal muscle degenerations [64]. Recent work described the generation of myogenic progenitors from iPS cells that were able to play an active role during muscle regeneration in pre-clinical models for both DMD and LGMD2D [65-66].

Deriving patient-specific iPS cells and expanding their differentiated progeny provides a unique tool for gene and cell therapies, even if further safety studies and improvements in protocols might be necessary to avoid any potential risk that could hamper the translation of these promising strategies into future clinical trials.

2.4 Utrophin up-regulation

Another promising pharmacological strategy for DMD is the up regulation of utrophin levels in muscle fibers. Utrophin is an orthologue of dystrophin and its expression can compensate for the absence of dystrophin, indeed utrophin shares many of the same binding proteins as dystrophyn.

Some studies in mdx mice demonstrated that increasing utrophin levels in dystrophic muscle fibers could restore sarcolemmal expression of the dystrophin associated protein complex (DAPC) members and ameliorated the dystrophic phenotype [67-68].

Utrophin expression is predominantly driven by two promoters: the promoter A and B [69]. The former is responsible for the skeletal muscle-specific expression of utrophin, and the latter regulates its expression in endothelial cells [70]. In particular, the knowledge of the utrophin-A promoter, started the research for small molecules that could stimulate the transcription of utrophin. [71].

Utrophin-based drug therapy for DMD shows many advantages as it could be effective for all DMD patients, regardless of the specific gene defect and could be administered to

patients systemically because utrophin overexpression in tissues other than muscles does not seem to cause detrimental effect [72].

High-throughput screenings allowed the identification of small molecules able to increase utrophin. These include the use of hergulin, which acts via the N-box motif of the utrophin A promoter, and L-arginine, which results in an increase in utrophin expression as a result of increased production of nNOS [71].

Other approaches have been developed such as calpain inhibition [73] and overexpression of the small GTPase RhoA that account for an increase in utrophin expression with no change in transcription levels [74], but none of them is able to increase the protein level substantially and then achieve a complete recovery of the dystrophic phenotype in mdx mice.

Also increased expression of integrins [75-76] and ADAM12 [77] have been shown to upregulate utrophin expression and at the same time to improve the pathological phenotype.

Furthermore, agents such as biglycan that stablize the DAPC also hold therapeutic promise, recruiting utrophin protein to the muscle membrane and so reducing muscle pathology in the mdx mouse model [78].

Other approaches include the use of glucocorticoids to stabilize utrophin in muscle, due to their ability to enhance the activity of an internal ribosome entry site located within the utrophin A 5′UTR [79] and custom-designed proteins with zinc finger motifs that have been shown to act as strong transcriptional activators of the utrophin A promoter [80].

Finally Ervasti and colleagues developed a direct protein-replacement therapy for the treatment of skeletal and cardiac muscle diseases caused by loss of dystrophin, delivering utrophin protein directly [81].

2.5 Read-through strategies for suppression of nonsense mutations

Read-through strategy is a molecular approach based on treatment of patients carrying a non sense mutation with chemicals that induce read through of premature stop codons and this is suitable for approximately 10-15 % of DMD patients [82]. Small molecules that are able to introduce a conformational change in the mRNA structure can be administred, allowing the ribosomal subunit to substitute a mutation-induced stop codon with a single amino acid thus resulting in an increase read-through of the premature stop codon and production of a full length protein [83].

Pre-clinical studies, in the *mdx* mice model, demonstrated that a treatment with the aminoglycoside Gentamicin, could suppress stop codons not only *in vitro* but also *in vivo* enhancing dystrophin expression [84]. These results led to clinical trials in DMD patients with stop codons, treated weekly or twice weekly for six months using gentamicin. In a most recent study, 3 out of 12 DMD patients treated with gentamicin for 6 months revealed 13 to 15% of normal dystrophin levels, associated with reduced serum CK, stabilization of strength and a slight increase in forced vital capacity [85]. However, the remaining patients revealed no or only moderate dystrophin expression.

Another agent for stop codon mutation is the orally bioavailable drug called Ataluren (also known as PTC124). Pre-clinical studies in *mdx* mice showed dystrophin restoration up to ~20% of normal levels and an improved contractile properties of treated skeletal muscles for a limited period of time [86]. Unfortunately, no convincing evidence has been presented regarding the increase of dystrophin expression in cardiac muscle after ataluren treatment.

A phase I study in healthy volunteers established the safety of Ataluren in humans [87] and led to a phase IIa proof-of-concept study in DMD/BMD patients and subsequently to a randomized, double-blind, placebo-controlled dose-ranging phase IIb clinical trial. Although a good safety profile emerged from these studies, the preliminary results demonstrated no significant difference in functional tests [83].

Further, it has been demonstrated that Ataluren was also able to induce read-through of a stop codon in cells derived from a LGMD2B patient with a R1905X mutation in the gene for dysferlin and to produce enough amount of protein to rescue myotube membrane blebbing [88].

Chapter 3: Nitric Oxide and Muscular dystrophies

3.1 Nitric Oxide and nitric oxide synthases

Nitric Oxide (NO) is a gaseous messenger, synthesized from L-arginine and oxygen by NO synthases (NOS) in almost all mammalian cells and tissues to regulate key physiological events of adult organisms [89-90]. Three distinct isoforms of NOS have been identified, two of which, namely the endothelial (eNOS) and neuronal (nNOS) isoforms, are regulated by second messengers, whereas the inducible one by cytokines and bacterial products (iNOS). All three NOS isoforms are expressed in skeletal muscle, but differ in their mechanisms of activation, regulation and catalytic activity. Neuronal NOS is abundant at the surface of type II fibers (fast twitch), whereas it is less represented in type I (slow twitch) fibers [91].

Neuronal and endothelial NOS are constitutively expressed and require the Ca^{2+}/calmodulin complex for their activation. Changes in the expression of these enzymes may occur in skeletal muscle, in fact expression of nNOS is increased by crush injury, muscle activity and ageing [92]; also changes in eNOS expression are described after exercise and shear stress [93]. These changes, however, are usually of small extent. Both nNOS and eNOS produce NO at low, physiological levels (in the pico to nanomolar range) for short periods. Conversely, iNOS is expressed in skeletal muscle primarily under severe inflammatory conditions, such as in the course of autoimmune inflammatory myopathies [94] and after crash injury. The activity of iNOS is independent of the Ca^{2+}/calmodulin complex and generates NO at high concentrations (micromolar range) for prolonged periods.

In the recent years, many studies have uncovered an increasingly important role of physical association of the NOS isoforms with several regulatory and structural proteins [95]. Interestingly, these protein–protein interactions, as well as regulating the activity of NOSs often target them to cellular membranes. The N-terminus of nNOS contains a PDZ (post-synaptic density protein-95, discs-large, Z0-1) domain that allows interactions of the enzyme with other PDZ containing proteins at the cellular plasma membrane. In skeletal muscle, the localization of nNOS to the dystrophin–glycoprotein complex at sarcolemma, due to the binding via one of these PDZ-containing proteins, α1-syntrophin

[96], is a key aspect that explains the coupling of NO generation with muscle contractile activity.

Pathological features of muscular dystrophies are, at least in part, due to the displacement of nNOS from the sarcolemma as a consequence of the disruption of the dystrophin–glycoprotein complex.

3.2 Nitric Oxide and the Therapy of Muscular Dystrophies

Whereas the genetic (exon skipping and antisense oligonucleotides) and stem cell approaches appear to be significant steps forward to a resolutive therapy for muscular dystrophies, they are expensive and still not really available for all patients as the results of the last clinical trials indicate (Pichavant, Aartsma-Rus et al. 2011; Cirak, Feng et al. 2012). Moreover, they are direct to specific subsets of population (e.g. about 13 % in the case of exon 51 skipping) and cannot restore fully the damage already caused by the disease to the muscle.

In this context, the need for novel therapeutic strategies that can limit muscle wasting and ameliorate the dystrophic symptoms is particularly urgent also considering the adverse side effects of the current therapy with corticosteroids. Therefore, classical pharmacological approaches appear therefore still attractive and of significant value, as they address all patients and at affordable costs [97].

Nitric oxide is known to exert many important functions in skeletal muscle physiology: for example it regulates the muscle force excitation–contraction coupling together with myogenesis and muscle repair, thus suggesting a possible use of NO as a tool for therapeutic strategies in Duchenne, Becker and limb-girdle muscular dystrophies, where genetic alterations in genes coding for structural muscle proteins lead to repeated and enhanced muscle damage during physiological activity.

The role of NO in therapy is also supported by the demonstration of a rescue in *mdx* and dystrophin/utrophin double-knockout mice by over-expressiong the nNOS transgene. Of interest, the transgenic nNOS acts without a strict localisation to the sarcolemma,

indicating that generation of NO per se was sufficient to exert the beneficial effect [98-99]. The efficacy of therapies based on the administration of NO has, thus, been investigated by several groups in mouse models of muscular dystrophy.

Approaches with only NO donors or the NOS substrate L-arginine yield some amelioration of the *mdx* mouse dystrophic phenotype; nevertheless, none of these studies report long-term observations. Thus, the assessment of the validity of NO donation/generation as a therapeutic strategy inducing persistent beneficial effects could not be defined [100-101].

The NO-based approaches that have been tested so far include:

(i) administration of the NO precursor aminoacid L-arginine, alone or in combination with corticosteroids;
(ii) NO donation *per se*;
(iii) NO donation combined with glucocorticoids;
(iv) NO donation combined with non steroidal anti-inflammatory activity;
(v) Administration of 5′ phosphodiesterases inhibitors (Tadalafil and Sildenafil) that inhibit hydrolysis of cGMP by phosphodiesterases prolonged NO effects.

NO donation *per se* has been tested using the organic nitrate isosorbide dinitrate and molsidomine.

Isosorbide dinitrate alone is not really effective, and molsidomine shows a moderate long term efficacy [1], but the reason why molsidomine is more effective than the other NO donors tested so far remains to be established. A possible explanation could be found in recent studies in which molsidomine has been demonstrated to be particularly effective in increasing satellite cells number and preventing the adipogenic differentiation of mesenchymal fibro-adipogenic progenitors (FAPs) residing in the muscle, thus reducing fibrosis. In particular, the inhibition of adipogenesis is due to NO-induced increased expression of miR-27b leading to down-regulation of peroxisome proliferator-activated receptors gamma (Pparγ1) expression in a pathway independent of cGMP generation [102]. It has also been shown that molsidomine treatment modulates effectively the characteristics of the inflammatory infiltrate within dystrophic muscles, enhancing its healing functions [103]. Distinct effects depend on the phase of the disease. Initially, the treatment with molsidomine contributes to a more efficient macrophage recruitment

inside the dystrophic muscle, in order to promote a more efficient clearance of cell debris and an effective tissue regeneration. Later molsidomine decreases the extent of the inflammatory infiltrate, whose persistence worsens muscle damage [103]. Molsidomine efficacy could also depend on the pharmacokinetic profile of NO release by the drug, or on the release of biologically active nitrites and nitrates [104], which have beneficial functions in skeletal muscle and have been shown to ameliorate the dystrophic phenotype [105-107]. Despite the positive results obtained with molsidomine, a strategy based on NO donation alone appears not endowed with the characteristics of clinical efficacy, needed for an effective pharmacological therapy.

An alternative strategy is to use NO donation in combination with anti-inflammatory drugs. For example, a study in *mdx* mice reports that the association of the corticosteroid deflazacort with L-arginine spares *mdx* dystrophic limb muscle from exercise-induced damage and induces a persistent functional improvement in distance run [108]. This approach, even substantial, still implies the use of corticosteroids.

In order to avoid corticosteroids, different strategies have been tested, in which the anti-inflammatory activity is obtained using non-steroidal anti-inflammatory agents (NSAIDs). Two approaches have been proposed: the combination of two different molecules isosorbide dinitrate and ibuprofen administrated together and a strategy in which a the two activities are combined together in a single molecular entity consisted of compounds of the CINOD class (cyclooxygenase-inhibiting nitric oxide (NO) donators) [109].

Using these drugs or the combination important therapeutic effects in both the α-sarcoglycan null and the *mdx* mouse models have been observed in the absence of relevant side effects or signs of toxicity. Of interest, the beneficial effects are persistent (up to 12 months, that is the observation period set to mimic a chronic treatment) and both approaches are able to slow down the dystrophic progression, maintaining the functional capacity of muscles. At biochemical level several mechanisms synergize to obtain the therapeutic effect of the combined therapy:

- reduced fibers damage and inflammation,
- significant increase in the number and differentiation capacity of myogenic precursor cells, which preserve the long-term regeneration capacity of muscle [110-111] (Figure 6).

- effects on blood flow. NO has vasodilating and angiogenic properties; the former contributes to reduce muscle ischemia induced by nNOS displacement and increase glucose uptake and energy generation and the latter can contribute in muscle repair [112-113].

An additional interesting aspect of these therapies is that they significantlyenhance the homing of exogenously added myogenic stem cells to dystrophic muscles [110-111], suggesting the possibility of obtaining synergic effects by combining pharmacological and cell therapy approaches.

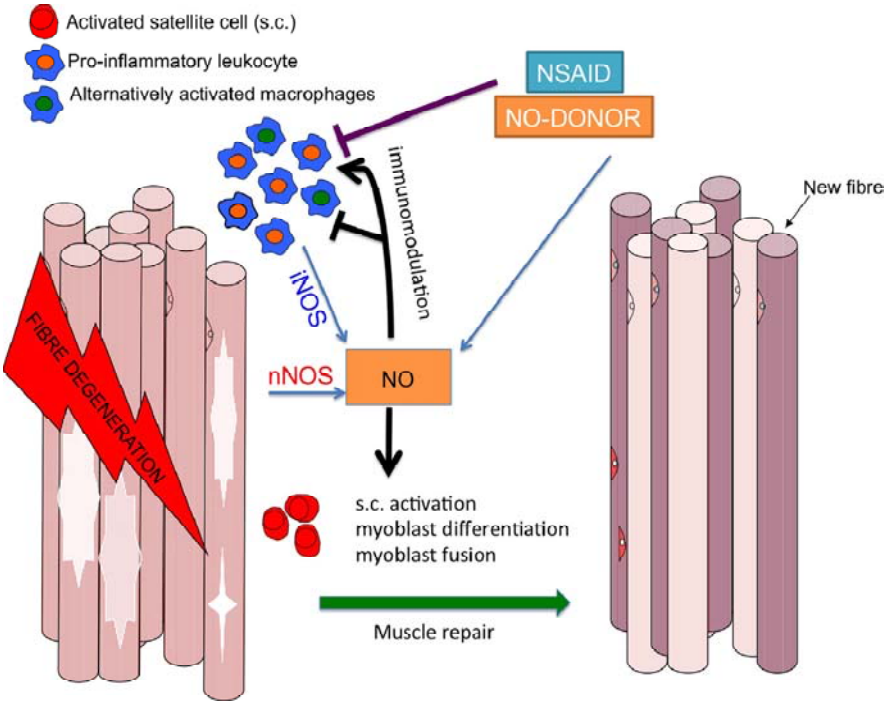

Figure 6: Nitric oxide and muscle repair: Multiple actions converging on therapeutic efficacy. Schematic diagram summarizing the different actions through which NO, endogenously generated by iNOS or nNOS, or administered through NO donors stimulates muscle repair, alone or in combination with NSAID. NO stimulates satellite cells activation, myoblasts differentiation and fusion to form new fibres. Both NSAIDs, and to a lesser extent NO, modulate the nature of the inflammatory infiltrates (mainlymacrophages) [114].

In spite of no significant differences in terms of efficacy between CINOD and the combination of NO-donating drugs plus NSAID have been observed, an advantage in therapeutic perspective of the use of a combination of drugs is the possibility of titrating the two active principles independently, thus optimizing their use of single patients [97].

The therapeutic potential of the combination o have also been tested on dystrophic patients in an open-label pilot study in a cohort of adult patients affected by Duchenne,

Becker and limb-girdle muscular dystrophies. The trial have been designed mainly to evaluate safety and tolerability of the drug combination, but exploratory measures of efficacy, such as the motor function measure scale, have also been applied. The combination shows good safety and long-term tolerability profiles with only few and transient side effects. Of importance, the exploratory efficacy measures point out a trend towards amelioration, in particular better performance in the Motor Function Measure scale [115]. These results strongly indicate that the systemic administration of a combination of NO-donating drugs and NSAIDs (and possibly of CINODs) is of potential clinical relevance and should be optimised and further investigated in future clinical studies.

Moreover, the combination of NO donation and anti-inflammatory activity ameliorates cardiac dysfunction in mdx mice, an event that characterizes advanced disease [116]. This effect is shared by sildenafil, which shows significant cardio protective actions [117]. Whether sildenafil has beneficial effect also on skeletal muscle needs to be clarified, even if some initial promising results have been reported [118]. However, many of the action of NO on muscle myogenesis and repair are independent of cGMP generation, including the effects of NO on the proliferation and differentiation of stem cells, on the control of inflammation and on the regulation of gene transcription and regulatory miRNA activity [103,119-121] distinguishing between NO-donating drugs and sildenafil.

Chapter 4: Mitochondria and Mitochondrial Dysfunctions in Muscular Dystrophies

4.1 Mitochondria: Structure and function

Mitochondria are membrane bound organelles involved in many important biological functions, for instance they are the major sites of oxidative energy production in eukaryotic cells. They in fact produce adenine triphosphate (ATP) as the main source of cellular energy through a process called oxidative phosphorilation (OXPHOS) [122-123].

According to the endosymbiont hypothesis, mitochondria arose from the engulfment of aerobic eubacteria by a primordial anaerobic eukaryote.[124]. Thus, the organelle has its own genetic system that exhibits several prokaryotic features including a compact circular DNA (mtDNA), multigenic RNA transcripts and bacteria-like antibiotic sensitivity of the translational apparatus. mtDNA encodes only 13 proteins and the 22 tRNAs and 2 rRNAs required for their translation within the mitochondrial matrix [125-126].

Mitochondria play essential and diverse roles in the physiology of eukaryotic cells, not only providing energy, but they also participate in numerous metabolic reactions and play central roles in apoptosis [127]. Impairments of mitochondrial functions have been implicated in several human pathologies, among which age-related diseases, cancer and neuromuscular diseases.

In the 1950s, electron microscopy studies led to the canonical view of mitochondria as bean-shaped organelles consisting in two double lipid membranes: the outer membrane that covers the organelle and the inner membrane that folds over many times arising structures termed *cristae*. The space between the outer and inner membrane of the mitochondria is called the intermembrane space and the volume enclosed by the inner membrane is called matrix that contains a highly-concentrated mixture of hundreds of enzymes. Electrons from oxidative substrates are transferred to oxygen, through a series of redox reactions, to generate water. In the process, protons are pumped from the matrix across the mitochondrial inner membrane by respiratory complexes I, II III, and IV. When protons return to the mitochondrial matrix following their electrochemical gradient, ATP is synthesized via complex V (ATP synthase) [128].

In the last years taking advantage from fluorescence microscopy techniques has been demonstrated that the mitochondrial structure is highly dynamic [129]. In mammalian cells mitochondria morphology depends on cell type and, even in the same cell, mitochondria can change morphologies, ranging from small spheres or short rods to long interconnected tubules [130]. To this end, they continuously and reversibly rearrange their structure through the tightly-regulated processes of fission and fusion. In particular, mitochondrial fusion is regulated by the activity of three large GTPase proteins, called mitofusin-1 and 2, and optic atrophy-1 (Opa1), whereas the fission process is mainly regulated by the GTPase dynamin-related protein-1 (Drp1) [131].

Mitochondria are also defined as dynamic organelles because they are actively transported in cells determining specific subcellular distributions. Depending on the cellular context, these transport processes can ensure proper inheritance of mitochondria or can recruit mitochondria to active regions of the cell. For example, in budding yeast, mitochondria are transported into and retained in the developing bud to ensure mitochondrial inheritance to the daughter cell [132].

Finally, the plasticity of mitochondria involves the internal structure of the organelle that can change in response to their physiological state. Purified mitochondria placed in low ADP conditions have limited respiration and have an 'orthodox' morphology, characterized by narrow *cristae* and few *cristae* junctions *per cristae* compartment. Under high ADP and substrate conditions, isolated mitochondria have high respiratory activity and a 'condensed' morphology, characterized by larger *cristae* and several *cristae* junctions per *cristae* compartment [133] (Figure 7).

It is known that the gaseous messenger NO play a crucial role in the regulation of mitochondrial function competing with oxygen and inhibiting complex IV. NO at physiological nanomolar concentrations reversibly binds the cytochrome c oxidase blocking the activity of the enzyme [134-136] and thus negatively regulating mitochondrial oxidative phosphorylation. Recently it is has been demonstrated that NO is also able to inhibit fission during myogenic differentiation allowing myogenic process to occur [137].

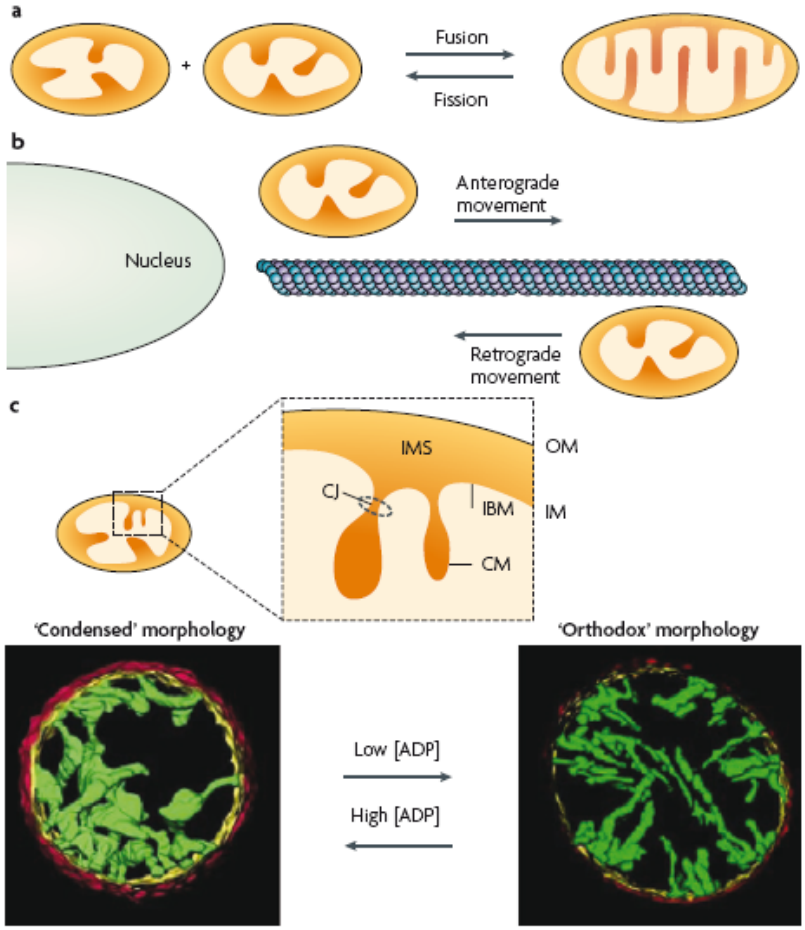

Figure 7: Mitochondria as dynamic organelles: A) Mitochondrial fusion and fission control mitochondrial number and size. B) In mammalian systems, mitochondria are distributed throughout the cytoplasm by active transport along microtubules and actin filaments. Distinct molecular motors transport the mitochondria in anterograde or retrograde directions. C) Inner membrane dynamics. The diagram indicates the different regions of the inner membrane. The bottom panels show electron microscopy (EM) tomograms of two mitochondria under different metabolic conditions (red, outer membrane; yellow, inner boundary membrane; green, cristae membrane).

4.2 Mitochondrial biogenesis

The renewal of mitochondria through the process of biogenesis is essential to maintain mitochondrial integrity and changes in mitochondrial mass have been documented in both normal and disease states. For instance, mitochondria differentiate post-natally to acquire increased respiratory capacity as an adaptation to oxygen exposure outside of the womb [138]. Moreover mitochondrial biogenesis increases in muscle cells upon exercise [139] or in response to contraction induced by chronic electrical stimulation [140]. Also thyroid

hormones have long been associated with increased mitochondrial mass and the elevated expression of PGC-1α gene [141] and finally, developmental signals induce the proliferation of mitochondria as occurs in the brown fat of rodents and other mammals during adaptive thermogenesis [142-143].

PGC-1α is currently considered to be the most important regulator of mitochondrial biogenesis [144]. The ability of PGC-1α to regulate mitochondrial mass is due to its capacity to co-activate multiple transcription factors involved specifically in the regulation of nuclear genes encoding mitochondrial proteins [145-146]. In particular, PGC-1α interacts with NRF-1 and 2 to transactivate genes of the respiratory chain, of mitochondrial import machinery and transcription factors of mtDNA, such as Tfam, TFB1M and TFB2M [147-148 149].

More recently, the estrogen-related receptor alpha (ERRα) has also been implicated as an important regulator of oxidative metabolism. Evidence suggests a correlation between ERRα and PGC-1α, in particular PGC-1α regulates ERRα activity , but inhibition of ERRα in cells decreases the ability of PGC-1α to induce mitochondrial biogenesis and cellular respiration [149-151]. The ability of PGC-1α to integrate the transcriptional activity of these and other several nuclear transcription factors provides the mechanism by which the coactivator can coordinate the large number of genes required for mitochondrial biogenesis.

PGC-1α levels depend on the oxidative capacity of the tissue, for instance it is highly expressed in oxidative tissues such as brown fat, heart and skeletal muscle.

PGC-1α is dynamically regulated at the level of mRNA and protein expression in response to a variety of signaling pathways involved in cellular growth, differentiation, and energy metabolism. Moreover, PGC-1α expression is induced by multiple stimuli involved in the regulation of mitochondrial biogenesis, such as chronic exercise or thyroid hormone treatment [141,152-153].

In skeletal muscle, the increase of Ca^{2+} concentration and calcium/calmodulin-dependent protein kinase IV (CaMKIV) activation, stimulate binding of CREB protein to the CRE region in the PGC-1α promoter.

It has also been shown that PGC-1α is involved in an autoregulatory loop controlling its own transcription, in fact, PGC-1α coactivates myocyte enhancer factor (MEF)-2, that in turn binds to the PGC-1α promoter regulating the expression of the coactivator [154] itself.

PGC-1α is also regulated at post-translational level (Figure 8). In fact, it is directly activated by a Sirt1-dependend deacetylation and by specific phosphorilations at threonine-177 and serine-538 by AMP-activated kinase (AMPK) in response to changes in nutrient or energy depletion [155-156].

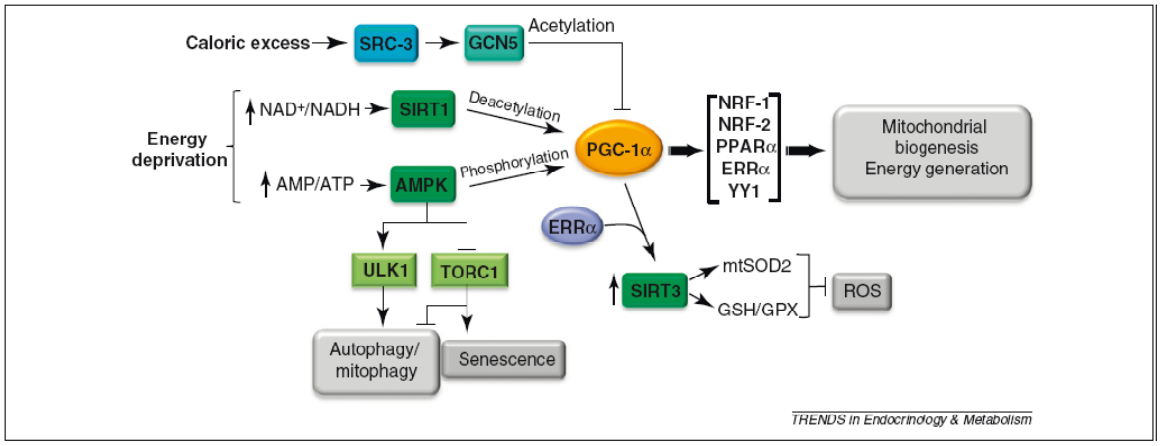

Figure 8: Post-traslational control of PGC-1α
PGC-1α is activated via post-transcriptional phosphorylation by AMPK or by deacetylation via SIRT1 in response to nutrient deprivation. Induction or activation of the coactivator can enhance mitochondrial biogenesis and oxidative function through the coactivation of multiple transcription factors involved in respiratory gene expression [157]

The importance of PGC-1α in regulating mitochondrial content in skeletal muscle has been demonstrated with the use of animal and cell culture models in which the expression of the coactivator has been markedly altered [158-159]. More specifically, over-expression of PGC-1α has been shown to increase the expression of nuclear encoded mitochondrial proteins involved in the expansion of the mitochondrial reticulum [160]. Ectopic expression of PGC-1α in C_2C_{12} myoblasts increases nuclear (cytochrome c, COX subunit IV) and mitochondrially (COX subunit II) encoded electron transport chain (ETC) mRNAs and amplifies mtDNA copy number [149].

The induction of mitochondrial biogenesis has been further studied with the use of PGC-1α transgenic mice. Gain-of function experiments establish that overexpression of the coactivator *in vivo* activates mitochondrial proliferation; furthermore, muscle-specific overexpression of PGC-1α enhances the expression of mitochondrial genes involved in the oxidative phosphorylation. In addition to the effects of PGC-1α on the steady-state of

mitochondrial mass in skeletal muscle, the transcriptional coactivator is also highly implicated in induced-organelle biogenesis for instance during endurance exercise [161-162]. Exercise enhances the PGC-1α-dependent mitochondrial biogenesis, whereas chronic muscle disuse results in a decrease of mitochondrial mass, along with concomitant reduction of PGC-1α level [163]. Similarly, the process of aging is associated with concomitant reduction of mitochondrial content, endurance performance and PGC-1α protein expression [164]. Although the full role of PGC-1α in aged-related deficits of mitochondrial content remains to be determined, some of the pathways involved in the transcriptional activation of PGC-1α are impaired with age. The importance of PGC-1α in maintaining basal mitochondrial content, as well as its potential involvement in physiological and pathological processes highlights the interesting of understanding in detail how its expression and activity are controlled.

4.3 Nitric Oxide Regulation of Mitochondrial Biogenesis

The hypothesis of an involvement of NO in the regulation of mitochondrial biogenesis came from observations by several groups about the role of NO, either exogenous or generated by eNOS, in the differentiation programme of the brown adipose tissue (BAT)[165-166]. It was found that treatment with NO-releasing drugs increases the mtDNA content[167]. The NO-dependent mitochondrial biogenesis occurs through activation of PGC-1α, NRF-1 and the mitochondrial transcription factor A (Tfam). Using a cyclic GMP (cGMP) analogue and a guanylate cyclase inhibitor, it has been demonstrated that mitochondrial biogenesis depends on this second messangers. Activation of guanylate cyclase and generation of cGMP are known to take place at physiological concentrations of NO indicating that mitochondrial biogenesis is a physiological process regulated by NO. In animals acclimatized to cold high levels of noradrenaline (NA) are released from sympathetic nerve terminals stimulating brown adipocytes, with an increase of eNOS expression, NO and cGMP production in both cytosol and nucleus and resulting in enhanced expression of uncoupling protein 1 (UCP1). UCP1 is an inner mitochondrial membrane proton channel that dissipates the inner trans-membrane potential to produce heat [168-169]. Interestingly, NA also induces expression of PGC-1α, which binds UCP1

promoter and increases UCP1 expression [170]. Interestingly, such function by NO is associated with PKG-mediated brown fat cell differentiation suggesting a key role of NO and cGMP in BAT differentiation [171]. Thus, NO and/or cGMP levels in brown adipocytes are linked to cell activation, mitochondrial biogenesis and heat production.

Experiments in mouse white-fat 3T3-L1, human U937 monocytic and HeLa epithelial cells, as well as rat L6 myoblasts and PC12 neurosecretory cells, show similar results to those obtained in brown adipocytes, revealing that the NO-dependent mitochondrial biogenesis is restricted neither to brown adipocytes and their differentiation processes nor to a specific cell lineage or species [167,172]. An important aspect of NO-dependent mitochondrial biogenesis is that it leads to formation of functionally active mitochondria. In particular, the newly generated mitochondria are capable of sustaining coupled respiration, and generate ATP, through the oxidative phosphorylation pathway. Interestingly, this increase in ATP generation by mitochondria is not accompanied by a decreased generation of ATP through the glycolytic pathway, yielding a steady state concentrations of ATP more higher than those observed in cells not exposed to NO.

The obligatory role of eNOS in mitochondrial biogenesis was further investigated by using eNOS-/- mice. Histological analysis indicated that BAT of eNOS-/- is functionally inactive, and indeed exposure of these animals to cold did not result in the mitochondriogenetic process that is commonly observed in wild-type animals [167]. In addition, deletion of eNOS was sufficient to reduce the mitochondrial mass even in tissues that have a basal expression of nNOS, and possibly iNOS, such as the brain, liver, muscle and heart. The reduced mitochondrial content in tissues from eNOS-/- mice was accompanied by reduction of both basal oxygen consumption and steady state levels of ATP associated with a significant reduction in the mitochondrial size. This occurred in tissues dependent on oxidative metabolism, such as the brain, and in glycolytic tissues, such as the gastrocnemius muscle, suggesting that the effect of NO on mitochondrial biogenesis is a general phenomenon. Interestingly, in the gastrocnemius muscle mitochondrial biogenesis is important for the conversion of type II (glycolytic) fibers into type I (oxidative) fibers [173]. The mitochondrial volume and network extension [174] have been shown to correlate positively with the efficiency of the mitochondrial respiratory function. Thus, it is likely that the decrease in mitochondrial size observed in tissues from eNOS-/- mice is a morphological feature that correlates with reduced mitochondrial function.

The importance of NO as a mitochondrial biogenetic stimulus has also broad implications in term of pathology. Impairment of mitochondrial function is associated with neurodegenerative diseases, neuromuscular disorders, liver and heart failure, and type 2 diabetes [159,175-178]. The possibility of generating new, metabolically active mitochondria might therefore improve the outcome of these pathologies.

4.4 Mitochondrial dysfunction in muscular dystrophies

Mitochondrial dysfunctions can be defined as alterations in the mitochondria, including mitochondrial uncoupling, mitochondrial depolarization, inhibition of the mitochondrial respiratory chain, mitochondrial network fragmentation, mitochondrial DNA depletion, mitochondrial or nuclear DNA mutations and the mitochondrial accumulation of protein aggregates.

All these dysfunctions are known to alter the ATP production and are observed in several pathological states or diseases, including cancer, obesity, muscle and neurological disorders.

Mitochondrial dysfunction has long been suspected to be an important pathogenetic feature also in muscular dystrophies even if their role in this kind of pathologies is not fully understood. Alterations of mitochondrial function have been described so far in the pathogenesis of several muscular dystrophies such as congenital muscular dystrophy (CMD), limb-girdle muscular dystrophy type 2 (calpainopathy) and Duchenne muscular dystrophy.

1. *Mitochondrial dysfunction due to dysregulation of mitochondrial permeability transition pore (mtPTP)*

The most described mitochondrial dysfunction in the biology of muscular dystrophy is exemplified in Collagen VI myopathies that share a dysregulation of an inner membrane high-conductance channel, the mitochondrial PTP [179-180].

In particular, Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) are muscle diseases due to mutations in the genes encoding the extracellular matrix

protein collagen VI and key events in their pathogenesis are dysregulation of the mitochondrial permeability transition pore and inadequate removal of defective mitochondria, which amplifies the damage.

Myoblasts obtained from patients with UCMD and BM exhibit a latent mitochondrial dysfunction, irrespective of the genetic lesion responsible for the lack or deficiency of collagen VI. In UCMD myoblasts, the threshold for PTP opening is very close to the resting membrane potential, hence a depolarization can trigger pore opening and ATP depletion. Collectively, myoblasts obtained from patients with UCMD display functional and ultrastructural mitochondrial alterations and increased apoptosis due to inappropriate opening of the mtPTP. These alterations can be normalized by treatment with cyclosporin A (CsA), a widely used immunosuppressant that desensitizes the permeability transition pore independently of calcineurin inhibition.

CsA is used in mouse models and also in clinical trials for patients affected by UCDM and BM demonstrated that collagen VI myopathies can be effectively treated with drugs acting on the pathogenic mechanism downstream of the genetic lesion, and they represent an important proof of principle for the potential therapy of genetic disease [181-182].

Interestingly, a mtPTP dysregulation is also observed in vitro in myoblasts obtained from muscle biopsies of patients with limb-girdle muscular dystrophy (LGMD) type 2B, but not in myoblasts from patients with BM, merosin-deficient CMD, LGMD2A or Duchenne muscular dystrophy (DMD) [183].

2. *Mitochondrial dysfunction in DMD and LGMD*

Abnormalities in mitochondrial function have been also implicated in the pathogenesis of DMD and LGMD.

Biochemical and ultrastructural abnormalities have long been recognized in DMD patients and carriers. Electron microscopic analyses performed on muscle biopsies from obligatory carriers of DMD show massive aggregates of subsarcolemmal mitochondria, some with paracrystalline inclusions [184]. In young DMD patients an increased fragility of the mitochondrial membranes is observed, indicating that mitochondria could be involved early in the disease process[185]. Furthermore, more recently, it has been documented a general metabolic crisis in dystrophic muscle of Duchenne and Limb girdle 2D patients

with severe downregulation of nuclear-encoded mitochondrial genes [186]. DMD patients also exhibit a reduced transcription of the genes involved in energy metabolism as demonstrated by genome-wide gene expression profiling of skeletal muscle [187].

In animal studies, *mdx* mice, compared with normal mice, exhibit abnormalities in mitochondrial respiratory function, greater ROS buffering capabilities, and susceptibility to Ca^{2+} inducing opening of the mt PTP complex [188]. Gene transfer and overexpression of PGC-1α in post necrotic dystrophin-deficient muscles restores mitochondrial function and exerts a moderating effect regarding the activation of proteolytic and apoptotic signaling associated with disease progression.

The mitochondrial abnormalities in DMD-associated heart failure support a critical role for dystrophin in maintaining optimal coupling between metabolism and contractile function. Mitochondrial abnormalities, which include changes in energy substrate utilization and cell death signaling due to increased permeability of mitochondrial membranes, disruption of Ca^{2+} homeostasis, and ROS production, could be responsible for the clinical manifestations of overt cardiomyopathy. It has been suggested that mitochondrial changes in the dystrophin-deficient heart may provide the rationale for mitochondria targeted therapies [189].

The extent of mitochondrial abnormalities across the genetic spectrum of autosomal recessive LGMD is currently unclear but it is an interesting field of study. There are isolated case reports of LGMD, that show some mitochondrial morphologic abnormalities, including ragged-red fibers (a histochemical hallmark of abnormal mitochondria in skeletal muscle fibers), decreased COX histochemical staining, and ultrastructural aberrations observed by electron microscopy [190]. For instance, ragged-red fibers have been reported in instances of dysferlinopathy [191].

3. *Mitochondrial dysfunction associated with Calpain3 (CAPN3) gene and other muscular dystophies*

Mutations in the non-lysosomal cysteine protease calpain-3 (CAPN3) gene cause a recessive form of limb girdle muscular dystrophy type 2A (LGMD2A).

Ultrastructural and biochemical mitochondrial abnormalities have been reported in skeletal muscles of calpain-3 knockout (C3KO) mouse associated with reduced oxidative ATP production [192].

These data suggest that mitochondrial abnormalities leading to oxidative stress and energy deficit are important pathological features of calpainopathy and might represent secondary effects of the absence of CAPN3.

Patients with facioscapulohumeral muscular dystrophy (FSH-MD) display derangements in the oxidative stress responses and reduced levels of systemic antioxidative molecules (low levels of zinc, selenium, and vitamin C). Systemic oxidative stress and mitochondrial dysfunction in patients with FSH-MD correlate with abnormalities in skeletal muscle functional parameters, providing the rationale for therapeutic use of antioxidants in maintaining FSH-MD muscle functions [193].

Abnormalities in mitochondrial proteins and the ubiquitin-proteasome system are potentially relevant also in DM2 (Myotonic Dystophy type 2) pathogenesis. Mitochondrial proteins, including elongation factor-Tu, heat shock protein 60, glucose-regulated protein 75, and dienoyl CoA isomerase, an enzyme involved in fatty acid degradation [194] are downregulated in DM2 myotubes.

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AIM OF THE STYDY

Muscular dystrophies are heterogeneous genetic diseases affecting skeletal muscle. At present there is no satisfactory therapy for muscular dystrophies, apart for the treatment with corticosteroids that, however, provides only temporary improvements and is associated with severe side effects. To this end elucidation of novel possible targets is necessary to develop new therapeutic strategies and is the goal of this project.

Since mitochondria are emerging as determinant in many muscle disorders including muscular dystrophy, the first aim of this study is the investigation of the role of mitochondria in the pathogenesis of Limb Girdle muscular dystrophy 2D , studying a-sarcoglican-null mice.

Moreover, during the years, nitric oxide donors have been identified as good candidate molecules for Duchenne Muscular dystrophy therapy and our group has recently demonstrated that the NO donor molsidomine is able to slow disease progression and to restore the functional capacity of damaged muscles, significantly enhancing spontaneous and forced motor activities.

In this context, the second aim of the study is the evaluation of the impact of molsidomine treatment on mitochondrial function of dystrophic mice.

All the experiments were carried out comparing wild-type mice to a-sarcoglican-null mice treated with standard diet or with diet containing the NO-donor molsidomine.

MATERIALS AND METHODS

Animals

α-Sarcoglycan (α-SG) null mice C57BL/6J were a kind gift of K. Campbell (Iowa University, Iowa City, IA, USA) and C57BL/6 WT mice (C57Bl10SnJ strain) were purchased from Charles River (Wilmington, MA, USA) and treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee. Animals were used when 5 months old, since from 4 months of age αSG-null mice are characterized by the development of histopathological features of muscle dystrophy with ongoing fibre degeneration and modest spontaneous regeneration [20].

The mice were housed in an environmentally controlled room (23°C, 12h light–dark cycle) and provided food and water ad libitum. Standard diet (STD) or a diet containing 3 mg/kg of (1-ethoxy-N-(3-morpholino-5- oxadiazol-3-iumyl) methanimidate (molsidomine) were prepared based on the daily food intake measured for these animals.

For in vivo miR treatments, 5 µg (every 3 days for a total of 4 consecutive injections) of miR-34a antagomiR in saline (50 µl) was intramuscularly injected into the right TA of dystrophic mice. The controlateral TA was injected with the same protocol with control antagomiR as negative control. Mice were sacrificed 4 days after the last intramuscular injection to avoid inflammatory infiltration.

High resolution respirometry

The respiratory chain capacity efficiency was determined by high-resolution respirometry (HRR) on permeabilized muscle fibers from 5 months wild type and dystrophic mice (standard diet or molsidomine treated). This technique allows to measure total mitochondrial VO2 and to suggest the role of each mitochondrial complex in the process.

After cervical dislocation, diaphragm or tibialis anterior muscles were excised and placed into a Petri dish containing ice-cold biopsy preservation solution (BIOPS: 15 mM Phosphocreatin, 5.77 mM ATP, 6.56 mM MgCl2, 10 mM EGTA, 0.1 mM free Ca, 20 mM imidazol, 20 mM taurin, 50 mM K-MES, 0.5 mM DTT),

The fibers were permeabilized by gentle agitation for 30 min at 4°C in solution supplemented with 50 μg/ml saponin. Fibers were washed in ice-cold respiration medium (see below) by agitation for 10 min and were kept in this medium until respirometric assay.

The respiration rates of single fibers obtained from TA and Diaphragm were measured using two-channel titration injection respirometers (Oroboros, Instruments Oroboros, Innsbruck, Austria) at 37 °C in a respiration medium containing 0.5mM EGTA, 3mM MgCl2 60mM lactobionate, 20mM taurine, 10mM KH2PO4, 20mM HEPES, 110mM sucrose and 1 g/l of BSA (bovine serum albumin). Before each experiment the medium was equilibrated with air in the oxygraph chambers at 37°C until a stable signal was obtained for oxygen calibration.

The software DatLab (Oroboros) was used for data acquisition and analysis.

We placed fibers (1–3 mg wet weight) into the O2K oxygraph chambers and we added glutamate (10 mM), malate (2 mM) and ADP (2.5 mM) to obtain state 3 respiration. Then, we injected succinate (10 mM) to have an estimation of the entire OXPHOS capacity (CI+CII activity). Then, CII activity was measured by injecting rotenone 0.5 μM and inhibiting CI after convergent stimulation of CI+II. Finally, inhibition of CIII with antimycin A 2.5 μM and of CII with malonate 5 mM was followed by CIV stimulation with TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine) 0.5 mM and ascorbate 2 mM.

Experiments were performed at 25 °C and all data were normalized to fiber weight or mtDNA content.

DNA, RNA and protein extraction

DNA Extraction: Total DNA was isolated from fresh-frozen muscles, heart, liver and brown adipose tissue (BAT) using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) as described in the protocol provided by the manufacturer. After eluition in water, total DNA was quantified by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

RNA Extraction: Total RNA was isolated from boath Tibialis Anterior and diaphragm using TRIzol reagent (Invitrogen, Life Technologies, Cergy, France) following the manufacturer's instructions. After solubilization in RNase-free water, total RNA was quantified by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). First-

strand cDNA was generated from 1 ug of total RNA using ImProm-II Reverse Transcription System (Promega).

Protein Extraxtion: Tissue samples from TA and diaphragm muscles were homogenised in a lysis buffer containing 20mM Tris-HCl (pH 7.4), 10 mM EGTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1% SDS supplemented with a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Samples were centrifuged at 1000 x g for 10 minutes at 4°C to discard cellular debris. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific-Pierce Biotechnology, Rockford, IL), according to the manufacturer's protocol.

Quantification of mtDNA copy number

The mtDNA content was measured by real-time PCR normalizing the quantity of a not-polymorphic mitochondrial gene (NADH dehydrogenase 1, ND1) with a single-copy nuclear gene (RNAse P). Real-time PCR was performed using the SYBR Green Supermix (Bio-Rad) on a Roche LightCycler 480 Instrument (Roche Diagnostics, Rotkreuz, Switzerland). For each assay, 10 ng of total DNA were analyzed in triplicate. Samples were analyzed using the Roche LightCycler 480 Software (release 1.5.0) and the second derivative maximum method. Specific sets of primer pairs, described in Table 1, were designed to hybridize to unique regions of the appropriate gene sequence.

	rward Primer	**verse Primer**
RnaseP (DNA)	5'-GAAGGCTCTGCGCGGACTCG-3'	5'-CGAGAGACCGGAATGGGGCCT-3'
ND1 (DNA)	5'-CCTATCACCCTTGCCATCAT-3'	5'-GAGGCTGTTGCTTGTGTGAC-3'

Tab.1: Primers used for quantification of mtDNA copy number

Immunoblotting and antibodies

Protein separation was performed following the standard protocol for western blotting. Samples were solubilized in Laemmli buffer, boiled 5 min at 100 °C and loaded on 4-20 % polyacrylamide precast gels (Mini-PROTEAN TGXTM; Bio-Rad) immersed in

running buffer (Tris-base, SDS, glycin) (Gels were run at 300V). Proteins separated in the gel were transferred onto a nitrocellulose membrane using a Bio-Rad's Trans-Blot Turbo SystemTM (7 min at 2.5 A) using Bio-Rad Transfer packTM.
The nitrocellulose membrane was blotted with Ponceau red, to determine the quality of the protein transfer. Then it was incubated for 1 hour at room temperature in a blocking buffer (TBS-T: Tris-buffered saline-Tween 20 (Tris-HCl, NaCl, Tween 20) and non fat dry milk 5%), to block the sites for unspecific binding of the antibodies to the membrane. Then, membrane was incubated with a primary antibody, specific for the protein of interest, and, after three washes in TBS-T, with a secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody, both diluted in Blocking buffer for 1 hour each. After these incubations the membrane was rinsed with TBS-T 3x10 min. Detection of the antigen was performed with the system of HRP/Hydrogen Peroxide catalyzed oxidation of luminol in alkaline conditions using the Clarity Western ECL Substrate by Bio-Rad. The light produced by this enhanced chemiluminescent reaction was detected using CemiDocMP Imaging SystemTM.

The membranes were probed using the following primary antibodies anti: phospho-AMPKα (Thr172) (2531), AMPKα (2532), obtained from Cell Signaling Technology (Billerica, MA, USA). Antibodies to LC3 (L7543) and p62 (P0067), BNIP3 (B7931) were from Sigma, antibodies to VDAC1 (ab15895) and mtCO1 (ab14705) were from Abcam and antibody to COX IV (A21348) was from Life Technologies. The secondary antibodies were from Bio-Rad (anti-rabbit and anti mouse Ab HRP-conjugated).

Mitochondrial biogenesis induction through cold exposure

A control group was kept at 23°C, while experimental groups were kept at 48°C for 24 or 72 hours. Animals were sacrificed and tissues were dissected and collected immediately.

Quantitative Real Time PCR

Real-time PCR was performed using the SYBR Green Supermix (Bio-Rad) on a Roche LightCycler 480 Instrument (Roche Diagnostics, Rotkreuz, Switzerland), according to manufacturer's recommended procedure. As shown in Table 2, a set of primer pairs were designed to hybridize to unique regions of the appropriate gene sequence.

All reactions were run as triplicates. The melt-curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. As to control experiments, gel electrophoresis was also performed to verify the specificity and size of the amplified qPCR products.

Samples were analysed using the Roche LightCycler 480 Software (release 1.5.0) and the second derivative maximum method. The fold increase or decrease was determined relative to a control after normalising to the internal standard 36b4 through the use of the formula 2-ΔΔCT [195-196].

	orward Primer	everse Primer
36b4 (mRNA)	5′-AGGATATGGGATTCGGTCTCTTC-3′	5′-TCATCCTGCTTAAGTGAACAAACT-3′
PGC1α (mRNA)	5’-ACTATGAATCAAGCCACTACAGAC-3’	5’-TTCATCCCTCTTGAGCCTTTCG-3’
Nrf-1 (mRNA)	5’-ACAGATAGTCCTGTCTGGGGAAA-3’	5’-TGGTACATGCTCACAGGGATCT-3’
Tfam (mRNA)	5’-AAGACCTCGTTCAGCATATAACATT-3’	’-TTTTCCAAGCCTCATTTACAAGC-3’
Sirt1 (mRNA)	5’-ACGGTATCTATGCTCGCCTTG-3’	5’-GACACAGAGACGGCTGGAAC-3’
CytB (mRNA)	5’-CTTCGCTTTCCACTTCATCTTACC-3’	5’-TTGGGTTGTTTGATCCTGTTTCG-3’
MCAD (mRNA)	5’-AGGGTTTAGTTTTGAGTTGACGG-3’	5’-CCCCGCTTTTGTCATATTCCG-3’
LCAD (mRNA)	5’-TCTTTTCCTCGGAGCATGACA-3’	5’-GACCTCTCTACTCACTTCTCCAG-3’
PDK4 (mRNA)	5’-AGGGAGGTCGAGCTGTTCTC-3’	5’-GGAGTGTTCACTAAGCGGTCA-3’

Tab.2: Primers used for quantitative RT real-time PCR analyses

Muscle mitochondria isolation

Muscles were minced with scissors in 1ml of ATP medium (100 mM KCl, 50 mM TRIS HCl ph 6.8, 5 mM MgCl2, 1.8 mM ATP, 1 mM EDTA) + Collagenase type II 0.1% in a 2 ml tube and then digested for 10 minutes at 37°C under strong agitation.

After centrifugation at 380 x g for 5 min, pellet was washed twice with ATP medium, resuspended in 700 µl of ATP medium and homogenized with ultraturrax.
Then we centrifugated at 380 x g for 5 min, 4°C in order to pellet nuclei, myofibrillar components and tissue fragments. Supernatant was collected and spun twice at 10000 x g. The final mitochondrial pellet was washed twice and suspended slowly into Mito Resuspension Buffer (225 mM Sucrose, 44mM KH2PO4, 12.5 mM Tris Acetate ph 7.75, 6 mM EDTA).

ATP production from isolated mitochondria

ATP concentration was determined using the luciferin-luciferase method. Briefly, isolated mitochondria were resuspended in Mito Resuspension Buffer. They were then washed by adding 1ml of buffer-A (150 mMKCl, 25 mM Tris HCl, 2 mM EDTA, 0.1 % BSA, 0.1 mM $MgCl_2$, 10 mM KH_2PO_4) and pelletted at 10000 × g. The pellets were resuspended in 160µl of buffer-A and plated in 96 wells in triplicate. The samples were treated with 30µl of a mix containing 1mM malate, 1mM pyruvate, 0.1mM ADP (Sigma), 10µl buffer-B (containing 0.8mM luciferin and 20mg/ml luciferase in 0.5M Tris-acetate (pH 7.75)) and either 1µg/ml oligomycin to assess OXPHOS ATP. ATP was measured using a GloMax luminometer (Promega, Madison, WI, USA).

Immunofluorescence assay

The animals were killed by cervical dislocation, and TA or diaphragm muscles were dissected and immediately frozen in liquid N2-cooled isopentane.
Frozen 10µm cross-sections of muscle were fixed with paraformaldehyde 4% for 10 min at 4 °C, blocked for 30 min with 2% goat serum, 0,5 % BSA-PBS at room temperature and then incubated with primary antibodies directed to BA-D5 for type 1 MHC and to SC71 for type 2a (1:400, Developmental Studies Hybridoma Bank). All primary antibodies were diluted in PBS, 2% goat serum, 0,5 % BSA and incubated at room temperature for 2 hours. After incubation with the appropriate fluorescent-labeled secondary antibodies (goat anti-mouse IgG2b conjugated to AlexaFluor 568 for BA-D5, goat anti-mouse IgG1 conjugated to AlexaFluor 488 for SC71, all from Invitrogen/Molecular Probes), nuclei were counterstained with Hoechst 33342 (Molecular

Probes, Life Technologies) and slides were finally mounted with the ProLong Gold antifade reagent (Invitrogen, Life Technologies). Stained tissues were photographed with a LEICA CTR4000 fluorescence microscope.

SDH staining

SDH staining was performed for 30 min at 37°C using succinate as a substrate and nitro blue tetrazolium as a redox indicator. Criosections were then washed in distilled water (3 changes), and fixed in neutral formalin 10% for 10 minutes. After 10 minutes in 15% ethanol sections were washed, mounted with mounting medium (glycerol 80% in PBS) and visualized using a LEICA CTR4000 microscope.

The darkest staining fibers (type I and II) were counted and their percentage of total number of fibers was calculated (150–200 total fibers/image, 5 images/mouse, 5 mice/ experimental group).

Fluorescent and phase-contrast image acquisition and manipulation

Fluorescent and phase-contrast images were taken on microscopes (Leica DMI4000 B automated inverted microscope; lenses: HCX PL FLUOTAR × 10/0.30, HI PLAN I × 40/0.59 and HCX PL FLUOTAR × 100/1.30). Images were acquired using a DFC490 digital colour camera (Leica Microscopy Systems, Heerbrugg, Switzerland) and the acquisition software LAS AF (Leica Microscopy Systems). Images were assembled in panels using Photoshop 7.0 (Abobe System Software, Dublin, Ireland).

PGC-1α acetylation assay

We analyzed PGC-1α acetylation in tibialis anterior and diaphragm muscles by immunoprecipitation of PGC-1α from 500 μg of protein extract with antibodies to PGC-1α (Santa Cruz, sc-13067, 2 μg per sample) followed by western blot analysis using antibodies to acetyl-lysine (1:2,000, Cell Signaling 9441).

Quantification of miR expression

The total RNA including the miR fractions was isolated using TRIzol reagent (Invitrogen, Life Technologies, Cergy, France) following the manufacturer's instructions.

The quantification of miR-34a was performed using Taqman assays for miR (Applied Biosystems) and Taqman Universal Master Mix (Applied Biosystems). miR levels were quantified using miR-U6B as the housekeeping miR.

Statistical analysis

Values were expressed as means ± standard error of the mean (S.E.M.). The statistical significance of the differences between means was assessed by independent Student's t-test or by one-way ANOVA followed by the Bonferroni post-test to determine which groups were significantly different from the others. A probability of <5% ($P<0.05$) was considered to be significant.

RESULTS

a-Sarcoglycan null mice have a lower mitochondrial content and capacity

Mitochondrial function was assessed as function of oxygen consumption in permeabilized muscle fibers from *Tibialis Anterior* (TA) and diaphragm (DP) of 5 month old a-SG null mice and compared to the respective age-matched wild-type mice. In both dystrophic muscles the respiratory rates, expressed per tissue wet weight and measured with substrates targeting complex I (CI), complex II (CII) and complex IV (CIV), were significant reduced (Fig. 1A). Of interest, real-time quantitative PCR analysis of mitochondrial DNA (mtDNA) revealed a marked reduction of mitochondrial content in both a-SG null muscles (Fig. B). We then expressed the respiratory rates relative to mtDNA copy number and we abolished the differences observed both in TA and diaphragm of dystrophic mice, suggesting that mitochondrial content could be the major determinant of the lower oxidative capacity of a-SG null muscles. However these data also indicate a decreased capacity of the entire respiratory chain, rather than a deficiency of a single complex, but this point will need further investigation, measuring the respiratory capacity of equal amount of isolated mitochondria. This allows us to definitively understand whether dystrophic muscle fibers display lower respiratory function associated with a decreased in mitochondrial content. Consistent with the reduced mtDNA content, the expression of both nuclear encoded mitochondrial proteins (VDAC and COX IV) and mitochondrial encoded protein mtCO1 were significantly lower in a-SG null mice as assessed by immunoblotting on whole TA and diaphragm homogenates and compared to age-matched wild-type.

Altogether these findings indicate that mitochondrial mass is significantly decreased in TA and diaphragm of dystrophic a-SG null mice, probably accounting for their reduced OxPhos capacity.

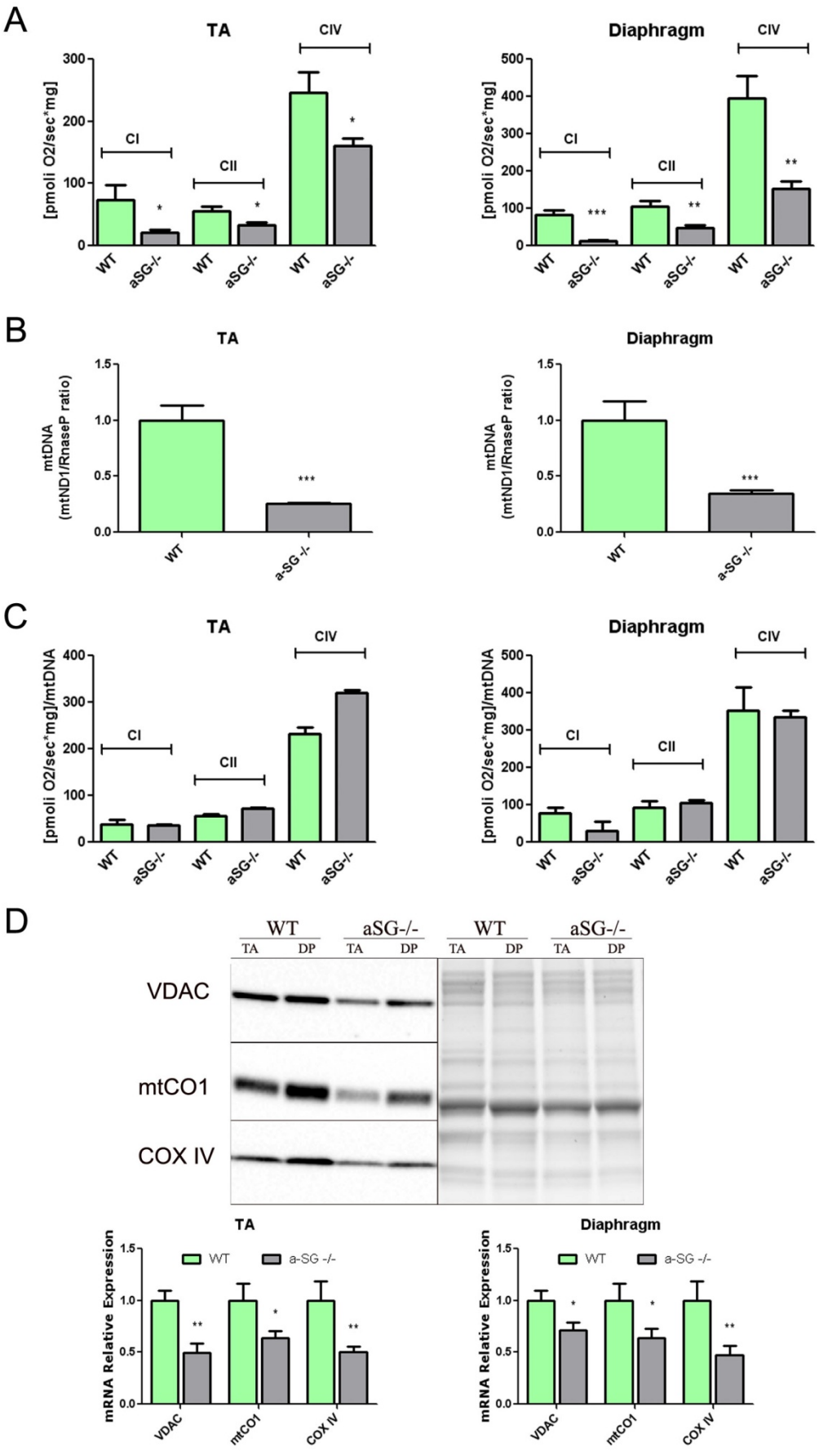
A
TA
CIV
CI
CII
[pmoli O2/sec*mg]
300
200
100
0
WT
aSG-/-
Diaphragm
500
400
B
mtDNA
(mtND1/RnaseP ratio)
1.5
1.0
0.5
0.0
a-SG -/-
C
[pmoli O2/sec*mg]/mtDNA
D
VDAC
mtCO1
COX IV
TA
DP
mRNA Relative Expression
WT
a-SG -/-

Figure 1: a-Sarcoglycan null mice have a lower mitochondrial content and capacity

A) Mitochondrial respiration in permeabilized TA and diaphragm muscle fibers from a-SG null and WT mice (n = 8 per genotype). **B)** Mitochondrial DNA (mtDNA) content (n = 8 per genotype). **C)** Mitochondrial respiration in permeabilized TA and diaphragm muscles fibers normalized for mtDNA copy number. **D)** Western blot analysis of mitochondrial proteins (VDAC; mtCO1 and COX IV) in TA and diaphragm homogenates from WT and a-SG null mice (n= 6 per genotype). The graph shows the densitometric values normalized on the total protein content (right panel) ± SEM.

The low mitochondrial content is a muscle specific defect in a-SG null mice and correlates with the disease progression

To understand whether there is a direct link between the genetic defect (the lack of alpha sarcoglycan from dystrophin complex at the sarcolemma level) and the mitochondrial content we assessed by qPCR the mtDNA copy number in different tissues and we found out that the deficiency in mtDNA content was only present in skeletal muscles (*tibialis anterior* and diaphragm), but not in heart, liver and brown adipose tissue (BAT) (Fig. 2A-B-C-D-E), suggesting that tissues not directly involved in the pathology do not show a mitochondrial damage.

We also analyzed mitochondrial content in muscle during the development (1.5, 3 and 5 months) of wild-type and a-SG null mice and we observed a marked increase in mitochondrial mass in both TA and diaphragm, between 3 and 5 months of age in wild-type mice. Conversely, in both muscles of dystrophic mice we did not observe any increase in mitochondrial mass during the development.

In particular, diaphragm from a-SG null mice showed differences in mitochondrial content starting from 3 months of age, while a-SG-/- TA, at each time considered, exhibited a significant lower mitochondrial content compared to wild-type (Fig. 2F-G).

These data indicate that the mitochondrial content defect correlates with the progression of the disease and the worsening of the muscular phenotype with a much higher impairment at 5 months of age compared to younger mice.

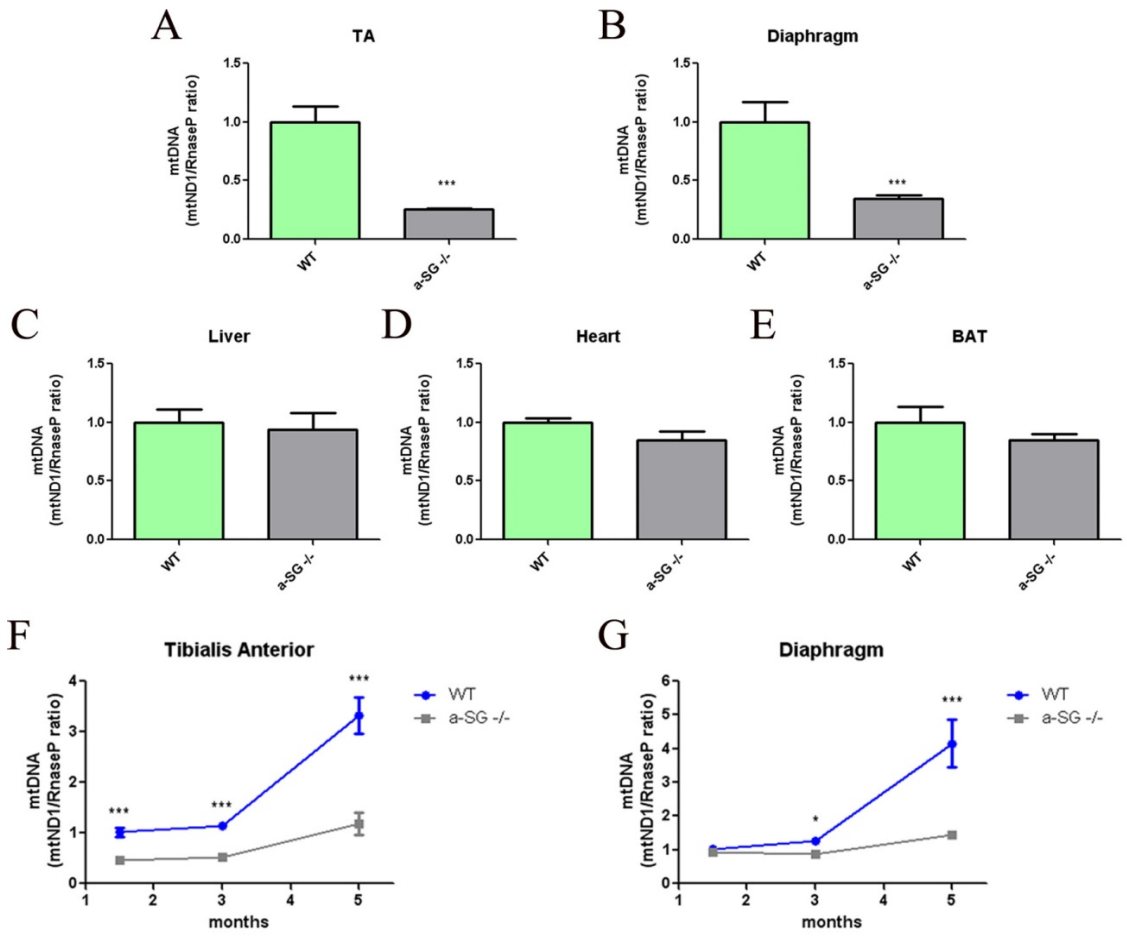

Figure 2: The low mitochondrial content is a muscle specific defect in alpha-sarcoglycan null mice and correlates with the disease progression

A-B-C-D-E) qPCR analysis for mtDNA content in different tissues (n = 5 per genotype). Values are expressed as mean ± SEM. F-G) Mitochondrial content in TA and diaphragm during development (1.5- 3 and 5 months of age). Asterisks indicate statistical significance between WT and a-SG -/- mice at each point considered. Values are expressed as mean ± SEM (n = 4 or 5 per genotype).

In alpha-sarcoglycan null mice the reduced mitochondrial content is the result of induction of mitophagy and repression of mitochondrial biogenesis

Mitochondrial abundance is directly correlated to the coupling between mitochondrial biogenesis and mitochondrial degradation by autophagy.

Autophagy is a self-digestion process that mediates the selective clearance of cytoplasmic components, such as damaged mitochondria (mitophagy) that could otherwise become deleterious.

To explore whether autophagy could be involved in the decreasing of mitochondrial mass we measured different markers. Compared to wild-type mice, TA and diaphragm from 5 months old a-SG null mice showed a significant increase in the lipidated form of microtubule-associated protein-1 light chain 3 (LC3)-II, which is a common marker of autophagy [197]. In agreement, BCL-2/adenovirus E1B–interacting protein-3 (Bnip3), a protein recognized to have a key role in the autophagic removal of mitochondria [198-199], was induced in TA and diaphragm of a-SG null mice. Finally p62, a cargo protein known to be incorporated into autophagosomes [200] and degraded, decreased in both dystrophic muscles (Fig. 3A). Taken together these results provide clear evidence that mitophagy process could be responsible for with the reduction of mitochondrial content likely in order to supply energy.

However, since mitochondrial biogenesis is the other main process directly regulating mitochondrial abundance, we analyzed the relative expression of the master genes involved in the mitochondrial biogenesis pathway. Interestingly the mRNA level of the master gene peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α), as the mRNA levels of nuclear respiratory factor 1 (Nrf1) and mitochondrial transcription factor A (Tfam), were significantly down-regulated in dystrophic muscles. Also the expression level of the mitochondrial gene Cytochrome B (CytB) was significantly decreased in a-SG null mice, suggesting that also the biogenetic process could be repressed in dystrophic condition (Fig. 3B).

To better clarify this point, we wondered whether mitochondrial biogenesis could be enhanced by a well-known inducer such as cold exposure, comparing wild-type and a-SG null mice at 5 months of age. After 72h of cold exposure, mitochondrial content significantly increases in wild-type TA and diaphragm, whereas it was unchanged in a-SG null muscles (Fig. 3D). In agreement with these results, we measured the mRNA levels of several genes involved in the biogenetic process and as expected, PGC-1α, Nrf1, Tfam and Cyt B increased in wild-type TA and diaphragm after 24h of cold exposure, but not in a-SG null mice (Fig. 3C).

Overall our data indicate that a-SG null muscles display a defective mitochondrial biogenesis process that in combination with the increased mitophagy explains the marked reduction of mitochondrial mass a-SG null mice.

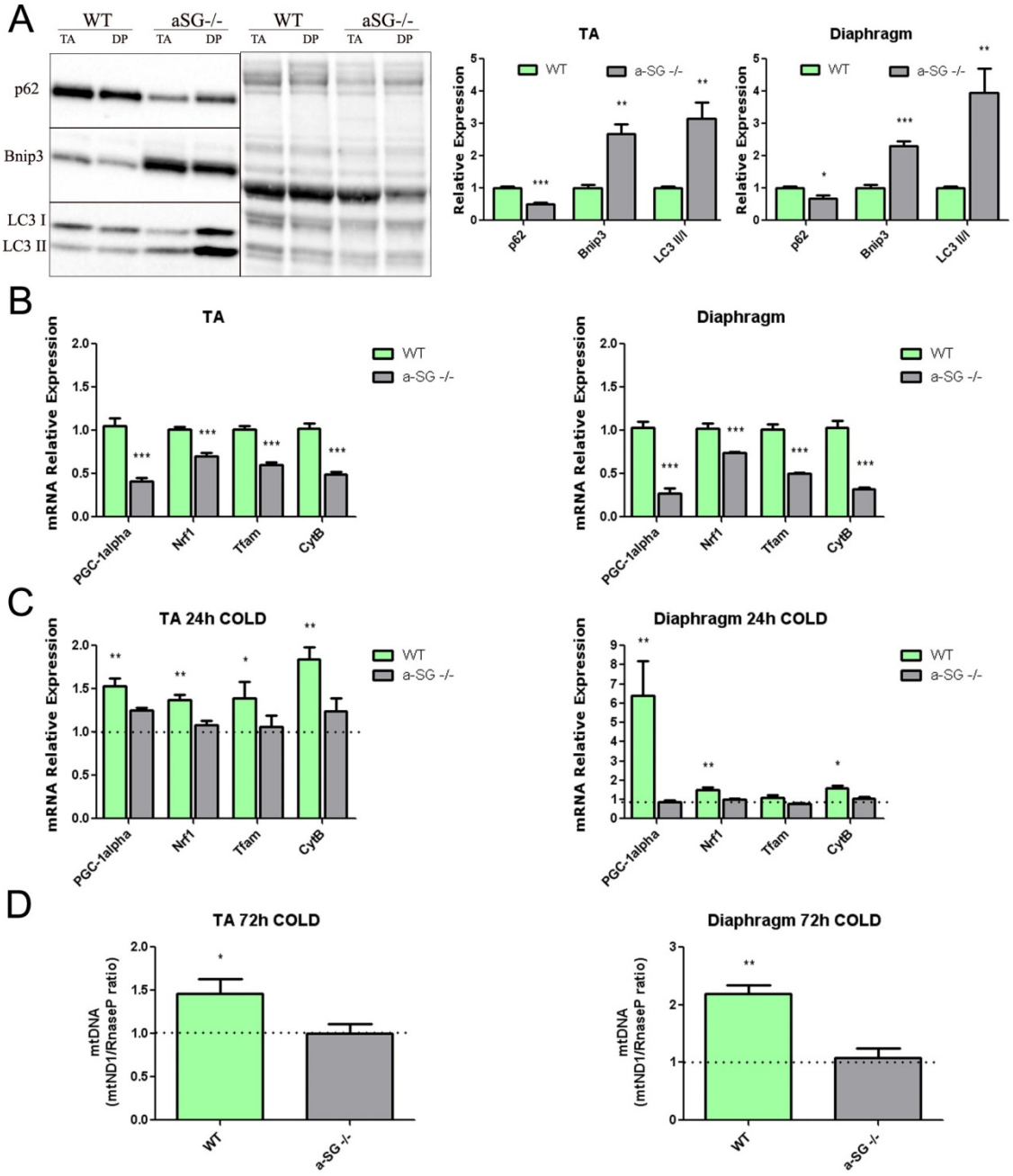

Figure 3: In alpha-sarcoglycan null mice the reduced mitochondrial content is the result of induction of mitophagy and repression of mitochondrial biogenesis

A) Representative immunoblot of muscle homogenates from WT and a-SG null mice (50 µg per lane). The graphs shows the densitometric values of p62 and Bnip3 normalized on the total protein content (right panel) ± SEM. Values for LC3 represent the ratio LC3II/LC3I ± SEM (n = 6 per genotype). B) RTqPCR analysis of mitochondrial biogenesis pathway genes in TA and diaphgram. Values are expressed as mean ± SEM (n = 8) C) RTqPCR analysis of mitochondrial biogenesis pathway genes in TA and diaphgram after 24 hours of could exposure (4°C). The graphs represent the fold induction of stimulated mice over basal condition. (n= 5 per genotype) D) qPCR analysis for mtDNA content after 72 hours of cold exposure. The graphs represent the fold induction of stimulated mice over basal condition. (n= 5 per genotype).

NO-donor Molsidomine modulates mitochondrial function in a-SG null mice

In a previous work we demonstrated the positive action of long-term treatment (at 5 and 9 months) with molsidomine on muscle function and recovery in the a-SG null mice. The NO-donor molsidomine is converted *in vivo* to its active metabolite SIN-1 and enhances significantly voluntary and forced motor activities, indicating that functional recovery of the muscle has taken place [1].

Since improved muscle function and endurance can result from activation of slow oxidative program [201] we measured whether molsidomine treatment of a-SG null mice for 5 months, could affect mitochondrial functions improving OxPhos capacity. Permeabilized muscles fibers from TA and diaphragm of treated a-SG null mice showed an increased respiratory capacity expressed per tissue wet weight and measured with substrates targeting complex I (CI),complex II (CII) and complex IV (CIV), compared to untreated a-SG null mice (Fig. 4A). In agreement with this, mitochondria from TA and diaphragm of treated a-SG null mice supplemented with CI substrates, produced more ATP over the time compared to mitochondria from untreated dystrophic mice (Fig. 4B). This shift toward more oxidative metabolism in molsidomine-treated a-SG null mice occurred in absence of changes in mitochondrial density in both muscles assessed by qPCR for mtDNA copy number (Fig.4C).

Next, we evaluated whether molsidomine could promote fiber type switching, assessing the contractile phenotype of muscle fibers. We quantified two markers of slow-twitch fibers (myosin heavy chains type I and type IIa) by immunofluorescence in TA and we observed a positive trend for the expression of type I, and a significant increase for type IIa myosin heavy chains (MyHC) in molsidomine-treated a-SG null mice. In addition the succinate dehydrogenase (SDH) staining revealed that the proportion of blue-stained oxidative fibres was higher in TA of treated a-SG null mice compared to untreated dystrophic mice (Fig. 4D). Interestingly this switch correlated with an increased expression of genes which promote the use of fatty acid over glucose; for instance molsidomine treatment enhanced the expression of medium and long-chain acyl- CoA dehydrogenases (MCAD and LCAD) and pyruvate dehydrogenase kinase 4 (PDK4) in both TA and diaphragm of a-SG null mice.

Therefore molsidomine induces a switch toward more oxidative muscle fibers mostly triggering fatty acid oxidation rather than improving mitochondrial function *per se*.

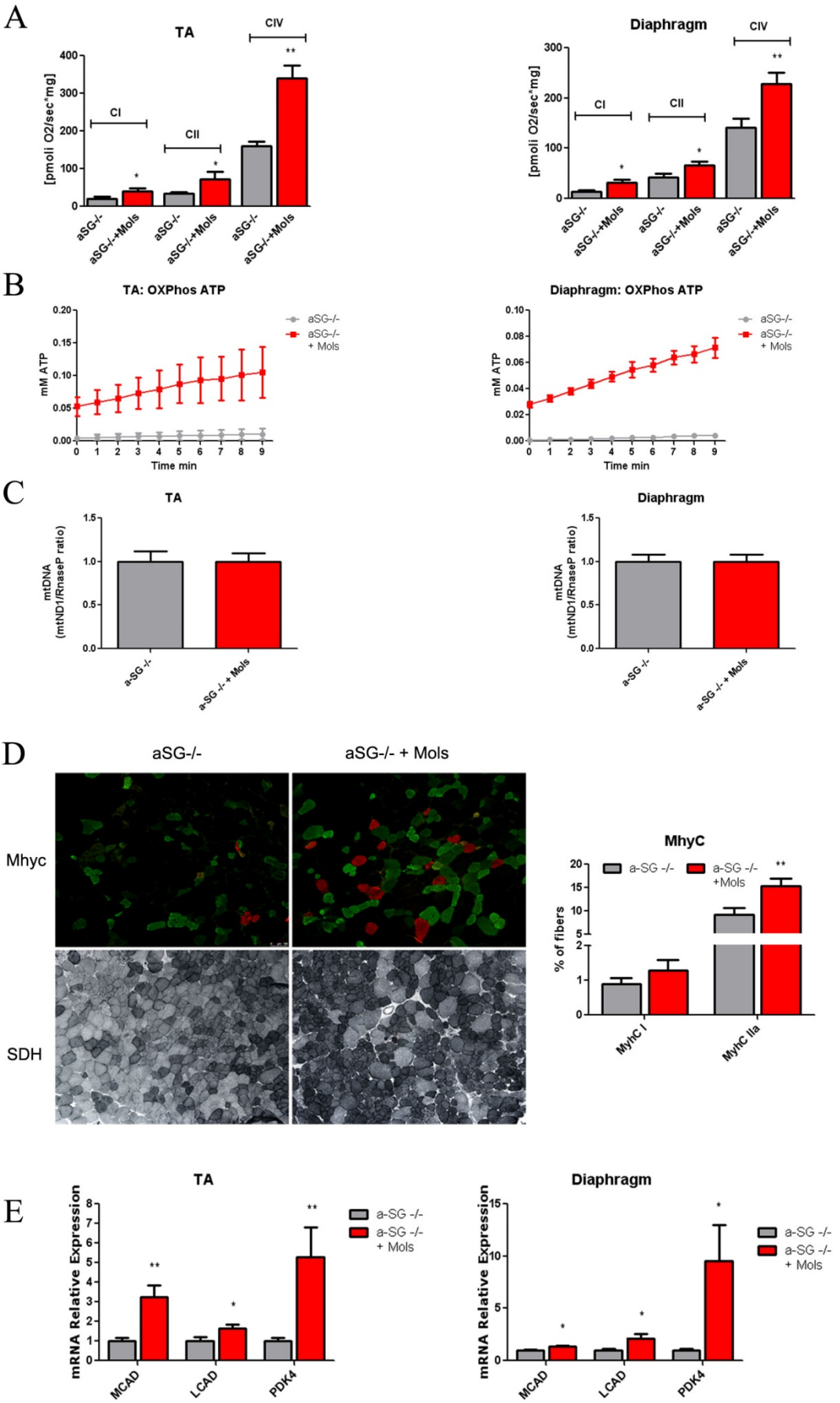
A
TA
CIV
CI
CII
[pmoli O2/sec*mg]
aSG-/-
aSG-/-+Mols
Diaphragm
B
TA: OXPhos ATP
Diaphragm: OXPhos ATP
aSG-/-
aSG-/- + Mols
mM ATP
Time min
C
TA
Diaphragm
mtDNA (mtND1/RnaseP ratio)
a-SG -/-
a-SG -/- + Mols
D
aSG-/-
aSG-/- + Mols
Mhyc
SDH
MhyC
a-SG -/-
a-SG -/- +Mols
% of fibers
MyhC I
MyhC IIa
E
TA
Diaphragm
mRNA Relative Expression
a-SG -/-
a-SG -/- + Mols
MCAD
LCAD
PDK4

Figure 4: NO-donor Molsidomine modulates mitochondrial function in a-SG null mice
A) Mitochondrial respiration in permeabilized TA and diaphragm muscle fibers from a-SG null mice untreated or treated with molsidomine (n = 8 per group). **B)** OxPhos ATP production over the time (10 minutes) in mitochondria isolated from both TA and diaphragm of dystrophic mice (treated or untreated with molsidomine) (n = 5 per group). **C)** Mitochondrial DNA content after molsidomine treatment in dystrophic mice (n = 8 per group). **D)** Histological analysis of a-SG KO *tibialis anterior* before and after molsidomine diet. Representative MyhCs immunostaining (upper panel) and SDH staining (lower panel). MyhC I (red) and MyhC IIa (green) were counted and their percentage of total number of fibers was calculated in the graph. Values are expressed as mean ±SEM. (n= 6 per group) **E)** RTqPCR analysis of fatty acid oxidation pathway genes in TA and diaphgram. Values are expressed as mean ± SEM (n = 8).

Molsidomine affects muscle metabolism through Sirt1 modulation

We aimed to gain insight into the molecular mechanism of nitric oxide action on mitochondrial metabolism observed in a-SG null mice. It has been reported that PGC-1α has a critical role in the control of mitochondrial OxPhos capacity and this cofactor is able to functionally convert fast-twitch fibers into more oxidative slow twitch fibers [160,202] , coupling the metabolism with the contractile apparatus of muscle fibers, therefore we examined whether it is involved in the effects of molsidomine in a-SG null muscles.
PGC-1α is known to be tightly controlled at transcriptional and posttranscriptional levels and in our system the mRNA levels of PGC-1α did not change after 5 months of molsidomine treatment in a-SG null mice, in both TA and diaphragm (Fig. 5A). Posttranslational modifications such as acetylation negatively correlate with PGC-1α activation and when we carried out an immunoprecipitation assay to evaluate the aceylation status of the protein we found out high levels of acetylated form of PGC-1α in a-SG null muscles compared to wild-type mice. After molsidomine treatment PGC-1α acetylation levels decreased at basal level in both TA and diaphragm, leading to enhanced protein activity (Fig. 5B and 5C).
PGC-1α is an established substrate of Sirt1, in particular Sirt1 associates with PGC-1α triggering its deacetylation [203] and considering our results we assessed Sirt1 in our model. We measured Sirt1 mRNA levels in TA and diaphragm comparing molsidomine treated and untreated a-SG null mice and we observed a significant increase of Sirt1 expression after molsidomine treatment in both muscles (Fig. 5D), likely suggesting Sirt1 as target of NO action, thus modulating PGC-1α activation and mitochondrial function.

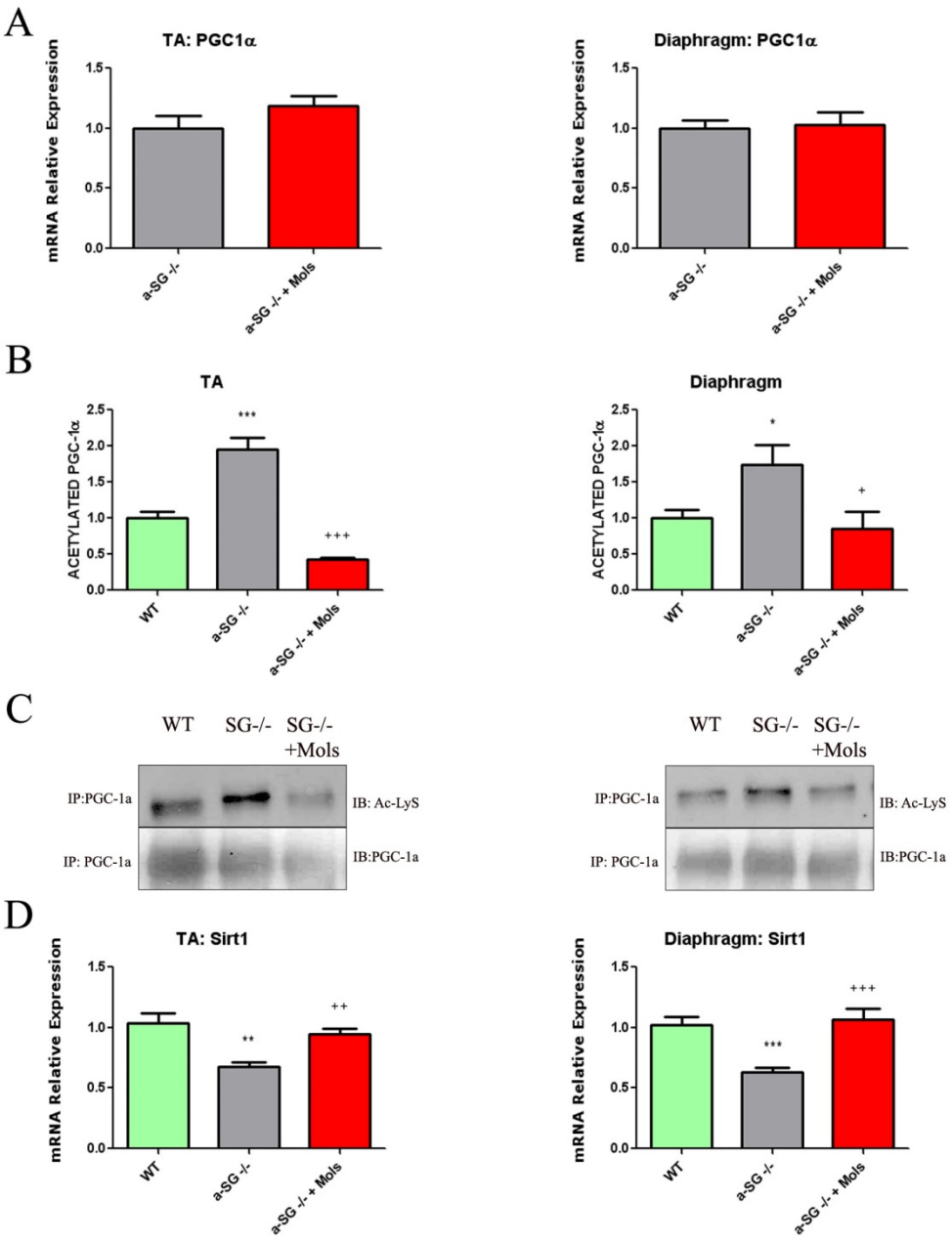

Figure 5: Molsidomine affects muscle metabolism through Sirt1 modulation

A) RTqPCR analysis of PGC-1α on mRNA extracted from TA and diaphgram of dystrophic mice (untreated or treated with molsidomine). Values are expressed as mean ± SEM (n = 8 per group). **B-C)** PGC-1α acetylation. Immunoprecipitation followed by western blotting analysis showing acetylation levels of PGC-1α in lysine residues compared to total PGC-1α in both TA and diaphragm (C). The graph represents the quantification as mean ± SEM in WT, a-SG-/- and a-SG-/- mice after molsidomine treatment. (*n* = 4 per group). **D)** RTqPCR analysis of Sirt1 on mRNA extracted from TA and diaphgram of WT mice and dystrophic mice (before and after molsidomine treatment).

Sirt1 modulation by nitric oxide is independent on miR-34a and dependent on AMPK

Published data show that Sirt1 expression can be modulated by microRNAs that can bind Sirt1 mRNA preventing its translation. In particular miR-34a negatively regulates Sirt-1 expression, increasing acetylation of its target proteins [204]. To test whether an aberrant expression of miR-34a correlated with a repression of Sirt1 in a-SG null TA and diaphragm, we determined the expression of miR-34a in both muscles but we found out a significant increase in a-SG-/- mice compared to wild type animals only in TA (Fig. 6A), whereas in diaphragm there was no difference between the two experimental groups (Fig.6B). Moreover molsidomine was not able to significantly decrease the miR expression in dystrophic muscles, indicating that the molsidomine-dependent increase of Sirt1 expression and function was not due to the modulation of miR-34a by nitric oxide (Fig. 6A and 6B).

To definitively exclude the role of miR-34a as a negative regulator of Sirt1 expression in dystrophic muscles, miR-34a levels were down-regulated by injecting the specific antagomiR intramuscular into the right TA (of 5 months old) a-SG null mice and Sirt1 mRNA level was examined afterward. After 4 injections of antagomiR, qPCR analysis demonstrated that miR-34a levels were diminished by 50% in right TA compared to the controlateral one injected with scramble oligomiR (Scramble) (Fig. 6C). Nevertheless antagomiR injection was not able to modulate Sirt1 expression, confirming that miR-34a is not responsible for the modulation of Sitr1 expression in a-SG null mice (Fig. 6D).

However adenosine monophosphate–activated protein kinase (AMPK) has been described as important activator of Sirt1 expression [205] and function [206]. Furthermore AMPK is known to be activated by NO in endothelial cells [207] thus we can hypothesize a role of AMPK in the NO-dependent Sirt1 activation in a-SG null mice.

Since the phosphorylation of threonine 172 in the α-subunit strongly correlates with the AMPK activity [208] we analyzed the phosphorilation status of AMPK in Molsidomine-treated a-SG-/- mice compared to untreated mice.

We found out a significant increase in AMPK phosphorilation after molsidomine treatment, suggesting that AMPK was the principal mediator of the NO-dependent effects on fiber type switching, improving mitochondrial function in a-SG null mice (Fig. 6E).

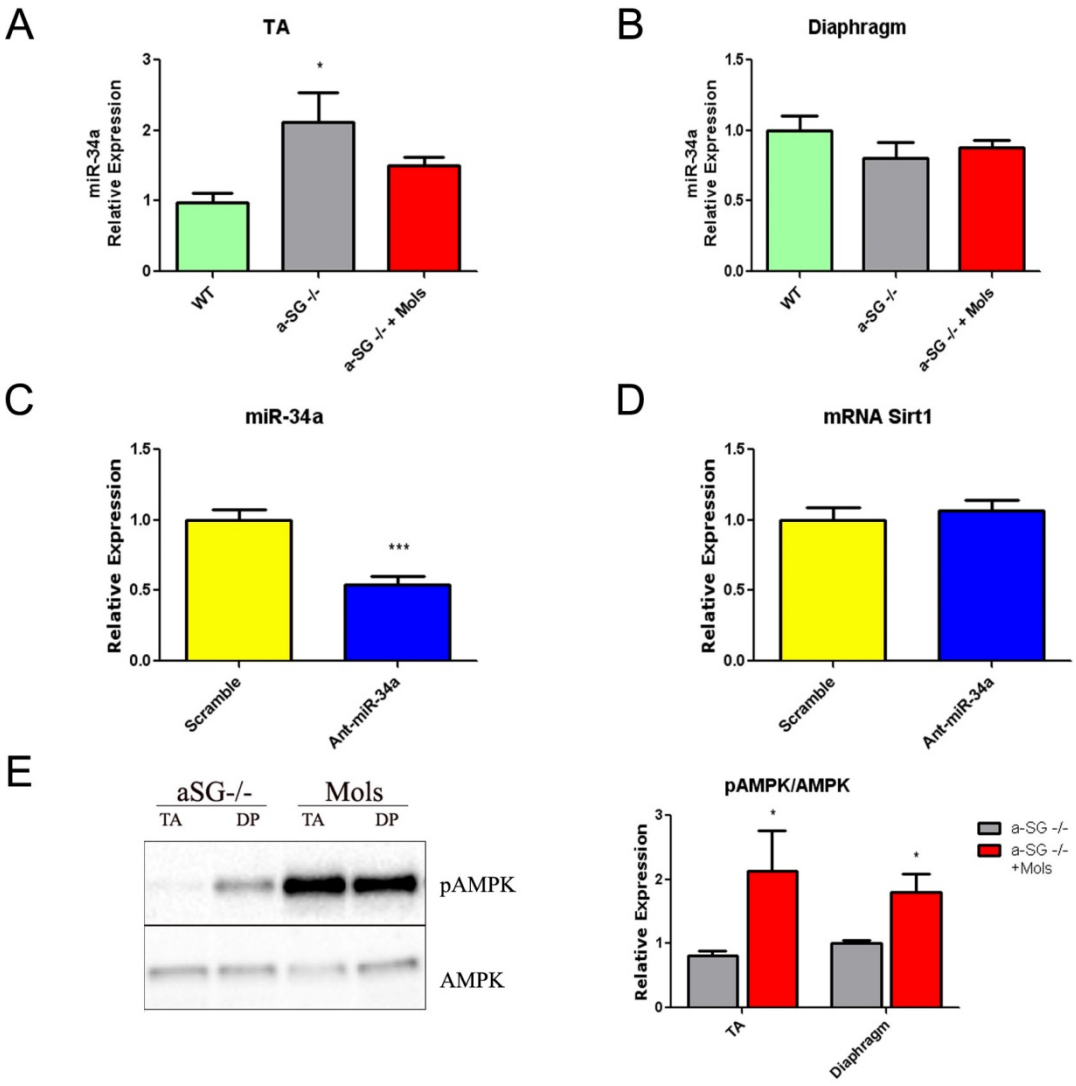

Figure 6: Sirt-1 modulation by nitric oxide is independent on miR-34a and dependent on AMPK

A-B) miR-34a expression levels analyzed in TA and diaphragm muscles of WT, a-SG-/- and a-SG-/- treated mice by quantitative PCR. Values of a-SG-/- mice (from untreated or form treated mice) were both normalized with respect to values of aged-matched WT mice (means ± SEM; *n* = 9 for each group). **C-D)** TA muscles of a-SG-/- mice were injected with antagomir for miR-34a (Ant-miR-34a) or with Scramble oligomiR (Scramble). qPCR analysis for miR expression (C) and for Sirt1 expression (D). Values are expressed as mean ± SEM (n = 9). **E)** Representative immunoblot for p-AMPK (Thr172) and total AMPK in TA and diaphragm of WT and a-SG KO mice. The graph shows the ratio pAMPK/ total AMPK and the values are expressed as mean ± SEM (n = 6).

DISCUSSION

In this study we describe, for the first time, abnormalities in mitochondrial content and function in skeletal muscles of a mouse model of Limb Girdle muscular dystrophy 2D.
Mitochondrial abnormalities have been reported for several different types of muscular dystrophy, even those not caused by mutations in mitochondrial DNA or in genes coding for mitochondrial proteins. The most relevant to our study is the documentation of a general metabolic crisis in dystrophic muscles of Duchenne and Limb Girdle 2D patients, with large scale down-regulation of nuclear-encoded mitochondrial gene expression [186].
In this case the mutated genes did not have a direct or obvious involvement in the mitochondrial metabolism, indicating that mitochondrial abnormalities could be considered as a secondary feature of many different types of muscular dystrophy.
Taking into advantage by the utilization of a mouse model of LGMD-2D (the a-sarcoglycan null mouse), we demonstrate a severe reduction the in OxPhos capacity of both *tibialis anterior* and diaphragm muscles of dystrophic compared to the respective age-matched wild-type mice. Since both muscles of a-sarcoglycan null mice show a reduced mitochondrial content than wild-type animals we hypothesize that the lower mitochondrial mass is the major determinant for the reduced OxPhos capacity of these muscles. This idea is supported by the fact that when we normalize the respiratory rates relative to mtDNA, instead of tissue wet weight, we eliminate the differences observed both in TA and diaphragm. However, to definitively exclude any defect in the respiratory chain complexes it will be necessary the analysis of oxygen consumption starting from equal amounts of isolated muscle mitochondria.
Taking into account the data reported above we decided to focalize our attention on the severe mitochondria depletion observed in the dystrophic model, and we observed an important correlation between the progression of the disease and the mitochondrial content that progressively decrease in a tissue specific manner.
It has been well established that mitochondrial abundance is directly regulated by two opposite pathways that cooperate to maintain an appropriate mitochondrial mass. These pathways are mitochondrial biogenesis and mitochondrial degradation by autophagy.

In spite of many papers that have documented an autophagy impairment in different models of muscular dystrophy (DMD, UCMD and Emery-Dreyfuss muscular dystrophy EDMD) [197,209-210], we demonstrate an activation of mitophagy in a-SG$^{-/-}$, as established by the induction of BNIP3 that is a clear marker of autophagic removal of mitochondria. This data, together with the analysis of respiratory chain function, support the idea that even if they are few, mitochondria of knock-out muscles are still functional and that up-regulation of mitophagy is a mechanism aimed to preserve their function.

But increased autophagy is not the only mechanism responsible for the low mitochondrial content in a-SG null mice. We also show a persistent impairment of the mitochondriogenesis pathway driven by PGC-1α co-activator in muscular dystrophy. The mRNA expression levels of all the genes involved in mitochondrial biogenesis are in fact reduced in a-SG null muscles and not even a well known activator of the pathway, such as cold exposure, is able to stimulate the process in knock-out mice as instead occurs in wild-type animals.

Taking into account that nitric oxide has been described as an important regulator of mitochondrial biogenesis in mammals [167] and that NO donors have been explored as new therapeutics for muscular dystrophy, we treated dystrophic mice with the NO-donor molsidomine to evaluate a possible impact on mitochondrial metabolism. Recent papers have demonstrated the ability of the NO-donor molsidomine to ameliorate the dystrophic phenotype in mouse models of DMD and LGMD, acting at different stages. The drug was able to stimulate satellite cells proliferation and self-renewal ability [1], to modulate the innate inflammatory response [103] and to regulate fibro-adipogenic precursors (FAPs) fate through inhibition of their differentiation into adipocytes [102], but the role of molsidomine on mitochondrial metabolism have not been investigated yet.

Surprisingly, our data show that NO-donor molsidomine fails to induce mitochondrial biogenesis but exerts a beneficial effect on muscle metabolism stimulating a switch toward more oxidative muscle fibers that are more resistant to contraction-induced damage. In particular treated mice show an increased respiratory capacity associated with increased of oxidative ATP production, compared to untreated controls both in absence of induction of mitochondrial biogenesis. The analysis of fiber type composition, by immunofluorescence for myosin heavy chains and by SDH staining, demonstrates that NO treatment triggers a fiber switch toward more oxidative phenotype. Our data are supported by several papers in which a fast to slow fiber type shift ameliorates the

dystrophic phenotype and therefore can be considered a rescue mechanism and a potential target for therapeutic intervention [201,211].

Unlike the studies mentioned above, our drug promotes the shift toward an oxidative phenotype controlling the oxidation of fatty acids rather than stimulating the mitochondrial functionality *per se*. Molsidomine induces the mRNA expression of MCAD, LCAD and PDK4, all genes promoting the flux of fatty acid and their utilization to produce energy.

It has been demonstrated that PGC-1α deacetylation, but not its upregulation, is required for activation of mitochondrial fatty acid oxidation genes program [155] so we hypothesize a role of molsidomine in deacetylating PGC-1α thus driving the oxidative fibers switch.

At the molecular level we find out that Molsidomine modulates PGC-1α not directly increasing its mRNA and expression, but modifying its acetylation status and activity. Our data indeed demonstrates that nitric oxide primarily regulates the expression level of the deacetylase Sirt1 which in turn controls PGC-1α acetylation status.

Sirt1 is an evolutionarily conserved NAD^+-dependent deacetylase that controls both energy homeostasis and metabolic adaptations [212] and functions to counteract metabolic and age-related disease. Of interest we demonstrate an up-regulation of mRNA level of Sirt1 in molsidomine treated muscles. Despite the extensive studies of Sirt1 downstream effectors, the upstream regulatory network is relatively less understood. A very important mechanism regulating Sirt1 expression is the control by microRNAs and it has been described that miRNA-34a binds to the 3'-untranslated region in its mRNA and represses its translation [204]. Nevertheless when we assess for miR-34a deregulation in our model, we find out the absence of any NO-dependent control of miR-34a expression, furthermore *in vivo* the down-regulation of miR-34a does not have any effect in the modulation of Sirt1 expression.

Looking for other modulators of Sirt1 expression, we pay particular attention on AMPK activity since it has been published that a chronic AMPK activation was able to drive the slow, oxidative myogenic program, triggering beneficial adaptations in *mdx* mouse model [201]. AMPK is an energy sensor that is activated when the cellular ratio of AMP to ATP increased [213] and it is also known to be activated by NO in endothelial cells [207]. Also, AMPK has been described as an important activator of Sirt1 expression [205] and function [206]. Our results show a significant increase of AMPK phosphorilation after molsidomine treatment, identifying AMPK as the target of NO thus increasing Sirt1 expression.

In agreement with a very recent paper that demonstrated how Sirt1 over-expression in muscle could reverse the dystrophic phenotype of *mdx* mice [214], our data suggest that the NO-based therapy with molsidomine is a pharmacological approach that combine the multiple beneficial effect already described on different cell populations to a prominent action on muscle metabolism, inducing a therapeutic fiber switch toward a more oxidative types. Improving oxidative capacity nitric oxide is able to counteract the low mitochondrial content and the persistent impairment of mitochondrial biogenesis in dystrophic muscles, ameliorating the pathological phenotype of a-SG null mice.

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