UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Molecular and Cellular Biology
XXX Cycle

“Study of oxidative stress and antioxidant response in the *Sgca* null dystrophic mouse model”

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PhD Thesis

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Academic year: 2017-2018

SSD: BIO/17

Thesis performed at the Department of Biosciences of Università degli Studi di Milano.

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Part I

During my PhD, I worked on a project focused on unravelling the role of oxidative stress during muscular dystrophy progression. To study this phenomenon, I took advantage of a dietary natural antioxidant, known as cyanidin, supplied to dystrophic mouse model *Sgca* null (Duclos et al.,1998). This study resulted in a manuscript (Part II) soon to be submitted. The use of a diet enriched in cyanidin helped us firstly to demonstrate that this compound is able to delay muscular dystrophy progression morphologically, but most importantly, functionally; secondly to identify the molecular pathways tuned by this natural antioxidant. Specifically, we demonstrated that cyanidin is able to promote the nuclear localization of Nrf-2, the master antioxidant transcription factor in mammals, through AMPK activity. We also investigated on the anti-inflammatory properties of this treatment, discovering that cyanidin is able to retain NF-kB in the cytoplasm modulating ERK activity.

Also, I collaborate with Andrea Barbuti's laboratory on a work aimed to study the role of miRNA in cardiac myocytes in a chronically exercised mouse model. I focus, in particular, on the role played by the mitochondrial homeostasis transcription factor PGC-1α.

Summary

During my PhD, I mainly focus on the of dietary antioxidant and the role of oxidative stress in the progression of Muscular Dystrophy (MDs). MDs are genetic human diseases which are hallmarked by a progressive muscle wasting of variable severity, in the most severe cases this condition leads patients to wheelchair life and premature death due to respiratory and cardiac failure (Emery 2002). Mutations, in these pathologies, mainly affect the Dystrophin-associated Glycoprotein Complex (DGC); this multiprotein complex is located in the myofiber sarcolemma and links the fibers to the extracellular matrix conferring stability to fiber structure. The absence or the malfunction of the DGC leads to myofibers instability, which leads to fibers death and in time compromise muscle functionality. In the most severe cases MD patients would die of respiratory and cardiac failure.

Nowadays there is no definitive treatment for MDs that can cure the root of the pathology, although among the different approaches, many efforts are directed to slow down the progression of the disease to counteract the progressive degeneration and to improve patients life quality (Cossu & Sampaolesi 2007).

It is now very well established that the DGC not only plays a structural role for the myofiber stability, but also its stretch during contraction is essential for the activation of important signalling pathways. In fact, in literature is known that accumulation of reactive oxygen species (ROS) and oxidative stress contribute strongly to the worsening of MDs, suggesting that muscles affected by these diseases display an impairment in antioxidant signalling (Rando 1998; Rando 2002). In this study, we show that an cyanidin enriched diet is able to delay MD progression in the dystrophic mouse model *Sgca* null. In particular we display a morphological amelioration of muscle tissue organization, more fiber stability and rescue of muscle performance.

Moreover, the antioxidant diet is able to interfere with the proinflammatory environment, typical of these pathologies. Specifically, cyanidin impairs NF-kB translocation into the myonuclei, and prevent the expression of typical pro-inflammatory genes such as *TNF-*α and *iNOS*.

Furthermore, we observe an increase of the antioxidant response in dystrophic mice fed with this particular diet. We found that the transcriptional levels of antioxidant genes (i.e. *HO-1* and *GCLC*), in this scenario, are increased through the activity a specific transcription factor known as Nrf-2. We investigate on the signalling pathway that promote Nrf-2 nuclei localization, finding that AMPK activity is the crucial factor.

Introduction

Heterogeneity of skeletal muscle

In order to fulfil different tasks, e.g. continuous low-intensity activity (posture), repeated submaximal contraction (movement) or strong and fast contraction (jumping) the muscle fibers display phenotypically, morphologically, metabolically and at the molecular level an intrinsic heterogeneity. These features are due to the expression of four different isoforms of myosin heavy chain (MyHC): MyHC-1, MyHC-2a, MyHC-2x and MyHC-2b, therefore muscle fibers are classified into four major groups (table 1) (DeNardi et al., 1993; Chakkalakal et al., 2012). The presence of a specific MyHC isoform lends to the fibers specific characteristics in terms of power and speed of shortening of each muscle; both features decrease in order MyHC-1<MyHC-2a<MyHC-2x<MyHC-2b (Raiser et al., 1985; Bottinelli et al., 1996). This commonly leads to subdivide the fibers in slow-twitching or type I fibers, which usually express MyHC I isoform; and fast-twitching fibers expressing MyHC-2a, -2x and -2b isoforms (Schiaffino and Reggiani, 2011).

Furthermore, fast and slow-twitching fibers differ in metabolic properties and mitochondrial content. Slow fibers are able to generate a great amount of ATP by oxidative metabolism via mitochondria and the ATP consumption rate is not very high during contraction, these allow them to extended contractile activity (Schiaffino and Reggiani, 2011).

On the other side, fast-twitching fibers produce ATP very quickly through glycolytic metabolism, generating a limited amount of ATP and a limited duration in contraction (Schiaffino and Reggiani 2011). Based on these metabolic features, myofibers can be divided into slow-oxidative (S), fast-oxidative-glycolytic (FOG) and fast-glycolytic (FG) fibers (Ashomor et al., 1972; Peter et al., 1972). The S fibers are usually type I fibers, expressing MyHC-I isoform, and display a great antioxidant complement to counteract the mitochondrial generation of reactive oxygen species (ROS), mainly resulted by the oxidative phosphorylation. On the contrary FG fibers, usually type 2x fibers and 2b, express respectively MyHC-2x and 2b isoforms, display more glycolytic

enzyme content. These fibers show less expression of antioxidant enzyme conveying them to more sensitivity to oxidative stress and ROS concentration (Schiaffino and Reggiani 2011).

	TypeI fibers	Type 2a fibers	Type 2x fibers	Type 2b fibers
Contraction time	Slow	Medium	Fast	Very fast
Resistance to fatigue	High	Medium	Intermediate	Low
Activity used for	Aerobic	Long-Term anaerobic	Short-term anaerobic	Short-term anaerobic
Maximum period of use	Hours	<30 min	<5 min	<1 min
Power produced	Low	Medium	High	Very high
Mitochondrial density	High	Intermediate	Low	Low
Oxidative capacity	High	Intermediate	Intermediate	Low
Glycolytic capacity	Low	High	High	High
Myosin heavy Chain expression	Myh7	Myh2	Myh1	Myh4

Table 1: Characteristics of muscle fiber type

Muscular Dystrophies

Muscular dystrophies (MDs) are a group of myogenic disorders, which have all in common the progressive muscle wasting and weakness of variable distribution and severity. This leads in time to different degrees of mobility limitation, heart and respiratory failure (Emery, 2002).

Muscular dystrophies can be divided in different groups, according to the distribution and severity of the phenotype. Most of them are due to mutations in genes encoding proteins belonging to the Dystrophin-associated Glycoprotein Complex (DGC). This multiprotein complex is located in the myofiber sarcolemma and links the fibers to the extracellular matrix conferring

stability to fiber structure (Figure 1A). The absence or the malfunction of the DGC leads to myofibers instability, event that promotes myofibers death and in time compromise muscle functionality.

Histologically, MDs are characterized by variation in fibers calibre, necrotic and apoptotic fibers, chronic inflammation and great macrophages infiltration (Emery et al. 2002). All these events activate the stem cell poll of the adult muscle tissue, called Satellite Cells (SCs; Mercuri & Muntoni 2013), which differentiate in newly regenerating muscle fibers. Since these cells share the same mutation, they will develop in fragile myofibers failing the regenerative process, thus driving the muscle homeostasis in a loop of regeneration-degeneration till the exhaustion of the stem pool (Mercuri & Muntoni 2013) (Figure 1B). The musculature is, therefore, in time replaced by adipose and connective tissue (fibrosis) compromising its functionality (Emery 2002; Mercuri & Muntoni 2013) (Figure 1C).

The most common form of MD is the Duchenne Muscular Dystrophy (DMD). DMD is an X-linked pathology, which mutation leads to the absence of the sarcolemmal protein dystrophin, with an incidence at birth about $300x10^{-6}$ and a prevalence in the total male population around $60x10^{-6}$ (Bushby et al. 2010; Gee et al. 2017). As DMD is the most common muscular dystrophy, great efforts have been done to study this pathology, through generating transgenic mice models. The most commonly used model, the *mdx* mice, is characterized by the most common mutation in the *dystrophin* gene observed in humans. Unfortunately, this model does not recapitulate properly the phenotype of the human pathology, showing a milder phenotype. This phenomenon could be ascribed to some difference between mice and humans, such as the compensation effects by utrophin, a dystrophin homologous, and the longer telomeres, which are present in mice compared to humans one (Deconick et al., 1997; Grady et al., 1997; Sacco et al., 2010).

In order to overcome this issue, novel models of MD have been generated, which better resemble the human phenotype.

Limb-girdle muscular dystrophy is an autosomal recessive disease characterized by a weakness that affects mainly the proximal limb-girdle

musculature (Darin & Tulinius, 2000). The α-sarcoglycan is part of transmembrane proteins, which together with β, γ, and δ take part of the DGC. This complex connects the cytoskeleton of the myofibers to the external cellular matrix, conferring sarcolemmal stability, but also the DGC is essential for triggering specific pro-survival signalling pathways (Durbeej & Campbell, 2002; Rando et al.; 2002).

The *Sgca* deficient mouse model develops progressive muscular dystrophy, with a severe phenotype showed by muscle necrosis with age, a hallmark of the human disease. Furthermore, this model displays a loss of sarcolemmal integrity, chronic inflammation at the muscle site recalling macrophage population, collagen and fat infiltration, and changes in terms of muscle performance (Duclos et al.; 1998).

When the aim of the study is not to restore genetically the *dystrophin* expression, models such as the *α-sarcoglycan* null mouse (a mouse model for limb-girdle muscular dystrophy 2D, *Sgca* null, Duclos et al.; 1998), are more indicated. In this study, we are interested in the effects of an antioxidant-enriched diet on the dystrophic phenotype, in the study we decided to use the *Sgca* null mouse model since better recapitulates the human pathology.

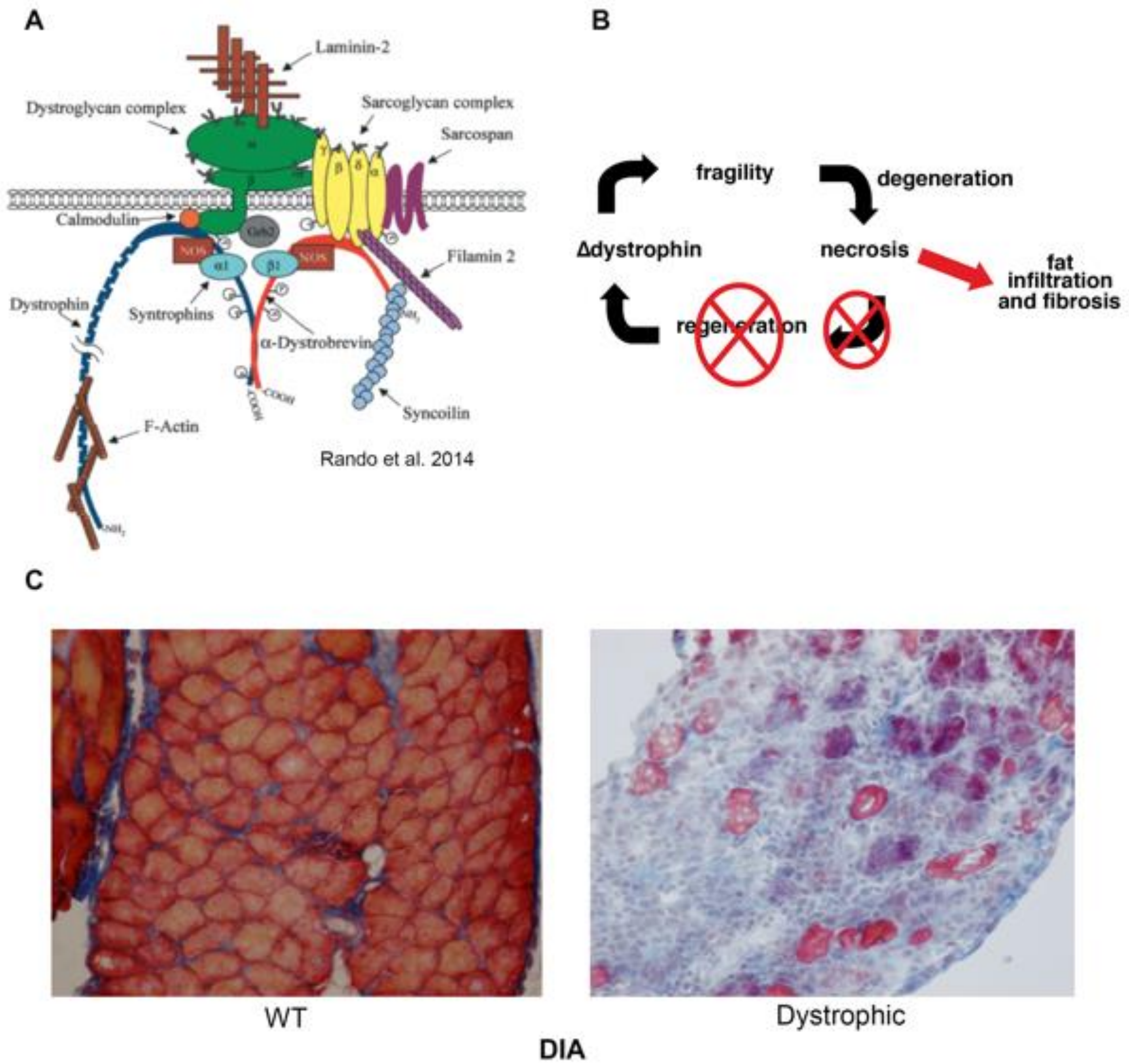

Figure 1 (A) Scheme of Dystroglycan complex. **(B)** Brief scheme of degenerative/regenerative cycles occurring during MDs onset. **(C)** Representative picture of wild type (WT) and dystrophic extra cellular matrix deposition in the diaphragm.

The state of art of therapeutic strategies for Muscular Dystrophies

Muscular Dystrophies and in particular DMD affects about 1:350 males, which are born apparently normal, but start to show symptoms around the first decade of life losing walking ability. Patients become, in time, dependent on wheelchair and aspiration around their 20s, and usually do not survive past the age of 30s caused by cardiac and respiratory failure (Bushby et al. 2010; Gee et al. 2017).

In these patients, as said above, lacking of functional dystrophin causes myofibers instability and cell death; overtime muscle fibers are replaced by connective and adipose tissue losing their primary function. Nowadays, typical therapies for DMD patients include corticosteroids treatment that can only delay the progression of this pathology and improve muscle force; however, several side effects are reported in literature including behavioural changes, fractures, cataracts, weight gain and cushingoid appearance (Mendell et al., 1989; Biggar et al., 2006; Gloss et al., 2016).

Unfortunately, up to date there are no successful therapeutic approaches to treat the root cause of these pathologies. Beside the drug therapy, there are several different approaches under investigation that aim specifically to treat the genetic defect.

Gene therapy

Gene replacement

Gene replacement aims to replace the defective dystrophin gene, delivering the physiological form through adeno-associated virus vector (AVV). This approach is very promising since AVV is a non-pathogenic virus and it could reach systemically the majority of the post mitotic nuclei in the muscles (Rodino-Klapac et al.; 2013). This strategy though showed some caveats, the most remarkable is the packaging capacity of the vector that is limited (less than 5kb) and it is not able to contain the large size of the whole *dystrophin* gene (Hoggan et al.; 1966; Rodino-Klapac et al.2013). To overcome this main issue, several laboratories developed a mini-*dystrophin*, deleting nonessential coding regions, such as spectrin repeats and C-terminus. This truncated dystrophin gene

allows to be correctly packed and delivered in the AVV vector, and studies demonstrated also a therapeutical efficacy of this strategy in the *mdx* mouse model (Harper et al., 2002; Wang et al., 2000).

Unfortunately, this is not the only disadvantage to take in consideration since is known that up to the 74% of human population display pre-existing antibodies against AVVs, which come from previous exposure that naturally occurs during childhood. Furthermore, this class of parvovirus cannot replicate in absence of helper viruses such as herpes simplex and adenovirus (Hoggan et al.; 1966; Boutin et al.; 2010).

Genome editing

This approach can be used to facilitate DNA repair after a double strand break is promoted by a programmable nuclease (Nakano and Hotta 2014). The main pathways involved in the DNA repairing are two: the homologous recombination pathway (HR), which requires the presence of DNA template with homologous overlapping regions (Harber 2000). The other pathway, knonw as nonhomologous end joining (NHEJ), leads to insertion or deletions introduced to path the DNA gap (Mao et al. 2008).

In light of these evidences the recently used CRISPR (clustered regularity interspaced short palindromic repeats) Cas9 (CRISPR associated protein) nuclease system uses RNA complementary guide to edit a specific genome DNA sequence, and for its high versatility has become the preferred nuclease of choice in the genome editing field (Gee et al; 2017).

This flexible tool can be applied to restore the *dystrophin* open reading frame (ORF), by AVV infecting CRISPR-Cas9. In fact, targeting one or more exons through CRISPR-Cas9 approach, *dystrophin* expression can be rescued. One example is deleting the whole exon 23 in the dystrophic *mdx* mouse model; it was reported that the deletion of the whole exon 23 is able to rescue the dystrophic phenotype (Nelson et al., 2015). Furthermore, it was demonstrated by Maggio and colleagues that *dystrophin* expression can be restored in immortalized DMD myoblasts by removing the splicing acceptor (SA) in front of exon 51 (Maggio et al. 2016).

Even if the growing body of evidences are promising there are still some caveats in the filed regarding using this approach. Unfortunately, this approach shares the same

limits encountered for the exon skipping, since AVV infections are required as well. Moreover, the possibility of nonspecific DNA cleavage by CRISPR-Cas9 is the major concern. It was reported by several groups that a high number of off-targets mutations could occur, this depends on the uniqueness of the target sequence (Fu et al., 2013). Another concern is the immune system response editing with CRISPR-Cas9. In fact, it is possible that newly expressed Cas9 nuclease might promote an immune response, it is documented indeed that Cas9 delivered into C57BL/6 mice triggered an increased number of T-cells reactive to CRISPR-Cas9 (Chew et al. 2016). Indicating the immune system could be another obstacle to establish permanent treatment.

Exon skipping

Another approach to take in consideration is to target the pre-messenger RNA level, promoting one or more exons to be omitted and restore in such a way dystrophin reading frame, this strategy is also known as exon skipping. This phenomenon is accomplished with antisense oligonucleotides (AONs), which are synthesized to hybridize in a complementary manner the pre-messenger-RNA, allowing splicing modification (Mann et al, 2001; Goyenvalle et al.; 2010).

Several clinical trials showed the safety of this treatment in human through either muscular injection either systemic delivery (Van Deutekom et al.; 2007; Geomans et al.; 2011). However, these treatments have limited transduction efficacy in the heart of the *mdx* mice, also in long term mdx mouse studies of AONs supplementation is able to worsened myocardial phenotype (Malerba et al.; 2004). This implies that rescuing proper dystrophin expression in skeletal muscles in the absence of cardiac expression might have negative effects on cardiomyocytes.

Cell therapy

A third remarkable approach is focused on interfering with the loop of regeneration/degeneration, impaired satellite cells differentiation, loss of muscle fiber which is replaced by fat and connective tissue. As a result, there are under investigation strategies to deliver normal or genetic corrected muscle cells or pluripotent stem cells.

SC transfer experiments, first promising in the *mdx* mice, was disappointing in clinical trials due to SCs impairment to cross the blood vessel wall, poor cell migration, fusion and survival (Patrige et al.; 1989; Mendell et al.; 1995; Skuk et al.; 2004). The use of atypical myogenic precursors overcomes these issues, by offering high proliferating capacity, and most importantly the ability to cross the vessel walls, these populations include mesoangioblasts (Asakura et al.; 2002; Cossu et al.; 2003; Tagliafico et al.; 2006). This last population demonstrated, in several dystrophic models, to be particularly promising as multipotent progenitors exhibiting potential to improve muscular dystrophy, ease isolation from blood vessels and their remarkable ability to arterial transmigrating (Tagliafico et al.; 2004). A series of study in *mdx* and *Sgca* null mouse models show that transplanting mesoangioblasts, genetically corrected to express dystrophin, resulted in dystrophin-positive terminally differentiated myofibers (Davies et al.; 2006; Tedesco et al.; 2012).

Recently a clinical trial just ended (Eduract 2011-000176-33), where intra-arterial transplantations of mesoangioblast were performed on children affected by DMD. In this study Cossu and collaborators demonstrated the feasibility of this strategy and also that is clinically safe. Unfortunately, only few amelioration data were collected from paediatric patients, indeed just the youngest patients displayed light amelioration of the pathology; when the others did not show changes in terms of disease progression (Cossu et al.; 2015).

We can conclude that important steps forward were made to cure muscular dystrophies; even if some clinical trials are promising, no efficient therapies are yet available. In this scenario, it is thus important to develop approaches that aim to delay MD progression for two main reasons: firstly, to improve the patient’s life quality; secondly to make those children eligible for future clinical trial, since any clinical trial has to be developed on high quality muscle with poor fibrosis and fat infiltration.

Oxidative stress and antioxidant response

Oxidative stress in skeletal muscle and Muscular dystrophies

Oxidative stress is defined as imbalanced between the generation of reactive oxygen species (ROS) and the removal of such species by enzymatic and non-enzymatic defences, also known as antioxidant response (Rando 2002). This imbalanced could arise from an overproduction of ROS, usually associated with inflammation, either from impaired antioxidant defences, as observed in some loss-of-function disorders. This event usually leads to oxidation of various molecules essential for cell survival (oxidized DNA, proteins carbonylation and lipids peroxidation) and ultimately causes cellular dysfunction and cell death. Such condition might lead to chronic tissue degeneration if these changes accumulate in time. Furthermore, several mouse models, that exhibit an impairment in ROS metabolism, such as the GSH knock out and the vitamin E deficient mouse models, (Kakulas et al.; 1966; Bradley et al.; 1980) show signs of muscle degeneration very similar to muscular dystrophies mouse models and patients.

Inflammatory signalling, that is very important in MD progression, is driven by the Nuclear Factor kappa B (NF-kB) and cytokine (e.g. TNF-α, iNOS and IL-1b) are suggested to be induced also by an oxidative environment (Haycock et al. 1996; Rando 2002; Tindball and Wehling-Henricks 2004).

Oxidative stress has been implied in many disorders including ischemia-reperfusion injury, cancer, degenerative diseases and in the physiological processes of aging and acute exercise (Halliwell et al.; 1999; Rando 2002).

Acute exercise has been shown to induce necrotic injury in skeletal muscle, and among the mechanism proposed behind this type of damage is the oxidative stress, resulting by a combination of several processes as ischemia-reperfusion, physical injury, consequent inflammation and altered metabolism (Irintchev et al.; 1987). However, it is important to establish whether oxidative stress is able to lead to the type of damages observed in muscular dystrophies.

There are several animal models, described in literature, in which oxidative stress is experimentally induced and it were observed dystrophic changes in skeletal muscles. One of the first insight, in this sense, was obtained from the vitamin E deficient animals, in which were reported signs of muscle degeneration with pathological features comparable to muscular dystrophies (Kakulas et al.; 1966; Bradley et al.; 1980). It also known that in humans the absence of vitamin E is usually associated with myopathic changes, and it is observed a selective involvement of type II (fast-twitching fibers) as in the inherited human muscular dystrophies (Lazaro et al.; 1986; Tomasi et al.; 1979; Webster et al.; 1988). Vitamin E is a group of compounds that act as lipid-soluble antioxidant, therefore deficiency of these molecules is associated with increased lipid peroxidation. The same observations were made on a different mouse model of systemic depletion of GSH, in which the skeletal muscles display signs of degenerative changes (Martensson et al.; 1989). GSH is a tripeptide present in most cells and is implied in a variety of antioxidant reaction (Anderson et al.; 1985). In this particular mouse model findings of necrotic degeneration highlighted the importance of antioxidant defence in skeletal muscle.

Furthermore ischemia-reperfusion injury model, both in cardiac and skeletal muscles, causes very similar lesions observed in muscular dystrophies (Mendell et al.; 1971). It is very well known that a period of ischemia followed by reperfusion, mimics what happens during intense contractile activity, leading to a burst of ROS, lipid peroxidation and protein carbonylation (Saez et al.; 1986; Grisotto et al.; 2000). Pre-treatments with antioxidant can prevent this type of injury (Jeroudi et al.; 1994; Bushell et al.; 1996). However, this model cannot be considered a model of oxidative stress causing dystrophic-like phenotype, but it may help understanding the mechanisms contributing the pathogenesis of muscular dystrophies. Moreover, elevated oxidative markers are a constant finding in DMD patients (Haycock et al.; 1996; Rodriguet and Tarnopolsky 2003) and *mdx* mice (Ragusa et al.; 1995; Kaczor et al.; 2007) as expired pentane and TBARS (lipid peroxidation products) and high level of protein carbonylation. Also, it is reported that in DMD patients and in the *Sgca* null mouse model fast-twitching fibers are preferentially affected by MD

phenotype, which are the fibers with less antioxidant defences, more glycolytic metabolism and less mitochondrial content; in respect to slow-twitching fibers which display a more oxidative metabolism, more mitochondrial content and greater antioxidant difence (Webster et al.; 1988, Danieli-Betto et al.; 2004).

These findings lead us to suppose that oxidative stress is not only a marker of MD progress, but it might be deeply involved in the pathogenesis of MDs, postulating that MD patients and mice models display an impairment in the antioxidant signalling pathways.

The Antioxidant response

Several sub-compartments within the cell produce ROS, either physiologically or in response to toxic or pathological insults. These dangerous species are metabolised by a complicate antioxidant system to maintain oxidative homeostasis. Major antioxidants enzymes are usually low-molecular weight proteins including GSH and HO-1 (heme-oxygenase 1) (Ma 2013), which are able to metabolize hydrogen peroxide in water and oxygen. The transcription of this class of genes is strictly regulated and their signalling pathway is still in part unknown, in particular in a dystrophic context. Emerging evidences in literature underlined a specific promoter sequence (5'-TGACnnnGC-3') upstream these genes, called ARE (antioxidant responsive element; Rushmore et al.; 1990; Wasserman et al.; 1997; Nioi et al.; 2003).

The ARE region is very similar to the DNA-binding site of a specific transcription factor: Nrf-2 (nuclear factor erythroid 2-related factor 2), which is a protein member of cap'n'collar subfamily of basic region leucine zipper (CNC bZip) transcription factors (Ma 2013). Studies from the past decade underlined the main role of Nrf-2 in response to oxidative stress and chemical toxicity. It has been shown that the knock out mice model for Nrf-2 (Nrf-2 KO) display an increased susceptibility to oxidative stress and conditions associated with oxidative pathologies (Motohashi et al.; 2004; Ma et al.; 2008; Klassen et al.; 2010 Walters et al.; 2010).

Nrf-2 is a broadly expressed 68-kDa protein that has two transcription activations regions in the N-terminal and C-terminal, and also displays peptides

for nuclear localization between residues 494 and 511, it contains also at the N-terminus a Neh2 domain that negatively regulates its activity through Keap1 suppressor (Kelch-like ECH-associated protein-1) (Itoh et al.; 1999; Ma 2013; O'Connell and Hayes 2015).

Under basal condition Keap1 is able to bind and retain Nrf-2 in the cytoplasm, through Neh2 domains, and acts also as scaffold for ubiquitin ligase proteins (i.e. Cullin 3, Cul3). The ubiquitination of the complex targets it to the proteasome resulting in Nrf-2 rapid degradation (He et al.; 2003; Ma 2013; Liam et al.; 2014).

Regarding the activation of the Nrf-2 dependent signal, instead, there are still now some opened questions. It is known that Keap1 displays 3 phosphorylation sites that promote, in an oxidative environment, the uncoupling from Nrf-2 and its consequent translocation into the nucleus, enabling then ARE-dependent genes transcription (Ma et al.; 2013; Liam et al.; 2014).

AMPK: a ROS-sensitive kinase

Cells homeostasis depends on the maintenance of ATP levels, whose hydrolysis is required for fundamental processes such as transcription and transport across membrane (Cardaci et al.; 2012). For this reason, cells have evolved mechanisms to sense changes in ATP concentration. AMPK (AMP-activated kinase) is able to monitor small changes in AMP concentration (the product of ATP hydrolysis) and represents the main metabolic regulator of the cell (Hardie et al.; 2011).

AMPK is a heterodimer serine threonine kinase, which is composed by a catalytic α-subunit, a regulatory β- and γ-subunits (Hardie 2007). The α-subunit contains at the N-terminal a serine/threonine kinase domain that include also the Thr172 residue, whose phosphorylation by upstream kinases is essential for AMPK activity. The auto-inhibitory region instead is placed at the C-terminus of the α-subunit that binds β– and γ–subunit (Crute et al.; 1998; Chen et al.; 2009). In particular in the β-subunit has been observed that myristoylation at Gly2 residue is pivotal for phosphorylation of the α-subunit at Thr172 (Mitchelhill et al.; 1997; Oakhill et al., 2011). Instead the γ-subunit contains

tandem repeats sequences, known as CBS motif (cystathionine beta-synthase), which works in pairs to provide nucleotide binding and show great affinity to AMP and ADP molecules (Xian et al.; 2007).

First findings suggested that AMPK activation through ROS injury might have been just a reflection of ATP imbalanced encountered during stressed condition, instead of a direct effect on the kinase (Choi et al.; 2001).

Zmijewskiand and colleagues provided evidences that exposing AMPK to an oxidative state results in the oxidation of cysteine residues in its $\alpha-$ and β-subunits, which are pivotal for AMPK activation. Furthermore, antioxidant treatment promotes as well the same modification (Emerling et al., 2009; Zmijewskiand et al.; 2010).

For these reasons, AMPK has been proposed as a good candidate as the upstream promoter of Nrf-2 signalling, since this kinase is particularly sensitive to ROS concentration (Cardaci et al.; 2012), sensitive to the mitochondrial status (Emerling et al.; 2009; Hardie et al.; 2014; Hayes et al.; 2014) and it is responsible also for macroautophagy and mitophagy activation signals (Kim et al.; 2010; Fortini et al.; 2016; Gracìa-Prat et al.; 2016; Pal 2016).

Interestingly these signals are reported to be all impaired in MD patients and dystrophic mouse models (Spitali et al.; 2013; De Palma et al.; 2014; Rybalka et al.; 2014; Pal et al.; 2016). Indeed, it has been shown also that mitochondrial biogenesis and slow-twitching fiber formation are under AMPK control, activating a transcription cofactor essential for mitochondrial homeostasis so called PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), a co-factor pivotal also for the switch to oxidative fiber metabolism and inhibits muscle atrophy (Renèe-Ventura et al. 2008;).

Inflammation in muscular dystrophies

The inflammatory mediator: NF-kB signal

It is now very well described that inflammation plays an important role in worsening the dystrophic pathology (Spencer and Tindball 2001; Hnia et al.; 2008). Broken myofibers free cytokines and ROS, in the extracellular matrix, which gradient recalls immune cells, and in particular macrophages, whose infiltration and activity at the muscle sites worsen tissue degeneration and fibrosis establishing a chronic inflammatory environment (Morrison et al.; 2000; Hodgetts et al.; 2006).

Accordingly, experiments of macrophages depletion or impairment of their activity, ameliorate muscle histology in dystrophic mice (Spencer et al.; 2001; Wehling et al.; 2001). This phenomenon is an early process in *mdx* and *Sgca* null mice models, which appears at first around 3-4 weeks of age (Duclos et al.; 1998; Spencer and Tindball 2001)

Indeed, activation of NF-kB (nuclear factor kappa B), oxidative stress and secretion of proinflammatory cytokines (e.g. TNF-α, IL-1β) appear to be essential and related in MD progression, in both humans and mice models (Monici et al.; 2003; Kumar et al.; 2004; Peterson and Guttridge 2008).

NF-kB is a family of transcription factors that usually is retained in the cytoplasm of the majority of cell types and exists as a homo- or heterodimers of structurally related proteins (May and Ghosh 1998). Every family member of this group of proteins contains a conserved N-terminal region known as Rel-homology domain (RHD) within lies the DNA-binding domain, the dimerization domain and the nuclear localization signal (NLS) (Baldwin et al.; 1996). Up to now there are described in literature five proteins belonging to NF-kB family in mammals: p65, c-Rel, RelB, p50/p105 and p52/p100.

Under resting conditions, dimers of NF-kB are sequestered in the cytosol through noncovalent interactions with a class of inhibitory proteins called IkBs (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitors). All known IkB proteins own multiple copies of 30-33 amino acid sequence called

ankyrin repeats; which specifically interacts with the RHD and this allows IkB molecules to mask the NLS of NF-kB, preventing its nuclear transfer (Thanos et al.; 1995; Verma et al.; 1995). Further findings underlined that the disruption of this complex is essential, in order to observe a NF-kB signal, and this is also due to phosphorylation of IkBs proteins (Kerr et al.; 1991; Link et al.; 1992; Shirakawa et al.; 1989). Mutagenesis experiments identified that in particular two Serine residues (Ser 32 and Ser 36) are pivotal for IkB-α phosphorylation; interestingly it was observed these phosphorylated residues induce conformational changes in the protein structure that expose lysine residues. It is very well know that lysine residues are often targets of ubiquitin ligase enzymes to target proteins to proteasome, in fact experiments show that once eliminated this specific lysine or the use of proteasome inhibitor IkB-α cannot be degraded, interfering with NF-kB signal under stressed condition.

To sum up, if cells are properly stimulated (e.g. with TNF-α, hypoxia or oxidative stress), IkB-α is rapidly phosphorylated; this allows two main events: the NF-kB/IkB-α complex dissociation, and the phosphorylation of IkB-α allowing its degradation via proteasome through ubiquitination.

There are several evidences in literature demonstrating that NF-kB binding activity is enhanced in mdx mice compared to wild-type (WT) (Kumar and Boriek 2003), showing greater protein levels in the nuclei fractions (Acharyya et al.; 2007) and this is linked also to lower protein levels of inhibitory IkB-α (Kumar and Boriek 2003). Many efforts have been done to understand which class of kinases could activate NF-kB signal pathway and orchestrate the inflammatory response in this specific scenario.

In primary myoblast culture and in myoblast stable cell line C2C12 has been observed that ERK kinases are directly involved in NF-kB activity, under hypoxia and oxidative stimuli, by phosphorylating its complex with IkB-α and promoting NF-kB translocation into the nucleus (Kefaloyianni et al. 2006; Osorio-Fuentealba et al. 2009), but there are still no findings on this specific pathway *in vivo* models of muscular dystrophy.

ERKs kinases

The insulin/mitogen regulated extracellular signal-regulated kinase 1 and 2 (ERK1/2) were the first mammalian MAPK pathways identified. MAPKs include four families and the most studied are ERKs, JNKs and p38-MAPK (Kyriakis et al. 2001; Kefaloyanni et al.; 2006). This class of kinases can be activated by a high number of ligand or self-activated tyrosine kinase receptor, such as EGFR, FGFR, IGFR, PDGFR and insulin receptor (Chang et al.; 2001; Karnoub and Weinberg 2008). Every MAPKs subfamily is composed by three kinase modules (MEEK, MEK and MAPK), each one activating the next via phosphorylation (Kyrianis et al.; 2001). Their substrates, usually is located in the cytoplasm, include other kinases, phospholipases, cytoskeletal proteins and transcription factors (Pearson et al.; 2001; Gosh et al.;2002).

In literature is described that the signalling cascade of ERKs is usually initiated by Ras activation, which pass the signal by phosphorylating MAPKK proteins. These proteins, in turn, activate MAPK/ERK kinases, known also as MEK (Ahn et al., 1991; Gomez and Cohen, 1991), that phosphorylate directly ERK kinases (Serger and Krebs, 1995). Once ERKs are active, are able to phosphorylate a large of substrate (Yoon et al., 2006), which are usually located in the cytoplasm and once phosphorylated could translocate into the nucleus to trigger gene transcription.

Nowadays is well established that the ERKs pathway is involved in anabolic processes, cell division, growth and differentiation (Widmann et al.; 1999; Kyrianis et al.; 2001). Recent studies, although, highlighted that oxidative stress is associated with an activation with kinases of the Src family, and various growth factors receptors such as EGFR (Abe et al.; 1999; Yoshizumi et al.; 2000; Chen et al.; 2001; Zhougang et al.; 2004; Purdom et al.; 2005).

In particular Kefaloyanni and colleagues show that MAPK kinases, specifically ERKs kinases, are able to enhance NF-kB binding activity under oxidative condition in myoblast cell culture of C2C12 (Kefaloyanni, et al. 2006). In this study it was evident that, upon H_2O_2 treatment, NF-kB signal is active and it is able to promote pro-inflammatory gene transcription (Kefaloyanni et al., 2006). Furthermore, they assume that ERK kinases are able to promote, via

phosphorylation, the disassociation of NF-kB from its inhibitor IkB-α. In fact, treatment with specific ERK inhibitors, show that C2C12 also under oxidative conditions display less NF-kB binding activity, on the other side the use of EGFR inhibitors do not lead to any effect on this specific pathway (Kefaloyianni et al. 2006; Osorio-Fuentealba et al. 2009).

Natural antioxidants

A natural antioxidant: Anthocyanin

Polyphenols are among the most important secondary phytoproducts displaying a great antioxidant power. This class of molecules are hydro-soluble and show a basic difenipropane structure (C6-C3-C6) with two aromatic rings (Fig2). In particular anthocyanin are colourful pigments, catabolites from phenylalanine (Bravo 1998). The anthocyanin antioxidant features stay in their structure: the aromatic ring protects photosynthetic tissue from oxidative stress, produced by the light. Several studies showed how cyanidin are able to promotes plant survival through a phenomenon called photoinhibition. This process reflects the light by photosynthetic tissues, establishing a new equilibrium between light absorbed, CO_2 produced and carbohydrates consumed. Furthermore plants, with a great oxidative insult, enhance the expression of antioxidant genes and, among them, those involved in anthocyanins metabolism (Stein et al.; 2002).

For these reasons, from the past decade anthocyanins have been the subject of different studies in vitro, in vivo and in clinical trials for treating particular conditions where oxidative stress plays a major role such as aging, cardiovascular diseases, neurodegenerative pathologies, cancer and obesity (Arts and Hollman 2005). Since anthocyanin bioavailability in mammals is very low (less than 1%), the protective effect it cannot come from its scavenging properties as observed in plants. It is hypothesized that this class of antioxidant in mammals might act on specific pathways in the antioxidant response (Manach et al., 2005).

In particular Cyanidin-3-O-glucoside (C3G) in rat hepatocytes promotes antioxidant genes expression (Shin et al.; 2007). There are also in vivo findings on murine model of obesity, which assume chronically a diet enriched in C3G (RD) show protective effects on terms of hyperglycaemia and fat deposition (Tsuda et al.; 2003). Furthermore, in heart ischemia-reperfusion rat model, long term treatment of enriched C3G diet show protection from oxidative damage (Martin et al.; 2008).

Up to now there are studies focusing on the effects of C3G on human health, specifically on breast cancer (Cerletti et al 2017).

Corn enriched in cyanidin: the red diet

The traditional corn (*Zea Mays*) is plant of the *Poaceae* family. From this cereal are obtain flours usually used for mice model food. In this corn anthocyanins production is regulated by two families of transcriptions factors: bHLH (basic Helix-loop-Helix) and MYB, once in the nuclei these factors are able to bind promoter regions of genes involved in this molecules metabolism (Toufektsian et al.; 2008). In particular gene *R1* (red colour 1) and *B1* (booster 1), from bHLH family; *C1* (coloredaleurone 1), *Pl1* (purple plant 1) and *P1* (pericarp colour 1), from MYB family, are responsible for anthocyanins both spatial and temporal profile production (Figure 2A and 2B). In fact, different genetic combinations give rise to different corn lines in terms of anthocyanins quantity and distribution in plant tissues (Petroni and Tonelli 2011); instead in the widely used line of Zea Mays these genes are transcriptionally silenced (Petroni et al.; 2014).

These corn lines were bred in such a way to obtain a hybrid line (*B1, Pl1*) with a high concentration of cyanidin and isogenic to Zea Mays (Yellow diet, YD) (Pilu et al.; 2011; Figure 2C). Therefore, from this particular corn line it is possible to produce food for murine models particularly enriched in cyanidin. A study from 2013 this diet is used to evaluate the effects of cyanidin on obesity in C57BL/6J mice, after 12 weeks of treatment it was observed a reduction in mice weight, adipocytes size and less inflammation at level of adipose tissue compared to control mice fed only with high fat diet (Tsuda et al.; 2003).

This is one of the many examples that proves dietary cyanidin could prevent pathologies where oxidative stress is at the base of the molecular (Hollman 2005; Shin et al.; 2007; Martin et al.; 2008).

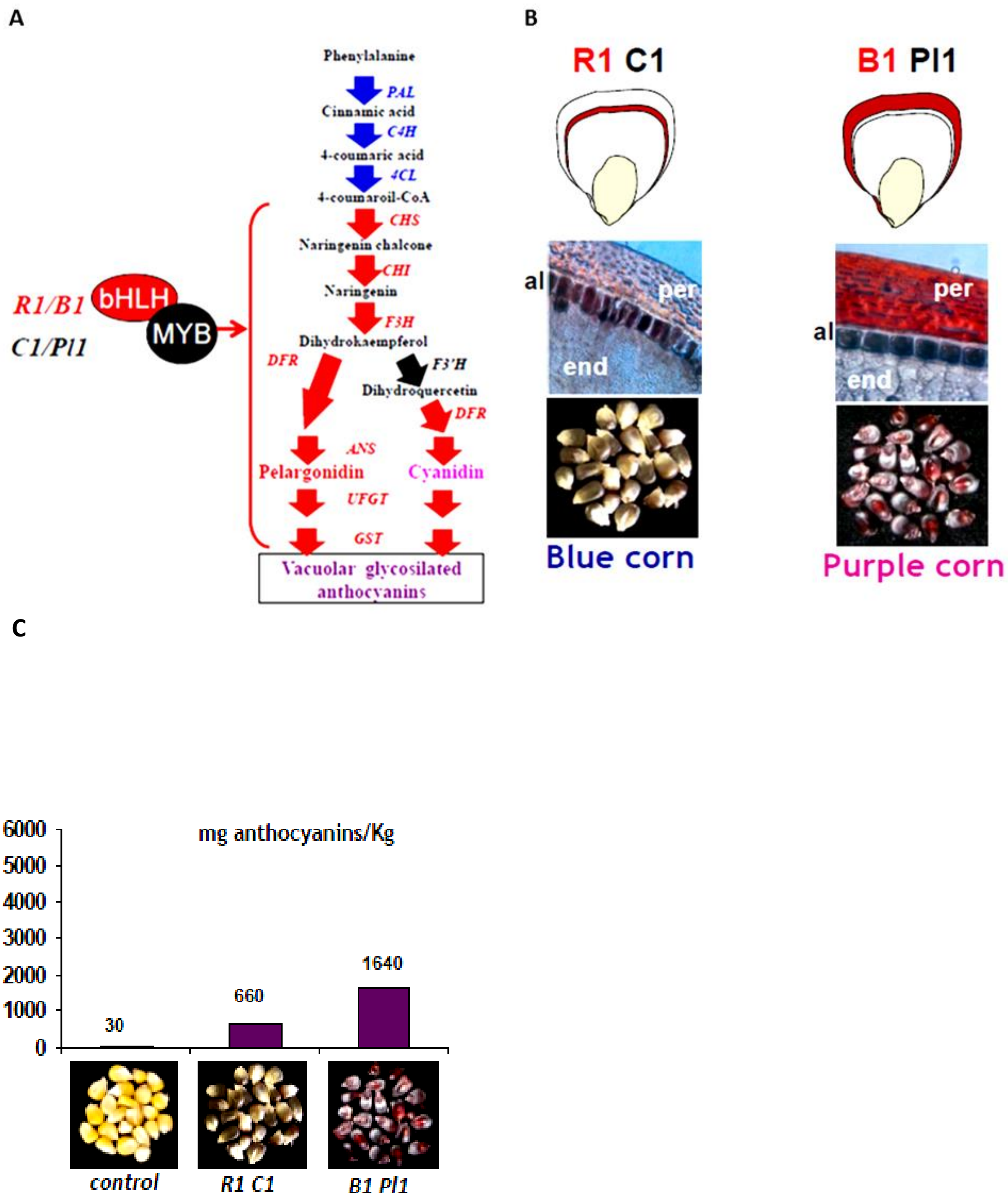

Figure 2: (A) Transcription factors involved in anthocyanin synthesis in Zea mays. **(B)** Phenotypical characterization of special distribution and accumulation of anthocyanins (Petroni et al. 2017). **(C)** Quantification of cyanidin levels produced by two Zea Maya varieties *R1 C1* and *B1 P/1* compared to the commercial variety *r1 b1 p/1* (Pilu et al., 2011)

AIMS OF THE PROJECT

MDs are hereditary diseases all characterized by progressive skeletal muscle wasting and weakness of variable severity, leading patients in time to wheelchair and premature death by cardiac and respiratory failure (Emery 2002). Mutations in these pathologies are all in genes involved in the dystrophin-glycoprotein complex (DGC). This structure is a sarcolemmal complex that connect the fiber plasma membrane to extracellular matrix and it is responsible for myofibers integrity during contraction. Lacking of DGC promotes fibers fragility and consequently cell death; myofibers are replaced by satellite cells (stem cells of adult muscle tissue) that however share the same mutation differentiating so in defective myofibers. In such a way, the muscle tissue falls in a loop of degeneration and regeneration until the exhaustion of the stem cells pool, which in time is replaced by connective and fat tissue (fibrosis). Furthermore, immune cells, in particular macrophage population, are recalled by broken fibers establishing an inflammatory environment at the muscle site, that in time it will become chronic, worsening the dystrophic phenotype. MDs are still severe orphan drug diseases.

Since oxidative stress is involved in muscle degeneration, one of the considered strategies is to use antioxidant molecules to counteract the oxidative stress generated. (Rando et al. 2002; Hori et al. 2011; Kuno et al. 2013; Bhuiyan et al. 2012; Ji 2008; Perveen et al. 2014; Sun et al. 2015).

This PhD project aims to evaluate the effects of an anthocyanin enriched diet (red diet, RD) on a dystrophic mouse model for the Limb Girdle Muscular Dystrophy 2D (LGMD 2D, an autosomal recessive disorder caused by mutations in the α-sarcoglycan gene, *Sgca* null) (Duclos et al. 1998). The choice of this mouse model is due to the fact that the *Sgca* null mouse, at variance with the most commonly used mdx mouse model, better recapitulates the progression of MDs as in human patients. Then, once investigated the effects of the RD on *Sgca* null mice, this study would focus on the possible pathways that RD could modulate.

Main Results

My PhD project was focused on the effects of antioxidant diet and the role of oxidative stress in muscular dystrophy progression. Specifically, we used a diet enriched in cyanidin (RD), a powerful natural antioxidant (Pilu et al.; 2011, Petroni et al., 2017), and, as a control, a cyanidin-free diet isogenic to the RD (yellow diet, YD) to firstly investigate on the phenotypical effects on the dystrophic mouse mode *Sgca* null (Duclos et al., 1998); and secondly on the pathway tuned by this diet. Here below are listened the main results of my project:

- RD fed mice show a morphological amelioration in terms of muscle organization, fiber integrity, macrophage infiltration and collagen deposits
- Muscle performance and endurance are rescued when dystrophic mice are supplied with RD
- Macrophage infiltrates are less present in dystrophic muscles of RD fed mice

This promising data allowed us to investigate deeper on how RD could trigger dystrophic muscle amelioration:

- Reduction of the inflammatory parameters, which is coupled to a less activity of NF-kB into the myonuclei, due to an enhanced expression of IkB-α and a decrease of the phospho-ERK protein level
- I observed a shift to a more oxidative fiber metabolism, more mitochondrial SDH activity, increased protein level of PGC-1α (master transcription factor of mitochondrial biogenesis) and its target genes
- I found out the activation of a particular antioxidant pathway in muscle of mice fed with RD, in which the antioxidant master transcription factor Nrf-2 plays the main role. Nrf-2 in these conditions is more localized into the nuclei and its target genes are transcriptionally active (i.e. *Hemeoxigenase-1, HO-1 and glutamate-cysteine ligase catalytic*

subunit, GCLC), triggering the antioxidant response. Furthermore, I observed that this translocation is due to AMPK activity.

Part II

Manuscript in preparation:

Cyanidin enriched diet delays muscular dystrophy in *Sgca* null mice

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Keywords: ROS, cyanidin, oxidative metabolism, antioxidant, muscular dystrophy, Nrf-2, NF-kB, oxidative stress

Abstract

Muscular Dystrophies are severe genetic diseases due to mutations in structural genes, which lead to wasting of skeletal muscle compromising patients' mobility and respiratory functions. Previous studies show that oxidative stress and inflammation play a great role in the worsening of these pathologies. Furthermore, it is known that myofibers that display oxidative metabolism and great antioxidant defence, such as type I fibers, are more protected from dystrophic signs. In this work, we show a morphological and functional rescue in *Sgca* null dystrophic mice fed with a diet enriched in a natural antioxidant known as cyanidin, as a consequence of both muscle fiber type switch and reduction of inflammation. In particular, we demonstrate that cyanidin reduces inflammation by allowing the retention of NF-kB into the cytoplasm through the inhibition of ERK kinases. Moreover, we identified Nrf-2 as the master transcription factor that, upon cyanidin stimuli, is able to promote the antioxidant response delaying muscular dystrophy progression. This work provides strong evidences that a cyanidin enriched diet protects and delays the progression of muscular dystrophies, demonstrating how much the oxidative stress might exacerbate the disease being detrimental for the successful of both gene and cell therapies.

INTRODUCTION

Muscular dystrophies (MDs) are a group of heterogeneous genetic diseases, characterized by wasting of skeletal muscle tissue, which in time compromises patient mobility and, in the most severe cases, respiratory and cardiac functionality leading to premature death (Emery 2002; Mercuri & Muntoni 2013). In many cases, the mutations affect one or more proteins that cluster in the dystrophin-glycoprotein complex (DGC) located at the basal lamina of the myofibers. This complex connects myofibers to the extra cellular matrix, and its role is essential for fiber integrity and cell signalling during contraction. The mutations result in the disassembly or a malfunction of the entire DGC, which leads to an increased fragility of sarcolemma and myofibers death. Damaged and death fibers can be replaced by satellite cells (SCs), the adult stem cells of skeletal muscle tissue. In this pathologic scenario, since SCs share the same mutation as well as the damaged myofibers, they differentiate in fragile myofibers, leading to a loop of degeneration and regeneration (Emery 2002). In time, the population of SCs is exhausted and the damaged muscle is replaced by connective and adipose tissue, impairing the physiological function of muscle tissue (Mercuri & Muntoni 2013; Konieczny et al. 2013; Emery 2002). Despite the molecular mechanisms behind MDs are very well known, this class of diseases is one of the most difficult to treat. Indeed, although several clinical trials have been carried on, no definitive cure is still available.

Skeletal muscle is the most abundant tissue in the human body and it is composed of large multinucleated fibers, whose nuclei cannot divide. Consequently, any cell or gene replacement strategy must restore a proper gene expression in hundreds of millions of post-mitotic nuclei, which are embedded in a highly structured cytoplasm and surrounded by a thick basal lamina. It is therefore evident that, although caused by a single gene defect, this group of pathologies could be considered as a multifactorial: misregulation of associated sarcoplasmic proteins, severe chronic inflammation and

consequent macrophage infiltration resulting in fibrosis. Among the different approaches, many efforts are directed to slow down the progression of the disease to counteract the progressive degeneration and to improve patients life quality (Cossu & Sampaolesi 2007).

Therefore, one of the considered strategies is to use antioxidant molecules to counteract the oxidative stress generated by muscle contraction and degeneration (Rando et al. 2002; Hori et al. 2011; Kuno et al. 2013; Bhuiyan et al. 2012; Ji 2008; Perveen et al. 2014; Sun et al. 2015). Several evidences in literature showed that oxidative stress and accumulation of reactive oxygen species (ROS) strongly contribute to the worsening of the dystrophic pathology (Rando et al. 1998, Rando et al. 2002). The models proposed suggest that myofibers cannot manage the physiological production of ROS during contraction, due to the lack of a proper antioxidant signal from DGC, leading to activation of apoptotic signals (Rando et al. 2002). Precisely, the accumulation of ROS in the myofibers leads to the carbonylation and alteration of several essential molecules as nucleic acids and lipids, consequently this phenomenon activates cell death programs (Bedard and Krause 2007; Cardaci, Filomeni, and Ciriolo 2012; Rando et al. 1998).

Another important aspect of MD progression is the chronic inflammation. Indeed, it has been shown that the presence of intracellular and interstitial ROS in MD patient, released by necrotic fibers, enhances the secretion of pro-inflammatory cytokines (such as tumour necrosis factor alpha, TNF-α, transforming grow factor beta, TGF-β and iNOS) in muscle extracellular matrix (Tidball & Villalta 2010; Rando 2002). This gradient recalls macrophages, which are essential players in acute muscle regeneration; but, in a chronic myopathic context, this population establishes in time a chronic inflammatory environment worsening the dystrophic phenotype (Haycock et al. 1998; Jackman et al. 2013) .

In this study, we provide evidences that a natural antioxidant, cyanidin, a polyphenol member of the anthocyanins family, is helpful in treating MD pathologies, by acting as both anti-oxidant and anti-inflammatory molecule.

We indeed provide evidences that cyanidin enriched diet (Red diet, RD) supplied to the dystrophic *α-sarcoglycan* null mouse model (*Sgca* null, Duclos et al.; 1998) at weaning and in adult period, when the first signs of the disease are already present, is able to morphologically and functionally rescue the pathologic phenotype. Specifically, we demonstrate that cyanidin acts by tuning the antioxidant pathway through modulation of Nrf-2 localization and the anti-inflammatory response through NF-kB-dependent activity.

METHODS

Maize production

Maize genotypes were originally in W22 background, homozygous dominant for the *a1*, *a2*, *c1*, *c2*, *bz1* and *bz2* genes, homozygous recessive for the *r1* gene and different *b1 pl1* constitution. To obtain cyanidin-rich and cyanidin free corn with an isogenic background, a novel maize cyanidin-rich hybrid was developed carrying the *B1* and *Pl1* alleles (red diet, RD), which confer cyanidin pigmentation in seed pericarp and all plant tissue (Radicella et al.,1992; Pilu et al. 2003; Petroni et al., 2011; Pilu et al., 2011). Plant and seed tissues carrying *b1 pl1* alleles are cyanidin-free (yellow diet, YD. To obtain ears with a high production of kernels, the homozygous inbred line *B1 Pl1* W22 and the *b1 pl1* W22 inbred line were crossed to a *b1 pl1* B73 inbred line and the F1 progeny seeds were used to produce two synthetic populations differing only in *b1 pl1* constitution (Petroni et al., 2017).

Mouse model

All mice were kept in pathogen-free conditions with 12-12 hours light-dark cycle. All the procedures on animals were conformed to Italian law (D. Lgs n 2014/26, implementation of the 2010/63/UE) and approved by the University of Milan Animal Welfare Body and by the Italian Ministry of Health. The genotyping strategies have been published in the references. *Sgca* null mice were previously described in Duclos et al. 1998. At three weeks of age, *Sgca* null mice were randomly divided in two groups: one fed with the control cyanidin-free diet (YD) and the other one fed the cyanidin enriched diet (RD). The diets were supplied *ad libitum* to the mice for 5 weeks or 25 weeks. Both male and female mice were used indiscriminately. In order to check the effects of the cyanidin enriched diet on mice which already show the first signs of the pathology, we also supplied the diets at 5 weeks for 15 weeks.

Hematoxylin and Eosin and Milligan's Trichrome

Hematoxylin and eosin staining was performed on 7µm-thick cryosections fixed with 4% paraformaldehyde for 10 min at 4°C. The staining was performed according to standard protocols. For Milligan's trichrome staining, sections were fixed for 1 h with Bouin's fixative (Sigma-Aldrich) and rinsed for 1 h under running water. Sections were then rapidly dehydrated to 95% EtOH in graded ethanol solutions, successively passed in 3% potassium dichromate (Sigma-Aldrich) for 5 min, rapidly washed in distilled water, stained with 0,1% acid fuchsin (Sigma-Aldrich) for 30 sec, washed again in distilled water, passed in 1% phosphomolybdic acid (Sigma-Aldrich) for 3 min, stained with Orange G (2% in 1% phosphomolybdic acid) (Sigma-Aldrich) for 5 min, rinsed in distilled water, passed in 1% acetic acid (VWR) for 2 min, stained with 1% Fast Green for 5 min (Sigma-Aldrich), passed in 1% acetic acid for 3 min, rapidly dehydrated to 100% EtOH and passed in Xylene before mounting with Eukitt (Bio-Optica).

SDH staining

For SDH staining, freshly cut 7µm-thick cryosections of *Tibialis anterior* were used. Sections were incubated in SDH incubating solution (1 tablet of nitroblue tetrazolium dissolved in 0,1M sodium succinate-0,1M phosphate buffer pH7.4, all from Sigma-Aldrich) for 1 h at 37°C, rinsed in distilled water, rapidly passed in 30%, 60%, 30% Acetone (VWR), and rinsed again in distilled water. Sections were then rapidly dehydrated in graded EtOH solutions, cleared in Xylene and mounted with Eukitt mounting medium.

Evan's Blue Dye uptake measurement

Evan's Blue Dye (Sigma-Aldrich) solution (10mg/ml) was injected systemically through the caudal vein 6 h before sacrifice (0,25µl per 10g of mice). Positivity for Evan's Blue Dye was revealed through its auto fluorescence, fixing sections with acetone (VWR) for 10 min at -20°C, permeabilizing them in 1%BSA (Sigma-Aldrich)-0,2% Triton X-100 (Sigma-Aldrich) for 30 min and incubating them O/N with rabbit anti-laminin antibody

(Sigma-Aldrich, 1:300) to reveal myofiber outlines. The day after, sections were washed, incubated with a goat anti-rabbit 488 secondary antibody (Jackson Lab, 1:250), together with Hoechst (Sigma-Aldrich, 1:250) to stain the nuclei, washed again and finally mounted with a Fluorescence Mounting Medium (Dako). Measurement of the percentage of Evan's Blue Dye Uptake was performed counting the number of Evan's Blue Dye positive fibers on total muscle section reconstructions, using Image J software.

Treadmill Test

For Treadmill test functional assay, 3 weeks old *Sgca* null mice were fed for 5 weeks with RD or YD diet and WT with YD diet, as control. Mice were trained 3 times once a week before recording the performance. Treadmill test was therefore performed starting from 8 weeks old mice, once a week for 21 weeks. The test was conducted on a treadmill (Bioseb) with a 10% incline, starting from a speed of 6 cm/sec and increasing it by 2 cm/sec every 2 minutes. For each test, the time to exhaustion of each mouse was measured.

RNA Extraction, RT-PCR and Real Time PCR

Real time PCR was performed starting from RNA extracts obtained from muscle tissues homogenized and extracted in Trizol Reagent (Invitrogen) following manufacturer's instructions. 1µg of RNA was retro-transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad) and 5µl of diluted (1:5) cDNA was used for each sample. Gene expression was quantified by comparative CT method, using GAPDH as a reference. Primers used are:

- *GCLC* FOR: CTACCACGCAGTCAAGGACC
- *GCLC* REV: CCTCCATTCAGTAACAACTGGAC
- *HO-1* FOR: AGGTACACATCCAAGCCGAGA

- *HO-1* REV: CATCACCAGCTTAAAGCCTTCT
- *TNF-α* FOR: CCACCACGCTCTTCTGTCTA
- *TNF-α* REV: AGGGTCTGGGCCATAGAACT
- *iNOS* FOR: CCACCAACAATGGCACATCAGGT
- *iNOS* REV: TAGGTCGATGCACAACTGGGTGAA
- *GAPDH* FOR: TTCACCACCATGGAGAAGGC
- *GAPDH* REV: GGCATGGACTGTGGTCATGA
- *CPT-1b* FOR: GACTTCCGGCTTAGTCGGG
- *CPT-1b* REV: GAATAAGCGGTTTCTTCCAGGA
- *UCP3* FOR: CTGCACCGCCAGATGAGTTT
- *UCP3* REV: ATCATGGCTTGAAATCGGACC

Protein extraction and Western Blot

Western blot was performed on protein extracts from muscles homogenized in Tissue Buffer (150 mM Tris-HCl, pH 7.5; 1mM EDTA, 1% Triton, 150 mM NaCl, all from Sigma-Aldrich) for 30 sec, followed by lysis on ice for 30 minutes and by centrifugation at 10000 rpm at 4°C to pellet cell debris. Supernatant was quantified using DC Protein Assay (Biorad), and 30-50μg of total protein extracts were loaded for each sample. Images were acquired using Chemidoc ImageLab software (Biorad). The following antibodies and dilutions were used: rabbit anti-PGC-1 (1:1000, Abcam), mouse anti-total ERK1/2 (1:500, Santa Cruz), rabbit anti-pERK1/2 Thr202/Tyr204 (1:1000, Santa Cruz), rabbit anti-total AMPK (1:1000, Santa Cruz), rabbit anti-pAMPK Thr162 (1:1000, Santa Cruz), mouse anti-IKBα(1:1000, Santa Cruz), muose anti-p65 (NF-kB, 1:1000, Santa Cruz), rabbit anti-Nrf-2 (1:1000, Abcam), mouse anti-Vinculin (1:2500, Sigma-Aldrich), mouse anti-GAPDH (1:5000, Biogenesys) IgG-HRP secondary antibodies (1:10000, Bio-Rad).

Nuclear and Cytoplasm fractioning

The protocol was taken from Dimauro et al. 2012. Quadriceps were minced on ice and homogenized in 300 µl of STM buffer (250mM sucrose, 50mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, all from Sigma-Aldrich and protease and phosphatase inhibitors). After 30 min on ice, samples were centrifuged at 800g for 15 min at 4°C and the supernatant, corresponding to the cytoplasmic fraction, recovered. The pellet, which is mainly composed by myonuclei, was washed in STM buffer and centrifuged at 500g for 15 min at 4°C twice and the supernatant discard. The pellet was washed again in STM buffer and centrifuged at 1000g for 15 min at 4°C and discard the supernatant. The pellet was crushed in 100 µl of NET buffer (20mM HEPES pH 7,9, 1,5 mM $MgCl_2$, 0,5 M NaCl, 0,2 mM EDTA, 20% glycerol, 1% Trito-X-100, all from Sigma-Aldrich and protease and phosphatase inhibitors) and let for 30 min in ice. The nuclei were then sonicated (Bioruptor) at maximum intensity for 5 pulses of 5 sec and pauses of 15 sec. Then, samples were centrifuged at 9000g for 30 min at 4°C, the supernatant obtained were the nuclei fraction.

In order to establish the purity of the two fractions, by Western blot, we used for the nuclei fraction rabbit anti-H3 antibody (Histone3, 1:7000, abcam), and mouse anti-GAPDH (1:5000, Biogenesys) for the cytoplasmic fraction.

Protein carbonylation content (PCC)

The level of oxidative stress in quadriceps protein extracts was quantified by measuring of protein carbonylation. Carbonyls groups were derivatized into their DNP adducts using 2,4-Dinitrophenylhydrazine (DNPH) (Mecocci et al., 1998). Quadriceps muscles were homogenized in tissue buffer adding 1mM DTT. After protein quantification 50µg of protein was derivatized with the same volume of DNPH (10mM in 2M HCl, Sigma-Aldrich) for 1h in the dark at RT. Afterwards, to precipitate corbonylated protein and stop the derivatization reaction was added a solution 30% of trichloroacetic acid (TCA, Sigma-Aldrich) and let sample rest for 15min in ice. Then,

samples were centrifuged at 15000g for 15 min at 4°C. After removing the supernatant, each pellet was washed three times with a solution of ethanol-ethyl acetate (1:1) in order to remove the excess of DNPH, then the pellet was solubilized in 1mL of guanidine (6M, Sigma-Aldrich) and incubated for 30min at 37°C. The content of corbonylated protein was measured by spectrophotometer at 370nm (Janway).

Immunofluorescence

Immunofluorescence was performed on 7µm cryosections. Slices were fixed for 10min at 4°C with 4%PFA, washed twice in PBS and permeabilized with a solution containing 1%BSA (Sigma-Aldrich) and 0.2% Triton X-100 (Sigma-Aldrich) in PBS, for 30min at room temperature. After a blocking for 30min with 10% donkey serum, slices were incubated O/N with primary antibodies in PBS-1,5% donkey serum. The day after, two washes in PBS-1%BSA-0,2%Triton X-100 were performed and samples were incubated for 45min at room temperature with secondary antibodies and Hoechst (1:500, Sigma-Aldrich). Excess of antibody was washed twice in PBS-0,2%Triton X-100 before mounting with Fluorescence Mounting Medium (Dako). The following antibodies and dilutions were used: goat anti Collagen I (1:200, Southern Biotech), donkey anti-goat 488 (1:250, Jackson Lab).

F4/80 immunofluorescence was performed on cryosections of Tibialis Anterior. Samples were permeabilized for 10 min at RT in a solution of PBS-0,5% Triton X-100 (Sigma-Aldrich), there washed three times in PBS. After blocking for 30 min with a solution of PBS-3%BSA (Sigma-Aldrich), slices were washed twice with PBS and incubated O/N with primary antibodies anti-F4/80 (rat, 1:400, Novus) and anti-laminin (rabbit, 1:300 Sigma-Aldrich) in PBS. The day after, three washes in PBS were performed and samples were incubated for 45min at room temperature with secondary antibodies and Hoechst (1:500, Sigma-Aldrich). Excess of antibody was washed five

times in PBS before mounting with Fluorescence Mounting Medium (Dako). F4/80 positive cells were normalized on the total number of fibers in the picture.

For slow Myosin heavy chain (slow MyHC) immunostaining, cryosections were retrieved in a Na-citrate solution (10mM pH 6) for 30 min in a steamer machine. Once equilibrated the section at RT, slices were washed twice with PBS for 10 min and then added the primary antibody mouse anti-slow MyHC 1:1000(Sigma) and rabbit anti-laminin 1:300 (Sigma-Aldrich) over night at 4°C Fibers positive for slow MyHC were normalized on the total number of laminin-positive myofibers in the cryosections.

Isolation of macrophages from skeletal muscle

Skeletal Muscles collected from hind limb were minced and digested enzymatically and mechanically in a single cell suspension with 0,2% of Collagenase B (Roche) in RPMI (Lonza) medium for 1h and 30min in water bath at 37°C under agitation. After filtration with cell strainers (70 and 40 µm, Grainer) and centrifugation at 272g for 10min at 4°C, the single cell suspension was resuspended in sterile PBS-0,5% BSA (Genespin)-2mM EDTA (Sigma-Aldrich) and incubated with anti-CD45 antibody conjugated with magnetic beads (Miltenyi Biotech) for 30 min at 4°C. Cells were then washed with PBS-0,5% BSA (Genespin)-2mM EDTA (Sigma-Aldrich) and $CD45^+$ cell isolation was performed by using magnetic columns (Miltenyi, Biotech) according to manufacturer instructions. After Fc blocking (Fc Buffer, Miltenyi Biotech), the fraction of $CD45^+$ cells were incubated with Ly6C-PE antibody (eBioscence), to discriminate pro-inflammatory ($Ly6C^+$) from anti-inflammatory macrophages ($Ly6C^-$), and with CD64-APC antibody (BD Bioscence) to discriminate neutrophils from macrophages Cell sorting experiments were then performed using a FACSAria IIu (BD Bioscience). Diva software (BD Pharmingen, San Diego, CA) was used for data acquisition and analysis

Image acquisition

Images were acquired with an inverted microscope (Leica-DMI6000B) equipped with Leica DFC365FX and DFC400 cameras. The Leica Application Suite software was used for acquisition while Photoshop was used to generate merged images.

Measurement of myofiber cross sectional area and Collagen I quantification

Measurement myofiber cross sectional area was performed on *Tibialis anterior* muscle sections using Image J software. Collagen I quantification was performed using a Macro in ImageJ to identify and quantify Collagen I positive areas.

Statistics

All data shown in graph are expressed as mean ± SD, apart from graphs showing cross sectional area distributions, which are expressed as mean±whiskers from min to max. Statistical analysis between two columns was performed using two-tailed unpaired Student's t-Test, whereas data containing more than two experimental groups were analysed with one-way ANOVA followed by Bonferroni's test. *P<0,05; **P<0,01; ***P<0,001; confidence intervals 95%, alpha level 0,05.

RESULTS

Cyanidin enriched diet leads to a general amelioration of histopathological signs of Muscular Dystrophy

In order to establish if cyanidin would be beneficial in protecting and delaying the different signs of muscular dystrophy, we fed *Sgca* null mice at weaning with a cyanidin enriched diet (named red diet, RD) for 5 weeks or 25 weeks, to evaluate a short and long-term benefits from this diet. As controls, we also provide to *Sgca* null and wild type mice (WT) a yellow diet (YD), an isogenic diet to RD without the enrichment in C3G, at the same time points (Figure Supplementary 1). The *Sgca* null mouse model (Duclos et al. 1998) was chosen for our analysis because of its very severe phenotype, resembling the main hallmarks of the Duchenne Muscular dystrophy (DMD) human pathology. Firstly, muscle histology was analysed through haematoxylin and eosin staining on *Tibialis anterior* and *Diaphragm* sections. We observed that dystrophic control mice fed with YD display at 5 weeks of diet first signs of muscle degeneration with inflammatory infiltrates and necrotic areas, hallmarks that worsen in the longer time point confirming that the control diet does not interfere with the pathology progression. On the other side, *Sgca* null mice fed with the RD display a better morphology of muscle tissue, with less infiltrates in particular at 5 weeks of diet (Fig.1A, B). In the 25 weeks of diet, YD group shows severe signs of muscular degeneration such as fibers disorganization, necrotic fibers and cellular infiltrates. Conversely, animals fed with the RD display a more preserved tissue, with less cellular infiltrates. Furthermore, to quantify the morphological amelioration, we measured the fiber calibre distribution (cross sectional area, CSA) in *Tibialis anterior* sections at both time points (Fig. Supplementary 2A), which underlined a non-homogenous distribution in dystrophic mice fed with YD. On the other side, animals supplied with the cyanidin enriched diet display a more homogenous distribution of the CSA at both 5 and 25 weeks of diet.

Collagen deposits in MDs are one the main signs of myopathy, which replace muscle tissue and compromise patients' mobility upon chronic inflammation environment (Zanotti et al., 2016). To establish if an anthocyanin-based diet might influence collagen deposits, we performed a Milligan's trichrome staining on *Tibialis anterior* and *Diaphragm* of YD or RD-fed *Sgca* null animals (Fig.2A). As the figure shows, *Sgca* null mice fed with control diet display abundant extracellular matrix in both muscles, in particular at 25 weeks. On the contrary, the dystrophic mice fed with the RD have less extracellular matrix deposition both in the tibialis anterior and in the diaphragm. Interestingly, this reduction persists also in the longer time point. Going further with analysis, we wanted to quantify what we observed with the trichrome staining, thus we performed an immunostaining for collagen I and measured the fluorescent positive area in tibialis anterior (Fig.2B). In both the time points analysed, we observed a decrease in collagen I deposits upon cyanidin stimuli compared to dystrophic mice supplied with the YD (Fig2C).

Antioxidant diet improves myofiber integrity and rescues muscle performance

To quantify the histological amelioration observed in *Sgca* null mice fed with a cyanidin enriched diet, we evaluated the main parameters that are normally altered in dystrophic muscles. The analysis of centrally nucleated myofibers, as a measure of muscle regeneration, did not lead to a significant difference between YD and RD-fed animals, indicating that regeneration process is not affected by cyanidin supplementation (Supplemental Figure 2B). In order to evaluate the integrity of muscle sarcolemmal membranes, we systemically injected Evan's Blue Dye (EBD) in dystrophic animal fed with yellow or red diet. As shown in Fig.3A and B, we observed a statistically significant decrease in the percentage of Evan's Blue positive fibers in

Sgca null mice supplied with RD compared to their littermates fed with the control diet at both 5 and 25 weeks of treatment.

Notably, we also verified whether this marked amelioration was also accompanied by an improvement of functional ability of RD-fed *Sgca* null mice, measuring muscle performance with a treadmill test. We measured the muscle performance as time to exhaustion for 21 weeks in *Sgca* null mice fed with RD or YD. As shown in the graph in Fig.3C, we observed a statistically significant increase in muscle endurance in mice supplied with cyanidin enriched diet, that lasts in time compared to their littermates fed with the control diet. Notably, the performance of *Sgca* null mice fed with the RD is comparable to the WT mice group.

Cyanidin prevents NF-kB nuclear localization inhibiting ERK phosphorylation

Inflammation in MDs plays an essential role in worsening the pathology. Macrophages are the immune population, which contributes the most in this phenomenon, establishing in time a chronic inflammatory environment. In order to evaluate macrophage infiltration, we sorted, by FACS, $CD64^+$ cells from hind limb muscle lysate from *Sgca* null mice fed with YD or RD. As shown in Fig. 4A, we observed a statistically significant decrease in the total macrophage population in muscle of dystrophic mice fed with cyanidin-enriched diet. Furthermore, we looked at the pro-inflammatory and anti-inflammatory macrophage sub-populations ($CD64^+$ $Ly6C^+$ and $CD64^+$ $Ly6C^-$, respectively), whose ratio is not altered with the diet in both the time points (Fig.4B). This indicates that cyanidin reduces macrophages infiltration but not interfere on their phenotype switching.

To further study on the possible modulation of the inflammatory pathway following Cyanidin assumption, we focused our attention on the ERK kinases. In fact, in literature it is reported that in primary myoblast culture, and in the myogenic stable cell line C2C12, under hypoxia and oxidative stimuli, ERK kinases are directly involved in NF-

kB translocation into the nucleus by phosphorylating its complex with IkBα (Kefaloyianni et al. 2006; Osorio-Fuentealba et al. 2009). Interestingly, we observed, through Western blot analysis, a decrease in ERK phosphorylation in quadriceps lysate of dystrophic mice fed with RD, which correlates with an increase in NF-kB canonical inhibitor IkBα protein level (Fig.4C). We also evaluated NF-kB translocation into the nucleus by analysing the nuclear and cytoplasmic protein fraction of quadriceps from YD or RD fed dystrophic animals (Fig.4D). Notably, we observed that when *Sgca* null mice were fed with the RD, NF-kB less translocates into the nuclei of muscle fibers in comparison to YD fed mice, in both time points. The impairment in NF-kB translocation into the RD-fed myonuclei resulted in a reduction of NF-kB functional activity as confirmed by the analysis of its target genes *TNF*-α and *iNOS,* which statistically decrease at both 5 and 25 weeks of diet (Fig4E).

Cyanidin enriched diet promotes a shift towards an oxidative metabolism

In order to further identify the mechanism(s) through which Cyanidin mainly acts, we tested its anti-oxidant power by looking at the protein carbonylation content (PCC), as a measure of the protein oxidation, in quadriceps protein extracts from *Sgca* null mice fed with the RD with respect to YD control fed animals. As shown in the graph in Fig.5A, we observed that the dystrophic condition in *Sgca* null mice fed with the control diet leads to high corbonylated protein contents that increases in time. More interestingly, when dystrophic mice are supplied with the RD, values of PCC strongly decrease to WT values. We thought that the decrease in carbonylation content might be due to an increase of oxidative metabolism and mitochondrial activity in animals fed with the RD (Schiaffino and Reggiani 2010). It is indeed well described that oxidative fibers, which express high levels of slow isoform of myosin heavy chain (MyHC slow, slow twitching fibers), are more protected from the dystrophy progression both in the *Sgca* null mouse

model and in human patients (Webster et al 1988; Danieli-Betto 2005). To confirm this hypothesis, we performed a SDH staining on cryosections of *Tibialis anterior* muscles from Sgca null mice fed with the YD and RD. This is an enzymatic staining able to colour fibers displaying oxidative metabolism (dark blue) by mitochondrial SDH enzymatic activity. The staining highlighted more fiber with oxidative metabolism in tibialis anterior of *Sgca* null mice fed with RD compared to their littermates who were supplied with the control diet (Fig.5B). This observation was molecularly confirmed by checking the presence of the pivotal transcription factor of mitochondrial biogenesis and oxidative metabolism in muscle: PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α) (Renèe-Ventura et al.; 2008; Villena 2014; Wang et al.; 2015). Western blot analysis on tibialis anterior protein extracts revealed a higher expression of PGC-1α in muscles derived from dystrophic mice fed with RD compared to those from fed with the control diet, with an increase at the later time point (Fig.5C). This observation was also confirmed by checking the expression levels of two genes target of PGC-1α, *CPT (carnitine palmitoytransferase)* and *UCP (mitochondrial uncoupling protein-3)* (Fig Supplementary 2C)

To further investigate whether the RD could, not only, promotes a metabolic shift, but also promotes a slow twitching muscle fiber phenotype, we performed an immunostaining for slow MyHC to quantify slow fibers in tibialis anterior sections (Fig.5D). The quantification of the immunostaining at both time points led us to conclude that a cyanidin enriched diet promotes a more switch towards an oxidative muscle metabolism.

Cyanidin promotes antioxidant pathway through Nrf-2 activity

To molecularly characterize the antioxidant response observed, we focused on the possible antioxidant pathway triggered by cyanidin. We interestingly observed, by Western blot, an activation of AMPK in quadriceps protein extract of *Sgca* null mice

fed with RD in respect to mice fed with YD (Fig.6A). It is indeed reported in literature that AMPK is sensitive to the cellular oxidative status and activate an antioxidant response (Emerling et al., 2009; Zmijewskiand et al.; 2010), suggesting that antioxidant pathway is triggered in our experimental model. We focused, then, our attention on the main transcription factor involved in mammals' antioxidant response: Nrf-2.

Under basal Nrf-2 is retained in the cytoplasm and its ubiquitination promotes proteasome-dependent degradation (He et al.; 2003; Ma 2013; Liam et al.; 2014). Instead, in an oxidative environment, Nrf-2 uncouples from its inhibitory complex and translocates into the nucleus, enabling then ARE-dependent (antioxidant responsive element) genes transcription, such as *heme-oxigenase-1(HO-1)* and *glutamate-cysteine ligase catalytic subunit (GCLC)* (Ma et al.; 2013; Liam et al.; 2014).

Indeed, Western blot analysis of nuclear and cytoplasmic protein fractions show that Nfr-2 is more present into the nuclei of dystrophic mice when cyanidin is supplied (Fig.6B), compared to their dystrophic littermates fed with the control diet, which indeed displays an impairment of Nrf-2 nuclear localization. This data was also confirmed by q-PCR looking at canonical targets of Nrf-2 *HO-1* and *GCLC,* which are up-regulated in mice fed with cyanidin for both 5 week and 25 weeks.

Red diet ameliorates already compromised dystrophic mice

The results shown so far have clearly demonstrated that cyanidin enriched diet in dystrophic animals causes a significant improvement of the disease. In light of a future translational approach, we wondered whether supplying the RD might lead to the same effects observed even when the dystrophic disease already occurred, which represents what would normally be feasible in patients.

To address this question, we provided RD and the control YD to *Sgca* null at five weeks of age for 15 weeks, when sings of dystrophy can be detected (Duclos et al. 1998). The Haematoxylin and Eosin staining on *Tibialis anterior* and *Diaphragm* muscles of *Sgca* null mice fed with RD highlighted amelioration in terms of muscle organization and morphology (Fig.7A), which appears more preserved with less cell infiltrates. This observation is further confirmed by the cross-sectional area data which shows in dystrophic mice supplied with RD a more homogenous distribution of the fiber calibre values compared to the YD fed ones (Fig. Supplementary 3A). Trichrome staining on *Tibialis anterior* and *Diaphragm* sections also showed a decrease in extra-cellular matrix deposits (Fig.7A) when RD is supplied, at variance with *Sgca* null mice fed with the control diet. Morphological amelioration was further confirmed by EBD assay (Fig.7B), where tibialis anterior sections of dystrophic mice fed with RD show less permeability to the dye compared to the control group (Fig. Supplementary 3B).

We also focused on inflammatory parameters by quantifying the number of F4/80 positive macrophages (a surface marker of this immune population) in *Tibialis anterior* sections from YD versus RD fed *Sgca* null mice. The data (Fig. 7C) displays less infiltrating macrophages in *Sgca* null mice fed with the cyanidin enriched diet compared to the data collected in the dystrophic mice supplied with control diet. We also checked whether cyanidin is also able to act on the ERK-NF-kB axis when the disease is already advanced. Fig. 7D shows that ERK phosphorylation is downregulated, whereas the IkBα protein level is increased. Moreover, the nuclear/cytoplasmic protein fraction analysis of muscle lysates from YD and RD fed animals displays less NF-kB nuclear translocation in mice fed with the RD compared to those fed with the control diet, which show an increased pro-inflammatory signal through ERK-NF-kB axis. These results were also confirmed by the analysis of the inflammatory genes *TNF*-α and *iNOS* which statistical significant decrease in dystrophic mice fed the antioxidant-enriched diet (Fig. Supplementary 3D).

Similarly, to what described in dystrophic animals fed with the different diet at weaning, we observed an oxidative fiber metabolism shift in *Sgca* null mice fed with the RD at 5 weeks of age, in terms of SDH activity (Fig. Supplementary 3C). Consistently, AMPK is more active also in mice with RD and nuclear and cytoplasmic protein fractions displays a more translocation of Nfr-2 in the nuclear compartment upon cyanidin dietary stimuli (Fig.7E)

DISCUSSION

Muscular dystrophies are severe degenerative diseases that still now lack of a definitive therapy. In particular, the main feature of this pathology is a loop of muscle tissue degeneration followed by attempts to repair muscle damage, which, failing, worsen the pathology. This is mainly reflected on the satellite cells population that, in time, it is exhausted and muscle replaced by connective and adipose tissue, thus compromising its function and leading patients first to wheelchair and, in the most severe cases, at premature death. Previous works in literature show that dystrophic muscle display an intrinsic susceptibly to oxidative stress, and it was suggested this process might be at the molecular basis of the MDs onset impairing myofibers viability and contributing in time strongly to the dystrophies progression (Bedard and Krause 2007; Cardaci, Filomeni and Ciriolo 2012; Rando et al. 1998), but literature still lacks of a detailed molecular mechanism that describe this phenomenon.

Proper nutrition, assuming an optimal intake of bioactive compounds, might be at basis of new therapeutic approaches (Speciale et al. 2014). Interestingly, there is a growing body of literature that show that consumption of natural antioxidant is associated with no side effects, and also despite their lower bioavailability (less than 2%) provide protective effects against oxidative stress in several models either *in vivo* and *in vitro* (Martin et al. 2010; He and Giusti 2010). Specifically, in this case, cyanidin enriched diet has already been proved its safety in humans in two, currently on-going, clinical trials on breast cancer (Cerletti et al., 2016)

In this study, we show that providing natural antioxidant (cyanidin, red diet RD) to dystrophic mice it is possible to delay muscular dystrophy sings. In particular, RD promotes, in *Sgca* null mice, a protective effect by MD progression, inducing important ameliorations both in the muscle morphology and, most importantly, in terms of muscle performance. We confirmed also that promoting an oxidative fiber metabolism is more

protective from this pathology as others have shown before (Webster et al., 1998; Danieli-Betto et al. 2004). Interestingly, in literature it is known that slow-twitching fibers, which show a more oxidative metabolism, are more protected from MDs degeneration in respect to fast-twitching fibers (Webster et al., 1998; Danieli-Betto et al. 2004). We reported that dietary cyanidin promotes a shift to a more oxidative metabolism, enhances mitochondrial biogenesis genes and slow-twitching fiber phenotype acting on MD progression in this mouse model. Further investigation on the upstream molecular pathway that might links molecular and histological amelioration observed, lead us to study Nrf-2 translocation pattern, a transcription factor which acts in response to oxidative stress. We show that when RD is supplied to *Sgca* null mice, Nrf-2 is more prone to translocate into the nuclei and promotes the activation of antioxidant genes such as *HO-1* and *GCLC.* More interestingly, for the first time, we reported that in dystrophic mice fed with the control diet, Nrf-2 nuclear protein levels are decreased compared to mice with RD, suggesting that *Sgca* null display an impairment in the antioxidant signalling due to Nrf-2 miss localization impacting, therefore, on dystrophy progression.

Moreover it is well described in literature how inflammation and in particular macrophages play an essential role in hastening the pathology course (Haycock et al. 1998; Jackman et al. 2013; Tidball & Villalta 2010; Rando 2002). We reported that macrophages infiltration in the muscle tissue is reduced when cyanidin is supplied to the mice, which acts on NF-kB reinternment in to the cytoplasm through both the activation of ERK kinases and the increased expression of IkBα protein levels. The cytoplasmic localization of NF-kB is crucial to the negative regulation of typical inflammatory genes, such as *TNF-α* and *iNOS* whose role in worsening muscular dystrophy progression is well described in literature (Haycock et al. 1998; Jackman et al. 2013; Tidball & Villalta 2010; Rando 2002).

Thus, this study gives a more detailed picture of how ROS accumulation, oxidative stress, antioxidant and anti-inflammatory signals cross-talk and impact on the MD progression, through the identification of the essential players in this process to tune in order to observe a morphological and functional on this pathology.

Summarizing, we demonstrated that cyanidin has a dual protective role in the amelioration of muscular dystrophy progression. Firstly, acting as a powerful antioxidant, and secondly interfering on the increased inflammation which is coupled with the progressive muscle wasting.

Overall, the results collected demonstrate for the first time that a dietary natural compound might be a powerful strategy to counteract the progression of a degenerative genetic disease. Since cyanidin enriched food is available also for humans (Ceretti et al. 2017), it could be good practice to couple this dietary protocol with molecular and genetic approach that aim to correct the genetic defects at basis of muscular dystrophies. Therefore, we suppose that cyanidin could be strongly affective not only on the different groups of muscular dystrophies but also for other pathologies, in which oxidative stress and inflammation play a crucial role.

Acknowledgements

We thank Marco Sandri and Pura Muñoz for the kind exchange of information and helpful discussion. We are also grateful to Professor Binelli and Dr. Camilla dalla Torre for the technical assistance.

This work was funded by the European Community, ERC StG2011 (RegeneratioNfix 280611).

AUTHOR CONTRIBUTIONS

E.C. designed and performed all the experiments with the assistance of S.A and V.T. C.B. F.S., V.F., M.S. performed the macrophage isolation and cell sorting analysis. C.T. and K.P. supplied the different diets. G.M. supervised the work and wrote the paper together with E.C.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests

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Figure and Figure legends

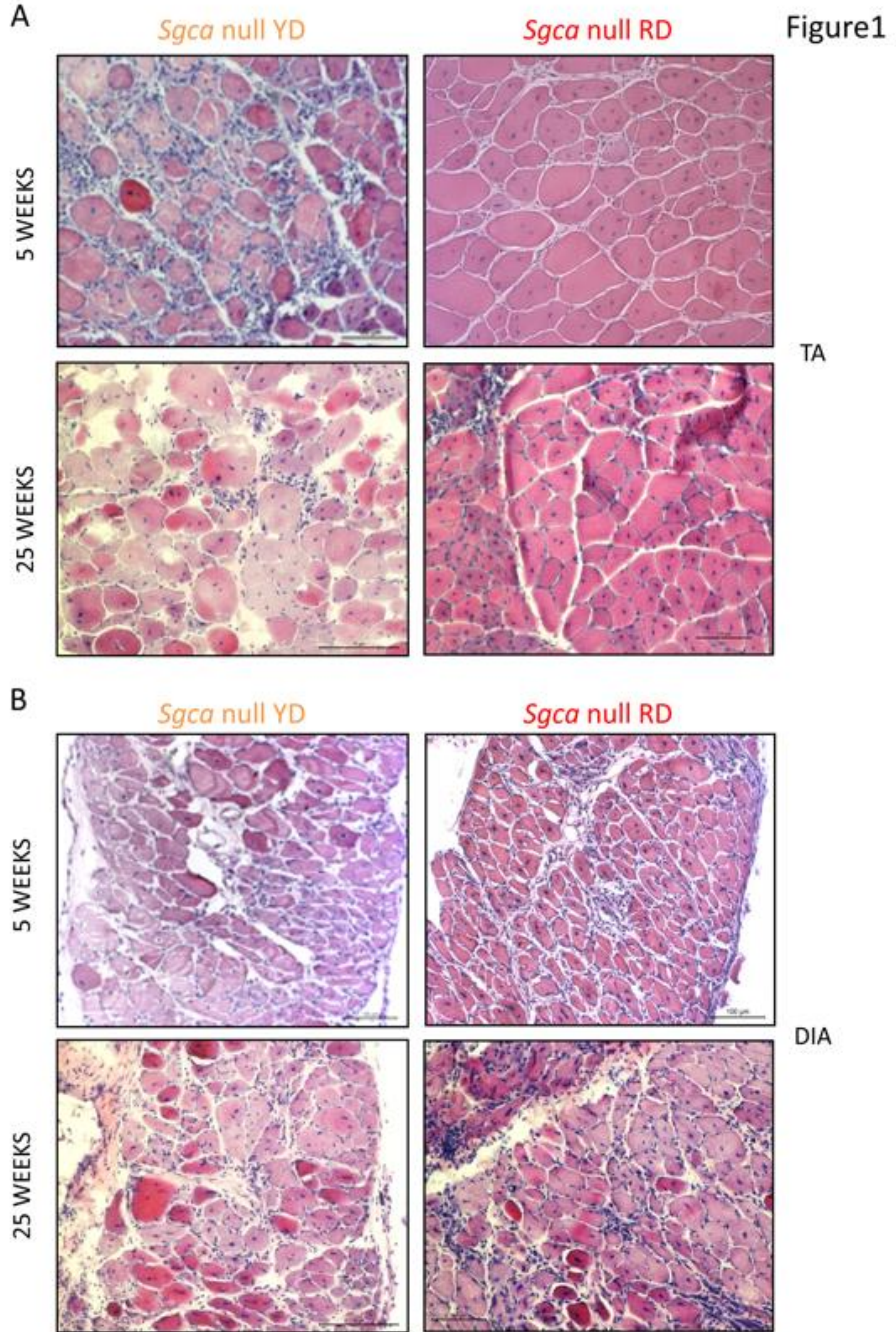

A
Sgca null YD
Sgca null RD
Figure1
5 WEEKS
25 WEEKS
TA
B
Sgca null YD
Sgca null RD
5 WEEKS
25 WEEKS
DIA

Figure.1 Cyanidin enriched diet ameliorates muscle morphology

(A) Hematoxylin and Eosin (H&E) staining of *Tibialis anterior* of *Sgca* null mice fed with YD or RD for 5 weeks or 25 weeks. Scale bar 100µm. N=16 *Sgca* null YD and N=16 *Sgca* null mice RD in the 5 weeks group. N=8 *Sgca* null YD and N=10 *Sgca* null mice RD for the 25 weeks group. (B) Hematoxylin and Eosin (H&E) staining of *Diaphragm* of *Sgca* null mice fed with YD or RD for 5 weeks or 25 weeks. Scale bar 100 µm. N=16 *Sgca* null YD and N=16 *Sgca* null mice RD in the 5 weeks group. N=8 *Sgca* null YD and N=10 *Sgca* null mice RD for the 25 weeks point.

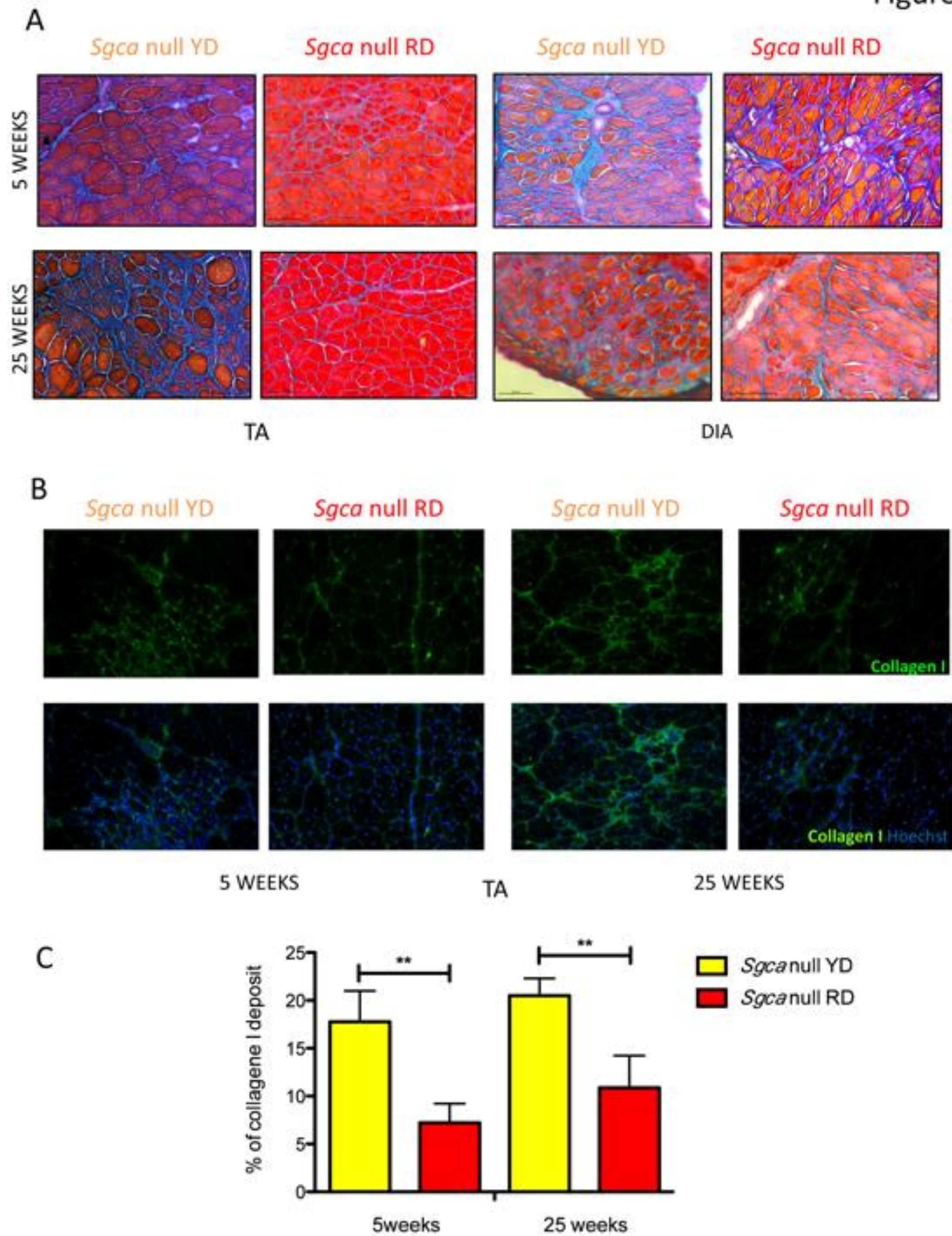
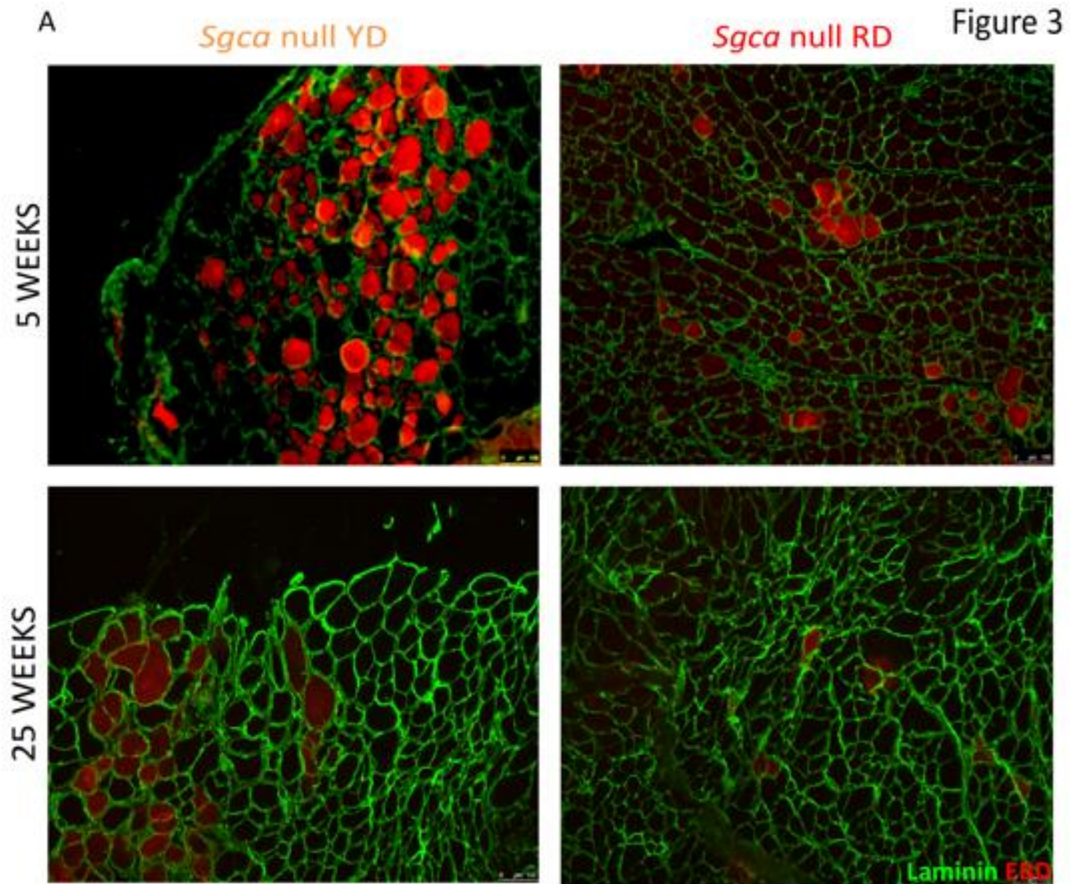


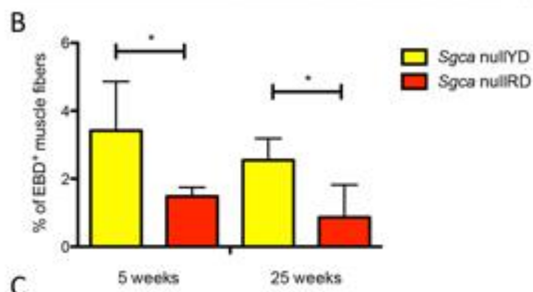
Figure 2
A
Sgca null YD
Sgca null RD
Sgca null YD
Sgca null RD
5 WEEKS
25 WEEKS
TA
DIA
B
Sgca null YD
Sgca null RD
Sgca null YD
Sgca null RD
Collagen I
Collagen I Hoechst
5 WEEKS
TA
25 WEEKS
C
% of collagene I deposit
25
20
15
10
5
0
**
**
5weeks
25 weeks
Sgca null YD
Sgca null RD

Figure.2 Cyanidin enriched diet promotes less collagen deposits

(A)Milligan's Trichrome staining of *Tibialis anterior* (left panel) and *Diaphragm* (right panel) muscles at 5 weeks (upper panel) and 25 weeks of diet (below panel), Scale bar 100 µm. N=16 *Sgca* null YD and N=16 *Sgca* null mice RD in the 5 weeks group. N=8 *Sgca* null YD and N=10 *Sgca* null mice RD for the 25 weeks group. (B) Immunofluorescence showing Collagen I deposits (green) and Hoechst (blue) in *Tibialis anterior* sections of *Sgca* null mice fed for 5 weeks (left panel) and 25 weeks (right panel) with YD or RD, Scale bar 100µm. N=4 *Sgca* null YD and N=4 *Sgca* null mice RD in the 5 weeks group. N=5 *Sgca* null YD and N=5 *Sgca* null mice RD for the 25 weeks group. (C) Quantification of fluorescent positive area for Collagen I at 5 weeks and 25 weeks of diet. N=4 for *Sgca* null YD 5weeks and N=4 for *Sgca* null RD. For the 25 weeks N=5 for *Sgca* null YD and *Sgca* null RD. Values are expressed as Mean±whiskers. Two-tailed unpaired Student's t-Test; **P<0,01.

Figure 3





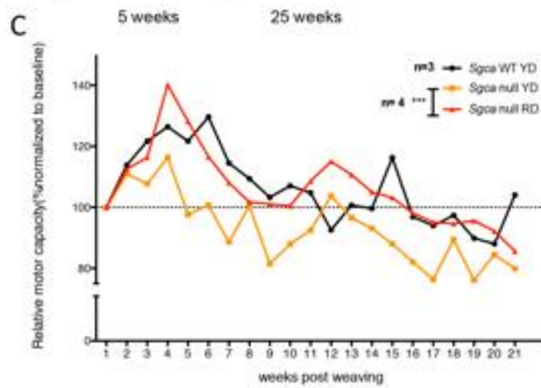


Figure.3 Red diet promotes fiber integrity and rescue muscle performance

(A) Immunofluorescence for laminin (green) and EBD (red) on *Tibialis Anterior* at 5 weeks (upper panel) and 25 (below panel) of diet. Scale bar 100m. N=7 *Sgca* null YD and RD for 5 weeks group, and N=10 for *Sgca* null YD and RD for 25 weeks group. (B) Percentage of EBD positive myofibers in *Tibialis Anterior* at 5 weeks and 25 weeks of diet. N=7 *Sgca* null YD and RD for 5 weeks group, and N=10 for *Sgca* null YD and RD for 25 weeks group; mean± SD. (C) Treadmill test on WT fed with YD, *Sgca* null YD, *Sgca* null RD mice. The plot shows the percentage of the time to exhaustion for each session compared to the mean of the first two sessions of training. N=21 measurements for 3 WT animals, 4 *Sgca* null YD and RD. Mean±SD. Two-tailed unpaired Student's t-Test; *P<0,05; ***P<0,001.

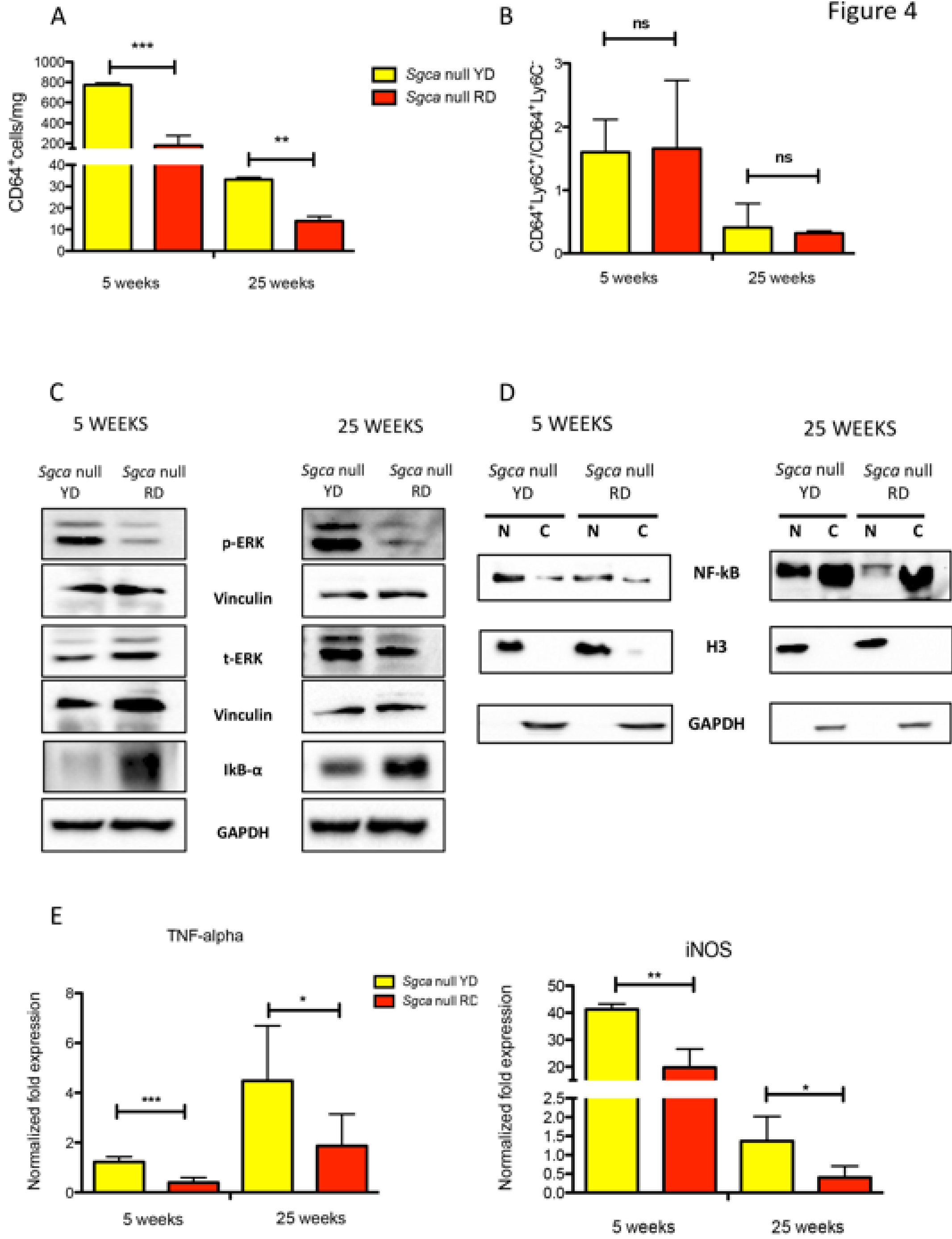


Figure 4
A
B
C
D
E
Sgca null YD
Sgca null RD
CD64⁺cells/mg
5 weeks
25 weeks
ns
5 WEEKS
25 WEEKS
p-ERK
Vinculin
t-ERK
IkB-α
GAPDH
NF-kB
H3
N
C
TNF-alpha
iNOS
Normalized fold expression

Figure.4 Inflammatory parameters decrease in *Sgca*-null mice fed with RD through a down regulation of NF-kB signalling

(A) Number of $CD64^+$ sorted cells from total hind limb extracts in *Sgca* null mice fed with YD or RD for 5 and 25 weeks. N=6 for *Sgca* null mice fed with YD and N=7 for *Sgca* null mice fed with RD for 5 weeks group. For the 25 weeks group, N=3 for *Sgca*-null YD and RD. (B) Ratio between $CD64^+LyC6^+$(proinflammatory) and $CD64^+LyC6^-$ (anti-inflammatory) sorted cells from total lysate of hindlimbs. N=6 for *Sgca* null mice fed with YD and N=7 for *Sgca* null mice fed with RD for 5 weeks group. For the 25 weeks group, N=3 for *Sgca*-null YD and RD.

(C) Western blot from protein lysate of mice fed with YD or RD for 5 and 25 weeks. The assay was repeated 4 times. (D) Western blot of nuclear and cytoplasmic fraction of *Sgca* null mice fed with YD or RD for 5 and 25 weeks. Blots show the localization of NF-kB into the nuclear and cytoplasmic compartments. Histone 3 and GAPDH were chosen as normalization for the nuclear and cytoplasmic fraction, respectively. The assay was repeated 5 times.

(E) Real Time PCR analysis of *TNF-α* (left panel) and *iNOS* (right panel) expression on N=5 *Sgca* null YD, N=5 *Sgca* RD null fed for 5 weeks. For the 25 weeks group N=4 *Sgca* null YD and N=4 *Sgca* null RD. Mean±SD;

Two-tailed unpaired Student's t-Test; *P<0,05; **P<0,01 ***P<0,001; ns: non significant.

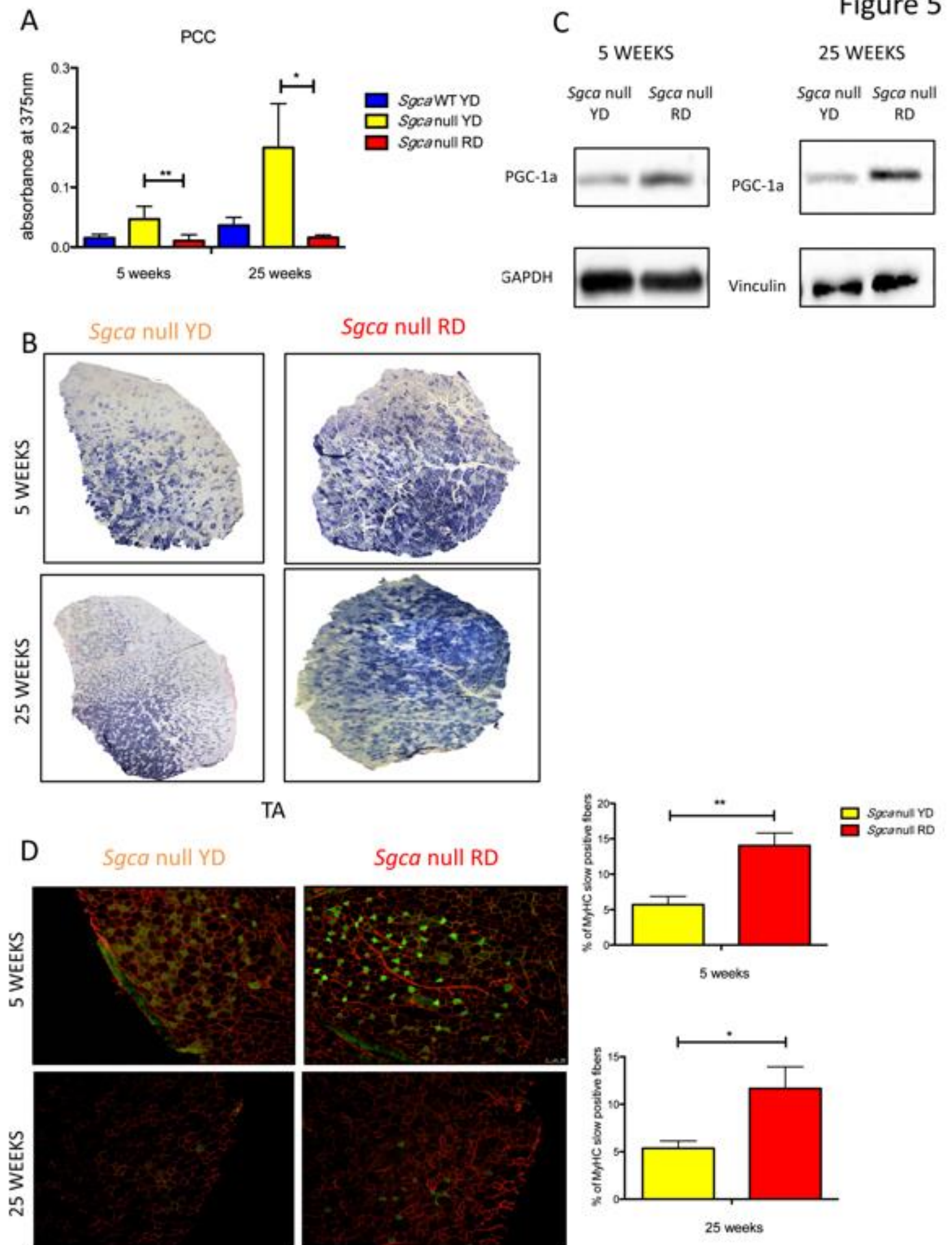


Figure 5
A
PCC
absorbance at 375nm
0.3
0.2
0.1
0.0
**
*
5 weeks
25 weeks
Sgca WT YD
Sgca null YD
Sgca null RD
C
5 WEEKS
25 WEEKS
Sgca null YD
Sgca null RD
Sgca null YD
Sgca null RD
PGC-1a
PGC-1a
GAPDH
Vinculin
B
Sgca null YD
Sgca null RD
5 WEEKS
25 WEEKS
TA
D
Sgca null YD
Sgca null RD
5 WEEKS
25 WEEKS
% of MyHC slow positive fibers
20
15
10
5
0
**
5 weeks
Sgca null YD
Sgca null RD
% of MyHC slow positive fibers
15
10
5
0
*
25 weeks

Figure.5 Cyanidin promotes a shift to a more oxidative fiber metabolism

(A) Quantification of protein carbonylation content (PCC) from protein lysate of WT, *Sgca* null YD and RD mice fed for 5 or 25 weeks. N=4 *Sgca* WT YD, N=4 *Sgca* null YD and N= 3 *Sgca* null RD mice fed for 5 weeks. For the 25 weeks, N=4 *Sgca* WT YD, N=3 *Sgca* null YD and N= 3 *Sgca* null RD mice (B) Entire *Tibialis anterior* muscle section reconstructions showing SDH staining at 5 weeks and 25 of diet; N=6 *Sgca* null YD and 5 *Sgca* null RD mice fed for 5 weeks. For the 25 weeks, group N=6 *Sgca* null YD and 6 *Sgca* null RD mice. Scale bar 100m. (C) Western blot underlying PGC-1α protein levels from protein lysate of *Sgca* null mice fed with YD or RD for 5 and 25 weeks. N=3 for *Sgca* null mice fed with YD or RD for 5 and 25 weeks. (D) Immunofluorescence showing slow MyHC positive fibers (green), laminin (red) and nuclei (Hoechst, blue) of Tibialis anterior sections of *Sgca* null mice fed with YD or RD for 5 weeks (upper panel) and 25 weeks (panel below). Scale bar 50µm. On the right panel percentage of slow MyHC-positive fibers were normalized on the total number of laminin-positive myofibers. N=7 *Sgca* null YD and N=7 *Sgca* null RD mice fed for 5 weeks. For the 25 weeks group, N=6 *Sgca*-null YD and N=6 *Sgca*-null RD mice. Mean±SD; Two-tailed unpaired Student's t-Test; *P<0,05; **P<0,01.

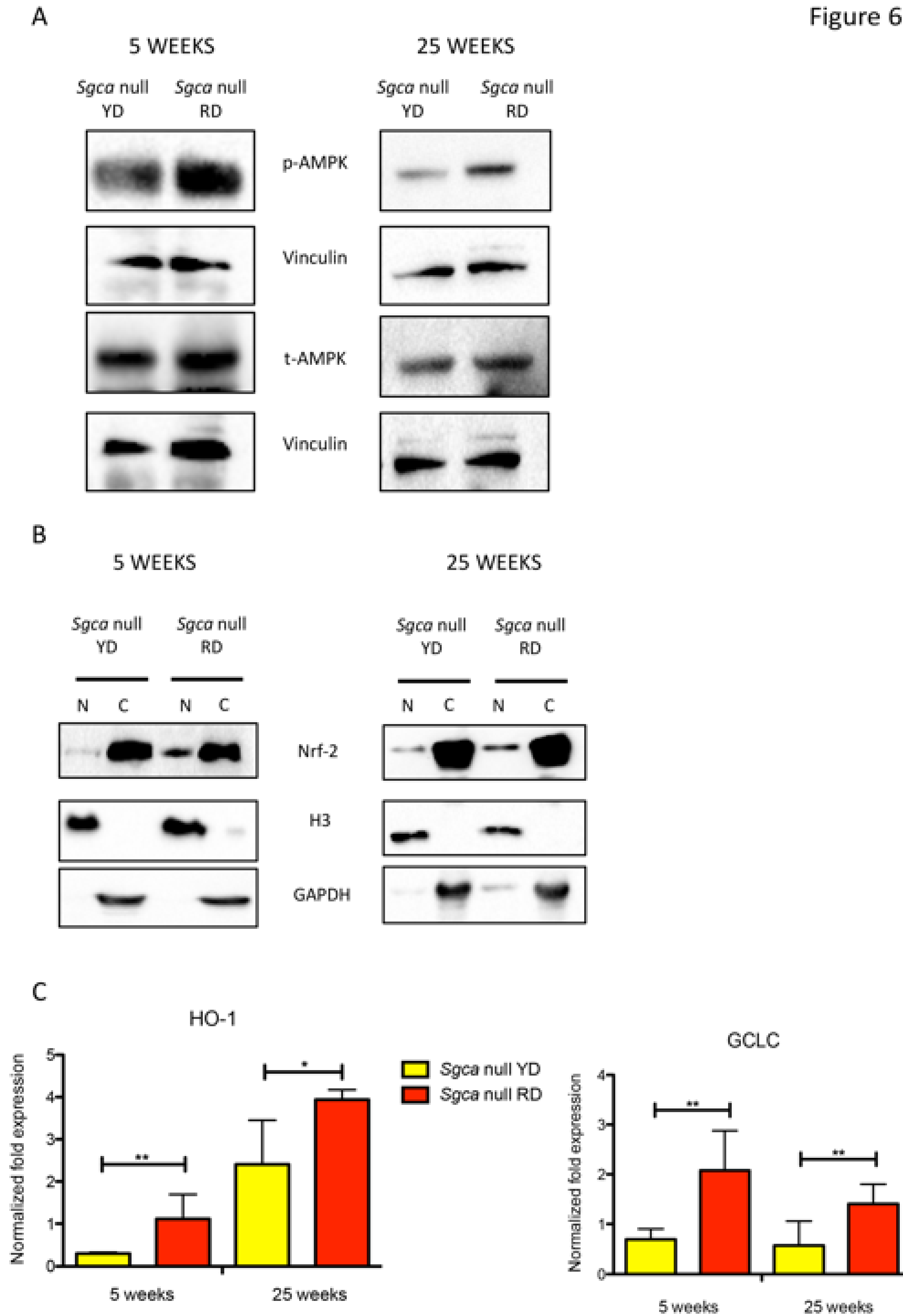


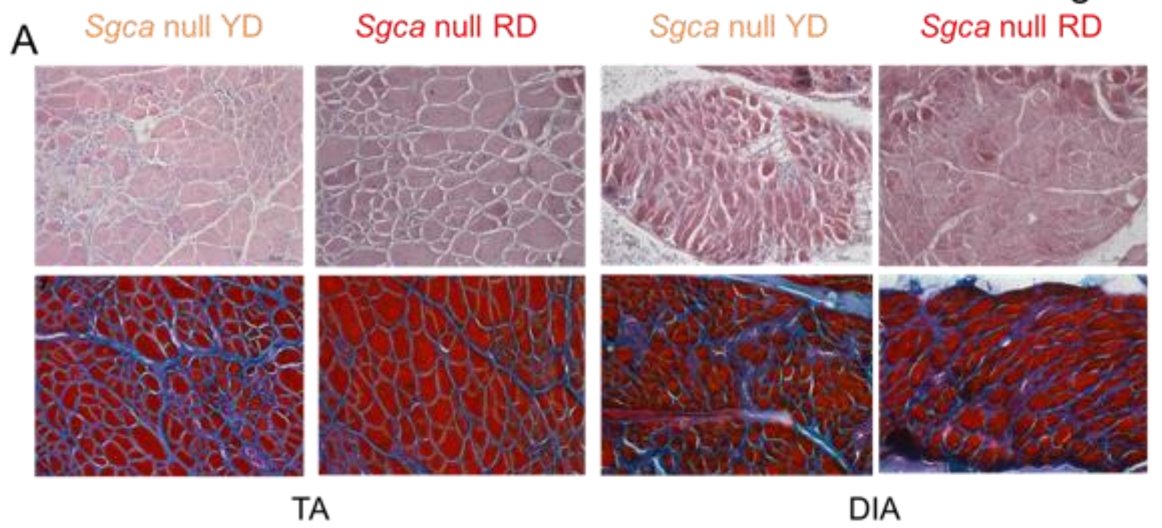
Figure.6 AMPK activity promotes the antioxidant response through Nrf-2 translocation into the nuclei

(A) Western blot analysis of AMPK phosphorylation from quadriceps protein extracts of *Sgca* null mice fed with YD or RD for 5 and 25 weeks. The assay was performed 4

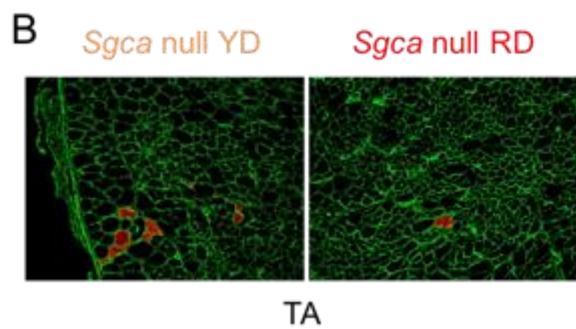
times for the 5 weeks group and 3 times for the 25 weeks group. (B) Western blot analysis of Nrf-2 localization from nuclear and cytoplasmic fraction of quadriceps of *Sgca* null mice fed with YD or RD for 5 and 25 weeks. Histone3 and GAPDH were chosen as normalization for the nuclear and cytoplasmic fraction, respectively. The assay was repeated 5 times. (C) Real Time PCR analysis of Nrf-2 target genes *HO-1* (left panel) and *GCLC* (right panel) expression on N=5 *Sgca* null YD, N=5 *Sgca* RD null fed for 5 weeks. For the 25 weeks group, N=4 *Sgca* null YD and N=4 *Sgca* null RD quadriceps muscles. Mean±SD; Two-tailed unpaired Student's t-Test; *P<0,05; **P<0,01.

Figure 7

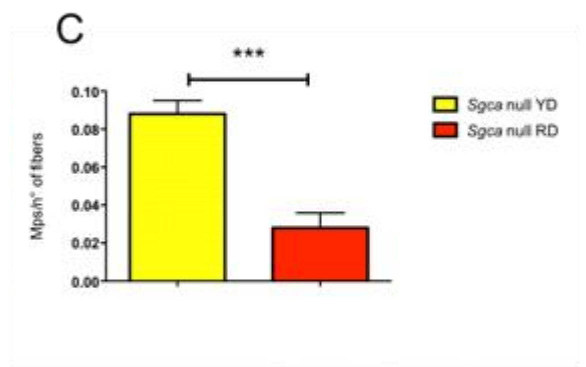
A



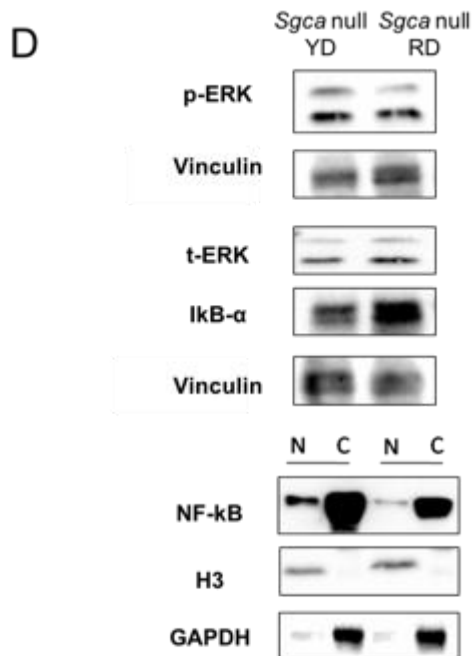
B



C



D



E

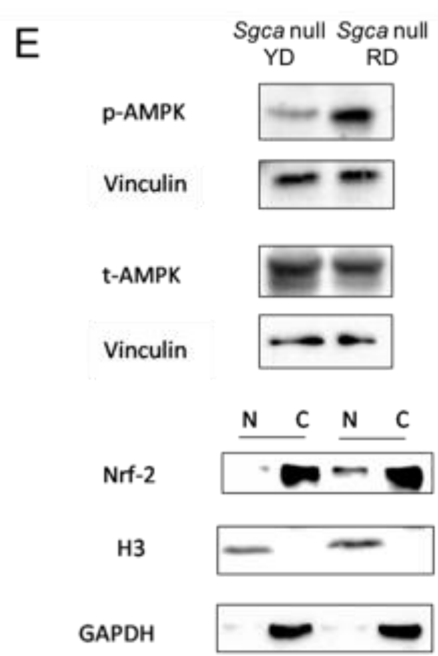


Figure.7 Cyanidin enriched diet ameliorates dystrophic phenotype also in already compromised *Sgca*-null mice

(A) Upper: Hematoxylin and Eosin (H&E) staining on *Tibialis anterior* (left panel) and Diaphragm muscles (right panel) of 5 weeks-old *Sgca* null mice fed with YD or RD for 15 weeks. Scale bar 100µm. Lower: Milligan's Trichrome staining on *Tibialis anterior* (left panel) and Diaphragm muscles (right panel) in animals in the different experimental conditions. Scale bar 100µm. N=3 *Sgca* null YD and N=3 *Sgca* null mice RD. (B) Immunofluorescence for laminin (green) and EBD (red) on Tibialis Anterior. Scale bar 50µm. N=3 *Sgca* null YD and N=3 *Sgca* null mice RD

(C) Quantification of F4/80$^+$ positive cells in Tibialis Anterior sections of 5 weeks-old mice fed with YD or RD for 15 weeks. N=3 *Sgca* null YD and N=3 *Sgca* null mice RD. (D) Western blots show phosphorylated ERK compared to total ERK, IKBα protein level (upper panel) from protein extract of *Sgca* null mice fed with YD or RD for 15 weeks. The below panel shows Western blot of NF-kB protein levels in the nuclear and cytoplasm fractions of *Sgca* null mice fed with YD or RD 15 weeks. N=3 *Sgca* null YD and N=3 *Sgca* null mice RD. (E) Western blots show AMPK phosphorylation level compared to total AMPK (upper panel) from protein extract of *Sgca* null mice fed with YD or RD 15 weeks. The below panel show Western blot of Nrf-2 protein levels in the nuclear and cytoplasm fractions of extracts of *Sgca* null mice fed with YD or RD 15 weeks. N=3 *Sgca* null YD and N=3 *Sgca* null mice RD. Mean±SD; Two-tailed unpaired Student's t-Test; ***p<0,01; ns: non significant.

Figure Supplementary 1

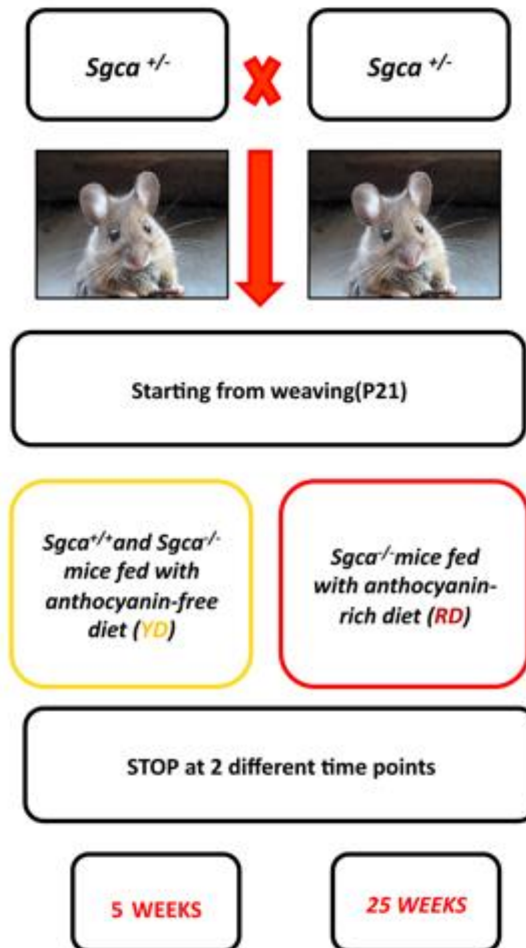


Figure supplementary 1

Scheme of the dietary protocol

Figure Supplementary 2

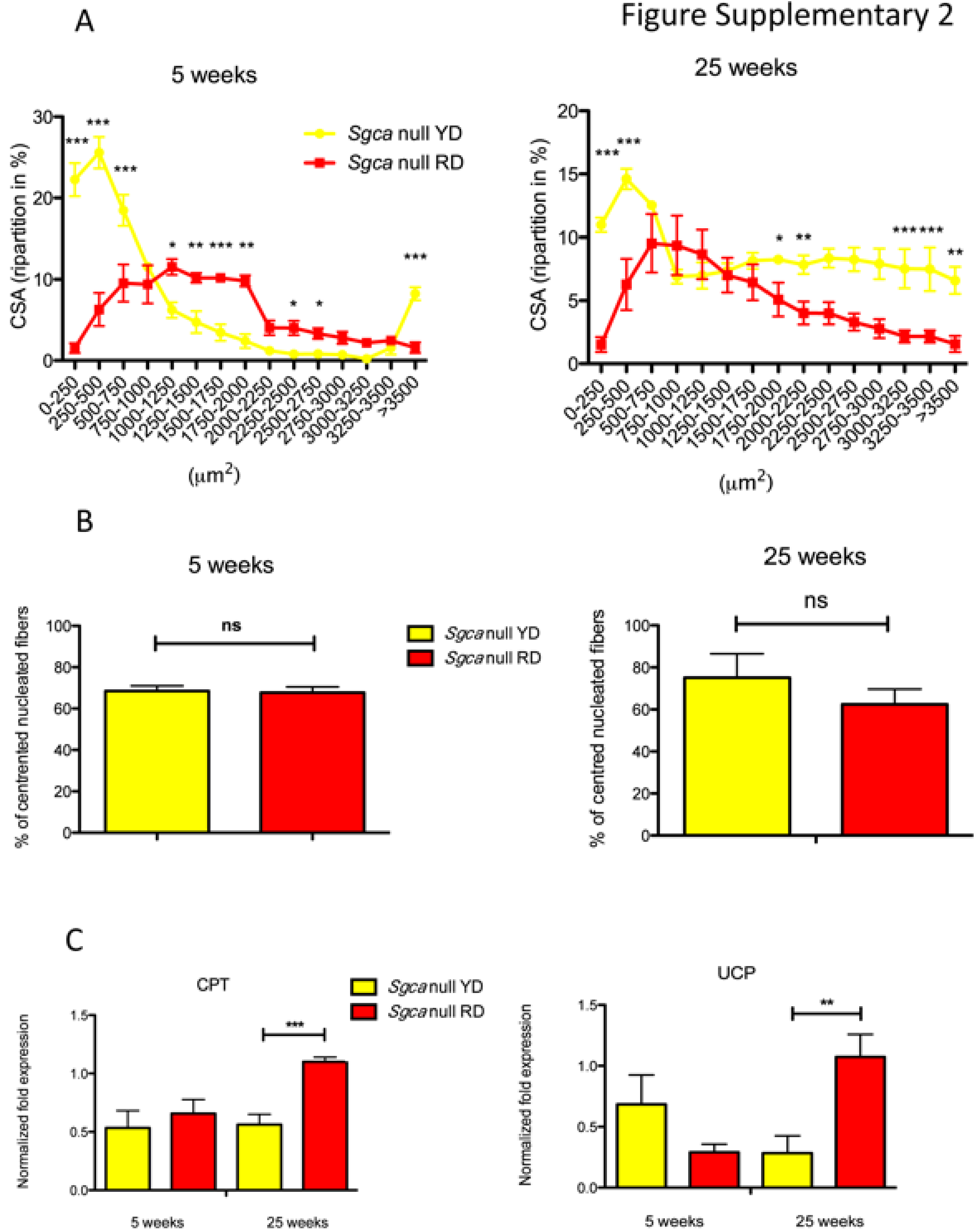


Figure supplementary2

(A) *Tibialis anterior* Percentage distribution of the fiber caliber (CSA) of *Sgca* null mice fed with YD or RD for 5 weeks (left panel) or 25 weeks (right panel). N=3 for *Sgca* null YD, N=5 for *Sgca* null RD in the 5 weeks group. N=7 for *Sgca* null YD and RD in the 25 weeks group. (B) Percentage of central nucleated myofiber in *Tibialis anterior* muscles. N=7 for *Sgca* null YD, N=7 for *Sgca* null RD in the 5 weeks group. N=7 for *Sgca* null YD and RD in the 25 weeks group. (C) Expression levels of two PGC-1a target genes *CPT* and *UCP*. N=7 for *Sgca* null YD, N=7 for *Sgca* null RD in the 5 weeks group. N=7 for *Sgca* null YD and RD in the 25 weeks group. N=5 *Sgca* null YD, N=5 *Sgca* RD null fed for 5 weeks. For the 25 weeks group, N=4 *Sgca* null YD and N=4 *Sgca* null RD. Mean±SD; Two-tailed unpaired Student's t-Test; **P<0,01; ns= non signficant.

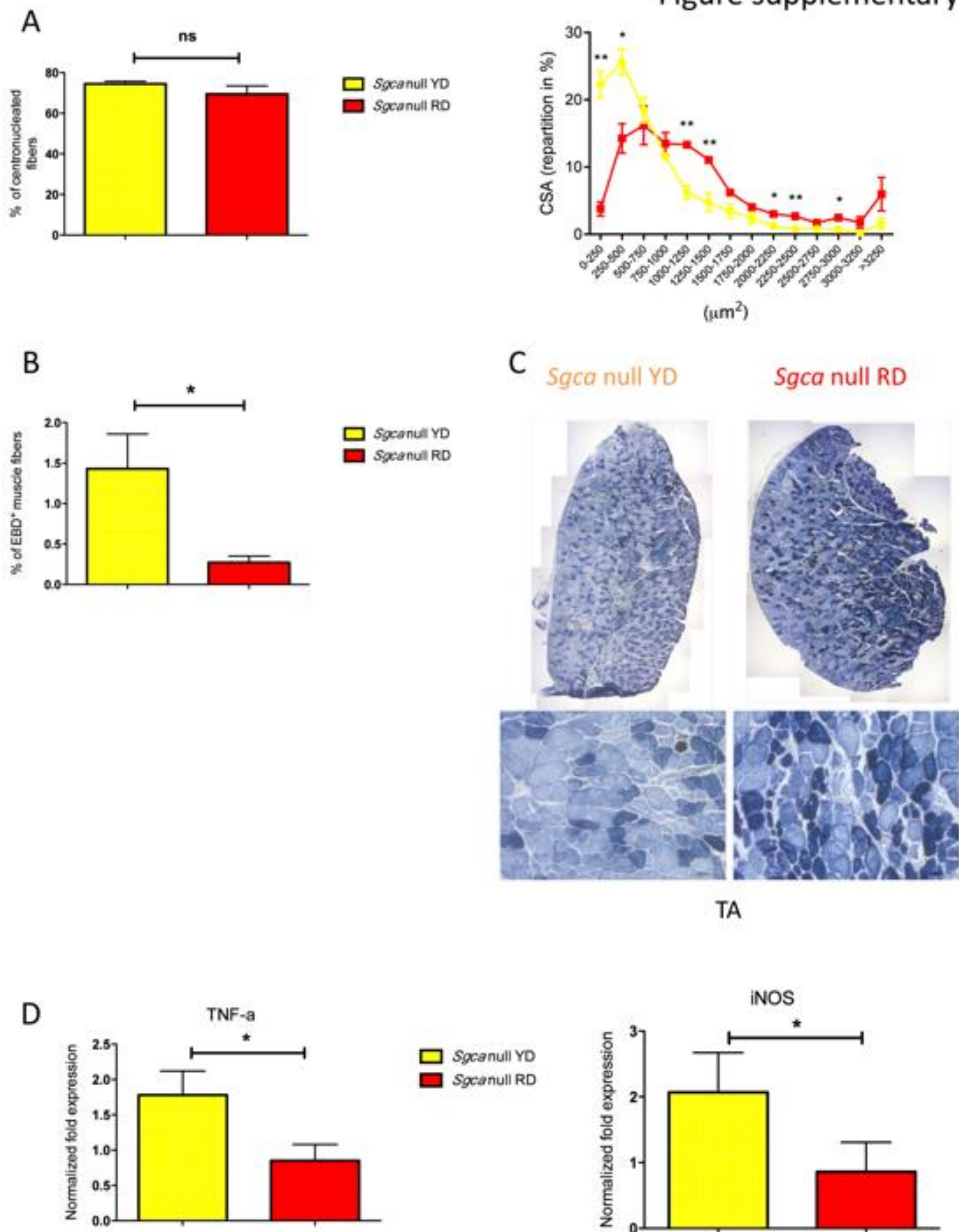


Figure supplementary 3
A
ns
% of centronucleated fibers
Sgca null YD
Sgca null RD
CSA (repartition in %)
(μm²)
B
*
% of EBD⁺ muscle fibers
Sgca null YD
Sgca null RD
C
Sgca null YD
Sgca null RD
TA
D
TNF-a
*
Normalized fold expression
Sgca null YD
Sgca null RD
iNOS
*
Normalized fold expression

Figure supplementary 3

(A)Percentage of centrally nucleated myofibers in *Tibialis anterior* muscles at 15 weeks of diet (left panel); N=3 *Sgca* null YD and 3 *Sgca* null RD mice; mean± SD; Distribution of fiber calibre (right panel) in *Sgca* null mice fed for 15 weeks with YD or RD; Mean± SEM. (B) Percentage of EBD positive myofibers in Tibialis Anterior at 15 weeks of diet; N=3 *Sgca* null YD and RD for 5 weeks group. (C)Entire *Tibialis anterior* muscle section reconstructions and higher magnifications showing SDH staining at 15 weeks of diet; N=3 *Sgca* null YD and 3 *Sgca* null RD mice. Scale bar 100µm. (D) Real Time PCR analysis of *TNF-*α (left panel) and *iNOS* (right panel) expression on N=3 *Sgca* null YD, N=3 *Sgca* RD null fed animals. Mean±SD; Two-tailed unpaired Student's t-Test; *P<0,05; **P<0,01; ns: non significant.

Part III

Cyanidin selectively promotes the antioxidant response through Nrf-2 signal in C2C12

In order to investigate if cyanidin is able to selectively promote the transcription of antioxidant genes through Nrf-2 dependent pathway, we infected the myoblast cell line C2C12 with a Short-hairpin targeting Nrf-2 mRNA (Sh-Nrf2, Sigma-Aldrich). The so modified cell line (fig. 8A) was exposed to an oxidative stimulus with 1mM of H_2O_2 (Sigma-Aldrich, Rando et al. 1997) with or without the cyanidin enrichment in the medium**.** After 4 hours, we checked the cells viability through Trypan blue counting, and observed that in the Sh-Nrf-2 C2C12 are more sensitive to an oxidative stimulus compared to scramble infected C2C12 exposed only to H_2O_2, or when in the double condition (fig. 8B). To consolidate the analysis, we checked by qPCR the expression levels of typical Nrf-2 target genes, such as *HO-1* and *GCLC,* in our four conditions. The assay highlighted that when cyanidin is present in the medium of scramble C2C12 group they express the same levels of antioxidant genes as the scramble C2C12 exposed only to H_2O_2, indicating that cyanidin in promote antioxidant response. More interestingly, when Nrf-2 is silenced, the expression of its target genes is decrease significantly, not only in presence of the oxidative stimulus, but also when this group is exposed to cyanidin (fig 8C), suggesting that no antioxidant response is triggered. Moreover, we might suppose then that cyanidin exhibits its antioxidant activity through Nrf-2 signalling.

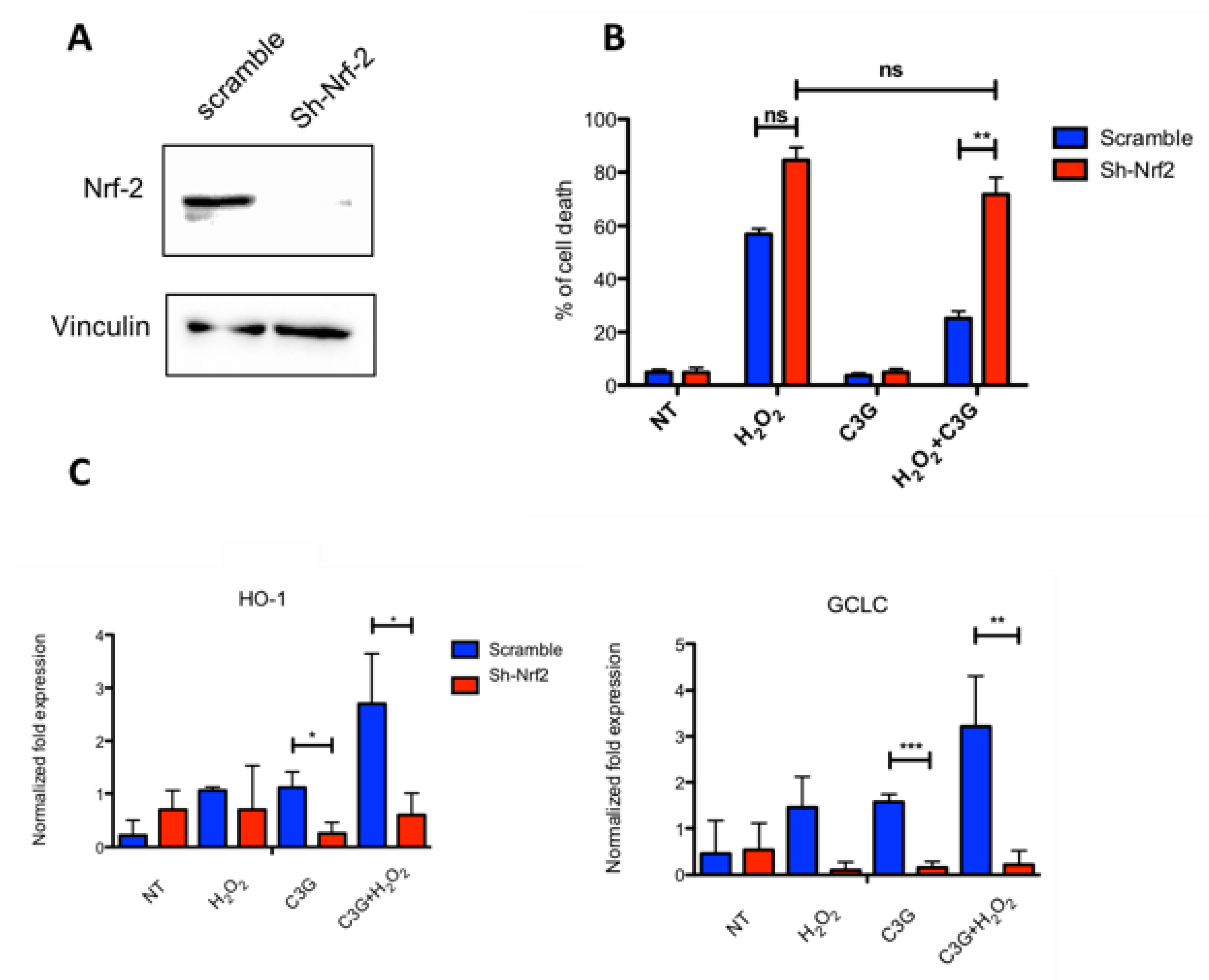


Figure 8: (A) Western blot for Nrf-2 from C2C12 protein extract infected with scramble (left) or Sh-Nrf-2 (right). **(B)** The plot showing the percentage of cell viability after 4h hours of treatment Mean±SD. **(C)** Real Time PCR analysis of *HO-1* (left panel) and *GCLC* (right panel) expression after 4 hours of treatment. N=3 for both the scramble and Sh-Nrf-2 group Mean±SD Two-tailed unpaired Student's t-Test; *P<0,05; ns: non significant.

Conclusion and future prospective

Muscular dystrophies are a wide group of genetic diseases, whose hallmark is a progressive muscle wasting which in time compromises patients' mobility, and the most severe cases respiratory and cardiac function (Emery 2002). Mutations, in these pathologies, affect genes involved in the dystrophin-glycoprotein complex (DGC) formation, whose role is pivotal for myofiber stability during skeletal muscle contraction.

Inflammation also plays an essential role in worsening muscular dystrophies. In fact, myofibers disruption promotes a proinflammatory environment recalling macrophages at the damage site, establishing in these patients a chronic inflammatory environment (Morrison et al.; 2000; Hodgetts et al.; 2006; Munoz-Cánovez and Serraro 2015).

Oxidative stress is one of the mechanisms proposed to contribute strongly to the physiopathology of muscular dystrophies and chronic inflammation environment (Rando 2002). Furthermore, evidences in literature show that type I fibers, that are known to display oxidative metabolism and greater antioxidant defences, are more protected from dystrophic phenotype both in humans and in the *Sgca* null mouse model (Webster et al.; 1988, Danieli-Betto et al.; 2004).

In this work I show that an cyanidin enriched diet (RD, Petroni et al.; 2017) supplied to *Sgca* null mouse model delays muscular dystrophy progression through specific molecular pathways. Specifically, providing the RD from the weaning time, I observe an overall amelioration of morphological parameters, such as the fiber calibre and less fragile fibers. Notably, this amelioration also persists when the diet is provided for longer time. Furthermore, this particular diet is able to rescue morphological parameters also when it is provided after the weaning when muscular tissue degeneration already occurred. Even more interestingly, this muscle improvement is coupled to an increase of muscle performance, indicating the delay of muscular degeneration is also present in terms of muscle functionality.

Chronic inflammation is an important process involved in muscular dystrophies progression, which display the involvement of macrophage as the main immune

population (Spencer et al.; 2001; Wehling et al.; 2001, Hnia et al.; 2008). When *Sgca* null mice are fed with the cyanidin enriched diet I observed also a decrease in macrophage infiltrates and inflammatory parameters, indicating that cyanidin acts also as an anti-inflammatory compound. The data collected show that in the *Sgca* dystrophic model, when the antioxidant diet is supplied, IkB-α protein levels are increased and there is a consequent recruitment of NF-kB in the cytoplasmic fraction, which is reflected also on its transcriptional activity. Furthermore, I demonstrate that NF-kB translocation in the myonuclei is ERK dependent, since phospho-ERK protein levels decreased in dystrophic mice fed with RD at variance with *Sgca* null mice fed with control diet. Specifically, ERK is able to promote the disassociation of NF-kB from its inhibitory complex, allowing so the NF-kB nuclear translocation and promotes the transcription of pro-inflammatory genes (such as *TNF- α* and *iNOS*), whose products recall macrophages during inflammation in the muscle tissue (Morrison et al.; 2000; Hodgetts et al.; 2006).

Since is known that fibers that display an oxidative metabolism, coupled with antioxidant defences, are more protected from dystrophic phenotype, I also investigated on the muscle metabolic status in mice fed with the RD. The SDH staining highlights that cyanidin promote a switch to oxidative metabolism, and since SDH is a mitochondrial enzyme, it is likely that also mitochondrial homeostasis is enhanced, data confirmed by PGC1- α increased protein levels, a transcription cofactor essential for mitochondrial homeostasis (Renèe-Ventura et al.; 2008; Villena 2014; Wang et al.; 2015). These observations let to suppose that mitochondrial homeostasis and oxidative metabolism might play an important role in muscular dystrophies amelioration; furthermore, it could be very interesting study deeper processes such as mitochondrial homeostasis, mitophagy and mitochondria turn over signalling since it was shown to be impaired in muscular dystrophies (Kim et al.; 2010; Fortini et al.; 2016; Gracìa-Prat et al.; 2016; Pal 2016).

Coupled with slow twitching phenotype, antioxidant defences are also enhanced in presence of cyanidin stimuli as shown by the qPCR data for typical antioxidant genes, such as *HO-1* and *GCLC*. To identify the player involved in the antioxidant pathway, I focused on the main transcription factor activated

under oxidative stress condition Nrf-2, in particular in endothelial cells models (Motohashi et al.; 2004; Ma et al.; 2008; Klassen et al.; 2010 Walters et al.; 2010). Western blot data from protein fractioning show that Nrf-2 protein levels are increased in the nuclear fraction of dystrophic mice fed with RD compared to YD fed one. This leads to suppose that, cyanidin can promote Nrf-2 translocation into myonuclei and display a specific transcriptional activity, but most importantly, *Sgca* null mice fed with the control diet show an impairment of Nrf-2 nuclear localization and this might be the cause of the mismanagement of oxidative stress. Going deeper with the analysis, I observed also that AMPK phosphorylation levels are increased when cyanidin is provided to dystrophic mice, and, together with some evidences in literature where AMPK is linked to oxidative stress, it might suggest a link between Nrf-2 nuclear localization and AMPK activity (Emerling et al., 2009; Zmijewskiand et al.; 2010).

Overall, I can conclude that, the use of dietary antioxidant in MD display an important effect delaying muscular dystrophy progression on different and important aspect of the pathology. Furthermore, I prove that also starting the RD at time points further than the weaning time, when the first signs of degeneration a fibrosis are already present, brings to beneficial effect on the pathology progression. Cyanidin enriched products are already present in successful human clinical trials, proving their safety but also offering cardioprotection, protection against cancer (Cerletti et al. 2017). The use of these products could be, therefore, applied to children affected by muscular dystrophies, firstly to improve patients' life delaying the pathology, and secondly to enlarge of cohort of patients eligible in clinical trials since any curative therapy must be performed on good muscle quality.

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