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## **Muscle redox disturbances and oxidative stress as pathomechanisms and therapeutic targets in early-onset myopathies**

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## Abstract

Because of their contractile activity and their high oxygen consumption and metabolic rate, skeletal muscles continually produce moderate levels of reactive oxygen and nitrogen species (ROS/RNS), which increase during exercise and are buffered by multiple antioxidant systems to maintain redox homeostasis. Imbalance between ROS/RNS production and elimination results in oxidative stress (OxS), which has been implicated in aging and in numerous human diseases, including cancer, diabetes or age-related muscle loss (sarcopenia). The study of redox homeostasis in muscle was hindered by its lability, by the many factors influencing technical OxS measures and by ROS/RNS important roles in signaling pathways and adaptative responses to muscle contraction and effort, which make it difficult to define a threshold between physiological signaling and pathological conditions. In the last years, new tools have been developed that facilitate the study of these key mechanisms, and deregulation of redox homeostasis has emerged as a key pathogenic mechanism and potential therapeutic target in muscle conditions. This is in particular the case for early-onset myopathies, genetic muscle diseases which present from birth or early childhood with muscle weakness interfering with ambulation and often with cardiac or respiratory failure leading to premature death. Inherited defects of the reductase selenoprotein N in SEP1N-related myopathy leads to chronic OxS of monogenic origin as a primary disease pathomechanism. In myopathies associated with mutations of the genes encoding the calcium channel RyR1, the extracellular matrix protein collagen VI or the sarcolemmal protein dystrophin (Duchenne Muscular Dystrophy), OxS has been identified as a relevant secondary pathophysiological mechanism. OxS being drug-targetable, it represents an interesting therapeutic target for these incurable conditions, and following preclinical correction of the cell or animal model phenotype, the first clinical trials with the antioxidants N-acetylcysteine (SEP1N- and RYR1-related myopathies) or epigallocatechin-gallate (DMD) have been launched recently. In this review, we provide an overview of the mechanisms involved in redox regulation in skeletal muscle, the technical tools available to measure redox homeostasis in muscle cells, the bases of OxS as a primary or secondary pathomechanism in early-onset myopathies and the innovative clinical trials with antioxidants which are currently in progress for these so-far untreatable infantile muscle diseases. Progress in our knowledge of redox homeostasis defects in these rare muscle conditions may be useful as a model paradigm to understand and treat other conditions in which OxS is involved, including prevalent conditions with major socioeconomic impact such as insulin resistance, cachexia, obesity, sarcopenia or ageing.

## Abbreviations

AP-1, activator protein 1 ; ARE, antioxidant response element ; CMs, congenital myopathies ; CMDs, congenital muscular dystrophies ; ColVI, collagen VI ; CPK, creatine phosphokinase; DMD, Duchenne Muscular Dystrophy ; ER, endoplasmic reticulum ; GPx, glutathione peroxidase ; GSH, glutathione ; MEF, myocyte enhancer factor ; MRF, myogenic regulatory factor ; NAC, N-acetylcystein ; NF- $\kappa$ B, nuclear factor kappa B ; NOX, Nicotinamide adenine dinucleotide phosphate oxidase ; NRF2, nuclear factor (erythroid-derived 2)-like2 related factor ; OxS, oxidative stress ; PTP, permeability transition pore ; ROS, reactive oxygen species ; RNS, reactive nitrogen species ; RYR1, ryanodine receptor type 1 ; SEPN1, Selenoprotein N ; SOD, superoxide dismutase ; SRF, serum response factor ; XO, xanthine oxidase

## Keywords

Congenital myopathies, SEPN1, RYR1, ColVI, dystrophinopathy, oxidative stress

## 1. Introduction

Early-onset myopathies (EOM) are inherited muscle conditions which present during infancy or early childhood and have so far no specific treatment. Aside from the metabolic, neuromuscular junction or systemic (congenital myotonic dystrophy) defects that can affect skeletal muscle, the two main groups of structural primary congenital muscle disease are congenital muscular dystrophies (CMDs) and congenital myopathies (CMs) [1]2. They typically present from birth or infancy with muscle weakness and hypotonia, delayed motor development, normal creatine phosphokinase (CPK) levels, difficult or absent ambulation and are often associated with orthopaedic complications, respiratory failure or heart disease that can lead to premature death [2]. The muscle biopsy pattern classically differentiates CMDs from CMs. CMDs present with endomysial fibrosis with or without muscle fibre necrosis and regeneration (dystrophic lesions), whereas CMs muscles are non dystrophic but are defined by characteristic changes in the internal fiber architecture. The early-onset myopathies present heterogeneous pathophysiological mechanisms, but typically involve proteins essential in muscle function, including components i) of the extracellular matrix or sarcolemmal membrane (such as collagen VI (ColVI), dystrophin or alpha-dystroglycan); ii) of the sarcomere (contractile and/or scaffolding proteins such as alpha-actin, myosin, nebuline or titin); iii) of the triadic junction, the structural basis of excitation-contraction coupling (such as the ryanodine receptor Ryr1 or Stac3); iv) of the redox regulation system of the endoplasmic reticulum (i.e. selenoprotein N encoded by *SEPN1*); v) of the inner nuclear envelope (i.e. lamin A/C) [1, 3]. In addition to CMDs and CMs, one of the most prevalent muscle diseases in childhood (1/3500 male newborns) is Duchenne muscular dystrophy (DMD) due to mutations in the DMD gene encoding dystrophin and typically presenting with muscle weakness and elevated CPK levels before the age of 3 years.

Deregulation of redox homeostasis has emerged in the last years as a common pathogenetic mechanism and potential therapeutic target in Collagen VI-related muscular dystrophies, in *RYR1*-related myopathies (*RYR1*-RM), in *SEPN1*-related myopathy (*SEPN1*-RM) and in DMD, as well as in other more prevalent processes such as age-related muscle loss (sarcopenia). This review will focus on the importance of muscle redox homeostasis, the methods to detect oxidative damage and the different origins of oxidative stress (including mitochondrial defects, calcium signaling alteration or reduction of antioxidant defenses) that represent hallmarks of these early-onset myopathies and potential targets for future therapeutic interventions.

## 2. Muscle redox homeostasis

Homeostasis is the property of a system to remain at equilibrium. Temperature regulation, balance between acidity and alkalinity or cell number regulation are all well known examples of homeostasis. Tight regulation of homeostatic processes at the cell, tissue, organ and organism levels is necessary for life, and disturbance of homeostasis is associated with many diseases. In particular, redox homeostasis is finely regulated, since low or moderate levels of reactive oxygen species (ROS) or reactive nitrogen species (RNS) are important physiological signaling molecules. Oxidative stress (OxS), an alteration of redox homeostasis consisting in an imbalance between the ROS and/or RNS production and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage, is frequently associated with pathological conditions. Mitochondria, described as the powerhouse of the cell, produce most of the cellular energy and are commonly considered as the main production site of free radical formation and oxidative damage. However, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), lipoxygenases or xanthine oxydase (XO) are other relevant sources of ROS [4].

Skeletal muscle, the largest type of tissue in the human body, has specific characteristics such as intense requirements for energy, calcium signaling and metabolism as well as the capacity to regenerate after lesion by recapitulating the myogenesis process, which depends on the activation of muscle stem cells (satellite cells) that can differentiate into myotubes and ultimately muscle fibers [5]. Maintenance of the muscle architecture, which is highly organized to optimize contraction and force generation, requires basal energy. In addition, muscle cells consume a high amount of oxygen that is significantly increased during exercise, and this O<sub>2</sub> consumption is associated with continuous ROS/RNS generation, although, contrary to early reports, the rate of ROS production by mitochondria during exercise has been probably overestimated [6]. These reactive species are important signaling molecules necessary for muscle function and for flexible adaptative responses to stress and/or effort [4]. Thus, they are involved in modulation of muscle cell growth regulation, proliferation or differentiation [6-8], contractile performance during exercise [9, 10], calcium signaling [11], glucose uptake [12] and mitochondrial biogenesis [6]. However, excessive ROS/RNS levels unchecked by antioxidant defenses have negative impact on muscle contractile proteins, mitochondrial phospholipids, or DNA, and have been involved primarily or secondarily in the pathophysiology of muscle ageing (sarcopenia) and of various muscular disorders like Duchenne Dystrophy, central core disease, malignant hyperthermia and muscle fatigue [13].

**2.1 ROS/RNS and the antioxidant defense system.** Most ROS/RNS are labile and their effects depend on multiple factors as local and/or dose environment, making it difficult to define a threshold between physiological signaling molecules and damaging factors leading to pathological conditions. Superoxide radical  $O_2^{\cdot-}$  is the “primary” ROS species produced mainly by mitochondria, converted into “secondary” ROS, directly or through enzyme- or metal catalyzed processes [14] (see Figure 1).  $O_2^{\cdot-}$  is rapidly converted into hydrogen peroxyde  $H_2O_2$  by spontaneous dismutation or by superoxide dismutases (SOD).  $H_2O_2$  can also be converted via the Fenton reaction in the presence of iron into the hydroxyl radical  $OH^{\cdot}$ . Compared to  $O_2^{\cdot-}$  and  $OH^{\cdot}$ ,  $H_2O_2$  has the lowest reactivity, the highest stability with its half-life values around  $10^{-5}$  seconds and the highest intracellular concentration, around  $10^{-7}M$  [14-16].  $O_2^{\cdot-}$  can also react with nitric oxide ( $NO^{\cdot}$ ) to generate peroxynitrite ( $ONOO^{\cdot}$ ) which is a ROS and a RNS species. To maintain ROS/RNS at non deleterious level, skeletal muscle and myogenic cells are equipped with strong enzymatic and non-enzymatic antioxidant defenses, making them responsive to redox environment change. Among enzymatic antioxidants, superoxide dismutase (SOD) catalyzes transformation of  $O_2^{\cdot-}$  to  $H_2O_2$ , which can then be converted to  $H_2O$ . Three isoforms exist in mammals, SOD1 in the cytosol, SOD2 in the mitochondria and SOD3 which is predominantly extracellular. SOD1/3 (Cu/ZnSOD) contains copper and zinc and SOD2 (MnSOD) contains manganese in the active site [17]. Exercise training increases superoxide dismutase activities specifically in soleus muscle, oxidative muscle characterized by a high mitochondrial content [18]. Absence of SOD1 imposes elevated oxidative stress and accelerated age-dependent atrophy in skeletal muscle [19]. Catalytic decomposition of  $H_2O_2$  can occur by several enzymes such as the heme-dependent enzyme catalase (CAT) or glutathione peroxidases (GPx). The latter belong to the family of selenoproteins, characterized by the presence of at least one selenocysteine, an aminoacid that represents the biological form of selenium. In mammals, there are at least five selenocysteine-containing GPx (GPx1 to-4 and GPx-6) [20]. GPx reduce lipid hydroperoxides to their corresponding alcohols and reduce free  $H_2O_2$  to  $H_2O$ , coupled to oxidation of reduced/monomeric glutathione (GSH, present in millimolar concentrations in muscle cells) to glutathione disulfide (GSSG). Reduced glutathione is then recycled from GSSG by glutathione reductase, the GSH/GSSG ratio being a good indicator of the cellular redox status [20]. Like for SOD, GPx activities are increased in muscle after exercise, while catalase tends to remain unchanged [18]. Almost all the other members of the selenoprotein family whose function is characterized have antioxidant capacities (based or not on enzymatic properties). This includes selenoprotein N (SEPN1, encoded by the *SEPN1* gene), whose defects cause SEPN1-related myopathy (see below and [20]). Defects in the transcriptional machinery required for selenocystein cotranslational incorporation and leading to deficiency of several selenoproteins have recently been associated in 3 families with

systemic diseases which include early-onset muscle weakness and, at least in one case, markers of systemic oxidative stress [21-23]. The antioxidant network also includes soluble non-enzymatic antioxidants like vitamin A, C and E, creatine, biliverdin and its derivate bilirubin and glutathione [4, 17].

Cellular response to oxidative stress also involves at least four key factors involved in transcription regulation: nuclear factor kappa B (NF- $\kappa$ B), activator protein 1 (AP-1), nuclear factor (erythroid-derived 2)-like 2 related factor (known as NFE2L2 or NRF2) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) [4, 6, 17]. The promoter of each of the three main antioxidant enzymes SOD2, CAT and GPx contains, among other specific sites, a NF- $\kappa$ B and AP-1 consensus sequence [24]. Oxidative stress upregulates NF- $\kappa$ B activity, and AP-1 dimer formation depends on the redox environment [8]. Similarly, physiological expression of the *PPARGC1A* gene encoding Pgc-1 $\alpha$  requires an optimal concentration of ROS in skeletal muscle [7, 25], and Nrf2 activity is determined by the redox status [8]. Under normal conditions, Nrf2 is rapidly degraded by the ubiquitin-proteasome pathway through its interaction with Keap1 (a substrate adaptor protein of Cul3-based E3 ubiquitin ligase). Under oxidative conditions, reactive cystein residues in Keap1 are oxidized leading to Nrf2 dissociation from Keap1 and activation. The free Nrf2 then translocates to the nucleus and forms heterodimer with Maf proteins. This complex activates the transcription of cytoprotective genes (HO-1, NQO1, PRDX1, GST, etc.) through binding to the antioxidant response element (ARE) present in their promoters [8, 17]. One stress indicator identified in the OKD48 mouse is based on this Keap1-Nrf2 pathway (see below).

**2.2 Redox homeostasis and myogenesis.** A central characteristic of the early-onset myopathies is the presence of muscle weakness before birth or in the first years of life, suggesting a potential alteration of myogenesis as part of the disease mechanism. The establishment of muscle through myogenesis is necessary during embryogenesis as well as for muscle regeneration. Satellite cells are multipotent cells able to proliferate into new progenitors or to differentiate into skeletal muscle cells upon activation. Non-proliferative quiescent satellite cells, which are identified by their location between the sarcolemma and the basal lamina and by expression of Pax7, once activated will proliferate as myoblasts and then fuse to become myotubes [26]. A decreased content of satellite cells has been reported in several EOM, in particular in CMS with minicore lesions due to mutations of the satellite cell gene *MEGF10* or of *SEPN1* [27, 28]. Although a clear relation between oxidative stress and loss of satellite cells has not been defined in early onset myopathies, transforming growth factor beta-activated kinase 1 (TAK1) has been involved in stem cell homeostasis and skeletal muscle repair [29]. Ogura and collaborators found that TAK1-deficient satellite cells have high level of



oxidative stress and undergo spontaneous cell death. More specifically, TAK1-mediated activation of JNK is essential to inhibit oxidative stress and to enhance differentiation of satellite cells.

Three main families of transcription factors are involved in the different steps of skeletal muscle proliferation and differentiation. Firstly, the myogenic regulatory factors (MRFs) MyoD, Myf5, Myf6 and Myogenin; secondly, the myocyte enhancer factors (MEFs), and lastly the serum response factor (SRF) [4]. There are controversial data about how the redox cell environment affects myogenesis, although oxidative stress appears to mostly reduce the efficiency of myogenic differentiation. ROS induces an important decrease of the intracellular GSH pool, which favors NF- $\kappa$ B activation thus contributing to reduce MyoD expression [30]. However, it is not well understood if this is a specific and direct effect of ROS or a consequence of cell suffering [4]. Exposure of differentiated primary human skeletal muscle cells and isolated rat skeletal muscle cells to H<sub>2</sub>O<sub>2</sub> led to a profound increase in MEF2 DNA binding via pathways that were dependent of p38 mitogen-activated protein kinase (MAPK), Protein Kinase C, PI 3-kinase and AMPK [31]. In *Drosophila*, over-expression of p38MAPK extends lifespan in a MnSOD-dependent manner through the transcription factor MEF2, while inhibition of p38MAPK causes early lethality and precipitates age-related motor dysfunction, that is rescued through muscle-restricted expression of p38MAPK [32]. Despite the important role of SRF in skeletal growth and maturation [33], so far the impact of oxidative stress on SRF function has not been extensively studied.

Nicotinamide adenine dinucleotide (NAD), which exists in an oxidized (NAD<sup>+</sup>) and reduced NADH form, is implicated in redox reactions, hauling electrons from one reaction to another [34]. The cellular redox ratio [NAD<sup>+</sup>]/[NADH] is dynamically modified in skeletal muscle [35]. Sirt1, a NAD<sup>+</sup>-dependent deacetylase, when overexpressed, impedes muscle differentiation and conversely cells with reduced Sirt1 level differentiate prematurely [36]. Sirt1, through its deacetylase activity, and other chromatin modifying enzymes, epigenetically reprogram muscle promoters at various stages, preventing or enhancing MRF and MEF2 activities [37]. Recently, in muscles from patients with recessive *RYR1* mutations or with nemaline myopathy, increased expression of class II histone deacetylases has been observed suggesting that epigenetic changes could be a common trigger of muscle weakness in early-onset myopathies [38].

### **3. How can oxidative stress be measured in muscle?**

Measuring redox status in a reliable and meaningful way in skeletal muscle is difficult, requires expertise and has been one of the main limiting factors in identifying the role of ROS/RNS in muscle function and disease. ROS signaling is doubtless complex: it can be multiphasic (having an immediate and a delayed phase), it depends on the reactive species involved, on their global and local

intracellular concentration, the tissue and cell environment and the stress exposure timing (chronic vs acute). Also, it is highly sensitive to technical artifacts depending on oxidation of the samples upon exposure to ambient O<sub>2</sub>. The “perfect” ROS indicator does not exist yet due to the long list of specific criteria required, which would include detection of one or several types of ROS species, high signal to background contrast, high dynamic range, non-toxic, not sensitive to environment parameters like pH or specific subcellular compartmentalization [39, 40]. However, important progress in ROS quantification has been made the last decade, particularly due to major advances in synthetic and genetically encoded fluorescent based ROS detectors and *in vivo* imaging technology [16, 39-42]. Table 1 summarizes the redox sensors commonly used and stresses their indications and limitations. As this is not primarily a methodological review, we will an overview of the main tools which are useful to measure reliably oxidative stress in muscle cells, and discuss their potential applications. [16, 39, 41, 43-53]

**3.1 Analysis of carbonylated proteins.** Oxidation of proteins can induce both structural and functional alterations which can be beneficial or harmful. Formation of carbonyl groups is the most frequent irreversible oxidative transformation, but no direct method exists to detect carbonylation as this is not associated with distinguishable fluorescence/spectrophotometric absorbance properties [41, 46]. Global analysis is mainly based on derivation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) followed by spectrophotometric analysis or immunodetection of DNP (2,4 dinitrophenylhydrazone) [41, 46]. More detailed analyses have been possible with proteomics and mass spectrometry approaches [54-56].

**3.2 Small-molecule fluorescent probes** (also called chemical reporter molecules). Hydroethidine (HEt) and its mitochondria-targeted variant Mito-HEt (known as MitoSOX-red) cross phospholipid bilayers and detect superoxide radicals [16, 44]. However, detection is difficult due to short half-life and conversion by SOD [14-16]. Moreover, Mito-HEt accumulation in mitochondria depends on its concentration and on the mitochondrial membrane potential.

CM-H<sub>2</sub>DCFDA, composed of a chloromethyl group, non-fluorescent 2',7'-dichlorodihydrofluorescein (H<sub>2</sub>DCF) and diacetate ester, optimized for cell permeability and reduced leakage, is oxidized by intracellular oxidants into fluorescent 2',7'-dichlorofluorescein (DCF) [16]. Thus, it is commonly used to measure global oxidant activity in living cells. , CM-H<sub>2</sub>DCFDA lacks specificity on the nature of the oxidants measured, as the probe has low reactivity toward superoxide radicals or hydrogen peroxide [45]. The formation of DCF should rather be considered as a general marker of cellular oxidant level [16, 45].

C11-BODIPY<sup>581/591</sup> is used to measure lipid peroxidation. Upon oxidation, the red fluorescent reduced form of the probe is converted into a green emitting oxidized form [57]. Because of the lack of lipid specificity (all membranes), a compound derived from C11-BODIPY<sup>581/591</sup> has been developed to target only mitochondria lipids [47]. This MitoPerOx contains the boron dipyrromethane difluoride (BODIPY) fluorophore conjugated to a triphenylphosphonium lipophilic cation to be selectively taken into mitochondria [47] and allows the measurement of mitochondrial lipid peroxidation.

**3.3 Genetically encoded ROS detectors.** Until recently, much of the data on ROS production have been obtained using isolated mitochondria, much less in intact cells and rarely at the organism level. In the last decade, promising *in vivo* systems for monitoring oxidative stress have been developed. Protein-based ROS reporters, first developed in single cells experiments, can target specific cell compartments since they contain targeting sequences that allow selective expression in nucleus, ER, mitochondrial matrix, mitochondria inner/outer membrane or plasma membrane [16, 39, 40]. Another advantage is that they can now be used for adenovirus-associated virus (AAV) mediated transduction or to generate transgenic animals. Indeed, genetically-encoded reporters of oxidative stress appear to be a highly convenient technology for noninvasive detection of oxidative stress in mice. Moreover, these tools will help with the measurement of bioactive molecules such as ROS for which it is important to determine where (subcellular origins), when (spatio-temporal distribution) and how (redox couple involved) they are produced [58]. One of the limitations of this approach is that the transgenic mice can only bear a unique redox indicator.

Five main families of genetically encoded redox indicators have been developed (cf. Figure 2):

1) Modified fluorescent proteins like the redox sensor roGFP, made by the introduction of two cysteine residues on the surface of GFP [59], have applied *in vivo* using AAV technology [60] and transgenic mice [61]. Formation of a disulfide bond between the two cysteines upon oxidation is reversible and induces an important decrease in the intrinsic fluorescence [62]. This green redox-sensitive (roGFP) as well as the yellow redox-sensitive YFP (rxYFP) based on the same principle [62] allowed to monitor the thiol/disulfide ratio [40].

2) These redox-sensitive proteins have also been combined to the redox-active enzyme glutaredoxin-1 to create the chimeric sensors rxYFP-Grx1p developed by Bjornberg *et al.* [63] and Grx1-roGFP developed by Gutscher *et al.* [64]. In these, glutaredoxin is oxidized by the disulfur bond-forming oxidized cysteine residues in rxYFP or roGFP and reduced non-enzymatically by GSH. These chimeric sensors allow improved specificity and faster kinetics to measure the redox process, and

their reactivity toward other oxidants remains low, giving almost absolute glutathione specificity and allowing to measure the GSH/GSSG ratio.

3) A superoxide biosensor has also been developed thanks to a circularly permuted yellow fluorescent protein targeted to the mitochondria (mt-cpYFP) [65]. In the transgenic mouse expressing the mt-cpYFP ROS biosensors, brief bursts of superoxide production also called superoxide flashes have been detected in skeletal muscle in basal conditions and are increased after glucose or insulin challenges [65]. These transient increases in fluorescence intensity (flashes) have some similarities with calcium sparks that represents elemental  $\text{Ca}^{2+}$  release signals mediated by the ryanodine receptors in the sarcoplasmic reticulum. Further analyses are required to understand the crosstalk between mitochondria and  $\text{Ca}^{2+}$  which probably represents a critical step in the activation of stress response.

4) A genetically encoded sensor for  $\text{H}_2\text{O}_2$  called HyPer has also been developed by the insertion of the cpYFP into the  $\text{H}_2\text{O}_2$ -sensing regulatory domain of the *E. coli* transcription regulator OxyR [66]. Despite similarities between HyPer and roGFP (fluorescence change upon formation of a disulfide bond between two cystein residues), the mechanisms of these reactions are different. In Hyper the cystein 199 is inaccessible to superoxide and GSSG because of its localization in the hydrophobic pocket of the regulatory domain of OxyR, but can be oxidized by the amphiphilic molecule of hydrogen peroxide [66]. So this probe allows targeted measuring of  $\text{H}_2\text{O}_2$ . Improved versions of HyPer (HyPer-2 and HyPer-3) have been developed and were used successfully *in vivo* in the zebrafish [67].

5) In addition, there is at least one biosensor not based on a protein but on a stress-inducible promoter, and is dually regulated by induction at the transcriptional level, and by protein stabilisation at the post-translational level in the Keap1-Nrf2 pathway. The OKD48 transgenic mouse (Keap1-dependent Oxidative stress Detector, No-48) expresses ARE stress-inducible promoters controlling a Nrf2 fragment fused to luciferase [68]. The OKD48 oxidative stress detector responds *in vitro* specifically to oxidative stressors such as sodium arsenite (ASN) and dimethylmaleate, and is barely responsive to ER stressors, reducing agents and cell death induced by DNA damage [68]. *In vivo*, the OKD48 transgenic mouse shows high level of bioluminescence after ASN intraperitoneal injection or UV-A irradiation [68].

These tools have been used to monitor oxidative stress *in vivo* in skin [69], in dopaminergic neurons [70], in red cells [61] or in whole body [68] with different transgenic mouse models. Muscles have not been extensively studied so far, although these genetically encoded ROS biosensors can bring important progress in our understanding of oxidative stress-related diseases such as the early onset

myopathies discussed below. Development of more sensitive oxidative stress detectors are required to allowed more precise ROS mapping. Indeed, most of the experiments above have been performed in anesthetized animals. Since exercise and muscle activity have a major impact in muscle redox homeostasis, monitoring oxidative stress in freely moving animals would give additional valuable information, but would necessitate precise tracking of the mouse and short time exposure for the ROS detection.

**3.4 Not to forget.** Analysing oxidative stress, despite all the available and developing tools, remains delicate and not straightforward. Precautions and expertise are required to avoid over- or underestimation of oxidative stress, artifacts and non-reproducible data [46]. Each technique has its own limitations but, independently of the methods used, careful ROS/RNS titration requires extensive controls to exclude background and interfering signals. A number of variables has to be taken into account to measure oxidative stress flux, such as the ROS/RNS species type, concentration, cell differentiation status (i.e. myoblasts versus myotubes) or intracellular compartmentation [4, 46], aside from the global environment (animal facility conditions, exercise, diet).

#### **4. Oxidative stress is a primary pathophysiological defect and therapeutic target in *SEPN1*-related myopathy**

*SEPN1*-related myopathy is so far and to the best of our knowledge the unique inherited muscle disease due to a primary defect in redox homeostasis. *SEPN1*, a member of the selenocysteine-containing protein family, is a transmembrane protein localized to the endoplasmic reticulum (ER) membrane, involved in redox-modulated calcium homeostasis and in protection against oxidative stress [20]. The precise function of this protein is still unknown. Sequence analysis revealed a potential EF-hand domain (calcium interacting site), a putative reductase catalytic site and several predicted glycosylated sites [71]. Several mutations in *SEPN1* gene are found in CMs or CMDs including rigid spine muscular dystrophy [72, 73], multimimicore disease [74], desmin-related myopathy with Mallory body-like inclusions [75] and congenital fiber-type disproportion [76], which are now considered to be part of the histopathological spectrum of presentations of the same, unique condition. All patients with *SEPN1* mutations have a severe weakness of neck and trunk muscles, leading to scoliosis, a variable degree of spinal rigidity and life-threatening respiratory insufficiency. In contrast, ambulation and limb strength are relatively preserved. The myopathological presentation of *SEPN1*-RM is quite large and heterogeneous, implying a polymorphic mechanism. Surprisingly, in postnatal tissues *SEPN1* is weakly expressed [77] but its

expression is more important in all proliferating cells as well as in muscle and spinal cord during embryonic development [78]. The *SEPN1* gene promoter contains predicted sequences for NF- $\kappa$ B, an ER stress response element and an AP-1 consensus sequence [79], underlying a potential gene expression regulation by cell stress [20]. *SEPN1* is completely dispensable during mouse development as the *Sepn1*<sup>-/-</sup> mice is viable, and its growth and lifespan is normal [80]. However, *SEPN1*-deficient mice displayed limited motility and body rigidity after physical exercise and stress conditions (forced swimming test). *SEPN1* has been shown to be involved in muscle regeneration and satellite cell maintenance [28]. In fact, the level of Pax7<sup>+</sup> satellite cells is reduced in uninjured adult muscle in *Sepn1*<sup>-/-</sup> mice and, after 2 cardiotoxin injections, muscle regeneration (efficient restoration of the muscle fibers) is impaired in *SEPN1*-deficient mice [28]. The mechanism underlying the loss of satellite cells is still unraveled and thus it is difficult to know if this is a cause or a consequence of muscle alterations. Antisense morpholinos against *sepn1* decreased zebrafish mobility without damaging muscle formation [78]. *SEPN1*-deficient myotubes from patients displayed increased basal oxidative activity and protein oxidation associated with enhanced susceptibility to H<sub>2</sub>O<sub>2</sub> treatment, all of which could be abrogated by pre-treating cells with the antioxidant N-acetylcysteine (NAC) [81]. These results are at the origin of the first clinical trial in this condition, using oral NAC (Pharmacological treatment of a rare genetic disease: N-acetylcysteine in selenoprotein N-related myopathy (SELNAC), ClinicalTrials.gov Identifier: NCT02505087). Moreover, *SEPN1*-deficient myotubes displayed an increased resting cytosolic calcium concentration and reduced sarcoplasmic reticulum Ca<sup>2+</sup> load compatible with redox-mediated abnormalities in calcium homeostasis [81]. In line with these abnormalities, *SEPN1* has been shown to interact with the redox-sensitive calcium release channel RyR1 [82] and the ER calcium import pump SERCA2 (sarcoendoplasmic reticulum calcium transport ATPase 2) [83]. Furthermore, *SEPN1* levels matched those of an endoplasmic reticulum oxidoreductin 1 and have been proposed to counteract peroxide formation and SERCA oxidation by this ER protein thiol oxidase [83]. Remarkably, the ryanodine receptor 1 has been involved in the same cellular differentiation events as *SEPN1* and is required for calcium fluxes in the zebrafish embryo [82]. Deficiency of either *SEPN1* or RyR1 leads to muscular disease with abnormal sensitivity to redox conditions [82]. Similar histological (namely core lesions) and clinical signs can be observed in *SEPN1*-RM and in myopathies associated with *RYR1* mutations. These data suggest that ER redox and calcium homeostasis are interlinked pathways essential in muscle function, and their deregulation by abnormalities of either *SEPN1* or RyR1 is a major cause of congenital muscle disease.

## 5. Oxidative stress due mitochondrial dysfunction or Ca<sup>2+</sup> handling alterations is a secondary pathophysiological defect in *RYR1*- and *COLVI*-associated congenital muscle diseases

Redox imbalance, associated with dysfunctions of mitochondria and calcium handling, have been observed in muscle disorders due to primary defects in the *COL6* and *RYR1* genes, and is thus emerging as an interesting drug-targetable therapeutic target for these untreatable diseases. This is particularly relevant since mutations in *RYR1* represent the most prevalent form of congenital myopathy (see review by Jungluth *et al* in this issue), and *COL6* mutations are also relatively common.

RyR1, also known as skeletal muscle calcium release channel, is an essential component of the excitation-contraction coupling apparatus. RyR1, which assembles as a homotetramer, is a key regulator of calcium homeostasis. Mutations in the *RYR1* gene are responsible for core myopathies, typically characterized by slowly progressive proximal weakness involving hip and axial muscles, but also of malignant hyperthermia (MH) [84]. Abnormal excitation-contraction coupling secondary to impaired calcium release or to uncoupling may be involved in *RYR1*-related myopathies ([85] and review by Treves *et al* in this issue). Oxidative and/or nitrosative stress can be regulated by calcium and conversely calcium homeostasis (via channels and transporters) can be a target of these stresses [86]. RyR1 is becoming an example of redox-sensor ion channel as the receptor contains the highest number of reactive cysteines strongly involved in calcium overload/leaking [87]. One RyR1 monomer comprises around 5000 amino acids, including more than 300 cysteines half of which are, under basal conditions, in the reduced form [88, 89]. Modifications of multiple key cysteine residues by S-glutathionylation, S-nitrosylation, S-nitrosoglutathionylation and unspecified S-oxidation induce functional modulation of the RyR1 channel properties [89-92]. Redox remodeling of the RyR1 complex causes “leaky” channels in *RYR1*-MH and in muscle fatigue after exercise [93, 94]. Consistently, the Y522S Ryr1 KI (a mouse model of MH) shows lipid peroxidation and reduced maximal developed force which was prevented by NAC treatment [93]. Interestingly, using the relatively relaxed zebrafish (*ryr*, a spontaneous mutant having defective expression of one of the *ryr1* fish isoforms) and cultured myotubes from patients with *RYR1*-RM, excessive production of oxidants by mitochondria and diminished survival under oxidant conditions have been observed [95]. Moreover, improvement of muscle function and histology and restoration of the myotube phenotype were obtained with the use of the antioxidant NAC. These results are at the origin of the first clinical trial in *RYR1*-related myopathy using oral NAC (Antioxidant therapy in *RYR1*-related congenital myopathy, ClinicalTrials.gov identifier: NCT02362425).

On the other hand, mutations in the genes encoding the extracellular matrix protein Collagen VI (*COL6A1*, *COL6A2* and *COL6A3*) cause four recognized clinical forms of collagen VI related myopathy: Ulrich Congenital Muscular Dystrophy (UCMD), Bethlem Myopathy (BM), congenital myosclerosis and limb-girdle muscular dystrophy [96]. While BM can have a later onset and displays a relatively mild and slowly progressive phenotype, UCMD is usually more severe with significant weakness of skeletal muscles in the first year of life, more rapid progression of symptoms and life-threatening respiratory failure [96, 97]. In fibers from skeletal muscles of ColVI null mice (*Col6a1*<sup>-/-</sup>) as well as in myoblasts from UCMD patients, mitochondrial dysfunction and apoptosis have been observed [98]. These alterations could be prevented by cyclosporine A (CsA), a potent inhibitor of the mitochondrial permeability transition pore (PTP) and a by a nonimmunosuppressive CsA, Debio25 [99]. However, in fibroblasts, the main producer of collagen VI [100], abnormal PTP opening was not either using cells derived from UCMD patients or from other forms of muscular dystrophy [101]. More work is needed to confirm the previous findings and to decipher the relationship between PTP dysregulation and UCMD pathogenesis. Nevertheless, accumulation of dysfunctional mitochondria due to defective autophagic degradation has been observed in *Col6a1*<sup>-/-</sup> mice [102]. Consistently, a strong decrease in Beclin1 and Bnip3, two key players in the autophagic process, was also found in muscle biopsies from UCMD and BM patients. Remarkably, forced activation of autophagy by dietary, genetic and pharmacological agents restored myofiber homeostasis and improved the dystrophic phenotype of *Col6a1*<sup>-/-</sup> mice [102]. Recently, monoamine oxidase (MAO) which catalyzes the oxidative deamination of neurotransmitters generating H<sub>2</sub>O<sub>2</sub>, has been suggested as responsible for mitochondrial dysfunction in myoblasts from patients affected by ColVI myopathies [103]. Inhibition of MAO by pargyline, which prevents ROS formation, led to recovery from the dystrophic phenotype. Obviously, interchange between calcium microdomains and mitochondria is essential for subcellular physiology. In RYR1- and ColVI-related myopathies, oxidative stress due to defects in calcium homeostasis or to altered mitochondrial function appears to be a common signature of the muscular disease and therefore a potential therapeutic target.

## 6. Oxidative stress: a pivotal role in dystrophin deficiency?

Duchenne muscular dystrophy is a severe degenerative skeletal muscle disease due to mutations in the large DMD gene, encoding *dystrophin* and localized in the X chromosome. Its clinical presentation includes skeletal muscle weakness, inflammation and fibrosis, elevated CPK levels or synaptic dysfunction, causing a premature death often due to respiratory and cardiac failure [104, 105]. Absence of dystrophin in skeletal myofibers induces sarcolemma damage after muscle contraction, leading to myofiber necrosis. Repeated cycles of damage/inflammation/regeneration



lead to important muscle weakness with irreversible damage over time [106]. Various dystrophin-deficient animals have been developed but the main model remains the *Mdx* mouse, first described in 1984 [107] and whose muscle presents necrosis, inflammation, fibrosis, although to a much lesser degree than that found in DMD patients [108]. Increase of oxidative stress markers has suggested ROS as a central mediator of these lesions [106, 108-110]. Various sources of ROS have been suspected, including the inflammatory cells (myeloperoxidase), NOX, XO, mitochondria or decoupling of NOS [106, 108, 110]. Nonetheless, NOX is suggested to be one of the most important sources of ROS in the development of the dystrophy pathophysiology. Indeed, activation of NOX2 by stretching in dystrophin deficient muscles induced ROS/RNS production that enhances Ca<sup>2+</sup> influx and activates Src kinase, which in turn activates further NOX2 by p47<sup>phox</sup> phosphorylation [111].

Several antioxidants have proven effective in ameliorating the dystrophin-deficiency phenotype, reducing muscle damage and loss of muscle strength, inflammation, fibrosis and muscle oxidative stress. These include antioxidants which act as ROS scavengers, such as NAC [112], epigallocatechin-3-gallate (a polyphenol extracted from green tea extracts) [113] or Idebenone (benzoquinone related to coenzyme Q) [114], and also those which prevent ROS formation like the MAO inhibitor pargyline [115]. Sirt1 muscle-overexpression results in increased levels of utrophin, a functional analogue of dystrophin, and reverses the phenotype of the *Mdx* mice [116]. Similarly, resveratrol, which activates Sirt1, improves muscle function in the *Mdx* model by reducing inflammation [117]. Moreover, the iron chelator deferoxamine, which decreases NF- $\kappa$ B levels [118], or low-intensity training which restores SOD1 levels [119] were able to counteract oxidative stress in *Mdx* mice. Although to date DMD remains without a successful single treatment, these findings are at the origin of a clinical trial using epigallocatechin-3-gallate in DMD (Sunphenon Epigallocatechin-Gallate (EGCg) in Duchenne Muscular Dystrophy (SUNIMUD), ClinicalTrials.gov Identifier NCT01183767).

Very recently, gene editing technology CRISPR/cas9 (clustered regularly interspaced short palindromic repeats)/Cas9) was able to generate a pool of endogenously corrected myogenic precursors in *Mdx* mouse muscle [120-122]. Correction was not a precise genomic modification by HDR (homology-directed repair) because HDR does not occur in post-mitotic adult tissues such as heart and skeletal muscle, but by “myoediting” non-homologous end-joining (NHEJ). To introduce CRISPR/cas9 able to skip the mutant dystrophin exon in postnatal muscle *in vivo*, the authors used AAV-9, which displays high tropism for muscle [120-122]. *Mdx*-CRISPR corrected mice partially recovered dystrophin expression in skeletal myofibers and cardiac muscle with significant enhancement of muscle force. It will be of great interest to see if these corrections are able to impact oxidative stress in DMD models and patients and if combination with antioxidant therapy will further improve dystrophin deficiency.

## **7. Conclusion: pharmacological correction of oxidative stress as an emerging therapy**

Significant advances have been made in the complexity of ROS signaling in a muscle context. Multiple lines of evidence showed that primary or secondary oxidative stress is a significant pathogenic abnormality in early-onset myopathies. Because redox balance is drug-targetable using drugs that are already in the market and approved for clinical use in patients, these findings and subsequent pre-clinical studies paved the way for the implementation of three pharmacological trials using antioxidants in SEPN1- and RYR1-related myopathies and in DMD. The result of these clinical trials will maybe allow discovering the first pharmacological treatment for these often devastating inherited disorders which have still no treatment. Thus, it will provide important proof-of-concept regarding the efficiency of pathophysiology-based drug therapies in genetic conditions. Furthermore, understanding and treating oxidative stress in these rare muscle conditions may be useful as a model paradigm to understand and treat other conditions in which OxS is involved, including prevalent conditions with major socioeconomic impact such as insulin resistance, cachexia, obesity, sarcopenia or ageing.

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**Figure 1 : Key players of redox homeostasis.** ETC, electron transport chain; GSH, reduced glutathion;  $H_2O_2$ , hydrogene peroxide;  $O_2^{\cdot-}$ , anion superoxide,  $OH^{\cdot}$ , radical hydroxyl;  $ONOO^-$ , peroxynitrite, ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase;  $NO^{\cdot}$ , nitric oxide; NOS, nitric oxide synthase, NOX, NADPH oxidase; XO, xantine oxidase.

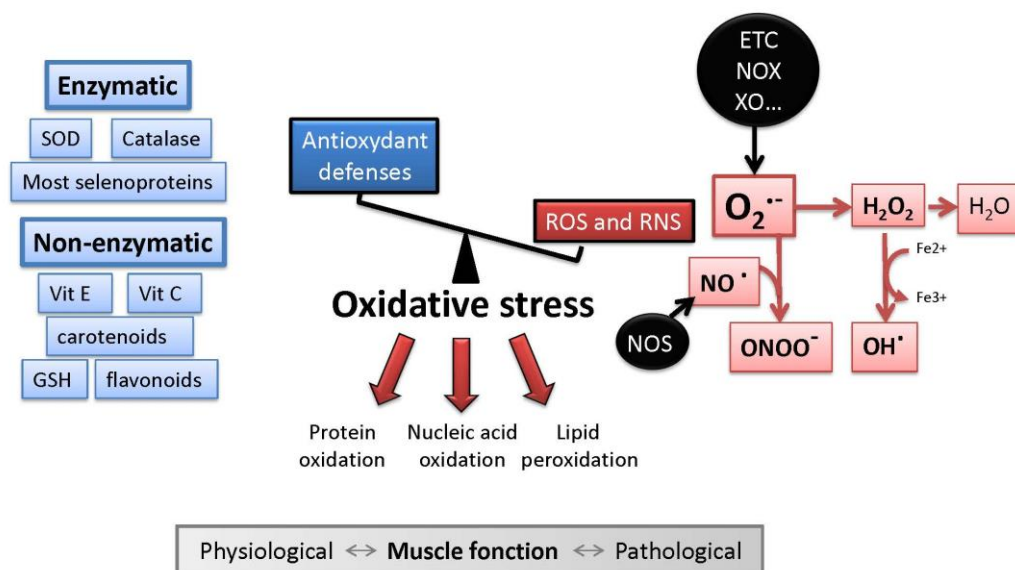
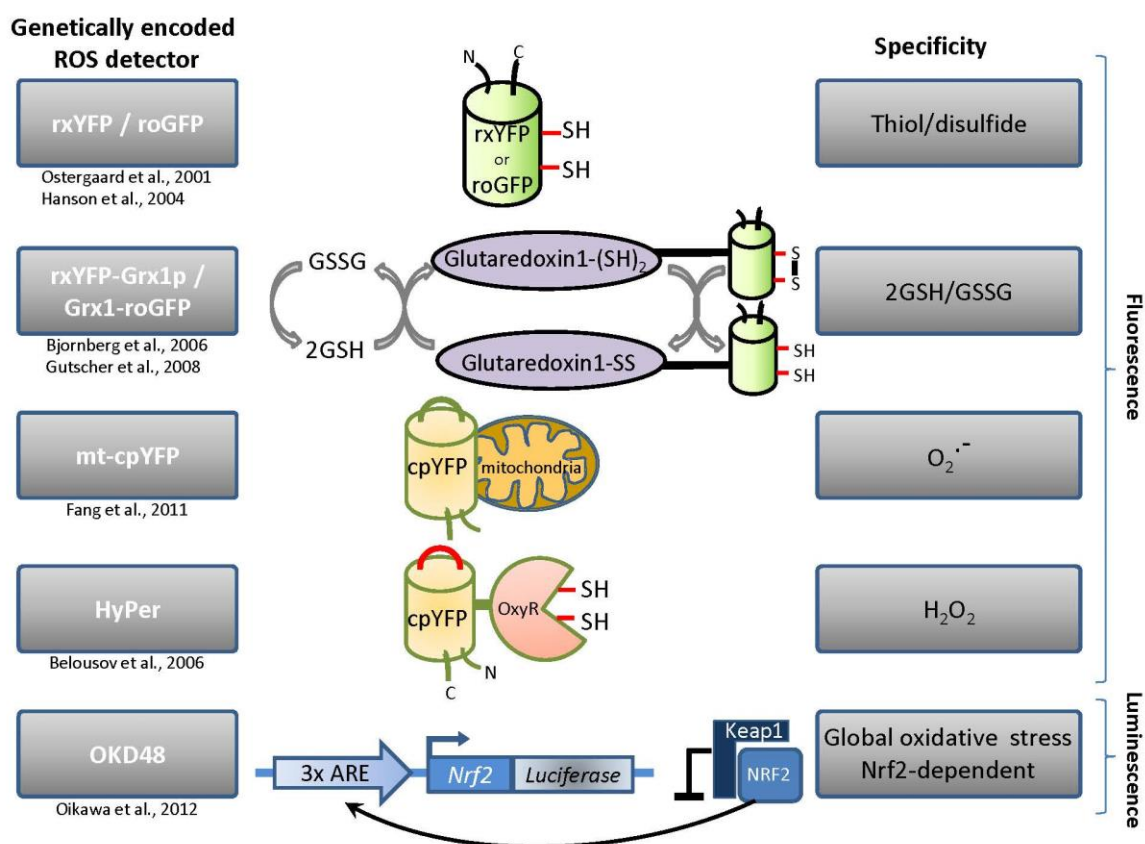


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**Figure 2** : Five main families of genetically encoded redox indicators.



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Table 1: Characteristics of the main redox markers

PROBE-ANALYTE	Readout	Used with	Limitations	Reference
<b>HET</b> Hydroethidine <b>(MitoSOX-red)</b>	Superoxide radical	Live imaging Flow cytometry	Short half life of $O_2^{\cdot-}$ Cytochrome C can oxidize HET	16, 39, 43, 44
<b>CM-H2DCFDA</b> 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate	Various ROS/RNS	Live imaging Flow cytometry	Global measurement	16, 43, 39, 45
<b>Amplex Red</b> N-Acetyl-3,7-dihydroxyphenoxazine	Hydrogen peroxyde	Live imaging Spectrophotometric analysis	Further oxidation of resorufin (the fluorescent product of Amplex red) to non fluorescent products can result in loss of fluorescence intensity	43
<b>DNP/DNP</b> 2,4-dinitrophenylhydrazine/ 2,4 dinitrophenylhydrazone	Carbonylated proteins	Spectrophotometric analysis Oxiblot	Global measurement	41, 46
<b>C11-BODIPY<sup>581/591</sup></b> <b>(MitoPerOx)</b>	Lipid peroxidation	Live imaging Flow cytometry	Light sensitive	43, 47
<b>MDA</b> Malondialdehyde	Lipid peroxidation	Spectrophotometric analysis Chromatography Western blot	MDA reactivity is pH-dependent. Different sensitivity depending on the technique used	48, 49
<b>4-HNE</b> 4-hydroxynonenal	Lipid peroxidation	ELISA Western blot Chromatography	Variable sensitivity depending on the method used	50, 51
<b>TBARS</b> Thiobarbituric acid reactive substances	Lipid peroxidation	Spectrophotometric analysis	Lack of specificity	48, 49
<b>8-OHdG/8-OHG</b> 8-hydroxy-deoxyguanosine 8-hydroxy-guanosine	DNA/RNA damage	ELISA Chromatography	Artificial oxidation of guanosine during sample preparation	52, 53