Myofibrillar myopathies through the microscope: From diagnosis to molecular pathogenesis

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SUMMARY

Myofibrillar myopathies (MFMs) are a group of neuromuscular disorders with the common histological features of myofibrillar dissolution, Z-disk disintegration and accumulation of myofibrillar degradation products. Mutations in genes encoding proteins of the Z-disk, a structural element of the striated muscle, have been reported in patients with MFMs. Nevertheless, in most of the cases the causative gene defect is still unknown. Microscopic studies conducted on skeletal muscle biopsy are fundamental to establish the diagnosis of MFMs and cannot be substituted by any other kind of laboratory investigation. Standard histological stains allow to visualize the granular or hyaline inclusions in the sarcoplasm, indicating the accumulation of misfolded proteins. Fluorescence microscopy analysis performed on muscle sections immunolabeled with antibodies against desmin, αB-crystallin and myotilin is used to detect protein aggregates. Transmission electron microscopy is crucial in the diagnostic workup to demonstrate the presence of Z-disk streaming and myofibrillar dissolution. Even if the pathogenetic mechanisms of MFMs are still under investigation, microscopic studies have played a key role to define the protein composition of the aggregates and to characterize the abnormal molecular processes occurring in the disease.

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Introduction

Myofibrillar myopathies (MFMs) are a group of clinically and genetically heterogeneous neuromuscular disorders sharing the histological features of Z-disk dissolution, myofibrillar degeneration and accumulation of myofibrillar degradation products (Selcen *et al.*, 2004; Ferrer and Olive, 2008; Vattemi *et al.*, 2011; Behin *et al.*, 2015; Kley *et al.*, 2016). MFMs are the paradigm of protein aggregates myopathies (PAM), diseases characterized by protein misfolding and aggregation in skeletal muscle tissue that clinically result in weakness of different muscles and that are frequently accompanied by non-skeletal muscle symptoms (Schroder, 2013). Considering a more comprehensive classification, MFMs can be included in protein aggregate or protein conformational disorders, a wide group of diseases characterized by protein misfolding and aggregation in different tissues and organs. Parkinson's and Alzheimer's diseases as well as prion diseases and amyotrophic lateral sclerosis are well known examples of widely studied hereditary or acquired central nervous system (CNS) diseases in which misfolded protein accumulation within or outside the neuronal cells is the key pathological event (Goebel and Muller, 2006; Sweeney *et al.*, 2017).

Clinical presentation of MFMs

The spectrum of MFMs clinical phenotype is wide and heterogeneous. Clinical picture consists mainly in progressive weakness of distal muscles even if proximal extremities are affected in some patients or may become involved in the later phase of the disease (Selcen *et al.*, 2004; Schroder and Schoser, 2009). Muscle weakness usually involves upper and/or lower limbs (Selcen *et al.*, 2004; Schroder and Schoser, 2009). Limb-girdle and scapuloperoneal phenotypes can be observed as well as the impairment of hand, facial and pharyngeal muscles (Schroder and Schoser, 2009). Cardiomyopathy, peripheral neuropathy, respiratory impairment/failure and cataract are frequent associated conditions (Selcen *et al.*, 2004; Schroder and Schoser, 2009). The first symptoms usually manifest in the adulthood (between 20 and 70 years of age), however infancy-early adolescence onset has been reported in cases with mutation in gene encoding BAG3, FHL1 and desmin (Schroder and Schoser, 2009). Due to their progressive clinical course and the lack of disease-modifying therapies, MFMs often lead to severe disability and premature death (Schroder, 2013).

Genetics of MFMs

Causative mutations of MFMs have been identified in several genes encoding proteins, the majority of which are component of or are associated with the Z-disk (Schroder and Schoser, 2009). Z-disk is a structural component of the sarcomere, the contractile unit of striated muscle (Frank *et al.*, 2006). This complex and highly organized structure is the result of the assembling and interaction between several proteins (Frank *et al.*, 2006). The main functions of Z disk are to provide anchorage for α-actin thin filaments and to link the actin-myosin contractile machinery to the cytoskeleton and the sarcolemma (Frank *et al.*, 2006). Beside the structural role, many of the proteins located at the Z-disk are involved in the transduction of mechanic signals in changes of gene expression (Frank *et al.*, 2006). Mutations in the following genes have been reported: desmin, αB-crystallin, myotilin, Z-band alternatively spliced PDZ-containing protein (ZASP), four-and-half LIM domain 1 (FHL1), Bcl2-associated athanogene-3 (BAG3) and filamin C (Schroder and Schoser, 2009). Nevertheless, at least in half of the patients the gene mutations remain to be identified, suggesting the existence of many other causative genes (Winter and Goldmann, 2015). The inheritance of MFMs is usually autosomal dominant, however X-linked, autosomal recessive and frequent sporadic cases have been described, further highlighting the genetic heterogeneity of these diseases (Schroder and Schoser, 2009).

Muscle biopsy analysis in MFMs: the key role of microscopic studies for the establishment of a proper diagnosis

The histological analysis of muscle biopsy with different microscopic techniques is the most important step in the diagnostic workup of MFMs. For a successful examination of muscle specimen, the appropriate collection, freezing/fixation and storage of the sample is mandatory.

Histological, histoenzymatic and immunohistochemical studies should be undertaken on well-preserved muscle specimen appropriately snap-frozen in liquid nitrogen-cooled isopentane bath to avoid freezing artifacts. The histological staining are performed on serial 8-µm-thick cryosections and usually include: Hematoxylin & Eosin (H&E), Engel-Gomori trichrome (Tri), adenosine triphosphatase (ATPase, pre-incubation at pH 4.3, 4.6 and 10.4), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), periodic acid Schiff (PAS) with diastase digestion, Sudan black and acid phosphatase (Vattemi

et al., 2011). For immunohistochemical studies, 6.5 µm-thick serial muscle sections are advisable to track the immunopositivity of the aggregates to different antibodies (Vattemi *et al.*, 2011). As it will be discussed later, antibodies against desmin, αB-crystallin and myotilin are used to highlight protein deposits. For ultrastructural examination, a small muscle fragment is fixed in 4% glutaraldehyde in phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Spurr resin. Semithin sections are stained with toluidine blue prior to the preparation of ultrathin sections that are stained with uranyl acetate and lead citrate and finally examined under the electron microscope (Vattemi *et al.*, 2011).

Histopathological features in MFMs

Light microscopy

Many are the light microscopic alterations that can be found in muscle of patients with MFMs. The major histopathological hallmarks are protein aggregates visible in the sarcoplasm of muscle fibers as eosinophilic and blue-red amorphous materials at the hematoxylin and eosin (Figure 1) and Engel-Gomori trichrome staining, respectively (Schroder and Schoser, 2009; Vattemi *et al.*, 2011). The same areas usually display loss or reduction of ATPase staining and of oxidative enzymes (COX, SDH) reactivity (Vattemi *et al.*, 2011). Uneven NADH reaction, with areas of increased or reduced staining, is usually observed (Figure 1) (Vattemi *et al.*, 2011). Other common myopathic features include fiber size variation, fiber splitting, atrophic angulated fibers, round muscle fibers, internal myonuclei, rimmed and non-rimmed vacuoles (Schroder and Schoser, 2009; Vattemi *et al.*, 2011). Cytoplasmic bodies, necrotic fibers, degenerating and regenerating fibers are less common (Schroder and Schoser, 2009; Vattemi *et al.*, 2011). Endomysial inflammatory infiltrates and increased endomysial connective and adipose tissue have also been reported (Vattemi *et al.*, 2011). By the way, the spectrum of light microscopy findings is quite broad and cases with mild histopathological changes have also been reported making immunohistochemical and ultrastructural investigations fundamental for the proper diagnosis of the disease (Schroder and Schoser, 2009).

Fluorescence microscopy

Immunohistochemistry with antibodies against αB-crystallin, myotilin and desmin is a sensitive diagnostic tool to highlight protein aggregation in MFMs (Schroder and Schoser, 2009). The presence of abnormal fibers with focal areas of increased reactivity for desmin, αB-crystallin and myotilin or at least for one of these antibodies is a common finding in all MFMs patients (Figure 2) (Schroder and Schoser, 2009). These areas are single or multiple, variable in shape and size and randomly located in the sarcoplasm (Vattemi *et al.*, 2011). Despite the high sensitivity of immunostaining, the presence of regions highly reactive to the described antibodies is not specific for MFMs and can be

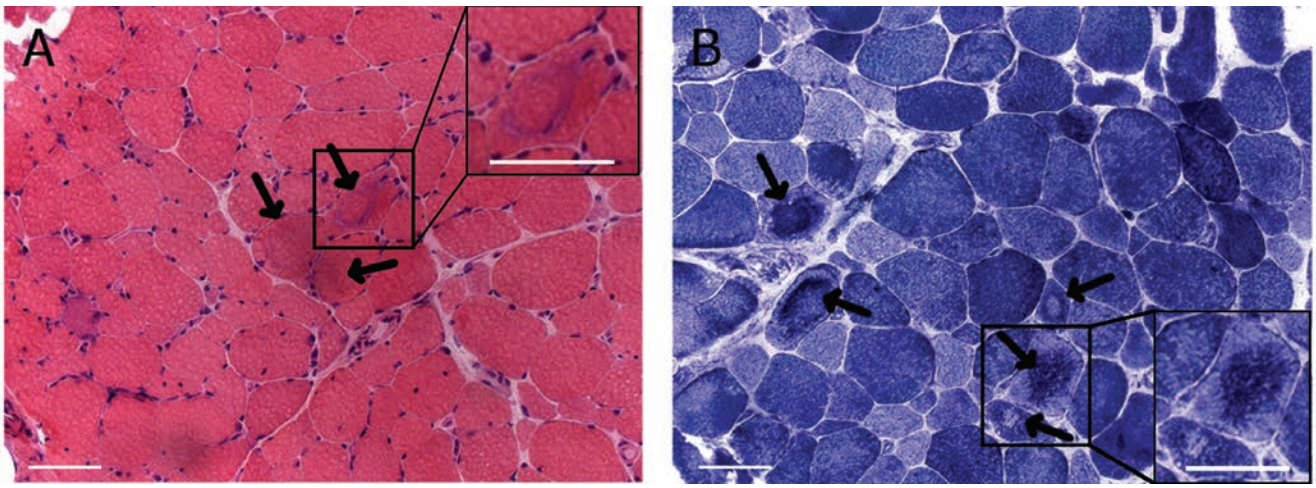

Figure 1. Histological features of MFMs. Hematoxylin and eosin (A) highlights amorphous material (arrows) in the sarcoplasm of abnormal fibers. NADH histochemical reaction (B) reveals fibers with loss or increased enzyme activity (arrows). Scale bars:100 µm.

displayed by other pathological lesions such as central core, minicore and neurogenic target fibers (Schroder and Schoser, 2009). It has been reported that the percentage of fibers showing abnormal immunoreaction ranges between 2% and 15% of the total muscle fibers (Vattemi *et al.*, 2011). Moreover, αB-crystallin immunopositive deposits seem to occur with a higher frequency compare to desmin and myotilin-reactive aggregates (Vattemi *et al.*, 2011).

Ultrastructural features

Ultrastructural analysis has a key role in the establishment of a definite diagnosis of MFMs and should be undertaken in suspected cases (Schroder and Schoser, 2009). Electron microscopy reveals remarkable signs of myofibrillar degeneration including: i) prominent disintegration of myofibrils; ii) alterations of the Z-disks such as streaming, aberrant structure and Z-disk loss; iii) accumulation of granulofilamentous materials in the sarcoplasm, among myofibrils and in the subsarcolemmal region (Figure 3) (Selcen *et al.*, 2004; Schroder and Schoser, 2009; Vattemi *et al.*, 2011).

To briefly summarize, the major morphological features in a muscle biopsy that point to the diagnosis of MFMs are: i) abnormal fibers containing in their sarcoplasm amorphous, granular or hyaline deposits of irregular shape and size visible at H&E and Trichrome stains and that display reduced or absent oxidative enzyme activity; ii) protein aggregates immunopositive to αB-crystallin, desmin and myotilin antibodies; and iii) myofibrillar disruption, Z-disk streaming and granulofilamentous material among the myofibrils (Guglielmi *et al.*, 2015).

Microscopic studies: A fruitful approach to identify new molecular players in MFMs

Microscopic techniques have played a central role in the characterization of the protein content of aggregates and in giving clues to the pathogenic mechanisms that could cause the disease. Several proteins with a structural role in sarcomeric organization and interconnection, such as component of the Z-disk and myofibrils (*i.e.*, actin, α-actinin, titin) or proteins involved in the formation of the intermediate filaments (*i.e.*, desmin, vimentin, plectin), have been detected within the deposits (De Bleecker *et al.*, 1996). Molecular chaperons (*i.e.*, αB-crystallin, HSP72, HSP73) and components of the ubiquitin-proteasome system (*i.e.*, 20S and 19S proteasome subunits, p62, ubiquitin, mutant ubiquitin) have also been found to be abnormally accumulated (De Bleecker *et al.*, 1996; Ferrer *et al.*, 2004; Olive *et al.*, 2008). Proteins with a role in the oxidative/nitrosative stress pathway (*i.e.*, nitric oxide synthases, SOD2), several cyclin-dependent kinases and proteins prone to aggregation that are involved in other conformational diseases (*i.e.*, phospho-tau, prion protein, β-amyloid precursor protein, amyloid-β, TDP-43) have been documented in the abnormal area of muscle fibers (De Bleecker *et al.*, 1996; Nakano *et al.*, 1997; Ferrer *et al.*, 2004; Kovacs *et al.*, 2004; Selcen *et al.*, 2004; Olive *et al.*, 2009). Among the components of the sarcoplasmic aggregates there are also nuclear proteins (*i.e.*, emerin, laminin B), proteins that shuttle between nucleus and cytoplasm (RPAP2 and RPAP4) and non-skeletal muscle proteins (*i.e.*, synaptophysin and SNAP-25) (Nakano *et al.*, 1997;

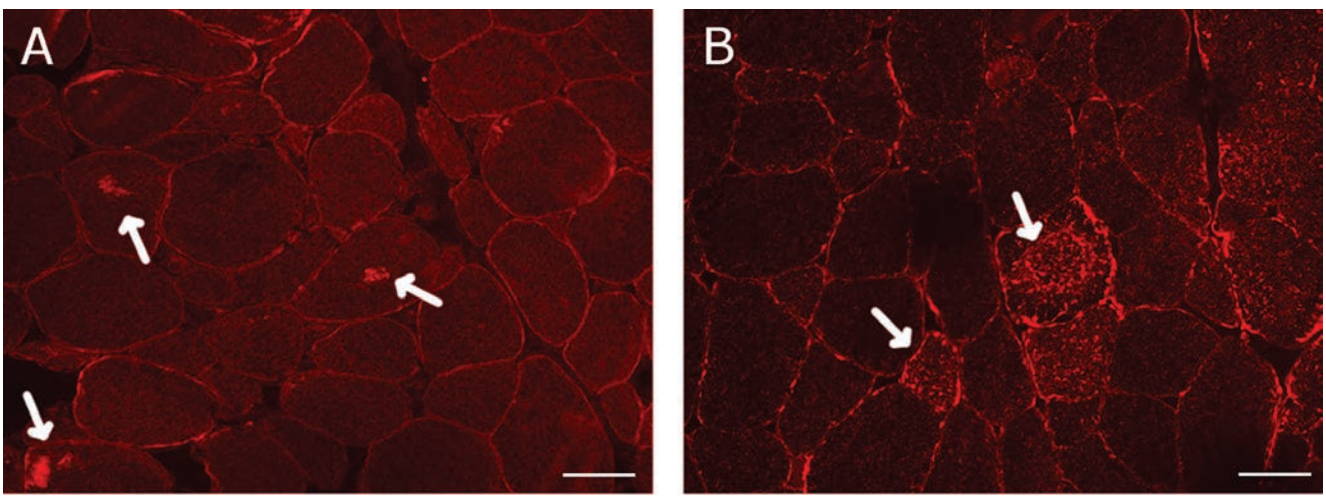

Figure 2. Immunofluorescence findings in MFMs. Focal areas with increased immunoreactivity to αB-crystallin (A) and desmin (B) occur in some muscle fibers. Scale bars: 50 µm.

Barrachina *et al.*, 2007; Guglielmi *et al.*, 2015). Recently, the use of laser microdissection technique to isolate aggregates from skeletal muscle sections followed by protein identification by mass spectrometry gave a major contribution in widening the spectrum of proteins known to be over-represented in the deposits (Feldkirchner *et al.*, 2012; Kley *et al.*, 2013; Maerkens *et al.*, 2013; Kley *et al.*, 2016; Maerkens *et al.*, 2016). The presence within the focal area of proteins identified with the latter approach have been confirmed by immunofluorescence, demonstrating the reliability of the innovative approach to better characterize aggregates composition (Feldkirchner *et al.*, 2012; Kley *et al.*, 2013; Maerkens *et al.*, 2013; Kley *et al.*, 2016; Maerkens *et al.*, 2016).

Overview on the pathogenetic mechanisms in MFMs

Despite several genes have been identified as causative of MFMs, the pathogenic mechanisms leading to protein aggregation and disease have not been completely elucidated.

In vitro protein assembly assays, cell transfection experiments and studies on transgenic mice have been performed in the attempt to uncover the molecular events that lead from gene mutation to disease (Wang *et al.*, 2001a, 2001b; Garvey *et al.*, 2006; Buhrdel *et al.*, 2015). The results of these studies suggest that the mutated proteins not only might disrupt the Z-disk organization, the cytoskeleton and/or intermediate filament network or affect the appropriate maintenance of these structures but also could act as a "sink" capturing other proteins in the insoluble aggregates (Yu *et al.*, 1994; Kaminska *et al.*, 2004). *In vitro* studies on cell cultures demonstrated that the alteration of protein-protein interaction network could contribute to the progressive accumulation of proteins in the aggregates (Yu *et al.*, 1994; Kaminska *et al.*, 2004). This model is further supported by the presence in the deposits of many *wild type* proteins indicating that the mutated protein could impair the proper localization and function of other proteins involved in the same network, in a sort of cascade mechanism (Schroder and Schoser, 2009).

Abnormalities in protein degradation *via* the ubiquitin-proteasome system (UPS) have been implicated in MFMs (Ferrer *et al.*, 2004). Accumulation of several proteasome (19S, 20S and PA28α/β) and immunoproteasome subunits (LMP2, LMP7, and MECL1) and their co-localization with ubiquitin and mutant ubiquitin within the aggregates have been reported (Ferrer *et al.*, 2004; Olive *et al.*, 2008). However, the increased chymotrypsin-like activity detected in skeletal muscle of MFMs patients suggests that the abnormalities of the UPS are not due to reduced proteolytic activity, but rather result from the inability of ubiquitinated proteins to enter the proteasome (Ferrer *et al.*, 2004). The impairment of protein quality control has been further supported by the presence of p62 in protein deposits. p62 has a key role in protein disposal acting as a bridge between the autophagy pathway and the ubiquitin-proteasome system; indeed, in response to certain conditions, p62 targets ubiquitinated cargoes either for autophagic or proteasome-mediated degradation (Liu *et al.*, 2016); p62 takes also part to the regulation of aggresome formation and degradation (Liu *et al.*, 2016). Aggresomes are aggregates of ubiquitinated proteins enclosed in an intermediate filament protein network that localize to the pericentrosomal area in conditions of impaired proteolysis (Ferrer *et al.*, 2005; Liu *et al.*, 2016). The occurrence of aggresomes and the increased level of γ-

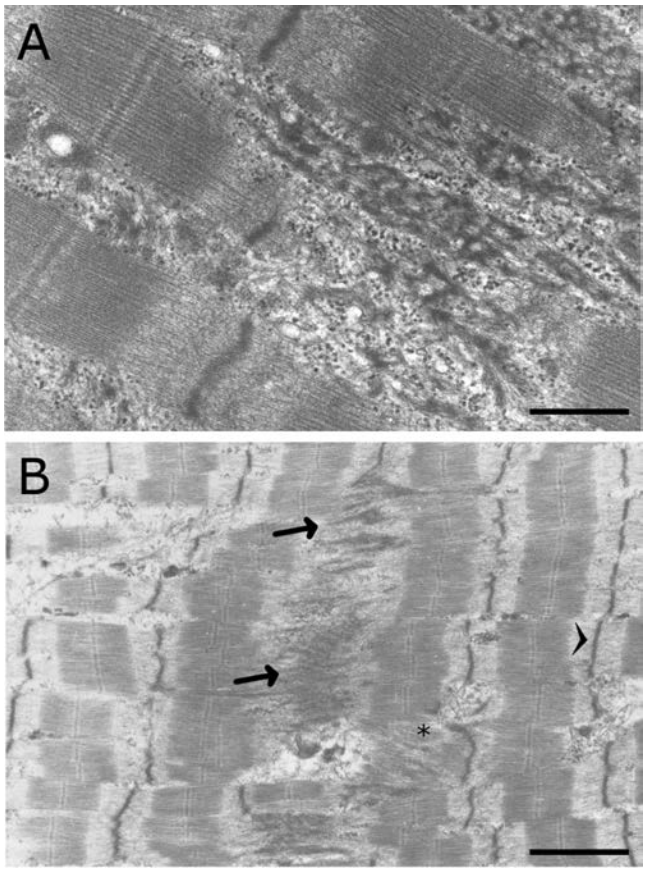

Figure 3. Ultrastructural features of MFMs. Amorphous granulofilamentous materials interspersed among myofibrils. The regular alignment between myofibrils is lost (asterisks) and normal-looking Z-disks (arrowhead) are replaced by "stretch-out" Z-disks and irregular electron-dense material (arrows). Scale bars: A) 1 µm; B) 3 µm.

tubulin, which is involved in the active transport of misfolded proteins to aggresomes, within protein deposits have been reported in skeletal muscle of patients with MFMs, indicating that aggresomes formation could contribute to protein accumulation (Ferrer *et al.*, 2005). Sequestration of p62 in MFMs protein aggregates might therefore alter the fine tuning between these processes.

Deregulation of transcription factors activity seems to take part in the pathogenesis of MFMs. Aberrant expression of neuron-related proteins such as synaptosomal-associated protein 25 (SNAP25) and synaptophysin and their accumulation in protein aggregates have been reported in patients with myotilin mutation (Barrachina *et al.*, 2007). The abnormal occurrence of neuronal proteins has been linked to the downregulation of the expression of NRSF/REST, a transcription factor expressed in non-neuronal tissues and involved in the repression of several neuronal genes (Barrachina *et al.*, 2007). Increased levels of glycoxidation and lipoxidation markers and of nitrotyrosin, a marker of protein nitration, have been detected in muscle of patients with MFMs (Janue *et al.*, 2007a; Janue *et al.*, 2007b). The three isoenzymes of nitric oxide synthase (NOS), namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), that are responsible for the synthesis of nitric oxide (NO), are abnormally distributed in myofibers containing the aggregates (Janue *et al.*, 2007b). These observations point to the occurrence of oxidative/nitrosative damage in MFMs (Janue *et al.*, 2007a, 2007b). It has also been shown that oxidized and nitrosylated proteins not only are more prone to aggregation but their presence above a certain threshold might affect proteasome activity and therefore facilitate protein accumulation in the myofibers, suggesting a possible link between oxidative/nitrosative stress and aggregates formation (Janue *et al.*, 2007b).

It has been reported that the RNA polymerase II-associated proteins (RPAPs) RPAP2 and, to a lesser extent, GPN1/RPAP4 are accumulated in the sarcoplasm of MFMs muscle fibers and co-localize with POLR2A/RPB1, the largest subunit of RNA polymerase II (RNA-PolII) (Guglielmi *et al.*, 2015). RPAPs are involved in assembling, folding and nuclear import of RNA-PolII and their accumulation in the cytoplasm of abnormal fibers suggest that an impairment of the nucleus–cytoplasm transport and transcription abnormalities might occur in the disease (Guglielmi *et al.*, 2015).

Mitochondrial alterations have also been reported in MFMs. Abnormal distribution of mitochondria is indicated by the presence of myofibers with absent or decreased oxidative stains and supported by ultrastructural studies showing the alteration of mitochondrial localization and the presence of mitochondria with paracrystallin inclusions (Schroder and Schoser, 2009). Patients displaying low respiratory chain complex I and complex IV and multiple mtDNA deletions have also been described and in rare cases, ragged red fibers and cytochrome c oxidase negative fibers have been documented (Reimann *et al.*, 2003; Joshi *et al.*, 2014). Transfection studies on different cell lines demonstrated that the expression of mutant desmin induces aggregate formation, disruption of the intermediate filament network and leads to changes in the subcellular distribution of mitochondria (Schroder *et al.*, 2003).

Conclusions

A major role in the diagnosis of MFMs is played by microscopic studies that cannot be replaced by any other laboratory investigation. The presence of the former described histopathological findings establishes the diagnosis of MFMs that is then accompanied by the genetic confirmation when the mutation occurs in one of the known causative genes. The clinical presentation is not specific, as in most of skeletal muscle diseases, and genetic analysis alone is not sufficient, indeed mutations in genes involved in MFMs have also been associated to other neuromuscular disorders such as limb-girdle muscular dystrophy (LGMD1A, myotilin mutations) (Hauser *et al.*, 2000). Laser microdissection followed by mass spectrometry has been useful to further characterize the protein composition of the aggregates and to identify novel molecular players in MFMs (Kley *et al.*, 2016). The application of next-generation sequencing in the field of MFMs is paving the way toward the identification of new causative genes (Hauser *et al.*, 2000; Schessl *et al.*, 2014; D'Avila *et al.*, 2016). Hopefully, the combination of different experimental approaches and techniques will help clarify the pathogenesis and define therapies for MFMs.

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