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Chapter

Clinical and Molecular Diagnosis in Muscular Dystrophies

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Abstract

Muscular dystrophies are a diverse group of inherited muscle disorders with a wide range of clinical manifestations from a severe form with early onset and early death to adult forms with later onset and minimal clinical manifestation that do not affect life-span. Overlapping clinical symptoms and the multitude of genes that need to be analyzed for an accurate characterization make the diagnosis hard. In next-generation sequencing era, a lot of used assay in molecular diagnostics must be taken into consideration for muscular dystrophy diagnosis. However, for more accurate diagnosis, muscle protein expressions analysis may have prognostic value. In this chapter, we present the most important clinical and laboratory findings in the most common forms of muscular dystrophies and molecular diagnostic approaches for a more accurate diagnosis.

Keywords: muscular dystrophy, multiplex Western blot, immunofluorescence, MLPA, hrMCA, dystrophin, calpain 3, *DMD* gene, CAPN3, genetic diagnosis

1. Introduction

Muscular dystrophies (MD) are an inherited group of genetic disorders clinically characterized by progressive muscular weakness and wasting [1] and reduced skeletal muscle mass until their destruction due to a primary defect in the muscle cell. To date, there are known more than 30 different forms of MD with specific signs, symptoms, and genetic basis but sharing common histological features like variation in fiber shape and size and the presence of degeneration and regenerating fibers and connective tissue proliferation [2]. The diseases are distinguished from one another by the age of onset, muscles affected, as well as rate of disease progression [3]. While for some forms of MD, the initial symptoms manifested begin with childhood and have a rapid progression of muscle weakness causing the death of the patients around the age of 20 years, the other forms debut later in adulthood [4, 5] and have a slow rate of progression and an almost normal lifetime [6, 7]. Also, heart disease and mental retardation accompany some types of MD [8, 9], suggesting a different pathogenesis of the disease. It also found that there are subtypes of MD that share similar clinical manifestations and different genetic defects with similar clinical manifestation [10, 11].

However, the progress made in the past 33 years, since the first protein involved in a type of muscular dystrophy was discovered, leads to identify a large number of the genes as well as novel proteins involved in these muscle disorders [12].

For a rapid and an accurate diagnosis improvement in analysis, methods have become a necessity. The combination of clinical signs with muscle histopathology

and protein and genetic analyses becomes the diagnostic gold standard for these disorders. Nevertheless, for many patients with yet unidentified muscular dystrophy, the diagnosis continues to be challenging.

In this book chapter, we draw attention on clinical manifestation and the most important laboratory investigations such us muscle histopathology, protein analysis, and genetic tests that can help in distinguishing between different forms of muscular dystrophy and could lead to an accurate diagnosis.

2. A general approach to the diagnosis of muscular dystrophy

The complexity and similarity of clinical manifestation of these conditions represent a challenge for getting an accurate diagnosis for patients. A complete diagnosis involved clinical examination and patient's medical history, blood tests (creatine kinase and serum transaminase levels), electromyography, muscle biopsy examination, and genetic tests [13–15].

Muscle biopsy had an important role in muscular dystrophy diagnosis and still provides essential information for diagnosis. Although in clinical observations, family history, muscle biopsy, and biochemical tests such as serum creatine kinase (CK) are still important tools for muscular dystrophy diagnosis, protein analysis and genetic study have an increasing importance in accurate establishing a diagnosis.

For several years, until the discovery of other muscle proteins, dystrophin was the only protein studied to establish a diagnosis of muscular dystrophy. It is also used today in the differential diagnosis between Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). In the last 10 years, genes involved in various types of autosomal recessive muscular dystrophy (LGMD) as well as in congenital muscle dystrophies have been identified [16–18].

Differentiation between recessive muscle dystrophies is much more difficult to achieve on the basis of clinical criteria because of phenotypic variability [19], different starting age of onset [20, 21], and a variable progression rate [22].

Identification of protein defect by immunohistochemistry and Western blotting allows firm and specific diagnosis in a wide variety of muscular dystrophies. However, while immunohistochemistry is very useful in identifying abnormal expression of primary protein deficiency, in genetically inherited recessive diseases, it is less useful for identifying primary defect in dominant diseases.

2.1 Clinical manifestations and symptoms for most common forms of muscular dystrophy

The patient's medical history and clinical examination allow the doctor to identify the signs and the specific symptoms of the diseases. A complete examination should include evaluation of movement and difficulty controlling movement, gait abnormalities, muscle strength, and the presence of weakness pattern, and also identification of the muscle groups affected.

While the most common sign for different types of muscular dystrophy is the progressive muscle weakness, the other features like age of disease onset, muscle group affected, and rate of progression are specific for each type of muscular dystrophy [21, 23].

2.1.1 Dystrophinopathies

Dystrophinopathies are recessive X-linked disorders caused by mutation in dystrophin gene [1]. Currently, they are recognized as a spectrum of disease with

involvement of skeletal and cardiac muscle in different degree [24] and include Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), involving mostly skeletal muscles, and DMD-associated dilated cardiomyopathy (DCM), affecting preferentially the myocardium. The clinical picture in males with dystrophinopathies ranges from mild to severe. The mild forms consist of high level of CK in serum and muscle cramps with myoglobinuria. The severe forms include progressive muscle diseases (Duchenne muscle dystrophy, DMD, Becker muscle dystrophy, BMD).

2.1.1.1 Duchenne muscular dystrophy (DMD)

In DMD, affected boys are clinically normal at birth. The onset of clinical features is in early childhood with delayed motor milestones, including delayed independent walking, with a mean age of walking of 18 months, and difficulties in standing up from the floor. The most characteristic clinical features of DMD are general motor delays (42%) and gait problems including persistent toe walking and flat-footedness (30%) [25]. Patients with DMD have a waddling gait and difficulties in climbing stairs, running, jumping, and standing up due to proximal weakness [26]. They rise from a supine position using their arms (Gower maneuver). The boys have hypertrophic and firm calf muscles [27]. The disease is rapidly progressive, at the age of 12 years, most boys being wheelchair bound [25]. Subsequently, the function of upper extremity will be lost, and, by the age of 14–15 years, cardiomyopathy is a common feature [26]; during the teenage years, the patients will require assisted ventilation [27]. Progressive cardiomyopathy and respiratory complications represent the most common causes of death in patients with DMD. Typically, the death occurs by 30 years of age, but currently the life expectancy of these patients has been improved through an improved management of cardiorespiratory function [28].

Intellectual disability can be present in up to 27% of boys with DMD, and 44% of patients have learning disability [28]. Other psychiatric disorders reported in these patients include attention-deficit hyperactivity disorder (ADHD) (32%), anxiety (27%), and autism spectrum disorder (15%) [29].

2.1.1.2 Becker muscle dystrophy (BMD)

BMD is a milder form of muscular dystrophy characterized by skeletal muscle weakness with a later onset and a preservation of the gait for a longer period (age of 40–50 years) [25]. The patients present usually with high serum CK concentration, calf muscle hypertrophy, muscle cramps, myalgia, or with muscle weakness in the pelvic and shoulder girdles [26]. Cardiomyopathy is a common complication of BMD, the mean age of diagnosis being around 14, 6 years [28]. Heart failure represents the most common cause of death in BMD, at an age of mid-40s [23, 24].

2.1.2 Emery-Dreyfus muscular dystrophy (EDMD)

EDMD is a muscular disorder with different inheritance patterns: X-linked recessive or autosomal dominant or autosomal recessive [25, 26]. The clinical picture includes the classical triad: (i) early joint contractures, (ii) slowly progressive muscle weakness and wasting in humeroperoneal distribution (upper arm, lower legs), and (iii) cardiac disease (atrial-ventricular conduction anomalies, atrial arrhythmias) [27]. Usually, the clinical presentation is characterized by Achilles contractures with toe walking in childhood. Later, arm weakness and elbow contractures develop, associated with biceps or triceps wasting with sparing of deltoid

muscle (a characteristic pattern called "Popeye arms." A rigid spine is also present causing a severe lumbar lordosis [27].

2.1.3 Limb-girdle muscular dystrophies (LGMDs)

LGMDs represent a group of muscular conditions with autosomal dominant or autosomal recessive inheritance, characterized by a typical pattern of slowly progressive, proximal weakness which involves shoulder and pelvic girdle muscles [18, 19]. Different subtypes of LGMDs have been described, with a wide clinical spectrum affecting various age groups. LGMDs with autosomal dominant inheritance are referred as LGMDs1 and recessive forms, as LGMDs2. LGMDs1 forms have, generally, a later onset and a milder course compared with autosomal recessive forms [29].

The most affected muscle groups are proximal muscles, namely, the muscles of the shoulders, upper arms, pelvis, and thighs. The clinical picture can vary among different subtypes of FSHD, even within the same family [29]. The onset of clinical features can be at any age and worsen with time. The first symptoms include abnormal gait (waddling gait, walking on the feet balls) and difficulties in running and standing up [29]. The muscle weakness slowly progresses, and, in later stage of the disease, the patients may be wheelchair bound. Other clinical features include scapular wings, lumbar lordosis, scoliosis, calf muscle hypertrophy and joint stiffness, that restrict movement of the elbows, hips, knees, and ankles [30]. Cardiomyopathy was reported in some forms of LGMD, and some patients may present respiratory difficulties which can vary from mild to severe. In some rare forms of LGMD, intellectual disability has been reported [31].

2.1.4 Facioscapulohumeral muscular dystrophy (FSHD)

FSHD is a genetic muscular disorder with autosomal dominant inheritance and a late onset; the disease has a slow progression and a high degree of phenotypic variability and side-to-side asymmetry [30]. The muscle weakness involves initial, facial, scapular, and proximal limb muscles (mimetic muscles, serratus anterior, rhomboid muscles, biceps, and triceps) [21]. The most frequent initial symptom is the inability to lift arms over shoulder height. Then, the weakness progress to lower limbs, typically the distal musculature first (tibialis anterior and gastrocnemius), and later more proximal muscles (quadriceps and hamstrings) and the pelvic girdle are involved [21]. The abdominal and paraspinal muscles can be affected, causing an exaggerated lumbar lordosis or camptocormia (bent spine syndrome) [22]. Pectus excavatum is another common feature in FSHD [22]. The risk to become wheelchair bound is high in the second decade for patients with a more severe infantile form and after the age of 50 years in about 20% of patients [23].

The respiratory involvement varies from 0 to 13% of patients with FSHD in different studies [24] and is caused by the loss of core/trunk muscles. It is present mostly in patients with pelvic girdle weakness who are wheelchair bound or with a marked paraspinal involvement or kyphoscoliosis. Between 1 and 8% of patients with FSHD require mechanical ventilation [23].

Cardiac involvement is not common in FSHD. 5–10% of patients can present supraventricular arrhythmias, mostly asymptomatic [25]; an incomplete right bundle branch block has been found in approximately one-third of patients in one study [26], with no significant progression.

Some extramuscular manifestations have been described in patients with FSHD, almost always in the cases with the smallest number of residual D4Z4 units. They include retinal vascular changes (peripheral telangiectasia); Coats disease, a severe

retinal vasculopathy characterized by aneurysmal dilatations and exudation, which can cause retinal detachment or blindness; loss of high-frequency hearing, usually asymptomatic; hearing loss; and intellectual disability and seizures in infants with FSHD [32, 33].

A careful, complete, and thorough clinical examination along with laboratory investigations provides more information necessary for management of patients with muscular dystrophy, differentiates between the type of muscular dystrophy, and directs to subsequent analyses.

2.2 Laboratory investigations

When a muscular dystrophy is suspected, blood enzyme test and a variety of laboratory test can be used for confirmation of clinical diagnosis. The blood serum samples are used to determine the level of specific enzymes known to have a high blood serum levels when a dystrophic process is present:

i. Creatine kinase (CK) also known as creatine phosphokinase (CPK), an intracellular enzyme found with relative predominance in skeletal muscle, is considered as the most specific and sensitive marker of muscle disease. Normal reference value of CK ranges between 60 and 174 IU/L into blood serum [13, 14]. Elevated level of CK could suggest a muscle disease before symptoms of muscular dystrophy become evident [33, 34]. In early stages of the muscle disease, CK levels are 20–300 times greater than normal levels and tend to decrease with the muscle damage [15]. In male DMD patients, the serum CK level is markedly elevated due to muscle degeneration [17] with less elevation level noted in BMD patients. Recent studies show that losses of lung function in DMD patients determine the high level of CK in blood serum [35].

The level of CK has been found higher in other types of MD like limb-girdle muscular dystrophy (LGMD) [17] and could serve as useful indicator being able to discriminate between autosomal recessive and dominant types of LGMD, knowing that CK level recessive types of MD are higher than dominant ones. Also, evaluation of CK level is a useful screening tool for female DMD carrier.

It is interesting to note that not all cases of MD show a high level of CK. For example, in Ullrich congenital muscular dystrophy, Emery-Dreifuss muscular dystrophy, and Bethlem myopathy, the level of CK may be normal or slightly increased [22].

ii. Aldolase, transaminases (alanine aminotransferase ALT and aspartate aminotransferase AST), and lactate dehydrogenase (LDH) are other muscle enzymes also reported with a rise level in blood serum [18] when a muscular dystrophy is suspected.

Also, from the blood collected on anticoagulant (EDTA), total genomic DNA is isolated for further genetic tests used to confirm the diagnosis.

Other laboratory tests like electromyography, magnetic resonance imaging (MRI), combined with muscle biopsy, and genetic tests contribute for toward a diagnosis.

2.3 Muscle biopsy

The assessment of skeletal muscle biopsy is an essential procedure for an accurate diagnosis when a muscle disease is suspected, providing evidence of pathological changes in muscle and guides for appropriate tests.

2.3.1 Muscle histopathological analysis

Even though the muscle biopsy is a highly invasive procedure, the data gained from it has the utmost importance for histopathological diagnosis and is an essential component in the diagnosis of muscle disorders that could identify the cause of the disease process and distinguish between different types of muscular dystrophy. The overall structure of the tissue as well as all specific histological features of dystrophic muscle can be observed by hematoxylin and eosin (H&E) staining performed on frozen sections sampled from the quadriceps or deltoid muscle. Generally, the features observed in all dystrophic muscle biopsy include fibers' size variation, round shape muscle fibers, the presence of atrophy, regenerating and degenerating fibers, splitting of fibers, proliferation of the connective tissue, and increased number of internal nuclei. In the end phases of the disease, the fibers are replaced by adipose tissue [36]. Histopathological changes differ widely in severity among the types of muscular dystrophy, as well as among allelic variants of the same genotype. Also, some features are specific for each type of muscular dystrophy. For example, lobulated fibers are characteristic for LGMD 2A; a high variability in fibers'size is specific for LGMD 1C, and increased internally nuclei are specific for myotonic dystrophy; the presence of rimmed vacuoles suggests a myotilinopathy, while prominent vacuoles are found in LGMD [36]. With all signs, none of the specific forms of muscular dystrophy can be diagnosed just based only on histological analysis.

Also, the muscle biopsy analysis can not only denote the specific genetic cause of the disease but can also provide clues for further investigation. In combination with protein analysis, the genetic investigations can provide an accurate diagnosis.

2.3.2 Protein analysis

The study of muscle protein expression is important for diagnosis, for genotype-phenotype correlations, and to identify possible genetic defect [37–39]. There are many methods used for the study of muscle protein expression [40], but the most used are immunostaining methods (immunohistochemistry or similar methods immunofluorescence and immunoblotting/Western blotting (WB)). Both methods use labeled antibodies for a specific muscle protein involved in a type of muscular dystrophy. While the immunohistochemistry/immunofluorescence method is used to identify the localization and relative abundance of the proteins, in tissue cryosections, the WB method is useful to detect the total amount of proteins as well as the normal or reduced size of the proteins in homogenized sample.

2.3.2.1 Immunofluorescence (IF)

In the past, the diagnosis of muscular dystrophy consisted only on clinical assessment, serum *CK levels*, and histological investigations of muscle biopsy [41, 42].

The discovery in 1986 of the first muscle protein involved in a type of muscular dystrophy, dystrophin [43], has later led to the identification of the dystrophin-associated protein complex (DAPC) [44] and other additional proteins from the muscle cytosol (calpain 3, TRIM32) [45], from extracellular matrix (a2-laminin, collagen VI) from the sarcomere (telethonin, myotilin, titin, nebulin) [46]. Each of these proteins is involved in a type of muscular dystrophy; so far over 40 types of muscular dystrophy are known [47].

The development of specific antibodies (Abs) for affected proteins has improved the diagnosis for these diseases, over time. Now, there are many

immunohistochemical protocols based on the use of specific antibodies for antigen localization through antigen-antibody interaction.

IF or fluorescent antibody staining is a helpful routinely technique widely used to determine the localization of fluorescent-tagged protein and changes in protein expression on a cryosection (presence, reduced, and absence). It is also used to investigate muscle architecture.

A large array of antibodies directed against different muscle protein is now available for current diagnosis and allows the use of these techniques for the diagnosis of many muscle disorders (see **Table 1**).

The use of antibodies directed against muscle protein *is useful to gain information about integrity of muscle complexes, secondary reduction of proteins, and also gene function by compared normal and affected patients* (see **Figure 1**) [48].

In addition, IF method identifies also the secondary reduction and overexpression of closely protein levels [49]. For example, utrophin, an autosomal protein which presents around 80% sequence homology to dystrophin [50, 51] and localized in normal skeletal muscle at the neuromuscular junction (NMJ) [52], is overexpressed in dystrophinopathies (see **Figure 2**) [53, 54]. Mutations in one sarcoglycan often lead to reduced expression of other sarcoglycans [49], and reductions of dysferlin (see **Figure 3**) are observed in other forms of muscular dystrophy such as calpainopathy, caveolinopathy, and anoctaminopathy [55].

However, IF is not always helpful in diagnosis of all forms of MD. For example, in LGMD 2A some available antibodies have no immunoreactions on the sections. Few studies tried to analyze calpain 3 on cryosections but, because of rapid degradation of calpain 3 after harvesting, concluded that immunostaining analysis alone does not predict the presence of CAPN3 mutations [40]. However, the staining of other proteins such us dystrophin and sarcoglycans which appear normal on cryosections by IF could be informative for further analysis.

Also, in dominant forms LGMD1B caused by changes in the *LMNA* gene which encode for lamin A/C, immunostaining of cryosection reveals normal expression even in the presence of a mutation [56].

Antibody	Manufacturer	Clone	Dilution for cryosection	Dilution for Western blot
Dystrophin C-terminus	Novocstra	Dy8/6C5	1:40	1:200
Dystrophin N-terminus	Novocstra	Dy10/12B2	1:50	1:50
Dystrophin rod domain	Novocstra	Dy4/6D3	1:20	1:200
Utrophin	Novocstra	DRP3/20C	1:50	1:200
Calpain 3	Novocstra	2C4	—	1:100
Calpain 3	Novocstra	12A2	—	1:50
Caveolin 3	Santa Cruz	A-3	1:50	1:200
Dysferlin	Novocstra	Ham1/7B6	1:50	1:100
α-Sarcoglycan	Novocstra	Ad1/20A6	1:50	1:100
β-Sarcoglycan	Novocstra	BSarc/5B1	1:50	1:100
γ-Sarcoglycan	Novocstra	35DAG/21B5	1:50	1:100
Merosin	Novocstra	Mer3/22B2	1:50	1:50

Table 1.
Details of the most used antibodies in muscular dystrophy diagnosis.

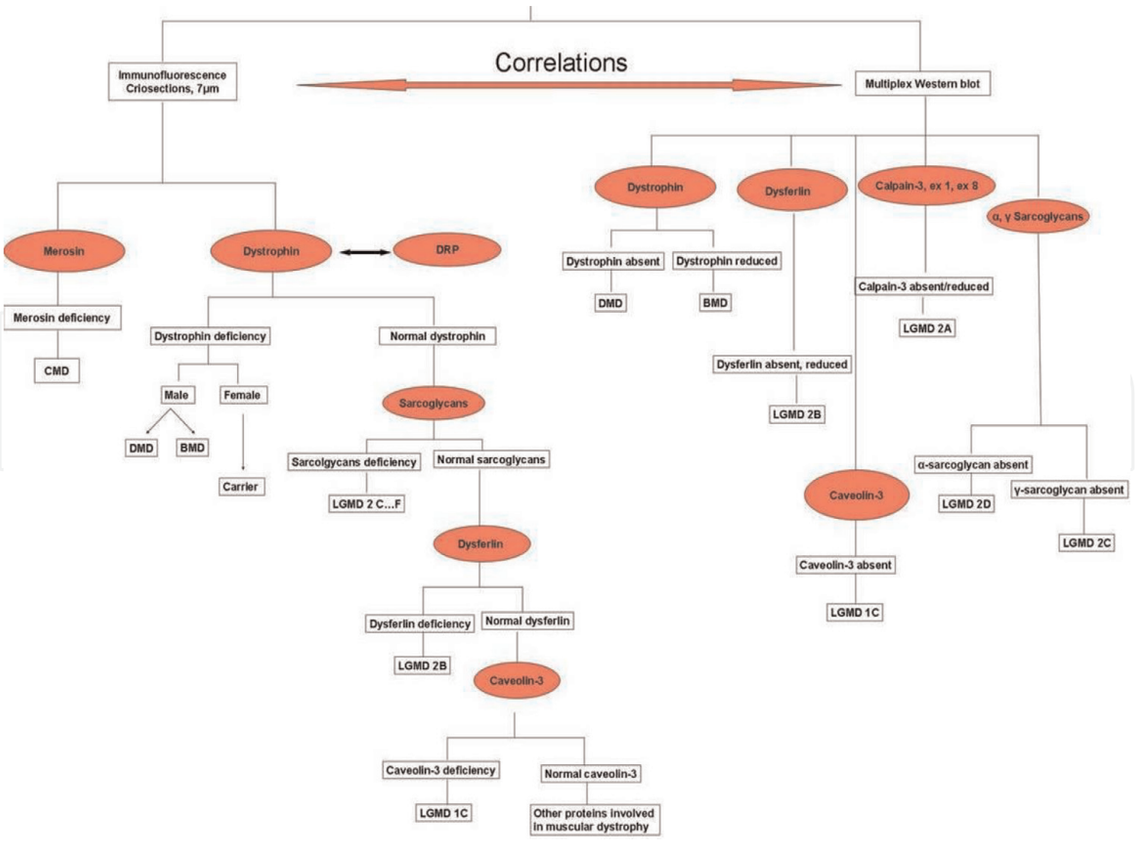


Figure 1.
Workflow chart for muscle protein analysis in muscular dystrophy diagnostic process.

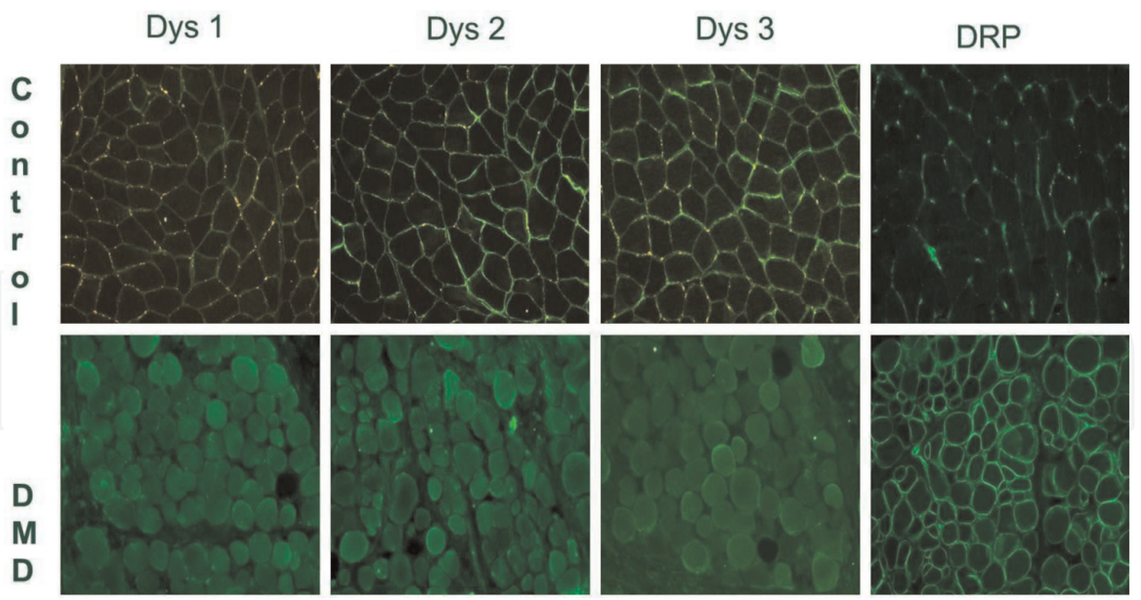


Figure 2.
Representative immunofluorescent staining from dystrophin and utrophin in normal human (control) and DMD and BMD skeletal muscle.

It is important to note that to obtain good results in protein evaluation by IF is a necessary investigation of the integrity of muscle fiber membrane by analysis of spectrin [54, 57].

All these facts suggest the difficulty of identification with accuracy of a type of MD based only on histochemical findings and immunofluorescence analysis and indicate a further investigation of proteins using immunoblot analysis.

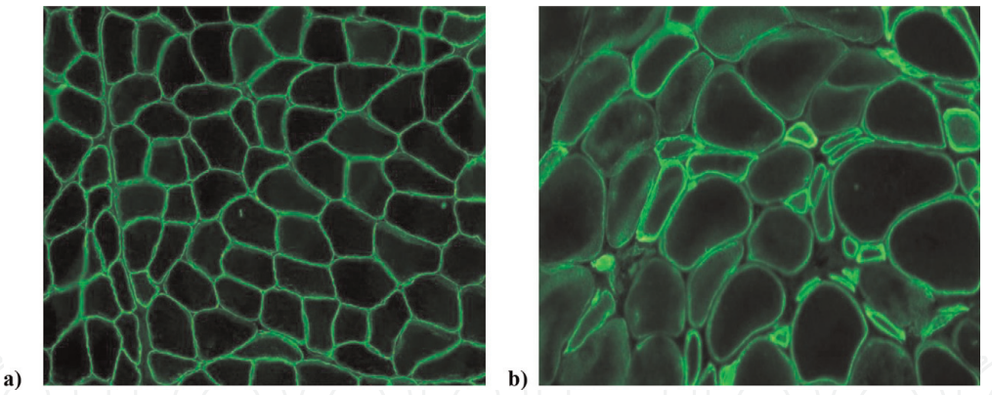


Figure 3.
Immunofluorescent staining of dysferlin in normal control muscle and patient with LGMD 2B: (a) expression of dysferlin from normal patients; (b) reduced expression of dysferlin around some muscle fiber; the presence of regenerating fiber.

2.3.2.2 Multiplex Western blotting

Western blot (WB), also called immunoblot, is a semiquantitative method used to detect specific proteins from cell lysate samples or tissue homogenates using specific antibodies [58].

Due to higher sensitivity and specificity of the method, WB brings more information about proteins that cannot be offered by other immunoassay techniques. The WB technique is an extremely powerful technique which gives information about the presence, absence, and size of the proteins, identifying the proteins with abnormal molecular weight [59, 60].

Usually, this analytical method involved three major processes [61]: (i) separation of proteins into a SDS-polyacrylamide gel based on their molecular weight; (ii) protein blotting on a nitrocellulose or polyvinylidene fluoride (PVDF) membrane; and (iii) visualization of the protein of interest by incubation of membrane with specific primary antibody and then labeled by secondary antibody conjugate with an enzyme.

For the preparation of tissue lysates, around 20–80 mg of muscle tissue are required which may be a significant part of a severely affected muscle. Cooper et al. [62] reported in 2003 obtaining of the lysate from 16 mg muscle tissue using a single cryosection (8 μm thick, 10 mm^2).

Given the complexity of muscular dystrophies, in recent years, has become a necessity in the analysis and comparison of the expression of multiple target proteins involved in a specific pathology.

Also, the technology has improved over time going from the detection of a single protein to identification of multiple proteins in complex samples using a biphasic polyacrylamide gel systems and a cocktail of primary antibodies [63]. In our laboratory, polyacrylamide gel system is performed as previously described [63] with some modifications [64, 65] which permitted separation of the large proteins more than 200 kDa (e.g., dystrophin) in the top part of the gel while and smaller proteins under 150 kDa (e.g., calpain 3), in the bottom. The intensity and thickness of the specific protein bands correspond to the relative abundance of protein of interest. The amount of target protein is determined by comparing stained band of control with the patients. Quantification of protein based on densitometry of bands using ImageJ software provides information about the relative level of protein in muscle.

In DMD patients, the total absence of the bands for dystrophin at 427 kDa is observed, while BMD patients show a reduced intensity of the bands for dystrophin (see **Figure 4**).

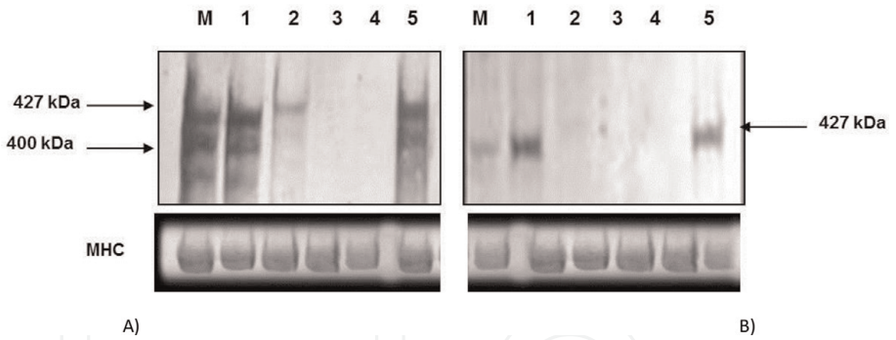


Figure 4.
Representative Western blotting analysis of muscle homogenates from four patients with muscular dystrophy: (A) nitrocellulose membrane labeled with antibody against rod domain of dystrophin (NCL-Dys1). (B) Blot labeled with antibody against C-terminus domain of dystrophin (NCL-Dys2); M-normal control; lane 1-MD with normal expression of dystrophin; lane 2-BMD; lane 3-DMD, absence of dystrophin band; lane 4-DMD; lane 5 MD with normal expression of dystrophin. MHC—Corresponding myosin heavy chain bands on the post-blotted gel, stained with Coomassie blue.

WB plays an important role in distinguishing between DMD and BMD patients especially for patients with discordant phenotype (do no respect reading frame rule).

Analysis by WB of calpain 3, protein found to be involved in LGMD 2A, could show a total or a reduced intensity of bands at 94 kDa. There are cases with LGMD 2A which displayed normal or almost normal bands for calpain 3 compared with control (see **Figure 5**).

This fact suggests a poor specificity of WB analysis for this protein [66]. Also, false-negative results provided by WB can be found in analysis of dysferlin when this protein is accumulated in the cytoplasm, and deficiency of lamin A/C could not be identified in all patients with LMNA mutations [67].

However, for these proteins, genetic analyses are required to confirm the exact diagnosis.

Western blot has the advantage of simultaneous analysis of several proteins which reduced cost and time for analysis. This method is useful in differential diagnosis of muscular dystrophies providing information on the relative location of mutation.

2.4 DNA diagnosis methods

The identification and characterization of genetic defect involved in pathology is often essential both for diagnosis and treatment options as well as in predicting

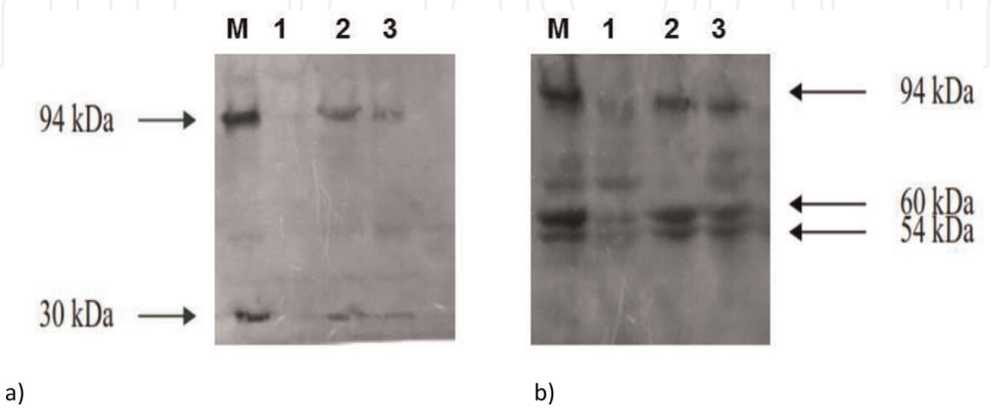


Figure 5.
Calpain 3 band patterns on WB. Representative Western blotting analysis of muscle homogenates from three patients analyzed with antibody against calpain 3. (a) Nitrocellulose membrane labeled with antibody against calpain 3 NCL-CALP-2C4 detects bands at 90 and 30 kDa in normal patients. (b) Nitrocellulose membrane labeled with antibody against calpain 3 NCL-CALP-12A2 detects bands at 90 kDa and two bands at 60 and 30 kDa in normal patients.

disease prognosis. Additionally, diagnostic accuracy leads to more specific genetic counseling for families and possible preimplantation prenatal diagnosis.

The great technological advances in molecular assays over the last 20 years have led to the identification of the molecular genetic cause for many forms of muscular dystrophy. This advance enabled, thus, the diagnosis of muscular dystrophy to evolve from the analysis of 20 exons out of 79 exons within hotspot region of *DMD* gene by PCR Multiplex [68–70] to multiple gene analysis using next-generation sequencing (NGS) technique.

Different forms of muscular dystrophy are caused by a variety of mutations that occurred in many human genes. Mutations that occurred in gene that encode for proteins from DAPC are responsible for many types of muscular dystrophy. The most common types of mutations involve large rearrangement (deletion and duplication) and point mutations. A correct characterization gene mutation for each type of muscular dystrophy represents the key for genotype-phenotype correlation.

Here, we describe some of the more used molecular techniques in the muscular dystrophy diagnosis.

2.4.1 Multiplex ligation-dependent probe amplification (MLPA)

Among the different methods used for detection of gene deletions and duplication, the MLPA assay is the most used due to rapid analysis up to 45 different DNA fragments in a single PCR amplification with only a single primer pair [71] and low amounts of genomic DNA. In addition to large deletions and duplications, MLPA may also identify single exon deletion. This apparent result should be always checked by an alternative method avoiding false-positive results that can occur in the presence of a single-nucleotide variation in a gene. This method proves its usefulness for female's carrier screening as well as for prenatal testing [72].

Because require equipments that exist in most molecular diagnosis laboratory (a thermocycler and a capillary electrophoresis), is a very cost-effective method widely used for both diagnostic and research.

Data analysis can be done by using the free MLPA data analysis software Coffalyser (MRC-Holland, Amsterdam, the Netherlands) or additional software such as MLPAinter [73] or GeneMapper v4.0 software [74].

The advantages of MLPA method for diagnosis are (i) low input of DNA required; (ii) the high specificity and sensitivity, the method being able to distinguish sequences differing in only one nucleotide; and (iii) plenty of MLPA kits available for different genes involved in muscle pathology such as CAPN3, DYSF, SGCA, SGCB, SGCD, SGCG, and FKRP.

2.4.2 High-resolution melting curve analysis (hrMCA)

High-resolution melting curve analysis (hrMCA) is a highly sensitive molecular post PCR method introduced in 1997 by Wittwer et al. [75, 76], to identify pathogenic variants in nucleic acid sequences. The improvement of real-time equipment regarding highly controlled temperature transitions, data acquisition software to monitor and analyze the melting, as well as the development of a new functional class of dyes have made this technology possible.

HRM analysis starts with PCR amplification of the region of interest with specific primers in the presence of a specialized double-stranded DNA (dsDNA) fluorescent binding dye.

The method is based on the DNA property of dissociating (or melting) from double-stranded DNA into single-stranded DNA (ssDNA) when exposed to a gradual temperature [77, 78]. The melting process can be real time monitored by

measuring the gradually diminishing amount of fluorescence during DNA strand dissociation. The presence of a mutation in PCR products determines a modification in the shape of DNA melting curves comparative with melting profile of the wild type (normal) DNA (see **Figure 6**).

HR-MCA is a rapid and accurate method for detection of genetic variation in population. Although HR-MCA is not locus specific, it can become a tool of choice for point mutations screening after identification of large mutation (deletion and duplications) by MLPA. The sequencing of the fragments with abnormal melting profile only, identified by HR-MCA, *reduces costs* and waiting *time* per archived results.

2.4.3 Sanger sequencing

All point mutations identified by HR-MCA method need to be confirmed by sequencing. Sequencing, the most widely used approach for DNA analysis, remains the "gold standard" for mutation analysis.

The Sanger DNA sequencing method is applied to *determine the* sequence of a DNA molecule and to identify the subtle mutations in samples compared with a reference sequence [79, 80].

Because a lot of muscle proteins associated with different forms of muscular dystrophy are extremely large, full gene analysis using Sanger sequencing can lead to higher costs and is time-consuming for analysis. The method finds its usefulness in the analysis of small gene composed of only few exons in which the frequency of point mutation is higher. For instance gene that encode for sarcoglycans (e.g., *SGCA* 10 exons, *SGCB* 6 exons, *SGCG* 10 exons), for calpain 3 *(CAPN3* 26 exons),

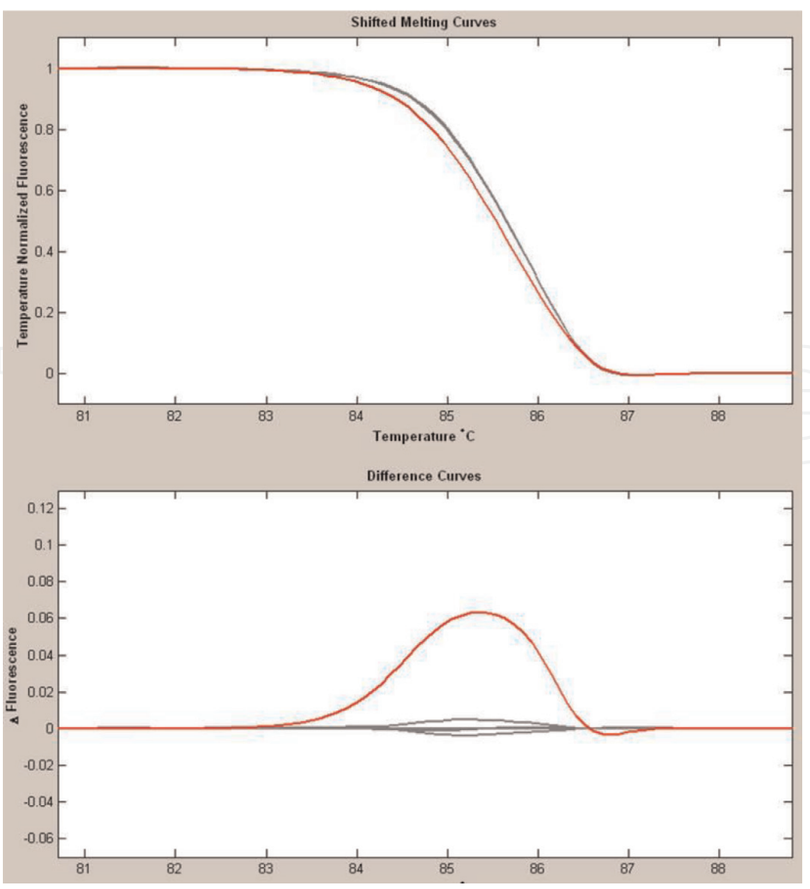


Figure 6.
Representative high-resolution melting curve analysis which reveals difference in melting shape between wild type and variant sequence.

and for dysferlin (*DYSF* 58 exons) [81] that are associated with LGMDs, is more suitable for sequencing.

Due to overlapping clinical symptoms and many possible genetic causes for LGMDs, obtaining a *diagnosis is* often *difficult*. Next-generation sequencing becomes a valuable option for an accurate diagnosis due to ability to analyze a large number of targets.

In *DMD* gene (79 exons), a high frequency of point mutations around 35% was reported [82]. Screening of this huge gene for point mutation is difficult to perform; therefore it is necessary to select only genomic regions that contain the variants [83].

However, the combined technology MLPA for identification of large deletions and duplication followed by HR-MCA and sequencing is a robust algorithm for diagnosis for male muscular dystrophy patients as well as for female carrier and also for prenatal diagnosis.

2.5 Diagnosis algorithm in the most common forms of MD

2.5.1 Dystrophinopathies

The presence of clinical symptoms in a male child presented in Cap 2.1 along with *increased* serum *levels of CK*, *transaminase* enzymes or aldolase should trigger the diagnostic investigation for dystrophinopathies [11]. When DMD or BMD is suspected, diagnostic recommendation as first investigation is the screening of *DMD* gene for deletions and duplications by MLPA. The presence of a mutation in gene confirms the dystrophinopathies diagnosis.

A large number of research studies highlight the utility of MLPA as detection methods for *DMD* gene [84–86] which is the largest gene (2.2 Mb) of the human genome.

Many different types of mutations that occurred in the *DMD* gene, encoding for the cytoskeletal protein, dystrophin, are responsible for both severe disease Duchenne muscular dystrophy (DMD) and the milder form of the disease Becker muscular dystrophy (BMD). The difference in disease severity between the two phenotypes, DMD and BMD, can be explained by the "reading frame rule" proposed by Monaco in 1988 [87]. According to this theory, DMD is caused by mutations which disrupt the reading frame, and no protein will be synthesized, while for BMD phenotype the mutations do not affect the reading frame leading to the synthesis of a smaller and semi-functional dystrophin protein.

Previously reported studies have shown that all over the world, deletions of one or more exons are most common mutation in dystrophinopathies (60–65%), followed by duplications (5–8%) and point mutations (30–35%) [88]. Around 90% from all dystrophinopathies cases present worldwide respect reading frame rule. For the remaining 10% as well as for patients with discordant phenotype, additional analyses are required for an accurate diagnosis.

Because the mutations have been observed across all exons of the gene with a higher incidence of mutation in two "hotspots" regions between exons 2–20 and 45–50 [85, 89], MLPA method proves its utility for the molecular diagnosis of dystrophinopathies, by simultaneous screening of all 79 exons of DMD gene for large intragenic rearrangements [90]. All deletion and duplications identified should be checked *for* the validity of the *reading frame rule on* http://www.dmd.nl.

If no deletions and duplication are identified, the *DMD* gene should be investigated for point mutations [91] by hrMCA followed by sequencing of exons with a modification of melting curve only. Full characterization of the mutation (type, size, and position) is important in identification of patients that are eligible for specific mutation gene therapy [89].

If a muscle biopsy is the tool of choice as first step in analysis routine, histochemical staining and dystrophin analysis by immunohistochemistry/immunofluorescence and Western blot confirm or not the dystrophinopathies diagnosis based on the difference in the expression of dystrophin.

Analysis by IF of dystrophin in muscle samples using three monoclonal mouse antibodies against three domains of protein (C-terminal, rod domain, and N-terminal) revealed the localization of protein at the sarcolemmal level of skeletal muscle fibers and displays a normal expression of intensity signal around each muscle fiber. In DMD patients, dystrophin is absent or severely reduced, while BMD patients displayed a variable expression of signal for dystrophin. Also, labeling of dystrophin on sections plays a critical role in identification process of the DMD female carriers which display a mosaic pattern of dystrophin expression.

Western blot as additional method confirms the diagnostic.

It's important to note that the protein result should be confirmed by genetic analysis.

2.5.2 Limb-girdle muscular dystrophies

Limb-girdle muscular dystrophies (LGMD) are a highly clinically and genetically heterogeneous group of muscle disorders that affect in both males' and females' voluntary muscles of the pelvic and shoulder areas [92].

Major advances in last decades, both in neuromuscular disorders field and diagnostic assays, made that many genes associated with LGMDs to be found such us: CAPN3 (encode for calpain 3), DYSF (encode for dysferlin), FKRP (encode for fukutin), SGCA, SGCB, SGCG, and SGCD (that encode for the α-, β-, γ-, and δ-sarcoglycan), and more than 30 forms of LGMDs to be characterized [11, 37]. However, a significant number of patients clinically diagnosed with LGMDs remain molecularly uncharacterized [93].

Based on their inheritance pattern were classified in dominant (type 1 LGMD) and recessive forms (type 2 LGMD) [94].

Giving the complexity of the clinical symptoms and different genes involved, a diagnosis algorithm for these pathologies is still waiting, but comprehensive guidelines for identifying these disorders have been published. The initial evaluation of a patient involves clinical examination, followed by laboratory test such as serum creatine kinase measurements, genetic tests, and muscle biopsy analysis. CK level can vary from lower level in dominantly inherited LGMD forms to very high level in recessively inherited forms [95].

All these diagnostic approaches should be sufficient to accurately predict the correct form of LGMDs. In the last years, the increasing use of next-generation sequencing technology for simultaneous analysis of known LGMD-related genes improved diagnostic rate and offered opportunity to identified new disease-related genes [96]. However, this technology is extremely expensive and is not yet available in all the molecular diagnostic laboratories.

If the clinical features, laboratory tests, and other investigations such as electromyography, magnetic resonance, or ultrasound imaging suggest a LGMD, muscle biopsy [97] may be considered an appropriate test to start the investigation.

Routine histochemical stains will display variable degree of typically dystrophic feature characteristic for LGMD: variation in fiber size, degeneration and regeneration fiber, *presence of internal nuclei*, and *increased* endomysial *fibrosis* [37, 40].

Disease prediction based on clinical and histochemical *tests alone* is difficult to achieve, so immunohistochemical stains (IHC or IF) of specific muscle proteins involved in LGMDs (calpain 3, dysferlin, caveolin3, the sarcoglycans, myotilin, lamin A/C, etc.) provides useful information about the presence, absence and

changes in protein expression. Muscle biopsy is not always informative taken into account the secondary protein reduction in addition to the primary protein deficiency but can guide to targeted genetic tests. Although genetic testing can be expensive, it will identify the exact defect of the disease. Being composed only few exons and considering that LGMDs could have many possible genetic causes, the sequencing of genes is the most suitable method of choices for diagnosis.

Autosomal dominant LGMDs' form is quiet rare in population. Recessive forms are more common and studied. The most commonly recessive form of LGMDs and the most studied are LGMD 2A (calpainopathy) and LGMD 2B (dysferlinopathy).

2.5.2.1 Limb-girdle muscular dystrophy 2A (LGMD2A)

LGMD 2A is the most common form of limb-girdle muscular dystrophy accounting for about 30% of all LGMDs, caused by mutation in CAPN3 gene which encodes for calcium sensitive dependent protease-calpain 3 protein.

Besides the clinical manifestation and laboratory investigation of serum enzymes presented above, for characterization of this condition, searching for mutations in CAPN3 gene in correlation with protein calpain 3 investigations by Western blot represents the "gold standard" in LGMD2A. The identification of the mutations in CAPN3 gene is most often difficult due to many genetic variations which appear in this gene and the position they have in the gene.

The improvement of next-generation sequencing technology (NGS), which screens genomic DNA for a large number of genes involved in neuromuscular disease, makes LGMD diagnosis easier to achieve. However, in most laboratories, the diagnosis starts with screening for mutation by direct Sanger sequencing analysis of the 24 exons of the CAPN3 gene. With this method more than 95% cases are diagnosed. Even if this analysis is successful, in most cases, there are not always identify large deletions and duplications as well as intronic splice mutations [98]. This fact shows importance of screening for large genomic rearrangements by MLPA, the most cost-effective techniques. Sanger sequencing combined with MLPA leads to an increase in the mutation detection rate and remains one of the most valuable diagnostic tools. Therefore, when DNA analysis is not conclusive, a muscle biopsy is required for protein analyses.

Until this moment, only few studies reported the success of immunohistochemical technique application for the diagnosis of LGMD using calp3d/12A2 and calp2C4 antibodies [40, 99] and demonstrated the localization of calpain 3 in myofibrils and myonuclei.

Nowadays, it is widely accepted that WB, even if it does not have a high accuracy, is the most suitable method for calpain 3 analysis.

In general, for analysis, two antibodies which produce characteristic patterns of bands are used: NCL-CALP-2C4 directed against exon 1 which recognizes the full-sized protein at 94 kDa and additional band at 30 kDa and NCL-CALP-12A2 against exon 8 that recognizes also to the full-sized protein at 94 kDa and additionally bands doublet at 60 and 54 kDa. An example of normal calpain on blot is shown in **Figure 5**.

Identification and interpretation of the pattern of bands obtained on WB by using the two antibodies for calpain 3 provide useful information about protein expression in muscle. Generally, it is widely accepted that the complete absence of all specific calpain 3 bands on blot is specific for LGMD2A diagnosis and is due to a primary defect in CAPN3 [100]. The presence of a normal amount of calpain-3 protein on blot does not exclude the LGMD 2A diagnosis. The normal amount of calpain-3 protein on blot in patients with LGMD 2A was reported [101] by several studies. The possible explanation of normal expression but functionally inactive

protein is due to a functional enzyme defect that impairs the autolytic or proteolytic activity of protein without elimination of protein from the muscle [102, 103].

The reduction is more difficult to interpret because calpain-3 appears to reduce in amount as secondary effect in other forms of muscular dystrophies such as dysferlinopathies [40] and titinopathies [81, 92].

Even if muscle calpain-3 results on blot should always be confirmed by mutation analysis, Western blot remains one of the most valuable diagnostic tools in LGMDs allowing for the simultaneous analysis of multiple proteins, identifying both the primary defect and the secondary reductions.

3. Summary and future directions

For most forms of muscular dystrophy, the diagnosis is still challenging, and a multidisciplinary approach is always required. Only a good knowledge of protein and gene involved in pathology can provide the correct diagnosis and is essential for therapeutic interventions. New genetic therapies under development like exon skipping which tried to restore the reading frame with antisense oligonucleotides and to transform severe DMD phenotype in a less severe phenotype require a good characterization of the mutation.

When clinical symptoms are combined with protein analysis by immunofluorescence and Western blot, and with high-throughput DNA molecular technique such us MLPA, hrMCA, and sequencing, the diagnostic capabilities greatly improve and can provide an accurate diagnosis.

A defined genetic diagnosis is important for an appropriate treatment and genetic counseling as well as inclusion of patients in further clinical trials.

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Conflict of interest

The authors declare that they have no competing interests.

Acronyms and abbreviations

BMD	Becker muscular dystrophy
CK	creatine kinase
CMD	congenital muscular dystrophy
DAPC	dystrophin-associated protein complex
DMD	Duchenne muscular dystrophy
DM	myotonic dystrophy (DM1 Type 1, DM2 Type 2)
EDMD	emery-Dreyfus muscular dystrophy
FCMD	fukuyama congenital muscular dystrophy
FSHD	facioscapulohumeral muscular dystrophy

hrMCA	high-resolution melting curve analysis
IF	immunofluorescence
IHC	immunohistochemistry
LGMD	limb-girdle muscular dystrophy
MD	muscular dystrophy
MEB	muscle-eye-brain disease
MLPA	multiplex ligation-dependent probe amplification
WB	Western blot

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