Advanced microscopic and histochemical techniques: diagnostic tools in the molecular era of myology

G. Meola
Dipartimento di Neurologia, Università degli Studi di Milano, Policlinico San Donato, S. Donato Milanese (MI) - Italy

Over the past two centuries, myology (i.e. the basic and clinical science of muscle and muscle disease) has passed through 3 stages of development: the classical period, the modern stage and the molecular era.

The classical period spans the last part of nineteenth century and the earlier part of the twentieth century. During this time, several major muscle disease were clinically and pathologically characterized, including *Duchenne muscular dystrophy* (DMD), *myotonic dystrophy* (DM) and *facioscapulohumeral dystrophy* (FSHD).

The modern stage in the second half of the twentieth century is characterized by the adaptation of histo and cytochemical techniques to the study of muscle biopsies.

These tools improved the diagnostic accuracy and made possible the identification of new changes and structures (Engel and Cunningham, 1963; Scarlato, 1975).

Examples of this are the demonstration of nemaline rods in nemaline myopathy (Shy *et al.,* 1963) and ragged red/blue fibers in mitochondrial diseases (Olson *et al.,* 1972). The advent of modern cytochemical techniques permitted the identification of various enzyme defects/storage diseases such as Pompe's disease (Meola *et al.,* 1984) or to study the intracellular lipids and membrane network in human muscle cultures (Santilli *et al.,* 1989). The histochemical techniques were also applied in de novo innervated human muscle cultures (Meola *et al.,* 1994), in myogenic clones from adult human muscle cell cultures (Meola *et al.,* 1991) to the show restoration and persistence of a cytoplasmic enzyme i.e. G6PDH in stable hybrid myotubes (Sansone *et al.,* 1993).

The molecular era was made possible by the development of molecular biology and its application to muscle diseases. This permitted the identification of gene defects in many inherited neuromuscular diseases, leading to accurate and specific diagnosis.

The best example of this, is DMD and the discovery in last 1980s of the gene at locus Xp21 whose mutation causes the deficiency of an absolutely essential protein, dystrophin (Figure 1) in muscle fibers (Hoffman *et al.,* 1987). Parallel with the spectacular development of genomic in relation to muscle disease, immunohistochemistry produced remarkable discoveries. A number of sarcolemmal proteins were identified whose deficiency causes different forms of limb girdle dystrophy, including dysferlin (Bashir *et al.,* 1998); sarcoglycan (Duggan *et al.,* 1997); calpain (Richard *et al.,* 1995); and caveolin (Minetti *et al.,* 1998; Royuela *et al.,* 2003).

Diagnostic advances also occurred in immunopathology using *in situ* hybridization, immunohistochemistry, immunofluorescence and westen blotting in the molecular diagnosis of non-genetic dysimmune muscle diseases (i.e. idiopathic inflammatory myopathies) (De Paepe *et al.,* 2004).

In the pre-molecular era, the classification of muscle diseases was based on characteristic clinical and/or microscopic pathological feature. For example, a disease with an early onset, x-linked recessive progressive proximal muscle weakness, large calves, and *dystrophic* microscopic pathology, was justified for the category of *muscular dystrophy*.

Furthermore, episodes of the profound hypotonic limbs muscle weakness along with reduced serum potassium level, would place such as a disease in the category of *periodic paralyses*.

In the molecular era, the classification of above mentioned diseases would change in *dystrophinopathy* in former case and *hereditary skeletal muscle channelopathies* in latter case (Meola *et al.,* 2003).

In the molecular era, the basis of classification has changed and is still evolving and includes: mutational characteristics, affected proteins, microscopic features, the nature of the abnormal cellular process(es), principal organelle involvement and distinctive clinical features. Three categories serve as basis for molecular classification:

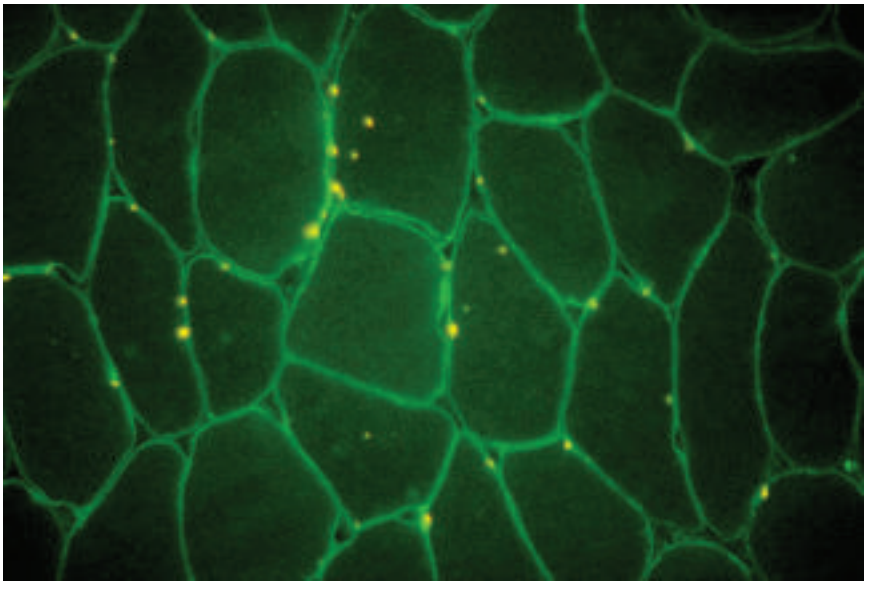

Figure 1. Immunofluorescence technique showing normal dystrophin ring in muscle fibres from normal control muscle labelled with an antibody (DY8/6C5) to the C terminus of dystrophin. Original magnification 20x.

Mutational profile plus organelle involvement

a. primary sarcolemmal diseases involving the plasma membrane or basal lamina: dystrophinopathies, sarcoglycanopathies, merosin-deficient disease, dysferlinopathies and caveolin-related diseases (Johnson, 2001);

b. diseases with primary myonuclear abnormalities: emerinopathies, lamin A/C-related diseases and myotubular related centronuclear myopathies (Maraldi et al., 2003);

c. *diseases with a primary involvement of myofibrils or cellular cytoskeleton:* actinopathies (Goebel *et al.,* 1997), core diseases (McCarthy et al., 2000), nemaline myopathies (Wallgren-Pettersson i 1999), plectin (Smith et al., 1996) and telethonin related myopathies (Moreira et al., 2000) myosin heavy chain type 2 syndrome (Martinsson et al., 2000) and desminopathy (Karpati and Sinnreich, 2004);

d. diseases with ion channel or ion transporter defects: chloride/calcium/potassium sodium channelopathies (myotonic or other periodic paralyses) (Davies and Hanna, 2003), sarcoplasmic reticulum (SR) calcium release channel (ryanodine receptor) and SR ATPase-related myopathy (Brody's disease) (Odermatt *et al.,* 1996).

Nature of the relevant cellular processes

a. muscle metabolism: catabolic metabolism, including lysosomal disorders (lamp-2 deficiency (Nishino *et al.,* 2000), α-glucosidase deficiency (Martiniuk, 2002) and x-linked myopathy with excessive autophagy (Kalimo *et al.,* 1988)) and nonlysosomal disorders (lamp-2 calpainopathy and proteosomal disorders); carnitine and fatty acid metabolism (Di Donato and Taroni, 2002); glycolytic pathways (Moxley *et al.,* 2001) and mitochondrial oxidative phosphorylation defects (Taylor *et al.,* 2004);

b. neuromuscular transmission: congenital myasthenic syndromes and autoimmune myasthenia gravis (McConville and Vincent, 2002);

c. glycosylation: inclusion body myopathy with GNE deficiency (Muntoni et al., 2002), muscle-eye-brain syndrome, and Fukuyama's congenital muscular dystrophy (Michele *et al.,* 2002).

Special complex molecular mechanisms

a. trinucleotide (CTG) repeat expansion: in DMPK (myotonin) gene in myotonic dystrophy type 1 (DM1) (Brook *et al.,* 1992);

b. trinucleotide (GCG) repeat expansion: in PABPN1 gene (oculopharyngeal muscular dystrophy) (Meola *et al.,* 1997);

c. tetranucleotide (CCTG) repeat expansion: in the gene encoding zinc-finger protein (ZNF9) in myotonic dystrophy type 2 (DM2) (Liquori *et al.,* 2001; Cardani et al., In press) (Figure 2);

d. large telomeric deletion: on chromosome 4 in the D4Z4 repeat zone (facioscapulohumeral muscular dystrophy) (Wijmenga *et al.,* 1992).

In the molecular era the diagnostic process of genetic or other myopathies must still start with obtaining a detailed history (including ascertainment of symptoms, pedigree, etc.) and performing a careful physical examination that is streamlined

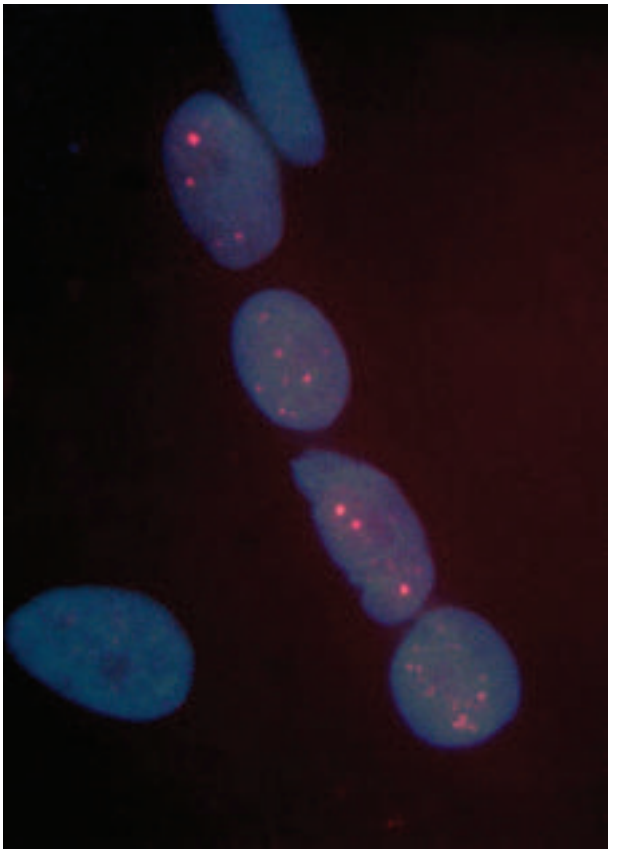

Figure 2. Fish (fluorescence in situ hybridization) of nuclei from myotubes in cultures obtained from muscle biopsy of patient with DM2 showing ribonuclear inclusions of mutant mRNA (red) in nuclei (blue). 21 days in vitro. Original magnification 40x.

for characteristic signs of muscle diseases. The next steps are electrodiagnostic studies and microscopic study of muscle biopsies, using advanced histochemistry, immunohistochemistry and immunoblotting analysis. However it should be emphasized that molecular testing is necessary at present. This includes mutational analysis and immunohistochemistry and immunoblotting on muscle biopsies.

For mutational analysis, one must focus on a highly suspected culprit gene. In many cases of genetic myopathies where a certain type of mutation is predominant, conventional technique with *polymerase chain reaction* (PCR) is the method of choice. In other instances, sequence analysis is necessary, which can be time consuming and expensive.

Some investigators give first preference to non invasive molecular analysis versus microscopic study of an invasive muscle biopsy (Muntoni, 2001), for example in case in which clinical and genetic history is highly suggestive for DMD, but confirmation is necessary for differential diagnosis from Becker or other forms of muscular dystrophy.

Others investigators advocate the demonstration of dystrophin deficiency by histochemistry/immunoblot on muscle biopsy. Another approach is to first perform mutational analysis by multiplex PCR of the dystrophin gene's coding sequence and to perform muscle biopsy if the former approach is not diagnostic (Flanigan *et al.,* 2003).

Another example for the absolute need for mutational analysis is carrier detection or prenatal diagnosis in DMD.

Despite the molecular discoveries pertaining the diagnosis of many myopathies, much remains to be explored.

In many genetic and non genetic muscle diseases, the culprit gene and its product remains unknown.

In some important diseases, where the genetic defect has been clarified, we still lack any understanding of the pathogenesis of muscle fibre damage. An example of this is FSHD.

Some basic pathogenetic mechanisms that are possibly operating in myopathies or have potential therapeutic usefulness need to be explored (Chaubourt *et al.,* 1999).

These include the role of signalling systems, apoptosis, oxygen radical-induced damage (Rando, 2001), muscle cell development and differentiation as well as related molecules, post translational processing of proteins, the interaction of nuclearly coded and mitochondrially-coded molecules, and perfecting gene therapeutic methods.

Giovanni Meola, Dipartimento di Neurologia
Università di Milano, Policlinico S. Donato
Via Morandi, 30 S. Donato Milanese (MI) – Italy
Tel: 02-52774480
Fax: 02-5274717
e-mail: giovanni.meola@unimi.it

References

Bashir R, Britton S, Strachan T, Keers S, Vafiadaki E, Lako M et al. A gene related to Caenorhabditid elegans spermatogenesis factor ser-1 is mutated in limb girdle dystrophy type 2B. Nat Genet 1998;20:37-42.

Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H et al. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 1992;68:799-808.

Cardani R, Mancinelli E, Sansone V, Rotondo G, Meola G. Biomolecular identification of (CCTG)n mutation in myotonic dytrophy type 2 (DM2) by FISH on muscle biopsy. Eur J Histochem. In press.

Chaubourt E, Fossier P, Baux G, Leprince C, Israel M, De La Porte S. Nitric oxide and l-arginine cause an accumulation of utrophin at the sarcolemma: a possible compensation for dystrophin loss in Duchenne muscular dystrophy. Neurobiol Dis 1999;6:499-507.

Davies NP, Hanna MG. The skeletal muscle channelopathies: distinct entities and overlapping syndromes. Curr Opin Neurol 2003;16:559-68.

De Paepe B, Schröder JM, Martin JJ, Racz GZ, De Bleecker JL. Localization of the ·-chemokine SDF-1 and its receptor CXCR4 in idiopathic inflammatory myopathies. Neuromuscl Disord 2004;14:265-73.

Di Donato S, Taroni F. Defects of fatty acid metabolism. In: Karpati G ed. Structural and molecular basis of skeletal muscle diseases. Basel: ISN Neurophat Press, 2002, pp. 189-201.

Duggan DJ, Gorospe JR, Fanin M, Hoffman EP, Angelini C. Mutations in the sarcoglycan genes in patients with myopathy. N Engl J Med 1997;336:618-624.

Engel WK, Cunningham GG. Rapid examination of muscle tissue: an improved trichrome stain method for fresh frozen biopsy sections. Neurology 1963;13:919-23.

Flanigan KM, von Niederhausern A, Dunn DM, Alder J, Mendell JR, Weiss RB. Rapid direct sequence analysis of the dystrophin gene. Am J Hum Genet 2003;72:931-9.

Goebel HH, Anderson JR, Hubner C, Oexle K, Warlo I. Congenital myopathy with excess of thin myofilaments. Neuromuscl Disord 1997;7:160-8.

Hoffman EP, Brown RH Jr, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-28.

Johnson MA. Immunocytochemical analysis. In: Bushby K, Anderson LVB, eds. Muscular Dystrophy: methods and protocols. Humana Press, Totowa, New Jersey, 2001, pp 339-67.

Kalimo H, Savontaus ML, Lang H, Paljarvi L, Sonninen V, Dean PB et al. X-linked myopathy with excessive autophagy: a new hereditary muscle disease. Ann Neurol 1988;23:258-65.

Karpati G, Sinnreich M. A clever road from myopathology to genes. The myotilin story. Neurology 2004;62:1248-9.

Liquori CL, Ricker K, Moseley ML, Jacobsen JF, Kress W, Naylor SL et al. Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science 2001;293:864-7.

Maraldi NM, Lattanzi G, Sabatelli P, Ognibene A, Columbaro M, Capanni C et al. Immunocytochemistry of nuclear domains and Emery-Dreifuss muscular dystrophy pathophysiology. Eur J Histochem 2003;47:3-16.

Martiniuk FT. Alpha glucosidase deficiency syndromes. In: Karpati G ed. Structural and molecular basis of skeletal muscle diseases. Basel: ISN Neurophat Press, 2002, pp.134-41.

Martinsson T, Oldfors A, Darin N, Berg K, Tajsharghi H, Kyllerman M et al. Autosomal dominant myopathy: missense mutation (Glu-706 -> Lys) in the myosin heavy chain IIa gene. Proc Natl Acad Sci 2000;97:14614-14619.

McCarthy TV, Quane KA, Lynch PJ. Ryanodine receptor mutations in malignant hyperthermia and central core disease. Hum Mutat 2000;15:410-7.

McConville J, Vincent A. Diseases of the neuromuscular junction. Curr Opin Neurol 2002;2:296-301.

Meola G, Scarpini E, Manfredi L, Velicogna M, Pellegrini G, Redi CA et al. Basic Appl Histochem 1984;28:245-55.

Meola G, Velicogna M, Brigato C, Pizzul S, Rotondo G, Scarlato G. Growth and differentiation of myogenic clones from adult human muscle cell cultures. Eur J Bas Appl Histochem 1991;35:219-31.

Meola G, Sansone V, Radice S, Rotondo G, Tremblay JP. Enzymatic activity and morphological differentiation in the de novo innervated human muscle cultures. Eur J Histochem 1994;38:125-36.

Meola G, Sansone V, Rotondo G, Tomè FMS, Bouchard JP. Oculopharyngeal muscular dystrophy in Italy. Neuromuscl Disord 1997;7(Suppl 1):S53-S56.

Meola G, Sansone V, Rotondo G, Mancinelli E. Muscle biopsy and cell cultures: potential diagnostic tools in hereditary skeletal muscle channelopathies. Eur J Histochem 2003:47:17-28.

Michele DE, Barresi R, Kanagawa M, Saito F, Cohn RD, Satz JS et al. Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. Nature 2002;418:417-22.

Minetti C, Sotgia F, Bruno C, Scartezzini P, Broda P, Bado M et al. Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy. Nat Genet 1998;18:365-8.

Moreira ES, Wiltshire TJ, Faulkner G, Nilforoushan A, Vainzof M, Suzuki OT et al. Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. Nat Genet 2000;24:163-6.

Moxley RT, Chinnery P, Turnbull D. The metabolic myopathies. In Karpati G, Hilton –Jones D, Griggs RC, eds. Disorders of voluntary muscle. 7th ed. Cambridge, UK: Cambridge University Press, 2001, pp. 580-603.

Muntoni F. Is a muscle biopsy in Duchenne dystrophy really necessary? Neurology 2001;57:574-5.

Muntoni F, Brockington M, Blake DJ, Torelli S, Brown SC. Defective glycosylation in muscular dystrophy. Lancet 2002;360:1419-21.

Nishino I, Fu J, Tanji K, Yamada T, Shimojo S, Koori T et al. Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). Nature. 2000; 406:906-10

Odermatt A, Taschner PE, Khanna VK, Busch HF, Karpati G, Jablecki CK et al. Mutations in the gene-encoding SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca2+ ATPase, are associated with Brody disease. Nat Genet 1996;14:191-4.

Olson W, Engel WK, Walsh GO, Einaugler R. Oculocraniosomatic neuromuscular disease with "ragged-red" fibers. Arch Neurol 1972;26:193-211.

Rando TA. The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle Nerve 2001;24:1575-94.

Richard I, Broux O, Allamand V, Fougerousse F, Chiannilkulchai N, Bourg N et al. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 1995;81:27-40.

Royuela M, Chazalette D, Rivier F, Hugon G, Paniagua R, Guerlavais V et al. Dystrophin and dystrophin-associated protein in muscles and nerves from monkey. Eur J Histochem 2003;47:29-38.

Sansone V, Rotondo G, Bottiroli G, Tremblay JP, Meola G. Cytoplasmic restoration and persistence of glucose-6-phosphate dehydrogenase activity in stable hybrid myotubes. Eur J Histochem 1993;37:241-8.

Santilli I, Prelle A, Geremia L, Scarlato G, Meola G. Nile red simultaneous staining of intracellular lipids and membrane network in human muscle cultures. Basic Appl Histochem 1989;33:49-52.

Scarlato G. Il contributo dell'istochimica alla nosografia delle malattie neuromuscolari. Rivista di istochimica 1975;XIX:76-94.

Shy GM, Engel WK, Somers JE, Wanko T. Nemaline myopathy. A new congenital myopathy. Brain 1963;86:793.

Smith FJ, Eady RA, Leigh IM, McMillan JR, Rugg EL, Kelsell DP et al. Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. Nat Genet 1996;13:450-7.

Taylor RW, Schaefer AM, Barron MJ, McFarland R, Turnbull DM. The diagnosis of mitochondrial muscle disease. Neuromuscl Disord 2004;14:237-45.

Wallgren-Pettersson C, Pelin K, Hilpela P, Donner K, Porfirio B, Graziano C et al. Clinical and genetic heterogeneity in autosomal recessive nemaline myopathy. Neuromuscl Disord 1999;9:564-72.

Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG et al. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet 1992;2:26-30.