# Histochemical and immunohistological approach to comparative neuromuscular diseases

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**Abstract:** The broad category of neuromuscular diseases covers conditions that involve the weakness or wasting of the body muscles. These problems may occur in the spinal cord, the peripheral nerves or the muscle fibers. Some may be hereditary, while others are acquired. Commonly recognized conditions fall into the categories of myopathies, which are diseases of the muscle like muscular dystrophy, disorders of the junction where the nerve impulses are transmitted to the muscle like myasthenia gravis, and neuropathies, which are diseases of the peripheral nervous system. The diagnosis of most neuromuscular diseases rest on careful clinical evaluation of the patient, electromyography, the muscle biopsy, and in some instances, molecular genetic studies. Muscle biopsy, associated to histochemical and immunohistological techniques, plays a key role in diagnosis of many neuromuscular disorders. A number of morphological abnormalities of muscle can be recognized on histological stains such as haematoxylin and eosin and Engel trichrome. Histochemical techniques are essential for the study of muscle biopsies for four main reasons. First, they demonstrate the non-uniform nature of the muscle highlighting the different biochemical properties of specific fibre type and their selective involvement in certain disease processes. Second, they may show an absences of a particular enzyme. Third, an excess of a particular substrate can be demonstrated. Fourth, they may show structural changes in the muscle which would not be apparent with routine histological stains, such as the enzyme-deficient cores in central core disease "mouth-eaten" fibers, and abnormalities in the distribution of mitochondria. In some neuromuscular disorders there could be only non-specific myopathological features. However, a number of proteins, including sarcolemmal, sarcomeric, and nuclear proteins as well as enzymes with defects responsible for neuromuscular disorders, have been identified during the past two decades, allowing a more specific and firm diagnosis of muscle diseases. Identification of protein defects relies predominantly on immunohistochemical preparations and on Western blot analysis. While immunohistochemistry is very useful in identifying abnormal expression of primary protein abnormalities in recessive conditions, it is less helpful in detecting primary defects in dominantly inherited disorders. Abnormal immunohistochemical expression patterns can be confirmed by Western blot analysis which may also be informative in dominant disorders. Besides identification of specific protein defects, immunohistochemistry is also helpful in the differentiation of inflammatory myopathies by subtyping cellular infiltrates and demonstrating up-regulation of subtle immunological parameters. This review will summarize and describe the impact that histochemistry and immunohistochemistry has had and the possibilities it has opened up in the diagnosis of neuromuscular disorders in human as well as in veterinary myology.

**Key words:** muscle pathology, veterinary myology, histochemistry, immunohistochemistry

## Introduction

The broad category of neuromuscular diseases covers conditions that involve the weakness or wasting of the body muscles in human as well as in animal.

These problems may arise in the spinal cord, the peripheral nerves or the muscle fibers. Some may be hereditary, while others are acquired. Commonly recognized conditions fall into the categories of myopathies, which are diseases of the muscle like muscular dystrophy, disorders of the junction where the nerve impulses are transmitted to the muscle like myasthenia gravis, and neuropathies, which are diseases of the peripheral nervous system like diabetic neuropathy.

The diagnosis of most neuromuscular diseases rest on careful clinical evaluation of the patient, electromyography, the muscle biopsy, and in some instances, molecular genetic studies.

Muscle biopsy, associated to histochemical and immunohistological techniques, plays a key role in diagnosis of many neuromuscular disorders.

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**Table 1.** Stains used in the diagnosis of selected neuromuscular disorders.

| STAIN | MAJOR USE |
|---|---|
| Haematoxylin & eosin | General structure: fiber size and contours, position of nuclei, fibrosis, inflammation, nerves, blood vessels |
| Engel Trichrome | Mitochondria red, nemaline rod red, membranous whorls of rimmed vacuoles red |
| Periodic Acid-Schiff | Checkerboard pattern of fibre types; fibers with excess glycogen heavily stained; fibers with loss of glycogen white |
| Reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) | Fiber type pattern; distribution of mitochondria; myofibrillar disruption |
| Succinic dehydrogenase (SDH) | Fiber type pattern; fibers with abnormal mitochondria |
| Cytochrome oxidase (COX) | Fiber type pattern; fibers devoid of activity |
| ATPase or myosin isoforms (different PH) | Distribution and involvement of fiber types and their subtypes |
| Phosphorylase | Absent in type V glycogenosis (McArdle's diseases) |
| Phosphofructokinase | Absent in type VII glycogenosis |
| Adenylate deaminase | Absent/deficient in exertional myalgia |
| Acid phosphatase | High in lysosomal storage disorders and vacuolar myopathies |
| Alkaline phosphatase | High in blood vessels in some inflammatory myopathies |
| Menadione-linked a-glycerophosphate dehydrogenase | Stains reducing bodies |
| Congo red | Shows presence of b-amyloid |

A number of morphological abnormalities of muscle can be recognized on histological stains such as haematoxylin and eosin and Engel trichrome. This last stain achieved  by W. King Engel (1963) is very important in the identification of red staining structures such as rods, membranous myelin-like whorls of rimmed vacuoles and  abnormal proliferation of mitochondria (the so called "Ragged Red Fibers").

Histochemical techniques are essential for the study of muscle biopsies for four main reasons. First, they demonstrate the non-uniform nature of the muscle highlighting the different biochemical properties of specific fibre type and their selective involvement in certain disease processes. Second, they may show an absences of a particular enzyme. Third, an excess of a particular substrate can be demonstrated. Fourth, they may show structural changes in the muscle which would not be apparent with routine histological stains, such as the enzyme-deficient cores in central core disease "mouth-eaten" fibers, and abnormalities in the distribution of mitochondria. In some neuromuscular disorders there could be only non-specific myopathological features. However, a number of proteins, including sarcolemmal, sarcomeric, and nuclear proteins as well as enzymes with defects responsible for neuromuscular disorders, have been identified during the past two decades, allowing a more specific and firm diagnosis of muscle diseases. Identification of protein defects relies predominantly on immunohistochemical preparations and on Western blot analysis.

While immunohistochemistry is very useful in identifying abnormal expression of primary protein abnormalities in recessive conditions, it is less helpful in detecting primary defects in dominantly inherited disorders. Abnormal immunohistochemical expression patterns can be confirmed by Western blot analysis which may also be informative in dominant disorders, although its role has yet to be established. Besides identification of specific protein defects, immunohistochemistry is also helpful in the differentiation of inflammatory myopathies by subtyping cellular infiltrates and demonstrating up-regulation of subtle immunological parameters. This report will summarize and describe the impact that histochemistry and immunohistochemistry has had and the possibilities it has opened up in the diagnosis of neuromuscular disorders in human as well as in veterinary myology.

## Histological and Histochemical Analysis of Muscle Fibers

A number of morphological abnormalities of muscle can be recognized on histological stains such as haematoxylin and eosin and Engel trichrome.

In this last stain achieved  by W. King Engel (1963) the muscle fibers stain a greenish-blue colour, and the collagen is lighter. The nuclei stain red and the myelin of the nerve stain a foamy red colour. Nerves my appear poorly stained in the absence of myelin. Abnormal accumulations of myofibrillar material may

appear to be a paler green -blue colour and cytoplamic bodies may appear more intensely stained. Engel trichrome is very important in the identification of red staining structures such as rods, membranous myelin-like whorls of rimmed vacuoles and abnormal proliferation of mitochondria (the so called "Ragged Red Fibers"). In Table 1 are reported additional stains which may yield valuable insights into the pathogenesis of selected neuromuscular disorders.

Histochemical techniques are essential for the study of muscle biopsies in human as well as in veterinary myology. Substantially there are three approaches to the histochemical analysis of the muscle fibers. Those include the examination of the pattern of fiber type; the measurement and recording of the diameters of each fiber type and the analysis of the various changes within individual fibers. Some of the most used histochemical techniques are reported in table 1. Further reference can be made to the excellent book on muscle biopsy of Dubowitz and Sewry (2007).

The pattern of fiber types can be studied accurately only by subjecting cryostat-sectioned unfixed, frozen muscle to myofibrillar adenosine triphosfatase (ATP) reactions at pHs 4.3, 4.6 and 9.4. The different pH sensitivity allows the differentiation of the fibers into four types. The type 1 that are base-labile and acid stabile, the type II fibers that possess the reverse properties. Type 2B and 2C fibers display activity in solutions containing a wider pH range than 2A and thus can be differentiated. Type 2C fibers are rare in normal human muscle but are present in developing muscle and may appear under pathological circumstances. In cat, type-2C fibers represent, approximately, 2 to 3% of the myofiber population [3].

In dog, masticatory muscles contain a unique adult fiber type composition and different contractile protein isoforms than do adult limb muscles. In addition, canine masticatory muscles are almostselectively involved in an autoimmune disease, named Canine Masticatory Muscle Myositis. Using histochemical methods, the myofibrillar adenosine triphosphatase (ATPase) staining of adult masticatory muscle resulted in two populations of fibers. The majority of fibers are stable after both acid and alkali preincubation, characteristic of type 2C fibers, whereas the remaining fibers are a variant of the type 1 fibers. However, further biochemical and immunocytochemical investigation has shown that the myofibrillar proteins of the masticatory type 2 fibers differ from those of type 2C: fibers in limb muscle. Hence, these fibers have been designated type 2M [4]. In animals the predominance of a fiber type is characteristic of a particular muscle. In the soleus, for example, type 1 fibers predominate; in the medial head of the gastrocnemius muscle, type 2 fibers predominate. In the anterior tibial muscle, two-thirds of the fibers are type 2. By contrast, in the human muscle the three fibers types occur in approximately identical proportions in a pattern that not often varies. Therefore in any study that assesses the distribution of the fiber types, it is necessary to compare identical muscles and identical areas (superficial versus deep) in a given muscle.

In certain congenital myopathies, the differentiation into fiber types may be delayed or never appear during development. This condition is called fiber-type predominance and it is only rarely described in foal (Paciello et al. 2008, unpublished data).

Fibers of some histochemical type may become involved when a disease selectively or predominantly affects the muscle of that type. Type 1 muscle fibers involvement is often observed in certain congenital myopathy instead type 2 muscle fibers atrophy can be caused by: disuse; aging; weight loss; systemic disease, such as endocrinopathy; paraneoplastic syndrome and childhood myasthenia (Fig. 1).

The checkerboard pattern of fiber type is altered also when denervated muscle is reinnervated. This morphological aspect is due to collateral sprouting of adjacent intact motor axons reinnervation of muscle after denervation. Such a histochemical change is termed fiber-type grouping.

There are various oxidative enzyme reactions in use to study muscle and some of them show similarity in the appearance of the sections. With reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) type 1 fibers are darker blue colour than type 2 fibers and some type 2 are of intermediate intensity. This stain is useful to study the intermiofibrillar network comprising mitochondria and sarcoplasmic reticulum. Abnormal internal fiber architecture is characteristic of target fibers, often seen with acute denervation and reinnervation. Target fibers may have clear, dark, or loculated centers with central clear regions with mitochondrial stains (Fig 2). Another example of abnormal internal architecture are lobulated fibers that may occur in myopathy or neuropathy Myopathies that commonly are associated with lobulated fibers are: LGMD 2A; Ullrich Congenital Muscular Dystrophy; Bethlem myopathy (Fig 3).

Succinic dehydrogenase (SDH) and cytochrome oxidase (COX) are purely mitochondrial enzymes. Sections stained for SDH have a bluish colour, it is the most sensitive stain for detecting mitochondrial proliferation. Muscle fibers with mitochondrial proliferation stain darkly for SDH and are named "Ragged Blue Fibers" (Fig 4). Staining for COX gives a brown end shade and type I fibers stain more darkly than type II. On SDH, COX- muscle fibers may be normal or have increased staining. It is characteristic that COX negative muscle fibers are present in mitochondrial myopathy (Fig 5).

To stains glycogen and polysaccharides the gold standard method stain is Periodic acid Schiff (PAS) that stain fibers in a deep-pink colour and highlights the intermyofibrillar network pattern. The specificity
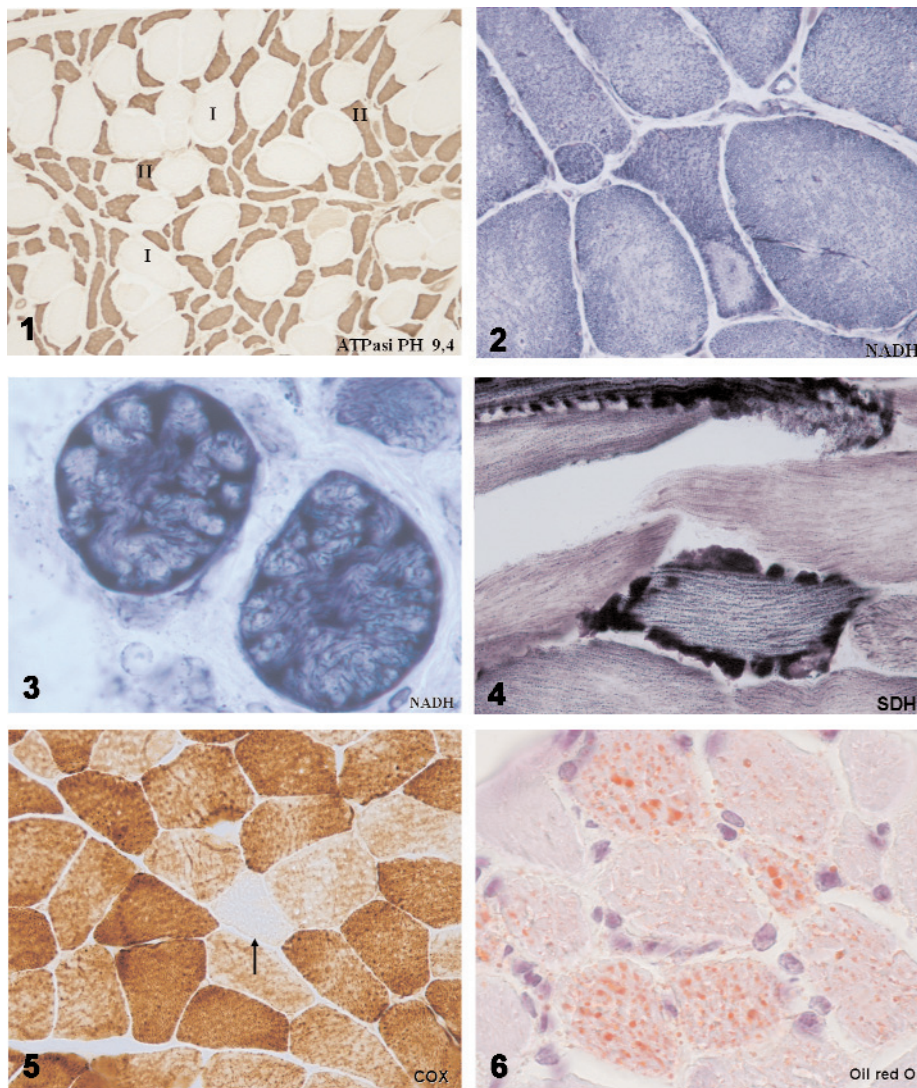
**Fig. 1.** Type II muscle fibers atrophy in a case of Cushing syndrome (Original magnification 20×). **Fig. 2.** Target fibers stained with NADH-TR . (Original magnification 40×). **Fig. 3.** Lobulated muscle fibers stained with NADH-TR. (Original magnification 40×). **Fig 4.** Fibers with abnormal mitochondria intensely stained for SDH (Original magnification 40×). **Fig. 5.** Fiber devoid of COX activity appear white compared with the fiber typing pattern of adjacent fibres (COX stain Original magnification 40×). **Fig. 6.** Accumulation of lipid in a "lipid storage myopathy " (ORO stain Original magnification 40×).

of the stain for glycogen can be checked by digestion with α-amylase prior to PAS staining. Polysaccharide storage myopathy is a common disorder of many breeds of horses and ponies [5].

Oil red O (ORO) and Sudan Black  stains lipid in red and black respectively. The intracellular lipid droplets of the fibers appear as a fine red (ORO) or black (Sudan black) dots of variable size and they are more abundant in type 1 fibers than in type 2.

In disorders affecting lipid metabolism, the excessive accumulation of lipid shows up as larger and more extensive droplets (Fig. 6).

Amyloid can be demonstrated in certain pathological condition within muscle fibers using Congo red stain. With this method amyloid is visible as a red deposit with normal bright field optics but also shows 'apple-green' birefringence with polarized light. An excellent method to enhanced  the positivity is using fluorescence with an excitation filter suitable for fluorochromes such as Texas Red [6] .

## Diagnostic Immunohistochemistry

Since the introduction of histochemical techniques in the 1960s, immunohistochemistry in the 1980s has had the most significant effect upon the interpretation of muscle biopsies. Overall, it is important to mention that, as for enzyme histochemical preparations, muscle specimen preservation by freezing is a prerequisite for accurate immunohistochemical and for immunoblot analysis as there are only a limited number of antibodies (especially against sarcolemmal proteins) that work on paraffin sections. Beside identification of specific protein defects, immunohistochemistry has turned out to be helpful also in the diagnosis of inflammatory myopathy.

A number of proteins, including sarcolemmal, sarcomeric, and nuclear proteins with defects responsible for neuromuscular disorders, have been identified during the past two decades, allowing a more specific and firm diagnosis of muscle diseases. Identification of protein defects relies predominantly on immunohisto-

chemical preparations and on Western blot analysis. Abnormal immunohistochemical expression patterns can be confirmed by Western blot analysis which may also be informative in dominant disorders, although its role has yet to be established

## Immunohistochemistry and Immunofluorescence

Immunohistochemical preparations are relatively simple to perform and represent a standard method in neuromuscular laboratories. There are numerous immunohistochemical methods that may be used to demonstrate specific protein. Immunohistochemistry consist in the localization of antigens in tissue sections by the use of antibodies as specific reagents through antigen-antibody interactions. This antibody-antigen complex is bound by a secondary, enzyme-conjugated antibody. Several techniques can be used to enhance the staining by forming a complex of peroxidase-antiperoxidase, avidin-biotin-peroxidase or avidin-biotin alkaline phosphatase. Finally, in the presence of substrate or chromogens, the enzymes form a coloured deposit at the site of the antigen-antibody complex. The colour of the deposit depends on the chromogen used.

There are several mono- and polyclonal antibodies commercially available that work on human as well as in several other animal species, and numerous detection systems.

Similar to immunohistochemistry, immunofluorescence can be used to detect the location and relative quantity of an antigen on muscle fibers. The technique use a fluorescent dye that is covalently attached to the primary or secondary antibody. When a light illuminates the fluorescent dye, the light is absorbed and the dye emits a different colour light which is visible under the microscope. Direct immunofluorescence requires only one single-step incubation with directly conjugated antibodies, while indirect immunofluorescence uses unconjugated primary antibodies which are labelled by fluorescently conjugated secondary antibodies in a second-step.

## Western blots

Western blots are used to detect the proteins in homogenized skeletal muscle samples by using specific antibodies. It gives information about the presence and the relative amount of that proteins, compared with a control. The proteins of the sample are electrophoresed into a gel and separated based upon molecular weight and charge. In the blotting process, they are transferred onto a membrane made of nitrocellulose or PVDF by applying a current. To detect the antigen blotted on the membrane, a primary antibody is incubated with the membrane and then labelled by a secondary enzyme

conjugated antibody. Finally, the enzyme substrate is added which will precipitate upon reaction with the conjugate so that the position of the membrane-bound-primary antibody will become visible. Size approximations can be done by comparing the stained band with that of a prestained protein size marker.

## Muscular dystrophies

Muscular dystrophies in animals are relatively uncommon and may be difficult to diagnose. A correct diagnosis is very important as the prognosis differs for the various muscle diseases. For most disorders that have been identified, neither a cure nor a specific therapy is yet available. Pharmacologic as well as cell and gene therapies that are being developed for similar human diseases may be used; and animals could be important models for clinical trials.

Since identification of the dystrophin gene mutation and protein defect responsible for progressive muscular dystrophy types Duchenne and Becker in 1987 [7] , many other protein defects have been identified allowing further classification and accurate diagnosis of muscular dystrophies [8]. These gene mutations and protein defects are subtyped in sarcolemmopathies with sarcolemmal protein deficiencies and in non-sarcolemmal muscular dystrophies with defects of nuclear, *i.e.* emerin, and sarcomeric proteins, *i.e.* myotilin and telethonin, as well as enzymes, *i.e.* calpain 3, and can be documented by immunohistochemistry and by immunoblot analysis.

Until recently, in veterinary medicine, only one form of muscular dystrophy have been detected, most likely, many of the same types of muscular dystrophy that occur in human beings also occur in companion animals.

In immunohistochemical examination, which alongside Western blot analysis are the gold standard in diagnosis of muscular dystrophies, the proteins show typical sarcolemmal expression patterns which cannot be distinguished among the different proteins. Loss or reduction of one of these sarcolemmal proteins results in increased fragility of the sarcolemma with destructive effects due to tension generated especially during contraction and exercise [9,10] which may lead to muscle fibre necrosis and apoptosis with subsequent muscle fibre loss and fibrosis. As the integrity of the entire dystrophin-glycoprotein complex is largely interdependent on each of its proteins, defects of one protein may be associated with secondary reduction or absence of other proteins, e.g. secondary loss of sarcoglycans due to primary dystrophin defects [11,12] . To exclude secondary absence of sarcolemmal proteins due to muscle fibre degeneration or necrosis inclusion of a control reaction for spectrin is recommended.
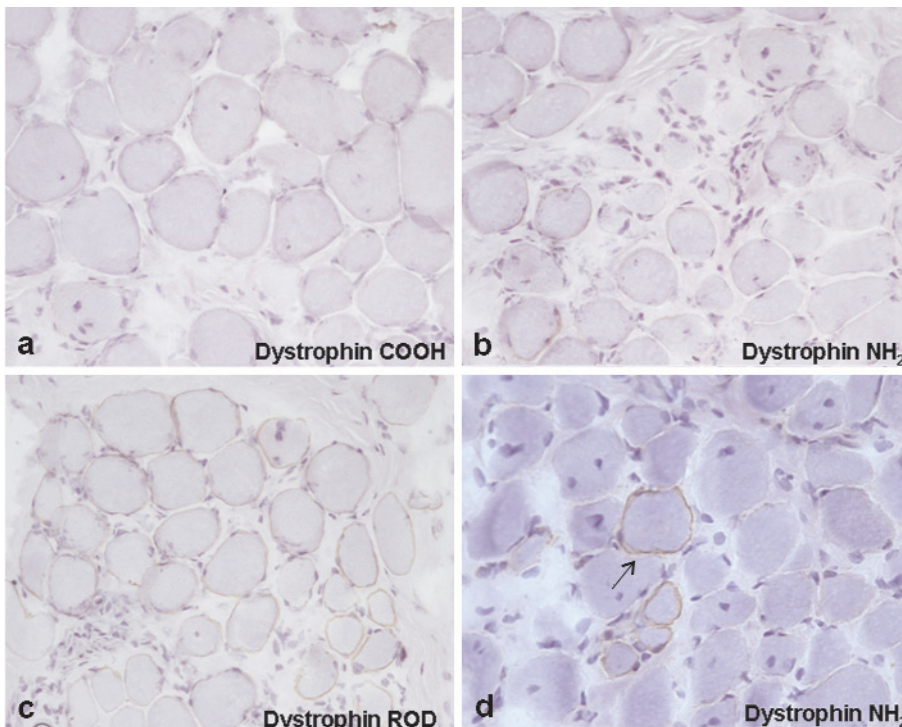
**Fig. 7.** Duchenne muscular dystrophy (**a**, **b**, **c**) with expression of dystrophin lacking, except in some 'revertant fibres' (**d**, arrow). Avidin-biotin-peroxidase complex method with Mayer's hematoxylin counterstain. Original magnifications 20× for all figures.

## Dystrophinopathy

Duchenne muscular dystrophy (DMD )associated with an absence of dystrophy and genetic mutation of the dystrophin gene is the most common and best studied of the muscular dystrophy in human beings and companion animals. In people, a less common and milder form (BMD) shows present but abnormal dystrophin. To date, BMD has not been identified in animals but likely occurs. In Duchenne like muscular dystrophy, there is a complete lack of normal dystrophin both in immunohistochemical and immunoblots study as mutations are out-of-frame mutations resulting in an early termination of protein synthesis due to newly created stop codons and an incomplete protein which cannot be bound and fixed onto the sarcolemma (Fig 7). Occasionally, can be seen revertant fibres which display immunoreactivity with antibodies to all or some domains and which are thought to arise by second site in-frame deletions which partly restore the reading frame (Fig 7) [13,14].

In immunohistochemical examination, antibodies against present and intact domains of the dystrophin molecule display regular sarcolemmal expression patterns while reactions with antibodies against the missing domains remain negative. In Western blot analysis, the usually smaller truncated molecule shows an increased migration; however, less frequently, duplication of the gene causes an elongated dystrophin molecule with a decreased migration capacity.

Primary dystrophinopathy affects typical secondary reduction or loss of sarcoglycans in immunohisto-

chemical preparations as well as in Western blots. This reduction is most striking in Duchenne muscular dystrophy and milder in the Becker type; in carriers of a dystrophinopathy the secondary reduction of the sarcoglycan subunits correlates with the dystrophin expression pattern and level in the individual carrier.

While sarcoglycans as proteins of the dystrophin-glycoprotein complex are down-regulated, utrophin, a 395-kDa protein with considerable homology to dystrophin, is up-regulated, which is suggested to be associated with muscle fibre regeneration and may compensate for dystrophin deficiency [15,16].

## Muscular Dystrophy with Merosin (Laminin $\alpha_2$) deficiency

Approximately 50% of human CMD patients have a deficiency of merosin (laminin $\alpha_2$) expression in muscle. Laminin $\alpha_2$ is the major component of the basal lamina that surrounds each muscle fiber. It is one of the extracellular ligands for the dystrophin-associated glycoprotein complex; it links dystrophin to the extracellular matrix and contributes to the stability of the muscle basement membrane. Therefore, analogous to dystrophinopathies, the use of two antibodies against different fragments of the laminin a-chain is recommended recognizing a 300-kDa fragment towards the N-terminus and a 80-kDa fragment towards the C-terminus. A defect of merosin which is closely associated with the sarcolemmal dystrophin-glycoprotein complex causes a defective or focally absent basal lamina which mediates injury of the sarcolemma [17]. In contrast to defects of
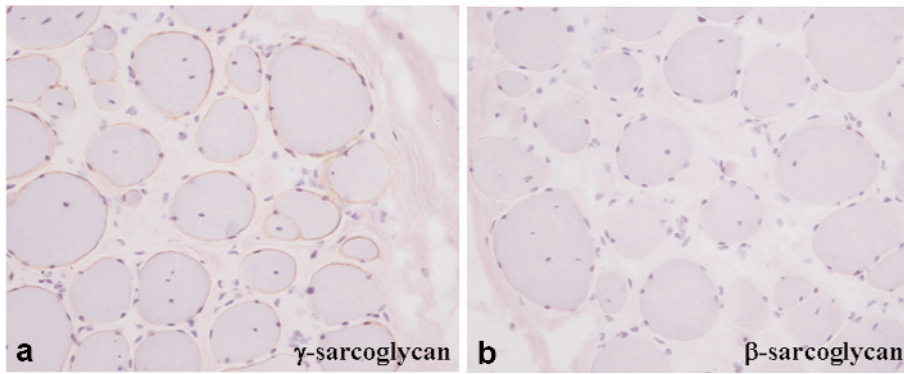
**Fig. 8.** Sarcoglycanopathy with reduced expression of γ- (**a**) and absence of β-sarcoglycan (**b**). Avidin-biotin-peroxidase complex method with Mayer's hematoxylin counterstain. Original magnifications 20× for all figures.
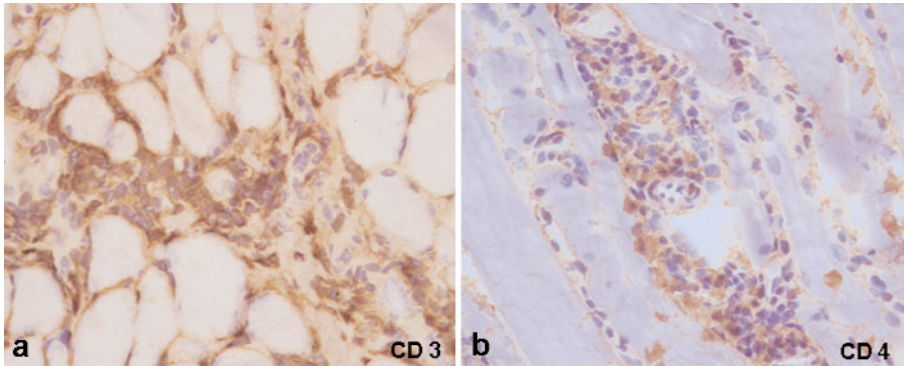


**Fig. 9.** Polymyositis with infiltration of predominantly T cells, identified as CD3 (a) and CD4 (b). Avidin-biotin-peroxidase complex method with Mayer's hematoxylin counterstain. Original magnifications 20× for all figures.

dystrophin or sarcoglycan subunits, defective expression of merosin causes no secondary defects or reductions in the expression of dystrophin or sarcoglycans.

As merosin is lacking not only in muscle but also in neural structures of the skin, skin biopsy provides a diagnostic alternative to muscle biopsy. However, differential diagnosis concerning other muscular dystrophies with sarcolemmal defects is not possible on epidermal tissue if the suspected merosin defect cannot be confirmed [18].

Congenital muscular dystrophy associated with absence of laminin $\alpha_2$ has been described in a young female Brittany-Springer Spaniel mixed breed dog [19]and in cats [20, 21].

## Sarcoglycanopathies

In humans, mutations in the components of the sarcoglycan (SG) complexes (α,β,γ and δ-SG) typically cause severe forms of limb girdle muscular dystrophy [22,23]. Numerous missense, splicing, and nonsense mutations have been identified in all four of the SG genes. Mutations in the fifth member of the sarcoglycan family, -SG, causes amyoclonus-dystonia syndrome [24].

A genotype-phenotype correlation in sarcoglycanopathies is very difficult because onset and progress are variable even with the same mutation and within the same family [12]. As has been mentioned for the whole dystrophin-glycoprotein complex, the integrity of the entire sarcoglycan complex is also

interdependent on each of its components. Theoretically, the mutant subunit is lacking; however, mutations of one of the sarcoglycan subunits cause secondary reduction or absence of any of the other sarcoglycan components in immunohistochemical preparations as well as in immunoblots. Sometimes there is a typical immunohistochemical expression pattern with primary β- and δ-sarcoglycan defects causing complete lack of the other subunits, while primary α- and γ-defects allow small remnants of the other sarcoglycan proteins (Fig 8). However, it is usually impossible to assess the primary defective subunit. Furthermore, Western blot analysis has been described to be technically difficult because the subunits are intermixed with and concealed by various cytoplasmic proteins with similar molecular weights and isoelectric points to those of each sarcoglycan subunit. As it is also virtually impossible to make a diagnosis of any specific sarcoglycan defect based solely on the clinical findings, genetic testing is required to differentiate between the sarcoglycanopathies [25].

While specific mutations have not yet been identified in any canine sarcoglycanopathies, SG-deficiencies have been identified in a young Boston Terrier, Cocker Spaniel, and Chihuahua [26]. Indirect immunofluorescence staining of muscle biopsy specimens from the three different breeds of dogs using monoclonal β and γ-SG and polyclonal α-SG antibodies showed varying patterns of SG loss. As so few cases have been identified in this species, a comparison of the incidence with that in humans cannot yet be
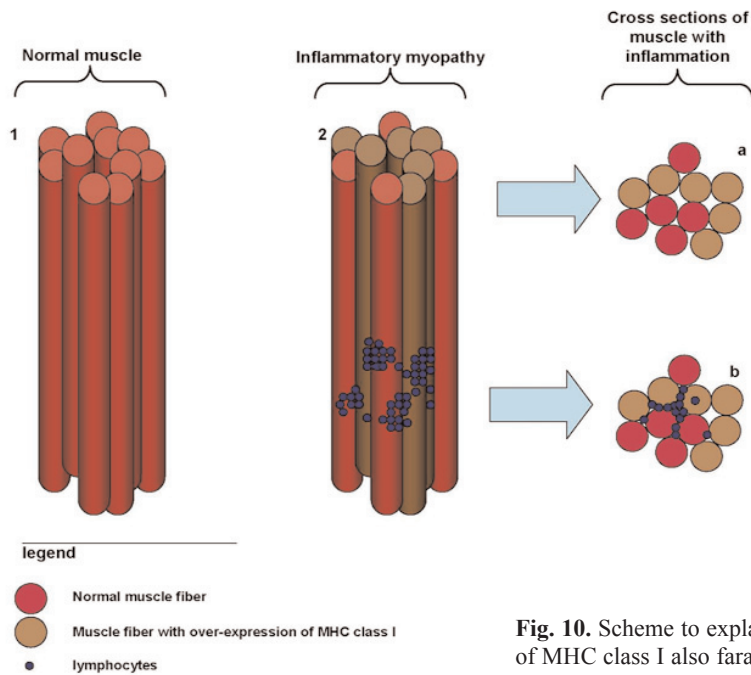
**Fig. 10.** Scheme to explain the anergization of muscle fibers and the expression of MHC class I also faraway that inflammatory cells.
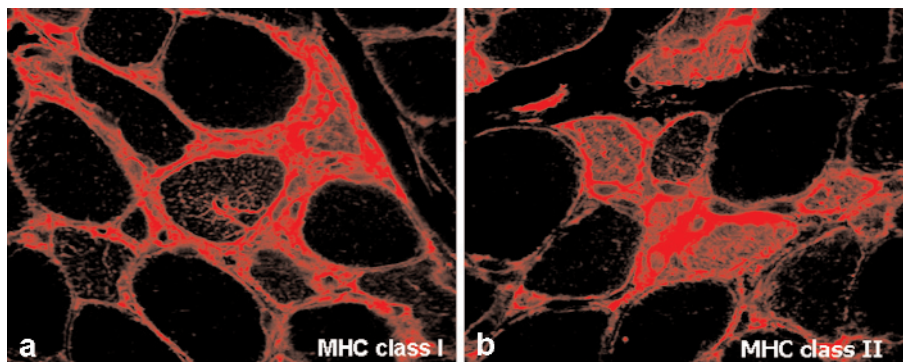


**Fig. 11.** Expression of  MHC class I (a) and MHC class II (b) on muscle fibers in CMMM. Original magnifications 400×. Nuclei counterstained with iodure propidium.

given. Sarcoglycan deficiencies have not yet been identified in the cat but are likely to exist. [26].

## Dystroglycan deficiency

Recent studies have identified a number of forms of muscular dystrophy, termed dystroglycanopathies, which are associated with loss of natively glycosylated alpha-dystroglycan. These disorders have been recently described in Sphynx and Devon Rex cats. Affected cats displayed a slowly progressive myopathy with clinical and histologic hallmarks of muscular dystrophy including skeletal muscle weakness with no involvement of peripheral nerves or CNS. Skeletal muscles had myopathic features and reduced expression of alpha-dystroglycan, while beta-dystroglycan, sarcoglycans, and dystrophin were expressed at normal levels. In the Sphynx cat, analysis of laminin and lectin binding capacity demonstrated no loss in overall glycosylation or ligand binding for the alpha-dystroglycan protein, only a loss of protein expression. A

reduction in laminin-alpha2 expression in the basal lamina surrounding skeletal myofibers was also observed. Reduction in the levels of glycosylated alpha-dystroglycan by immunoblot was also identified in an affected Devon Rex cat [27].

## Inflammatory myopathies

Another group of neuromuscular disorders where immunohistochemistry is very helpful in diagnosis and differential diagnosis is inflammatory myopathies, displaying a typical morphological features in distribution and proportions of inflammatory infiltrates which can easily be identified by immunohistochemical typing of infiltrating mononuclear cells (Fig 9).

In addition to typing of immunocompetent infiltrating cells which is predominantly helpful in differentiation of myositis subtypes, there have been great efforts to find other markers that are indicative of an immunological muscle disease. This is of special importance, as up to 20% of patients with inflammato-
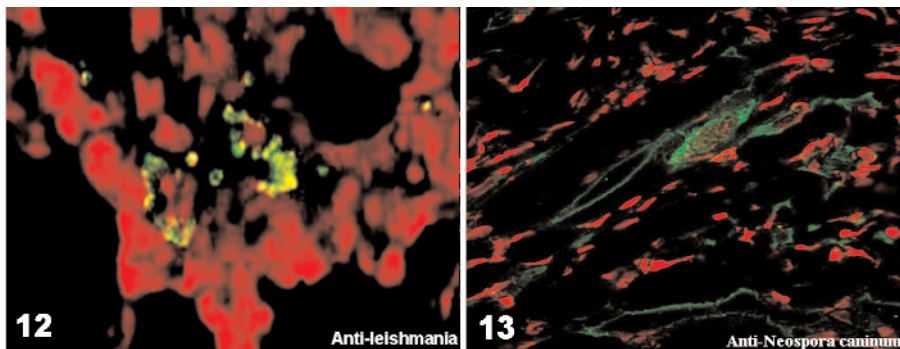
**Fig. 12.** Double immunofluorescence staining analyzed by confocal laser scanning microscopy. Numerous leishmania amastigotes in the inflammatory infiltrate (green fluorescence). Original magnifications X 400×. Nuclei counterstained with iodure propidium. **Fig. 13.** Immunofluorescence staining of protozoan parasite in muscle fibres. A three-dimensional reconstruction of a stack of images along Z axis. Numerous tachyzoites of Neospora caninum are visible in affected muscle (white arrow). Bar = 100 mm.

ry muscle disease lack any inflammatory cellular infiltrate in their first muscle biopsy, thus presenting a false-negative result. Further immunological parameters which are up-regulated in inflammatory muscle diseases are cell adhesion molecules and cytokines. Classical examples of cell adhesion molecules are MHC class I molecules binding T8 cells as well as MHC II molecules binding T4 cells.212-214 MHC class I molecules are usually extensively up-regulated in inflammatory myopathies (Fig 10). They are expressed by inflammatory cells and muscle fibres and represent a necessity for cytotoxic attacks by T8 lymphocytes. MHC class I + muscle fibres may display sarcolemmal as well as sarcoplasmic expression (Fig 11) [28].

Finally immunoistochemistry/immunofluorescence can be used to identify specific etiological agents such as Leishmania spp. (Fig 12), Neospora caninum (Fig 13) or Toxoplasma gondi that can cause inflammatory myopathies in animal as well as in human [29, 30] .

## Conclusion and future perspectives

Many of the other forms of muscular dystrophy identified in human medicine have not yet been discovered in animals. However, most if not all forms of muscular dystrophy in humans are certain to exist in other species; one just has to know how to recognize them.

Identification of protein defects by immunohistochemical as well as Western blot analysis allows a specific and firm diagnosis in a wide range of muscular dystrophies.

However, it is likely that in these disorders also, immunocytochemical analysis may widen the understanding of muscle fibre pathology as well as help in the development of therapeutic strategies.

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