Gulden Diniz
Editor

Clues for Differential Diagnosis of Neuromuscular Disorders

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This book is dedicated

to Ismet, Eylul, and Nazim Unlu, who enrich my life.

to Nazim Kemal, Gunsel, and Ahmet Riza Diniz, who taught me to fly with my own wings and always have encouraged me to go on.

to my dear friends who warm my heart with their presences or memories...

Gulden Diniz

Preface

I am sure you expect a satisfactory explanation of the purpose for which this book was written. You took the book in your hand and weighed it, flipped through the pages, you liked how illustrated it is, but that also made you doubt the book for it seemed too simple. You thought: "What could such a colourful and chirpy looking book teach me?" At this point I will explain what you should expect from a medical book today, and thereby try to convince you to read this one.

When I graduated from medical school as a medical doctor in 1985, what would come to mind when computers were mentioned were crude devices that were not available to the public and occupied huge areas in large research centres. Instant access to information with the Internet was still a dream. That's why medical books were incredibly comprehensive. Among the volumes of data, you attempted to find solutions to your patient's problems. Undoubtedly, you could also access scientific journals and current research results, but the journals were only available in large university libraries for reading or photocopying. In the past 37 years, accessing information has become so easy. Now, you can access the research results published on the same day via the Internet without leaving your seat. As such, the function of medical books has changed a lot. For instance, it is no longer possible for a medical book to be up to date because the stages of writing, editing, and printing can take at least 8–10 months, depending on the volume of the book. Additionally, medicine is the most researched branch of science, and therefore, there is constant movement and change. For example, the dystrophin gene deletion, which is the cause of one of the most common muscle diseases, was identified in 1985. This means that even in the most comprehensive books written during my medical education, Duchenne muscular dystrophy is described as a disease of unknown cause.

As a medical doctor, I continued my education in different subjects. During my microbiology and pathology residency training and basic oncology doctorate, I realized that my methods of accessing information were gradually changing. Earlier in my studies, I used comprehensive microbiology, pathology, and oncology books as much as possible. Eventually, I started to benefit more from articles in the context of daily diagnosis and treatments, as well as simpler books that summarize the underlying mechanisms of diseases and offer clues about the diagnosis and treatment of diseases over the years. In fact, I now think that medical education and literacy are no different from each other. When you first start primary school, no one tries to teach you Shakespeare's sonnets. First, you learn the alphabet and you start writing by scribbling lines and then creating letters from them. Therefore, the main purpose of this book is to provide condensed and concise information with useful clues about muscle and nerve biopsies to aid your education of a rapidly evolving discipline.

I suppose that there is no subject more untouched, that fewer pathologists are interested in and known as little about, as muscle and nerve pathology, a subject which I have been interested in for almost 20 years. Think about it, even electron microscopy, which covers an important part in muscle and nerve pathology books, can be used in only a few centres. In the old editions of the pathology textbooks, muscle and nerve pathology were mentioned in short chapters, and these chapters are no longer available in the new editions. As such, pathologists do not learn much about muscle and nerve biopsy during their residency training. In fact, as you will see in the following chapters, muscle biopsy is a much more comprehensive examination used to investigate differential diagnoses which can yield a wide variety of findings. On

the other hand, nerve biopsy is an examination which yields fewer and more precise findings, but it causes more morbidity in the patient. Today, the indications for muscle and nerve biopsy are becoming more limited. However, in some cases, they can be very important for the patient and the clinician. Apart from the old editions of pathology textbooks, I have not come across a book that deals with both muscle and nerve biopsy together. An important purpose of this book is to teach the alphabet of muscle and nerve biopsies to medical doctors dealing with neuromuscular diseases, especially pathologists. After that, the distance they will travel and the path they will take is entirely up to the readers.

Ever since I became interested in neuromuscular diseases, every patient reminds me of the "*Star Thrower*" story by American anthropologist Loren Eiseley (1907–1977), a short essay from the book *The Unexpected Universe*, published in 1969. A later modified story, also known as the "*Starfish Story*", tells of a man walking along the ocean shore. While walking along the beach in the early hours of the morning, he notices that someone constantly bends down and picks something up after which they throw it into the sea. When asked what he is doing, the man explains that when the sun rises, the starfish on the shore will die so he throws them back into the sea to keep them alive. The man walking finds this very absurd and says: "well, look, there is a lot of starfish on the beach, you can't save them all, you cannot possibly make a difference". The other man bends down, picks up another starfish from the sand, and throws it back into the ocean while saying, "it made a difference for that one!". The goal during muscle and nerve biopsy examination is to identify the elements that can make a difference in the patient's life. For example, by identifying that dystrophin proteins are partially present within the muscle biopsy of a child with dystrophin gene deletion, we can predict that the child has gained almost 30 years to their estimated life expectancy. Today, highly sophisticated treatment methods have been developed and continue to be developed. We can never predict where the therapy of dystrophinopathies will be 30 years from now. The main purpose of writing this book is to contribute to potential "star throwers" that can make a difference for individual patients.

As 29 medical doctors who grew up and were educated in the secular and democratic Republic of Turkey founded by Mustafa Kemal Atatürk, we set out to provide you with the clues we gained from our experience in the differential diagnosis of neuromuscular disorders. The artist and illustrator, Esmanur Emekli; medical doctors, Bahattin Erdogan, Rahul Phadke, Fatih Sarı, and Philipp Ruiz, who shared their patients' photos; the medical students and English editors, Annie Stack and Leonardo Buttice, have provided invaluable contributions to the creation of this book. It is our greatest wish that our book will make a difference in your professional life.

Izmir, Turkey Gulden Diniz

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Editor and Contributors

About the Editor

Gulden Diniz is professor of pathology at the Izmir Democracy University. She serves as chief of the Pathology Laboratory of Izmir Buca Seyfi Demirsoy Research Hospital as well as director of the Medical Microbiology Department at the Health Sciences Institute of Izmir Democracy University. She graduated from Ege University Faculty of Medicine in 1985. She became a microbiologist and an infectious diseases specialist in 1991 and a pathologist in 1998. In 2011, she completed her basic oncology doctorate education at the Oncology Institute of Dokuz Eylül University.She is especially interested in muscle and nerve biopsy, transplantation pathology, paediatric pathology, molecular pathology, and basic oncology. During her education, she has voluntarily studied for training on muscle, nerve, and transplantation pathologies from Professor Carolina Sewry, Professor Sebastian Brandner, and Professor Phillipe Ruiz in 2010 and 2018. She is the author of a Turkish pathology book titled *Autobiography of a Live Cell* (2009), a Turkish biostatistics book titled *Basic Statistics for Dervishes* (2015), and a Turkish pathology book series titled *Around the Pathology in 380 Days* (2017). She has also been the editor of the *Journal of Izmir Behcet Uz Children's Hospital* since 2011. In addition, she is the author of numerous national and international published articles and book chapters. Finally, her almost 20-year experience with muscle and nerve biopsies has led her to write this book on the diagnostic usefulness and limitations of muscle and nerve biopsies .

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A Quick Glance at the Neuromuscular Disorders

1

Gulden Diniz

This section presents the main questions that will be answered in this book:

- What is a neuromuscular disorder?
- Where do neuromuscular disorders originate?
- How are neuromuscular disorders classified?
- Why is the differential diagnosis of neuromuscular disorders necessary?
- Do muscle and nerve biopsies contribute to the differential diagnosis of neuromuscular disorders?
- Does a differential diagnosis contribute to the treatment of neuromuscular disorders?
- What will the future management of neuromuscular disorders be like?

What Is a Neuromuscular Disorder?

The term "neuromuscular disorder (NMD)" covers several diseases which predominantly affect the neuromuscular system with the inclusion of motor neurons, peripheral nerves, neuromuscular junctions, and muscle fibers. In childhood and early adolescence, many of the disorders are due to hereditary causes. However, during adulthood, the incidence of hereditary conditions decreases, whereas the incidence of acquired and mostly treatable diseases, such as inflammatory disorders, increases [1–3]. Since the identification of dystrophin gene dysfunction in Duchenne muscular dystrophy (DMD) found in 1985, enormous numbers of genes which cause different NMDs have been identified. These discoveries led to the improved understanding of the mechanisms underlying NMDs. However, as large numbers of new genes are identified, common genes causing the same phenotype and common phenotypes caused by different genes are also discovered, resulting in an increasing challenge for clinicians. Multigene panels partially help find the solution to this problem, but they have also brought about new problems. The results of these panels include a giant amount of data that is hard to analyze and interpret. Furthermore, the most common challenge is the reality that some variations of genes never cause disease because these variations are polymorphisms with different incidences according to geographic distribution. Therefore, genetic differentiations are mostly named variation instead of mutation [4, 5].

Bearing in mind that a group of patients with mild symptoms may not be diagnosed at all, it is almost unfeasible to estimate the real prevalence of NMDs [2]. In addition, the prevalence of NMDs may vary according to geographic regions, race, gender, and age. For example, in peripheral neuropathies, one of the most frequently diagnosed NMDs has an estimated prevalence of 2.4% in the general population, while the prevalence reaches 8% in older people [3, 4]. Similarly, muscular dystrophies, a group of well-known childhood NMDs, affects roughly 1 in 3500 children worldwide with the highest prevalence in younger boys. This is because the most common muscular dystrophy is dystrophinopathy, which has X-linked recessive inheritance [5–7]. However, in the population aged 60 and over, inflammatory processes, endocrine disturbances and toxic etiologies play a superior role in the development of NMDs [8]. Up until today, not only have relatively scarce manuscripts related to the prevalence of NMDs been published, but also idiopathic or unspecified cases have constituted an important percentage of these articles (Figs. 1.1, 1.2, and 1.3). Therefore, epidemiological surveys have an essential importance to aid deeper understanding of NMDs [4–9].

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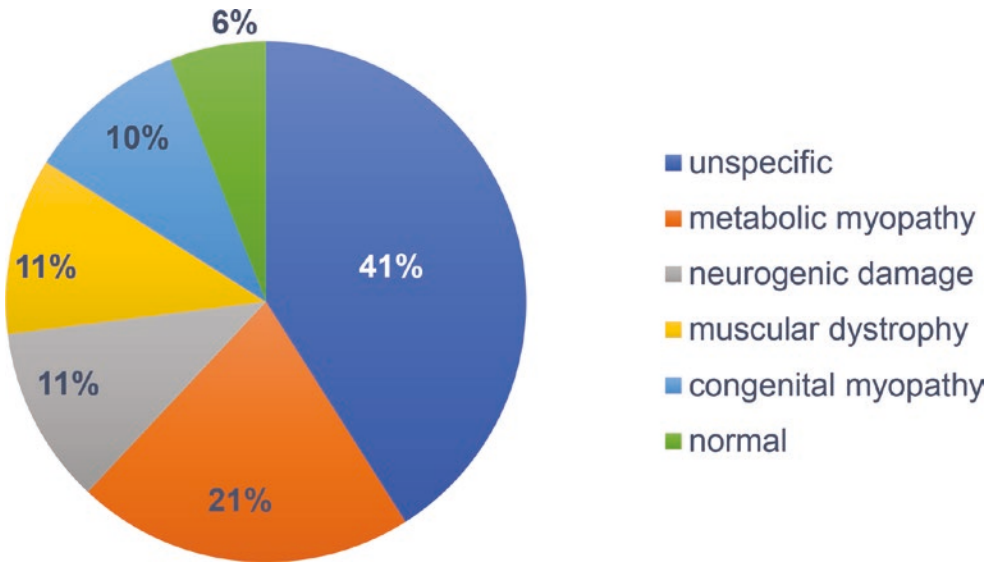

Fig. 1.1 Diagnoses of muscle biopsies in the 197 floppy infants aged 0–18 months [4]

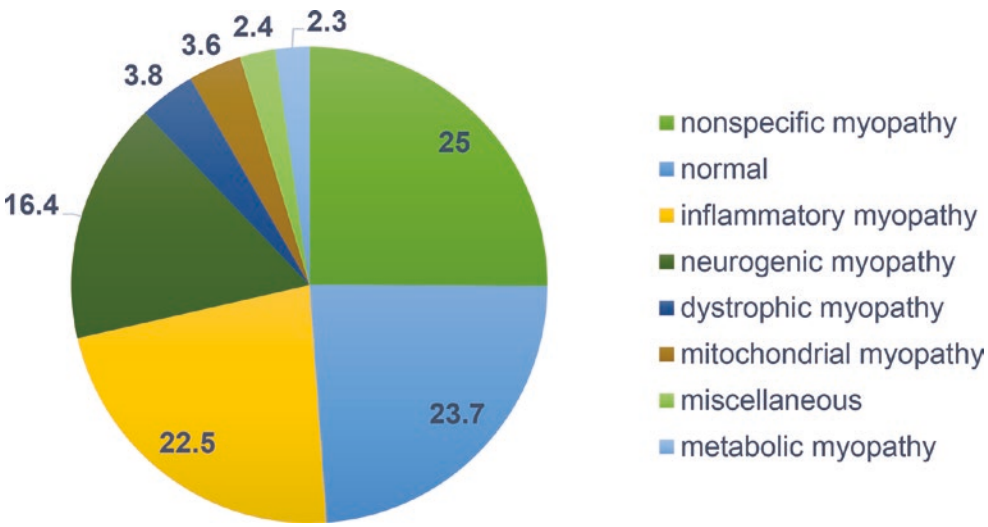

Fig. 1.2 Diagnoses of muscle biopsies in 698 adults with the mean age of 55.3 ± 15.5 years [5]

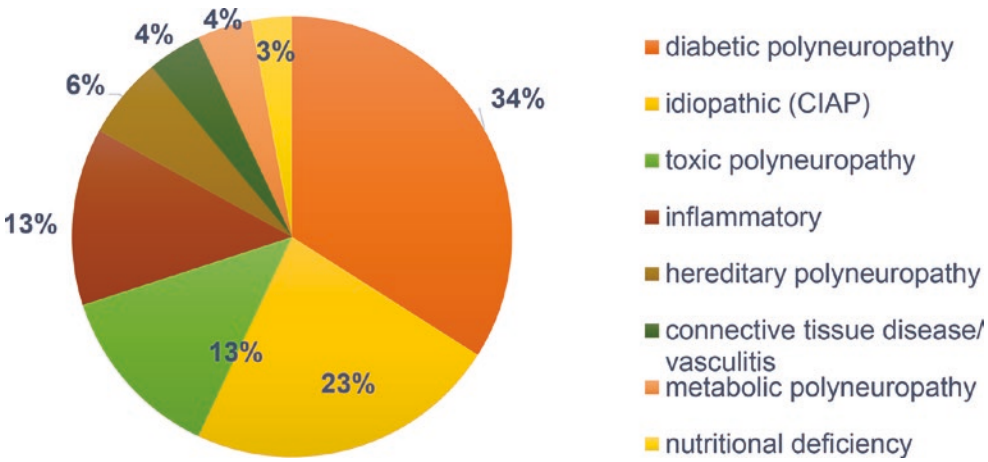

Fig. 1.3 Final diagnoses of 2613 patients with peripheral neuropathies [6]

Where Do Neuromuscular Disorders Originate?

NMDs are characterized by the ailment of peripheral nerves or skeletal muscles. The site of pathology can be in neurons, like amyotrophic lateral sclerosis (ALS); axons of neurons, like axonal neuropathies; Schwann cells, like chronic inflammatory demyelinating polyneuropathy (CIDP); neuromuscular junctions, similar to myasthenia gravis; skeletal muscle, similar to myopathy; and in any combination of these areas (Fig. 1.4). In some disorders, such as Prader-Willi syndrome, illness of the central nervous system (CNS) is also seen, but most NMDs are limited to the peripheral nervous system (PNS). The multitude of possible regions of dysfunction can make it difficult to differentially diagnose NMDs. Some clues in the clinical symptoms can help localize the site of injury [1, 2, 8, 10–14].

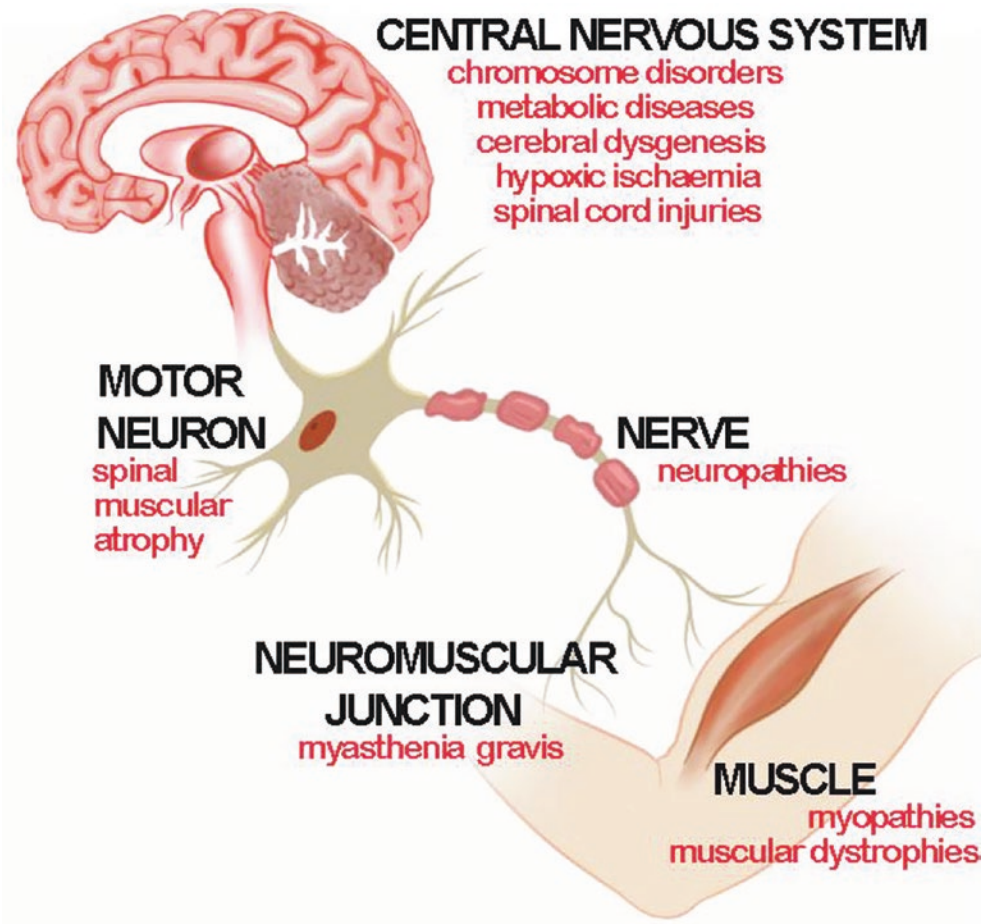

Fig. 1.4 Origination of neuromuscular disorders

How Are Neuromuscular Disorders Classified?

The classification of NMDs is usually made according to the affected area and the symptoms that occur due to this problem. It is possible to consider NMDs in three main groups in the context of the affected area: myopathy, neuropathy, and neuromuscular junction diseases. Correct classification is very important for nomenclature and diagnostic standardization. However, this classification depends on the underlying pathogenesis of the diseases, and it changes as new knowledge of underlying mechanisms is discovered. For instance, molecular studies have had a deep impact on the classification of both hereditary myopathies and neuropathies. If the connection between acquired and genetic origin in most NMDs is completely understood, there will be major changes in the current classification of NMDs [3, 8, 10, 15].

Why Is the Differential Diagnosis of Neuromuscular Disorders Necessary?

It is very important to make a differential diagnosis of neuromuscular diseases for two reasons. Firstly, diagnosing the disease provides prognostic prediction. If the diagnosis can be made, it will become clear how the disease will progress, whether its progression can be prevented, which measures can be taken to slow the course of the disease or reduce complications as well as which treatment options are available. Secondly, unnecessary or even harmful treatments are prevented. For example, lymphocyte infiltration may accompany the picture in some muscular dystrophies. There are many cases diagnosed as inflammatory myopathy by biopsy and receiving unnecessary treatment [16, 17].

Knowledge of NMDs has increased dramatically during the last 35 years thanks to the development of genetic tests. The impact of molecular genetics on the diagnosis and understanding of NMDs has been tremendous. For example, dystrophinopathies, the most prevalent group of childhood muscle diseases, are now easily diagnosed with Multiplex ligation probe amplification (MLPA) tests in most patients. Similarly, the CMT1A duplication of the PMP-22 gene which is a major causal defect for Charcot-Marie-Tooth (CMT) disease was discovered in 1991. Since that time, 76 causal genes and several other genetic locations were identified in patients with CMT [3, 8, 13, 18–21]. In recent years, special genetic analysis panels have been developed that can detect the most common genetic mutations for common muscular dystrophies, congenital myopathies, and hereditary neuropathies with scans that can be easily performed from a few milliliters of peripheral blood samples. In short, most NMDs are now diagnosed through genetic analysis. Knowing the gene associated with the disease and understanding the functions of the gene is a cornerstone for the development of new treatment options [8, 13, 18–22].

Do Muscle and Nerve Biopsies Contribute to the Differential Diagnosis of Neuromuscular Disorders?

By applying next-generation sequencing (NGS) techniques, some causal genes of NMDs have been identified. Consequently, the necessity for muscle and especially for nerve biopsy in the diagnosis of NMDs has sharply declined over recent years. Nonetheless, muscle biopsy still represents the gold standard for diagnosing in certain situations including discrimination of Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy (DMD). Similarly, nerve biopsy is still essential for the examination of interstitial pathological processes like vasculitis limited in the peripheral nerves. In addition, histopathological evaluation remains essential for the differential diagnosis if the phenotype is atypical or molecular studies are not helpful [8, 13, 23, 24].

Muscle and nerve biopsies also play a crucial role in guiding the management of NMDs [23]. However, it should be recalled that histopathological evaluation is only one aspect of all diagnostic processes for patients with NMDs [24]. Therefore, reaching the differential diagnosis requires links between more than one discipline. The clinician should reach a potential diagnosis by evaluating the information gained from the clinical history, physical, and laboratory examinations [3]. These findings are used to determine the details of biopsy procedures, such as choosing the right time for biopsy and choosing the muscle or nerve to sample. In addition, the surgeon should have the necessary skills to provide untraumatized material [3, 9, 14].

The evaluation of muscle or nerve biopsy specimens is a replaceable approach in some cases. Nerve biopsy, which is much more invasive and morbid than muscle biopsy, is almost always performed only once. A small number of peripheral nerves, such as the sural nerve, the superficial peroneal nerve, and the superficial radial nerve, are suitable for sampling because they consist of only sensory fibers. Local hypo- or hyperesthesia is usually permanent after biopsy sampling. Similarly, muscle biopsy sampling is usually done once, after which the tissue is frozen and stored. Frozen tissue is then used when further examination is required. Therefore, repeat biopsy examinations are rarely necessary. In any muscular dystrophy, recurrent biopsies show a progression to end-stage disease, and eventually the muscle tissue is replaced by fibrous and adipose tissues.

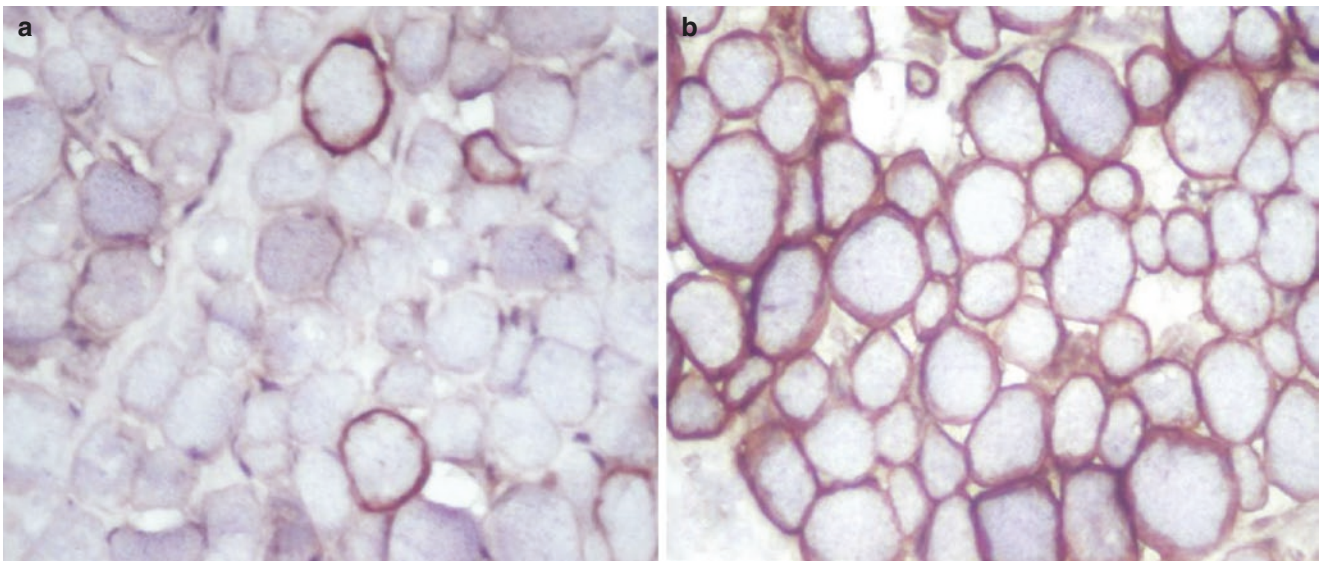

Fig. 1.5 Muscle biopsy from a genetically proved case of dystrophinopathy: (**a**) Focal presence of sarcolemmal dystrophin expression excludes the diagnosis of Duchenne muscular dystrophy (DAB ×200). (**b**) Diffuse sarcolemmal merosin expression in the same biopsy (DAB ×100)

There are centronuclear myopathies, in which histopathologic examination of repeat muscle biopsy specimens may have diagnostic value. In these myopathies, the nuclei are located peripherally at birth but migrate to the center of muscle cells over years. Therefore, the first biopsy specimens obtained during the infantile period may be nondiagnostic. Such cases are generally diagnosed with an examination of new biopsy specimens harvested around the age of 4 [23–26].

Today, many muscular dystrophies such as dystrophinopathies and limb-girdle muscular dystrophies (LGMDs) are diagnosed with immunohistochemical analysis of muscle tissues [3, 8, 23]. The two clinical types of dystrophinopathies, DMD and BMD, are very different from each other. DMD starts in early childhood, and patients with DMD can almost never live beyond their 20s. Contrarily, patients with BMD have milder clinic features and can reach their 50s thanks to close surveillance [3, 8]. Genetic changes are the same in both diseases and cannot be discriminated by molecular analyses [8]. The presence of partially functional dystrophin protein can confirm the diagnosis of BMD (Fig. 1.5). Therefore, demonstration of sarcolemmal dystrophin in muscle biopsy is very important [3, 8]. The immunohistochemical examination of muscle biopsy specimens with commercially available primary antibodies can be helpful in the differential diagnosis of dystrophinopathies and of many other muscular dystrophies. Additionally, enzyme staining of muscle biopsy specimens is the most trustworthy way to diagnose mitochondrial diseases with muscle involvement (Fig. 1.6). For instance, mitochondrial myopathies caused by mitochondrial DNA defects cannot be diagnosed with routine genetic analysis performed on nuclear DNA extracted from peripheral blood cells. Oil red O and PAS stains are also very beneficial for the differential diagnosis of metabolic diseases. This is especially useful in children older than 6 weeks where the presence of neonatal myosin expression in muscle cells provides further evidence for the presence of a muscle disease (Fig. 1.7). Contrarily, if there are no fibers expressing neonatal myosin, unnecessary treatments can be avoided. In summary, histopathologic evaluation of muscle and nerve biopsy specimens is an important step in the diagnosis of NMDs. Biopsy find-

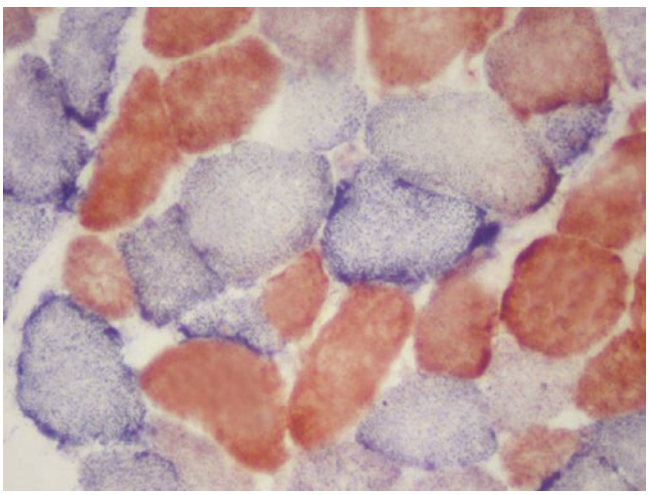

Fig. 1.6 Pathological blue fibers in a patient with mitochondrial myopathy (combined COX-SDH enzyme stain ×200)

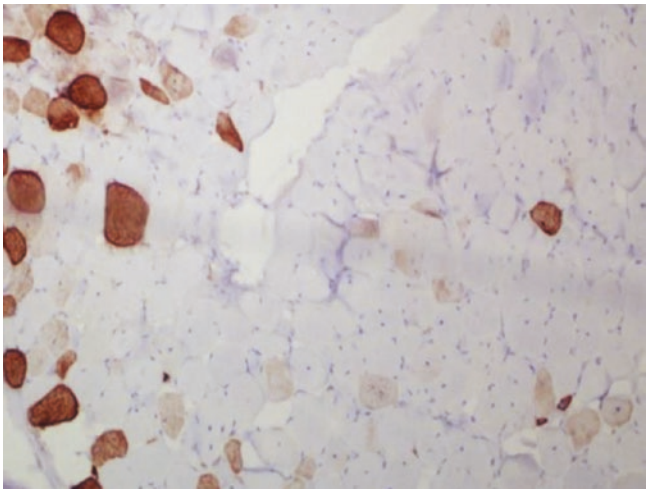

Fig. 1.7 Pathological neonatal myosin expressions in many fibers (DAB ×40)

ings may discriminate the disease group and may determine the most useful genetic analysis for further examination [3, 8, 23, 25–27].

Does a Differential Diagnosis Contribute to the Treatment of Neuromuscular Disorders?

During the past three decades, an increased understanding of the underlying mechanisms of NMDs has given rise to many gene-specific and mutation-specific animal models enabling therapeutic research. Currently, many therapeutic principles have been formulated during preclinical studies using these animal models, and some of them have already entered the stage of clinical trials. Even a few newly developed drugs are being used in the therapy of some NMDs. In the future, more effective diagnostic and therapeutic modalities can be created with the help of these comprehensive trials. However, there is often a certain group of diseases for which these drugs are ineffective. For this reason, it is necessary to make a differential diagnosis of NMD and confirm that it is a type which would benefit from a certain drug therapy. For example, some new drugs developed for dystrophinopathies only work for certain exon deletions. For this reason, making a definitive differential diagnosis of the disease with a multidisciplinary team will provide a treatment regime that the patient can benefit from [1–3, 11–13, 28–30].

What Will the Future Management of Neuromuscular Disorders Be Like?

Due to the absence of a curative therapy for most genetic NMDs, treatment has mainly been supportive with the use of physiotherapy, respiratory support, and cardiac care. During clinical trials, these supportive measures are also used in the control group to avoid study biases. Recently some principles were developed known as "care standards" for relatively common disease groups such as DMD, spinal muscular dystrophy, and congenital myopathies. Along with all these measures, life expectancy in DMD has been prolonged by more than a decade. However, establishing a diagnosis is essential for identifying appropriate treatment and care standards. Currently, there are huge scientific and clinical efforts to develop new treatment techniques and agents used for the treatment of NMDs. These new therapeutic approaches may improve patient survival worldwide [11, 12, 20, 28–30].

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Understanding of the Muscle and Nerve Tissue

2

Gulden Diniz and Bahattin Erdogan

Neuromuscular disorders (NMDs) describe different situations that disrupt distinct components of the neuromuscular system, which manage body movements. The deterioration of movement is generally determined in NMDs, and more rare, fatal complications like cardiac and respiratory insufficiency can occur. Clinical findings of patients with NMDs mainly develop due to the dysfunction of the nervous system and/or locomotor system including motor neurons, peripheral nerves, neuromuscular junctions, and skeletal muscles. NMDs can also cause overlapping and non-specific symptoms challenging the differential diagnosis [1, 2].

To better understand NMDs and the symptoms observed in these diseases, it is essential to know the two basic cells affected by these diseases, muscle and nerve [2]. For this reason, general features of muscle and nervous tissues from embryology to histology, from molecular properties to physiology, will be explained with original drawings. In addition, its relationship with the symptoms created by the disruptions in normal functioning will be emphasised.

Embryologic Development of Muscle Tissue

Embryonic development of the muscle and nerve is illustrated in Fig. 2.1. An embryonic midline structure named the notochord plays a vital role in developing vertebrates, which exists transiently in higher vertebrates but permanently in Chordata. This structure is a source of important signals modifying encompassing tissues and is an essential component for embryo vertebrate development [3]. Among the signals secreted by the notochord, Sonic hedgehog (Shh), one of the hedgehog proteins, plays a fundamental role in developing somites [4] and the neural tube (NT). It has been demonstrated that the removal of Shh in the sclerotome impacts myotome and motor neuron differentiation. The notochord induces the development of a graded-style ventral spinal cord due to several genetic transcriptions. For example, notochord signals play a role in setting left-right (LR) asymmetry and specifying slow-twitch muscle fates. Similarly, it is responsible for the normal development of the pancreas and the early endoderm [3].

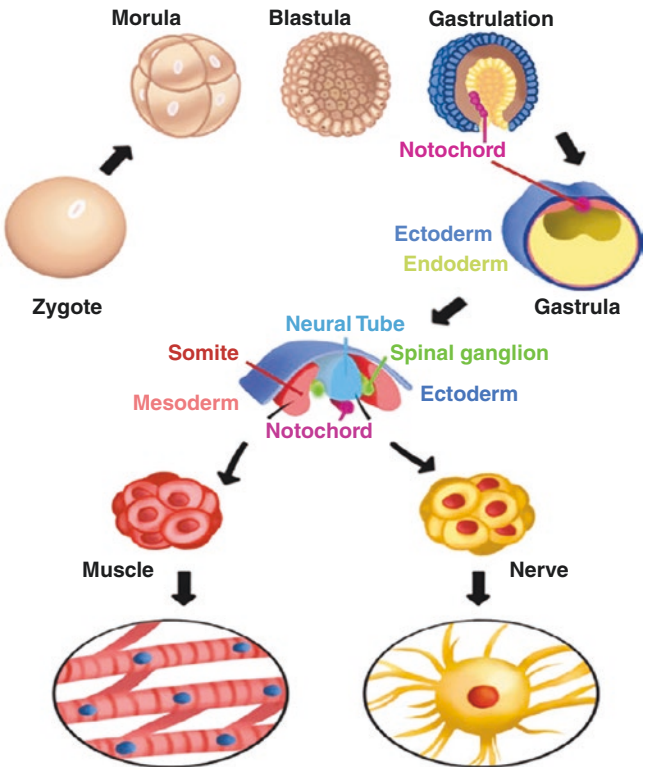

Fig. 2.1 Embryonic development of the muscle and nerve

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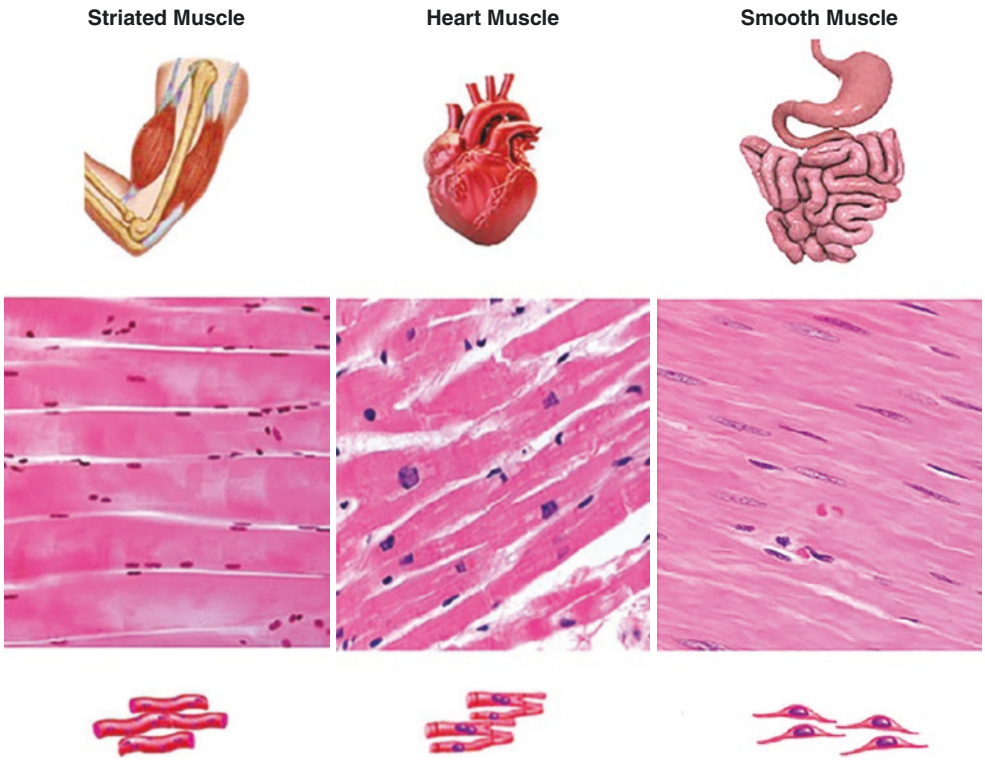

Fig. 2.2 Types of muscle tissue

Muscle tissue constitutes of the skeletal muscle, heart muscle and smooth muscle (Fig. 2.2). These three types of muscle tissue differ from each other in many ways. For example, skeletal muscles have multiple peripheral nuclei, while others have single centrally located nuclei. There is no intercellular connection in skeletal muscle, but cardiac muscle cells are connected by intercalated discs and smooth muscle cells by gap junctions. Skeletal muscle is somatically innervated and voluntarily contracted, whereas cardiac muscle and smooth muscle cells are autonomously innervated and involuntarily contracted. Skeletal muscle makes up most of the muscle tissue and is a locomotor's crucial component. In this chapter, skeletal muscle development and histology will be discussed.

The embryologic development of skeletal muscles is much more circuitous than most tissues [5, 6]. Somites are transient embryonic structures which originate from paraxial mesoderm and form the body's skeletal muscles. Head and neck muscles originate from anterior paraxial mesoderm. During gastrulation, the paraxial mesoderm is formed in the blastopore, and then during embryonic axis elongation, it forms in the tail. The developing paraxial mesoderm consists of presomitic mesoderm, which is a transient tissue at the embryo's posterior tip. This mesodermal unit is segmented into somites during embryogenesis, and this process continues from head to tail. After forming dermomyotome, somites differentiate into dermatome and then into myotome. The skeletal myogenesis initiates with the specification and differentiation of the myogenic progenitors in the myotomes. The multinucleated myofibers form from mononucleated myoblast fusion, which occurs during phases of proliferation and differentiation. Mesenchymal cells first transform into internal mononucleated myoblasts [7]. At the beginning of the foetal period, between 10 and 13 weeks of gestation, myotubes are formed by the end-to-end fusion of myoblasts. Primary myotubes with central nuclei are produced by the fusion of myoblasts, and then the second generation of myotubes are formed, requiring active innervation [5–7].

During embryogenesis, molecular mechanisms and gene expressions differentiate according to the stages of myogenesis. For example, the development and differentiation of the paraxial mesoderm is mainly under control by the Wnt and fibroblast growth factor (FGF) signalling pathways [8]. The Wnt/FGF pathways impact various transcription factors such as T, Tbx6 and Msgn1, which are expressed in the presomitic mesoderm. Paraxial mesoderm specification and patterning require these transcription factors. These pathways also control the exit of myogenic precursors from the progenitor zone and their entry to the posterior presomitic mesoderm. Another factor, bone morphogenetic protein (BMP), impacts mesoderm specification and fate. As a member of the transforming growth factor-β (TGF-β) superfamily, BMPs are a

group of signalling molecules. At first, their signalling was considered to cause the formation of the bone, but they are now accepted as important factors in embryogenesis and the development of the entire mesoderm [9].

Somite specification results from the presomitic mesoderm's dynamic molecular processes. The periodic production of somites is controlled by an automatic embryonic segmentation clock generating signals of Notch, Wnt and FGF [10, 11]. Muscle progenitor cells (MPCs) are separated from around the dermomyotome under the regulation of Shh, Notch and Wnt signalling pathways. Somites differentiate into sclerotome and dermomyotome in response to morphogenetic changes [12]. At this stage, Myf 5 and Mrf 4 regulate the MPCs' entry into the myogenic program independently of Pax3/7. MPCs migrate to the limbs and trunk by expressing Pax3 and Pax7 genes [13, 14]. Satellite cells (SCs) are a subpopulation of postnatal muscle stem cells which differentiated from MPCs. SCs and MPCs cause the myoblasts complete myogenesis. After proliferation, myoblasts exit the cell cycle and express myogenic regulatory factors (MRFs). Myogenesis, during postnatal and embryonic development, is a complex biological procedure controlled by transcription factors and distinct signalling paths. In addition, different noncoding RNAs (ncRNAs), such as miRNAs, circRNAs and lncRNAs, which make up most of the total RNA in cells, also have an essential regulatory role in myogenesis [7, 13, 15]. In recent studies, much attention has been paid to the role of the Pax3/7 cell population in skeletal muscle regeneration and growth. All Pax genes represent a key role in forming organs and tissues during embryogenesis. Especially Pax3 and Pax7, expressed in myogenic progenitor cells, regulate the MPCs differentiation toward skeletal muscle [16].

The role of the main regulatory function in the intrauterine skeletal muscle growth program lies with MRFs as a group of basic helix-loop-helix (bHLH) transcription factors. Myogenic factor 5 (myf5), myogenic differentiation 1 (myoD), MRF4 (myf6) and myogenin can activate differentiation of the skeletal muscle. These separate and interconnected MRF functions have been examined, and it has been identified that myoD and myf5, which are primary MRFs, seem to be necessary for myoblast formation and survival. In contrast, myogenin and MyoD/MRF4, which are secondary MRFs, appear to have a role in controlling the terminal differentiation of the myotubes. Previous studies have shown that although differentiation was not observed, normal numbers of myoblasts are formed in triple mutant mice (with insufficient MRF4, myoD and myogenin) [15, 16]. The regeneration of the muscle after an injury is similar to muscle development during embryogenesis, and reparation is orchestrated by SCs (Fig. 2.3).

In myogenesis, mononucleated myoblasts fuse together end to end to develop multinucleated myotubes and finally myofibers. Myofibers then group into bundles to form the muscles. Each myofiber is surrounded by a distinct layer of connective tissue called endomysium, and groups of myofibers inside the muscle are surrounded by perimysium, another layer of connective tissue. Surrounded by a cell membrane called sarcolemma, a myofiber has a maximum length of 20–30 cm and approximate dimensions of 20–100 μm. The mature myofibers consist of repeated sarco-

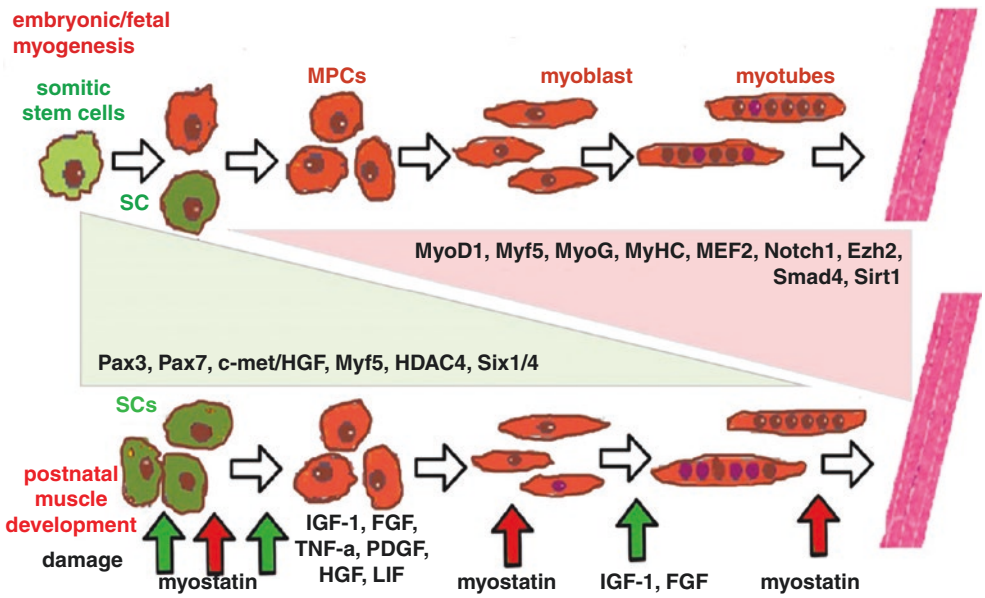

Fig. 2.3 Myogenesis and regeneration of striated muscles

meres to enable muscle contraction dependent on calcium. The skeletal muscle has a limited capacity to regenerate in response to injury. This regeneration capacity is possible for only a few mononucleated stem cells, which are 2–10% of all nuclei in a healthy human muscle fibre. These cells were identified 60 years ago and are called satellite cells due to their anatomical area on the muscle fibres' surface, between the sarcolemma of the myofiber and the basal lamina (Fig. 2.4). It is possible to distinguish SCs with electron microscopy through their cellular characteristics like high nucleocytoplasmic ratio, nuclei with dense peripheral heterochromatin and lack of organized contractile myofilaments. To date, many proteins have been introduced as markers of SCs in different studies. In the nuclei of SCs found in adult muscles, Pax7 is strongly expressed, whereas Pax3 is weakly expressed in most muscles. In quiescent SCs, other transcription factors like MyoD and Myf5 are not expressed. Markers of SCs can also include various proteins of the cell surface membrane, such as α7- and β1-integrins, M-cadherin, C-X-C chemokine receptor type 4 (CXCR4), c-Met, calcitonin receptor, syndecan-3 and -4, CD34, caveolin-1, neural cell adhesion molecule-1 (NCAM1) and vascular cell adhesion molecule-1 (VCAM1). Some of these markers are helpful when discriminating SCs. For example, by using the antibodies against laminin and M-cadherin, SCs

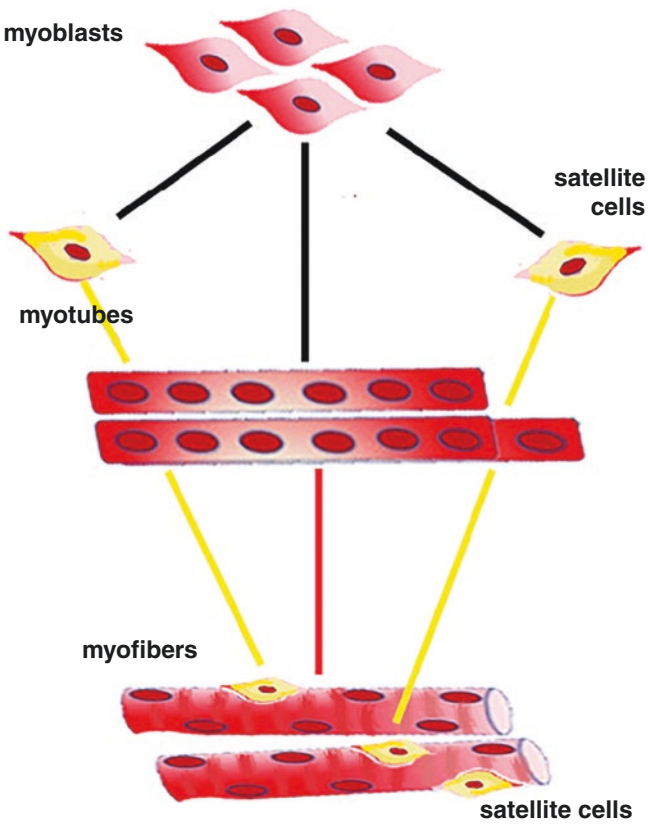

Fig. 2.4 Location of satellite cells

can be immunohistochemically identified. Similarly nuclear Pax7 is expressed in activated SCs, but it is not a quiescent SC marker.

Although SCs are mitotically inactive and usually quiescent, they can rapidly reenter the cell cycle after injury or in response to growth signals. After activation, SCs proliferate to support the differentiation of myoblasts into mature muscle cells. They then fuse alone or together to repair muscle fibres. This process creates many new multinucleated myofibers within a few days. Myoblasts are myogenic cells that express Myf5 and MyoD [16, 17]. The terminal differentiation of myoblasts into myocytes occurs due to the upregulation of secondary MRFs (MRF4 and myogenin). In addition to MRF4 and myogenin, myosin heavy chain (MHC) and muscle creatine kinase, which are important genes in the muscle cell, are also expressed. Following this, multinucleated syncytium forms by the fusion of mononuclear myocytes, which eventually develops into contractile muscle fibres [15, 17]. Specific signals secreted by the microenvironment drive SC proliferation and differentiation. Biologically active molecules are released in the extracellular space through muscle injury. There are two modes for the release of these molecules. They are either synthesised and secreted by other cell types, including neutrophils and macrophages, at the wound site; or they are endogenous in the damaged tissue. FGF-2, FGF-6, insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), leukaemia inhibitory factor (LIF) and platelet-derived growth factor (PDGF) stimulate the proliferation of SCs. On the other hand, myostatin, transforming growth factor-α (TGF-α) and transforming growth factor-β1 (TGF-β1) and all members of the TGF-β superfamily are major inhibitors of skeletal muscle regeneration [18–20].

For muscle regeneration after muscle injury, a subset of activated SCs undergo skeletal muscle differentiation. In the meantime, others return to a quiescence state, thus allowing future regeneration. However, the regeneration capacity of striated muscle is limited, and if there is a disease that causes continuous muscle destruction, the SCs inevitably exhaust after repeated regeneration processes. After this stage, muscle tissue is no longer repaired with new myofiber production, but with fibroadipose tissue [15].

Early myoblasts do not express desmin, the cytoskeletal proteins dystrophin or the dystrophin-associated protein complex, which consists of dystroglycans, sarcoglycans and sarcospan. Contractile protein synthesis follows myoblast fusion. From the 20th week of the intrauterine term, foetal fibres occurring in a cylindrical structure with central multiple nuclei, showing strong vimentin and desmin expression, are observed. Concerning sarcoglycan, beta-sarcoglycan is first expressed around the seventh week of gestation, whereas other sarcoglycans appear a few weeks later. At 9 weeks of

gestation, spectrin and dystrophin expression can be seen in most myotubes' sarcolemma. Another sarcolemmal protein of foetal myofibers, utrophin, appears at the same time. Before 10 weeks of gestation, the vimentin and desmin expression is observed in the myotubes; however, immunohistochemical staining becomes strongly positive at 15 weeks of gestation [21].

The myosin heavy chain (MyHC or MHC) has four main isoforms including embryonic/developmental, foetal/neonatal, fast and slow myosin. Smaller or secondary generation myotubes express fast, embryonic and foetal MHC, while larger or first-generation myotubes express slow, embryonic and foetal MHC. Although smaller tubules remain reactive, vimentin immunoreactivity is no longer present in the largest myotubes at around 15 weeks of gestation. Large muscle fibres, known as Wohlfart B fibres, are on average 10–15 μm in diameter, and they are visible at 20–21 weeks of gestation. The other large fibres are named Wohlfart A fibres are visible by 21–25 weeks. Both are composed of type I fibres with ATPase enzyme stain. From 21 to 25 weeks of gestation, 98% of the fibres are small undifferentiated fibres, and 2% are type I large fibres. By 24 weeks, perimysium surrounds compacted and grouped fibres. At this stage, utrophin fades, whereas dystrophin stains intensely, indicating a large quantity of the protein is present. In the last trimester of intrauterine life, striated muscles consist of five different foetal myofibers. These include Wohlfart B fibres; a small number of large fibres of type I nature; many small myofibers of type I nature and many type II myofibers (type IIA, IIB and IIC which constitute 66% of all fibres). At 35–37 weeks of gestation, embryonic MHC expression disappears completely, as opposed to foetal MHC expression, which is still present in the first few months of life. At birth, Wohlfart B fibres are quickly lost. Most type IIC myofibers turn into type IIA and type IIB fibres. This transformation is completed within 6 postnatal weeks (Fig. 2.5). For this reason, muscle fibres showing foetal or neonatal expression of MHC in muscle biopsy, after the neonatal period, are indicative of muscle pathology, as they show the persistence of foetal type myofibers or the presence of regenerated fibres. Desmin remains weakly present at birth, but by 15–30 weeks vimentin disappears. In an untrained and healthy adult, six different fibre types can be distinguished based on their staining features with myofibrillar adenosine triphosphatase (mATPase) histochemistry. This analysis uses preincubation pH values of 4.3, 4.6 and 10.4. These fibre types are I, IC, IIC, IIA, IIAB and IIB [21]. In other words, skeletal muscle cell types that exist in the embryonic period and show minimal differences from each other continue to exist. However, this distinction, which can be made by the ATPase enzyme histochemical studies at different pH levels, as well as by looking at different MHC profiles, is not very important in medical practice and in the differential diagnosis of NMDs. Today in practice, it is physiopathologically accepted that human skeletal muscles, regardless of their minimal variation, are composed of cells with type 1 and type 2 features, like a checkerboard pattern. In healthy adults, a third of every skeletal muscle is composed of type 1 myofibers, and two-thirds is type 2 myofibers. Type 1 myofibers predominantly contain MHC 1, while type 2 myofibers contain MHC 2. These fibres are also known as slow and fast myosin, respectively [22]. Skeletal muscles also have a considerable ability for adaptive modifications in reaction to different factors such as regular exercise or disuse. These changes can be summarised as differences both in fibre size and in fibre type, as well as the variations in muscle force and fatigue resistance. Various factors are well known to influence muscle fibre type arrangement, which are mechanical loading, nerve activity patterns and various hormones [21–23].

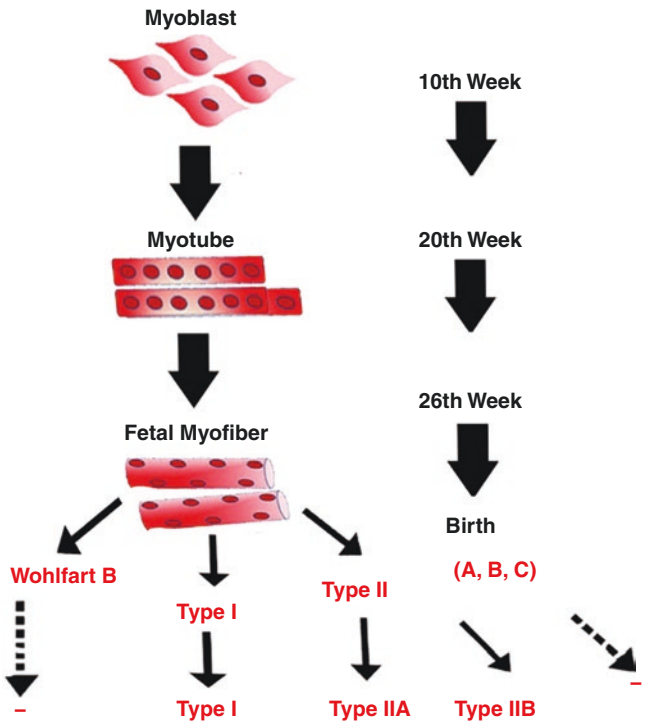

Fig. 2.5 Developmental stages of striated muscles

Embryologic Development of Nerve Tissue

The notochord develops from mesodermal cells just after gastrulation is complete. Sonic hedgehog (Shh) signals play a fundamental role in developing the NT. The notochord does not contribute to the last form of the nervous system but is vital to the developmental model. Signals from the notochord cause an internal folding of the neural plate, which is located next to the blastopore of the ectoderm, forming a neural groove and consequently a NT is formed. Neurulation refers to the folding of the neural plate. The columnar part of

the ectoderm builds up the midline of the neural plate forming the NT and neural crest (NC), while its cuboidal part, the lateral surface ectoderm, makes the epidermis. Before the formation of cerebral vesicles, three major branches of the brain can be differentiated, while the neural groove remains fully open. After a few days, neural crest cells (NCCs) often develop from the neural ectoderm, and near the junction between the spinal cord and brain, the neural folds begin to fuse. This premature NT is originally open at each end, and these pores are named the neuropores. On the 24th day of intrauterine life, the cephalic neuropore, also named the rostral neuropore, closes within a few hours. The caudal neuropore closes after a day. Normally the NT is entirely closed by the fourth week. The failure of these pores to close results in significant neural abnormalities known as NT defects [3]. All central and peripheral nerves develop from the NT [3, 4]. The central nervous system (CNS) (the brain and spinal cord) is formed from the NT, which has developed from the neuroectoderm. The brain is developed from the cranial two-thirds of the NT, and the spinal cord is developed from the caudal one-third of the NT [3].

A structure at the neural plate edge that lies in the dorsolateral regions of the NT, as a pair of streaks, is called the neural crest (NC). Epithelial-mesenchymal transition takes place during the separation of the NT from the surface ectoderm in the cells located along the dorsolateral sides of the NT to form the neural crest cells (NCCs). It has been proven that induction of the NC requires the presence of nonneural ectoderm, the neural plate and mesoderm below the ectoderm. The NCCs are pluripotent and have the capacity of migrating throughout the embryo to form many different cell types. The cells derived from NCCs are neurons, sheath cells, the glia of dorsal root ganglia, autonomic ganglia, sympathoadrenal cells, pia-arachnoid sheath, skin melanocytes, cardiac outflow cells, parafollicular thyroid cells, Schwann cells and connective tissue cells like adipocytes, osteoblast, chondroblast, odontoblast and smooth muscle cells of the head and neck region [24].

The BMPs produced by the ectoderm, Wnt produced by both the ectoderm and mesoderm and FGF produced by the mesoderm mainly support the generation of the NCCs. Retinoic acid, Notch/Delta, endothelin, and hedgehog signalling also play a role in this process. NCC induction can be separated into two phases. Firstly, FGF assists induction through Wnt signalling or directly. BMP at this stage must have an inhibitory effect. Secondly, FGF inhibition leads to BMP activation, associated with Wnt, to create a pathway for signalling. As a result, transcription factors like Pax3/7, Msx1/2, Dlx3/5, Zic1, Ap2, Id3 and Hairy2 appear. Then the second transcription factors like Snail2, Snail FoxD3, Sox9/10, Twist, Id3cMyc and Ap2 are produced. This latter set is very important for the maintenance of the NC and controlling NC behaviours such as epithelial-mesenchymal transition, migration and differentiation [25–29].

Originating from epithelial nerve cells, the peripheral nervous system (PNS) moves from the pia mater to the spinal cord ventricular layer. After differentiation in the spinal cord ventricular layer, migration takes place to form supporting Schwann cells, ependymal cells and neurons. The myelin sheath, composed of support cells, wraps around axons for isolation and increases the speed of neuronal conduction. Myelination of peripheral axons is produced by Schwann cells, while the myelination of CNS axons occurs via oligodendrocytes. Both are derived from neuroepithelium [3, 25].

Since it is not ethically acceptable to conduct experimental studies in human embryos, the embryological development of the human nervous system has generally been simulated through animal experiments. Therefore, the molecular mechanisms that occur in nervous system development are less clear [24, 30–32].

The biological process of neuronal migration during development ensures the correct organisation of the cerebral cortex. Failure of this biological process results in the risk of permanent complications such as lissencephaly, incurable epilepsy and mental retardation. Lis1, Dcx, Ywhae and Ndel1 are accepted as the major contributors of neuronal migration. These genes are part of the LIS1 complex, and therefore, their functions can overlap in neuronal migration and neurogenesis regulation [24].

The four members of the N-Myc downstream-regulated gene (NDRG) family are NDRG1, NDRG2, NDRG3 and NDRG4 which are expressed in different organs and carry out essential processes such as cell proliferation and differentiation. Research has been done on the NDRG1 gene, and its functional relationship with the PNS and CNS is limited. However, there are reported relationships with stress response, nerve myelination, differentiation, exocytosis and both lipid metabolism and biosynthesis [29, 30]. NDRG1 is exclusively expressed by myelinating cell types such as Schwann cells and oligodendrocytes. Also, NDRG1 mutations were reported to lead to demyelination, causing peripheral neuropathy known as Charcot-Marie-Tooth disease type 4D (CMT4D) [30–32].

Histology of the Skeletal Muscle

Skeletal muscles are responsible for movement of the body and can contract due to the complex organisation and interactions of myofilaments. Two types of myofilaments, actin and myosin, are especially associated with cell contraction, which are mainly used for the mechanical work. They are also present in most other cell types and play a role in some

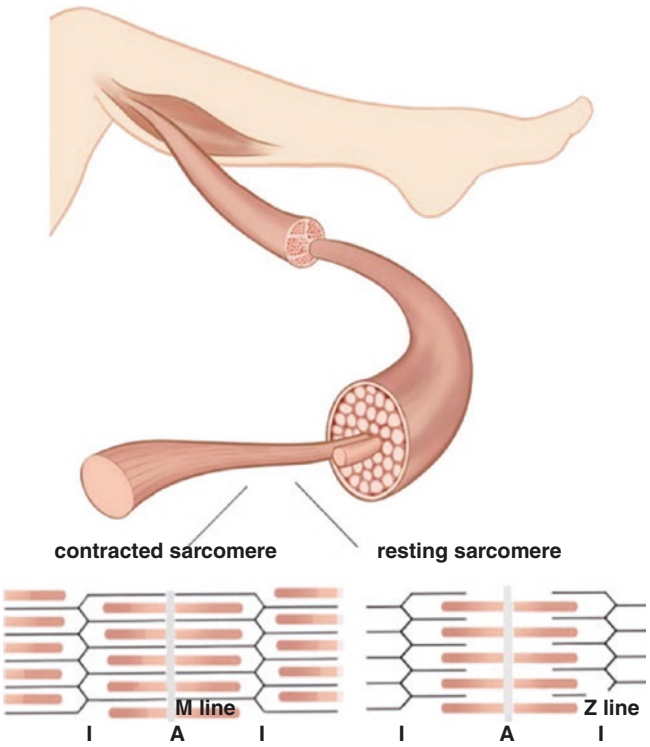

Fig. 2.6 Organisation of skeletal muscle and sarcomeres in resting and contracted states

cellular activities like cytokinesis, cell migration and exocytosis [33]. For example, the contractile proteins of smooth muscles are found in smaller amounts, and they do not arrange as rhythmic repeats like sarcomeres [20, 33]. Most cellular organelles of striated muscle are specifically named. For example, the muscle cell cytoplasm is known as the sarcoplasm; the endoplasmic reticulum is called the sarcoplasmic reticulum (SR); and cell membrane is called sarcolemma and so on. The sarcoplasm includes a regular array of contractile units, known as sarcomeres (Fig. 2.6). These contractile units are mainly comprised of myosin-containing thick filaments and actin-containing thin filaments [20].

The other regulatory and structural proteins are also known as myofibrils, which are arranged longitudinally to the muscle cell axis. Substalk protein complexes called costameres connect all myofibrils to the sarcolemma along the Z discs (Fig. 2.7). By transferring contractile forces from the sarcomeres of one myofiber to another and coordinating the contraction of myofibers in the muscle, these structures prevent sarcolemmal tears. The sarcolemma is attached to the extracellular matrix (ECM), a kind of connective tissue in the cell microenvironment. The ECM of muscle cells includes three layers: the epimysium, the perimysium and the endomysium, which is known as the basal lamina. ECM is required for maintaining the skeletal muscle morphology, mechanical force transmission, performing the muscle cells' physiological functions, neuromuscular junction formation and muscle fibre regeneration [33, 34]. For example, it was demonstrated that type I collagen, a major component of the ECM, enables the myogenic differentiation and migration of C2C12 myoblasts, which is an immortalised mouse myoblast cell line, through the release of IL-6, mediated by FAK/NF-kappaB p65 activation [35]. Failure between the interactions of myofibrils, costameres and the ECM results in sarcolemmal disturbance, which is the reason for some muscular dystrophies [34].

Myosin is a motor protein which plays an essential role in muscle contraction and in other motility processes. Although myosin was originally supposed to be restricted to muscle cells, later it has been determined that myosins are members of a large superfamily of proteins capable of actin binding, ATP hydrolysis with ATPase activity and force transduction. In addition, almost all cells contain a variable amount of different myosin isoforms. In striated muscles, myosin is mainly located in the thick filaments and is responsible for ATP-dependent actin-based motility. Class II myosin, also known as conventional myosin, is the most common myosin type responsible for producing muscle contraction in striated muscle cells [36, 37]. The physiological mechanism of striated muscle contraction is named the actomyosin cross bridge cycle (CBC). Troponin (TN) and tropomyosin (TM), two important Ca^{2+}-sensitive protein complexes of the CBC, are associated with the thin actin filaments. During resting, the myosin head is prevented from binding with actin molecules as myosin-binding sites on actin molecules are covered with TM. The contraction of muscle is regulated by Ca^{2+} released from the SR, which binds to TN on the actin filament. This event removes TM from its place, which was preventing the interaction of the myosin head with actin. Contractions of striated muscle cells require both calcium and ATP. ATP supplies the energy, and calcium is needed to regulate TN and TM. Myosin binding to actin is blocked by TM in a resting sarcomere. The rotation of TM around the actin filaments reveals the binding sites of myosin, and in this way, binding myosin to actin is possible. The position of TM changes with this rotation, and it moves away from the myosin-binding sites on actin, which causes the binding site to open. If the binding sites of myosin are identified and there is enough ATP, the connection of myosin to actin is formed and the CBC is ready to begin. After that, the muscle contracts by shortening the sarcomere [36–38].

Free calcium is an essential regulator of muscle contraction because this binding does not occur without calcium. In addition, for this cycle to end, ATP hydrolysis is required. Breaking the ATP molecule into adenosine diphosphate (ADP) and phosphate (P) releases energy used for the action of the myosin S1 head. In this extended conformation, myosin binds actin, while the released phosphate empowers the contraction of the myosin S1 region. Myosin remains bound

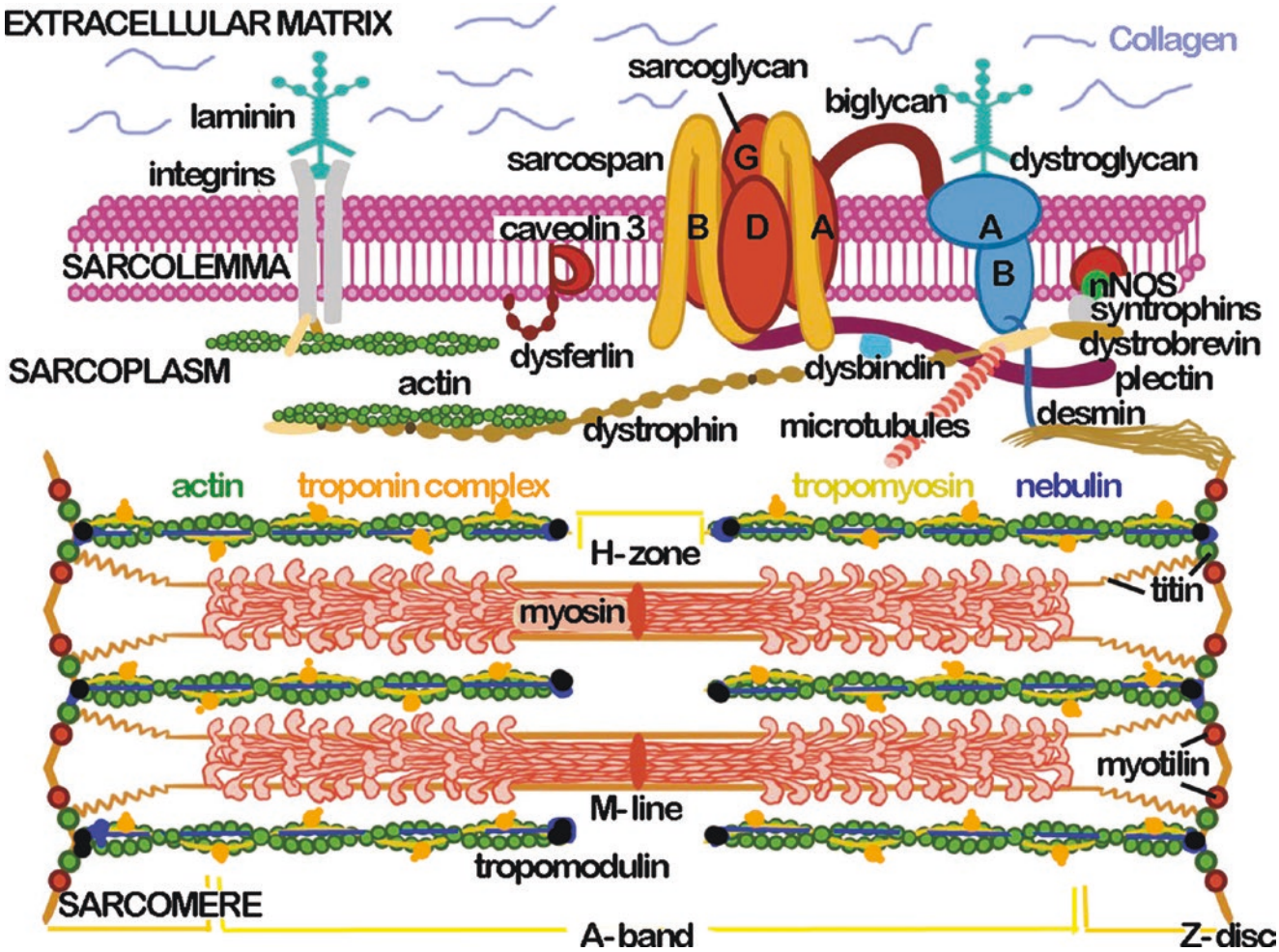

Fig. 2.7 Organisation of the sarcomere and sarcolemmal proteins

to actin indefinitely in the absence of new ATP molecule, causing a phenomenon known as postmortem rigidity [38].

Another two organelles that have important functions in the contraction of skeletal muscle are the transverse tubules (t-tubules) and the SR. The t-tubules are the cell membrane extensions present only in the striated muscle cells. The phospholipid bilayer, similar to the sarcolemma, forms t-tubules. They include an extensive amount of pumps, transporters and ion channels. Therefore, they enable quick communication of the activity in the cell. In addition, they also have an impact on the regulation of cellular calcium concentration. As the tubule network extends throughout muscle cells, the SR wraps around without contact with the myofibrils. SR of striated muscle cells is like the smooth endoplasmic reticulum in other cells. Storing calcium ions (Ca^{2+}) is the primary function of the SR. SR, the main reservoir of calcium ions, is longitudinally arranged as a repeating channel network which extends from one A-I junction to the next A-I junction of the sarcomeres around the myofibrils. There is a connection between the terminal reservoir in the skeletal muscle in the SR and the t-tubules over a short distance via three canals known as a triad, and these are found at the level of the A-I junction. This site is the main source of calcium discharge. The plasma membrane of the terminal cisternae contains a huge amount of gated Ca^{2+}-release channels called ryanodine receptors (RYR). In the terminal cisternae, calcium ions are released from the SR through the RyR in a process called the calcium spark. RyRs comprise three types: RyR3 in the brain, RyR2 in the cardiac muscle and RyR1 in the skeletal muscle. RyRs, found on the SR in different muscles, release calcium in different ways. In smooth muscles such as the heart, the electrical impulse brings calcium ions into the cell from the membrane of the t-tubules in the heart muscle or via the L-type calcium channel in the cell membrane. Intracellular calcium increases by binding these calcium ions to the RyR and activating it. The L-type calcium channel in skeletal muscle is connected to the RyR, which then releases calcium through the activation of the L-type calcium channel following an action potential. Caffeine stimulates RyRs and increases their sensitivity [33,

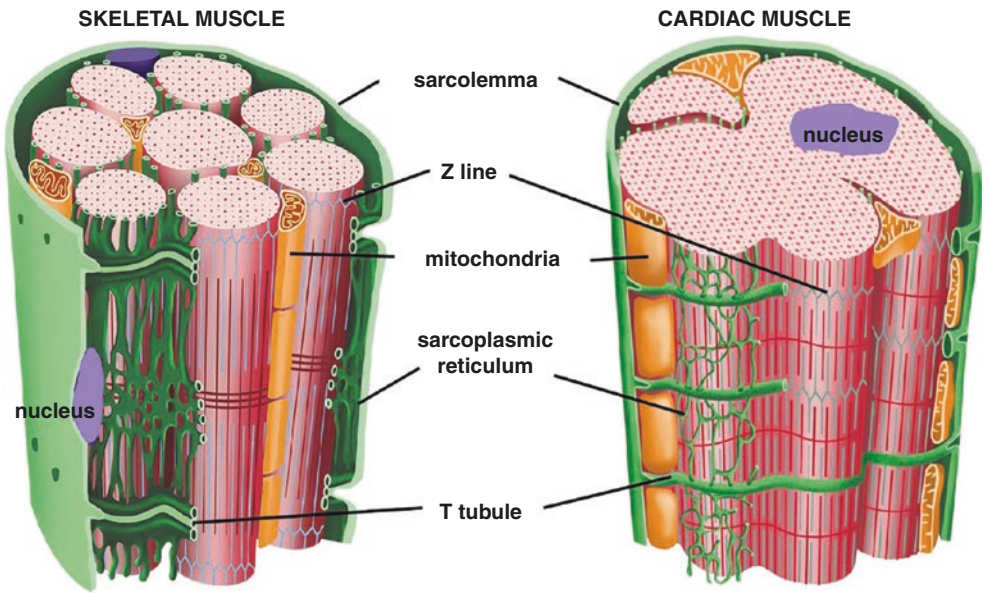

Fig. 2.8 Organisation of t-tubules and sarcoplasmic reticulum in skeletal and cardiac muscles

34]. A protein called calsequestrin in the SR can reduce the amount of free Ca^{2+} in the SR by binding to a large amount of Ca^{2+}. In the SR membrane, Junctin and triadin proteins bind to RyRs. Their primary role is to anchor calsequestrin to the RyRs. These proteins are involved in anchoring calsequestrin to the RyRs, and binding calsequestrin to RyRs, junctin and triadin prevents RyR opening. As the concentration of calcium in the SR decreases, the amount of calcium bound to calsequestrin also decreases. On the contrary, in the case of the excessive increase of calcium in the SR, the amount of calcium bound to calsequestrin also increases, and free binding to the junctin-triadin-RyR complex is possible. Therefore, the opening of RyRs and the release of calcium is possible [38]. However the contraction event of skeletal muscle depends on a nerve impulse arriving at the neuromuscular junction.

Although both are striated muscles, cardiac muscle has some structural and functional differences from skeletal muscle (Fig. 2.8). Firstly, heart muscle has the ability to spontaneously contract or beat, which can be detected in vivo in the embryo and in vitro in tissue culture. The initiation and regulation of the heartbeat is performed by adjusted and specialised cardiac muscle cells called cardiac conducting cells or Purkinje cells. They are organised into nodes, and they generate and rapidly transmit contractile impulses to all of the myocardium. Autonomous nerve fibres terminate in these nodes. The stimulation received by these nerves do not provoke the contractions, but function to alter the heartbeat rate [39–41].

The SR, which is the main calcium reservoir in skeletal muscle, is called the smooth endoplasmic reticulum in both cardiac muscle and smooth muscle. Additionally, skeletal muscle smooth endoplasmic reticulum is more organised than that of cardiac muscle because smaller terminal cisternae are close to the t-tubules and form a diad at the level of the Z line. However, t-tubules are absent in smooth muscle. It should also be noted that t-tubles are both larger and more numerous in cardiac ventricular muscle compared to skeletal and cardiac atrial muscle. Cardiac muscle also has the RYR2 isoform of RyRs [33].

Smooth muscles are very different from striated muscles as they contain different contractile proteins. Their contractile proteins are not in a special rhythmic arrangement, and their contractions are almost always regulated by the autonomic nervous system. Additionally, their contractions occur with the calmodulin myosin light chain kinase system mediated by calcium. Since they are not directly related to the physiopathology of NMDs, they will not be discussed in detail in this chapter [33].

Histology of Peripheral Nerves

The nervous system mediates the basic regulation of human activity. Functionally, the neuron network is divided into peripheral and central parts. The brain and spinal cord are in the CNS, and all spinal and cranial nerve fibres are in the PNS which provides innervation to the end organs. The PNS is subdivided into somatic and autonomic divisions (Fig. 2.9). The autonomic nervous system can be either sympathetic or parasympathetic. The parasympathetic is a rest-and-relax response, while the sympathetic system is a fight-or-flight response. Motor and/or sensory innervation to target organs is provided by peripheral nerves, a complex network throughout the body. Stroma and a functional unit or parenchyma are the two main elements of peripheral nerves. Three layers of

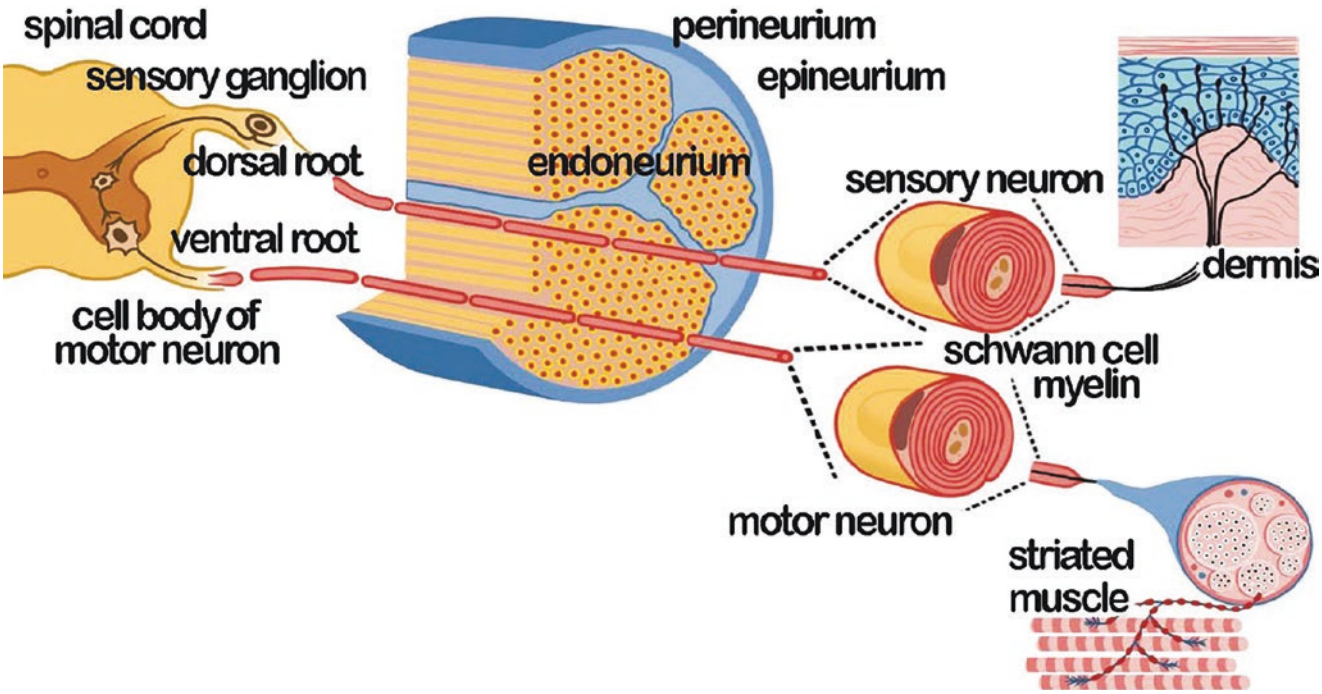

Fig. 2.9 Organisation of peripheral nerves

specialised connective tissue form the stroma, and nerve fibres along with axons and Schwann cells form the parenchyma. Schwann cells have the ability to myelinate a single axon forming myelinated nerve fibres. Interactions between a small group of thinner axons forming unmyelinated nerve fibres with a single Schwann cell are also possible. The fatty sheath called myelin surrounding the nerve fibres, in which there are periodic gaps called the nodes of Ranvier, is responsible for the separation of electrical signals and the acceleration of impulses in the axons. Impulses can move faster by jumping from one node to another compared to moving along the entire length of the nerve fibre. The speed of impulses in myelinated nerves reaches 100 m/s. The stroma regulates the compartmentalisation of the peripheral nerves. In the connective tissue of the endoneurium, located in the transverse sections of peripheral nerves, nerve fibres are scattered, forming separate fascicles surrounded by the perineurium. Additionally, the entire length of nerve is externally surrounded with a vascularised connective tissue called epineurium [42–46].

Nerve signal transmission is carried out by two mechanisms. The first mechanism involves transmitting electrical signals inside cells and along cell membranes. The second mechanism is used for communication between cells, and electrical signals are transmitted by neurotransmitters after being converted into chemical signals. Nerve impulses control all bodily functions. Electrical changes across the neuron membrane cause the transmission of nerve impulses along neurons. The neurons use chemical energy to create a form of electricity. Neurons enter a polarised state when the membrane of an unstimulated neuron has a difference in electrical charge between the inside and outside of the cell membrane. The inside is negative compared to the outside. Excessive retention of potassium ions (K^+) inside, along with excessive retention of sodium ions (Na^+) outside, causes this polarisation. Nucleic acids inside the cell and negatively charged proteins are other ions that play an auxiliary role in the overall negative charge inside the cell membrane. Additionally, despite the leakage of a small amount of K^+ and Na^+ through leaky channels in the membrane, the Na^+/K^+ pumps in the membrane direct the ions in the appropriate direction, and therefore, the resting membrane potential of a neuron remains roughly −70 mV [42, 46–48].

If a stimulus is strong enough, and with the opening of the Na^+ gates, the Na^+ current increases causing complete depolarisation (−70 mV to +30 mV). This stimulates neighbouring Na^+ gates further along the axon to open. Thus, the action potential moves along the axon by stimulating neighbouring Na^+ gates. No action potential is generated if the initial stimulus fails to depolarise neurons beyond the threshold value. As K^+ channels open in response to Na^+ influx, K^+ can leave the cell. The release of K^+ from the cell causes the return of the initial polarisation of the membrane, and thus this is called repolarisation. In contrast to what happens at the resting potential, during repolarisation Na^+ moves in and K^+ moves out. The Na^+ gates close after the K^+ gates open, and the cell membrane enters an abnormal state after the action potential has passed. K^+ and Na^+ are on the wrong side of the

membrane, but the membrane remains polarised. The axon will not respond to new stimuli during this refractory period. Na^+/K^+ pumps in the cell membrane transfer the K^+ and Na^+ to their respective resting potential locations to re-establish the original distribution of these ions. Neurons are ready for another stimulus when all these ions have returned to their resting potential location [42, 46–48].

There are a wide variety of neurotransmitters in the central and peripheral nervous systems, distributed in accordance with the different functions and different localisations of the nerves. A neurotransmitter releases from the presynaptic region and interacts with a specific receptor on the postsynaptic membrane. The action of the neurotransmitter depends on its chemical nature and on the characteristics of the receptor. The receptors can be classified into two main classes, metabotropic and ionotropic. The metabotropic receptors do not only act to open membrane ion channels but also to activate G-protein signalling cascades involved in intracellular signalling. The ionotropic receptors act to open membrane ion channels. Small peptides such as substance P, beta endorphins, enkephalins and free radical NO only activate G-protein [42, 44, 48].

The physiological events at the neuromuscular synapse can be summarised as follows. At first, an action potential arrives at the axon terminal causing the Ca^{2+} channels to open, releasing a Ca^{2+} influx into the axon terminal. At this time, the neurotransmitters are extracted by exocytosis and bind to receptors on target cells. The opening of Na^+ channels then causes the depolarisation of the target cells. Lastly, neurotransmitters are removed from the synaptic region [42].

Monitoring changes in muscle length is the responsibility of particular sensory organs, such as muscle spindles in skeletal muscles. These are mechanoreceptors with the ability to detect stretch. These spindles generate signals which reflect the stretching of muscles and transmit them by afferent neurons to the spinal cord. With this mechanism, the appropriate contractile responses are generated due to monosynaptic connections between motor and sensory neurons. The spindles of muscle consist of several tiny muscle fibres called intrafusal fibres. The central intrafusal fibres are innervated by sensory neurons, and the polar fibres are innervated by motor neurons [23].

Understanding Mitochondria

Mitochondria are known as the energy production centres or power plants of the cell. Mitochondria is an oval-shaped organelle like a silkworm cocoon surrounded by a two-layer membrane (Fig. 2.10). The outer membrane is smooth and surrounds the entire organelle, while the inner membrane is curved and has many folds called cristae. Cells with high energy production have a high number of cristae. For example, the number of cristae in heart muscle cells is three times that found in liver cells. Between the cristae and inside the mitochondria is a fluid called the matrix. It has specific mitochondrial DNA (mtDNA), ribosomes, tRNA and respiratory enzymes [49].

Most aerobic respiration reactions take place in the mitochondria. In this way, energy storage molecules called ATP

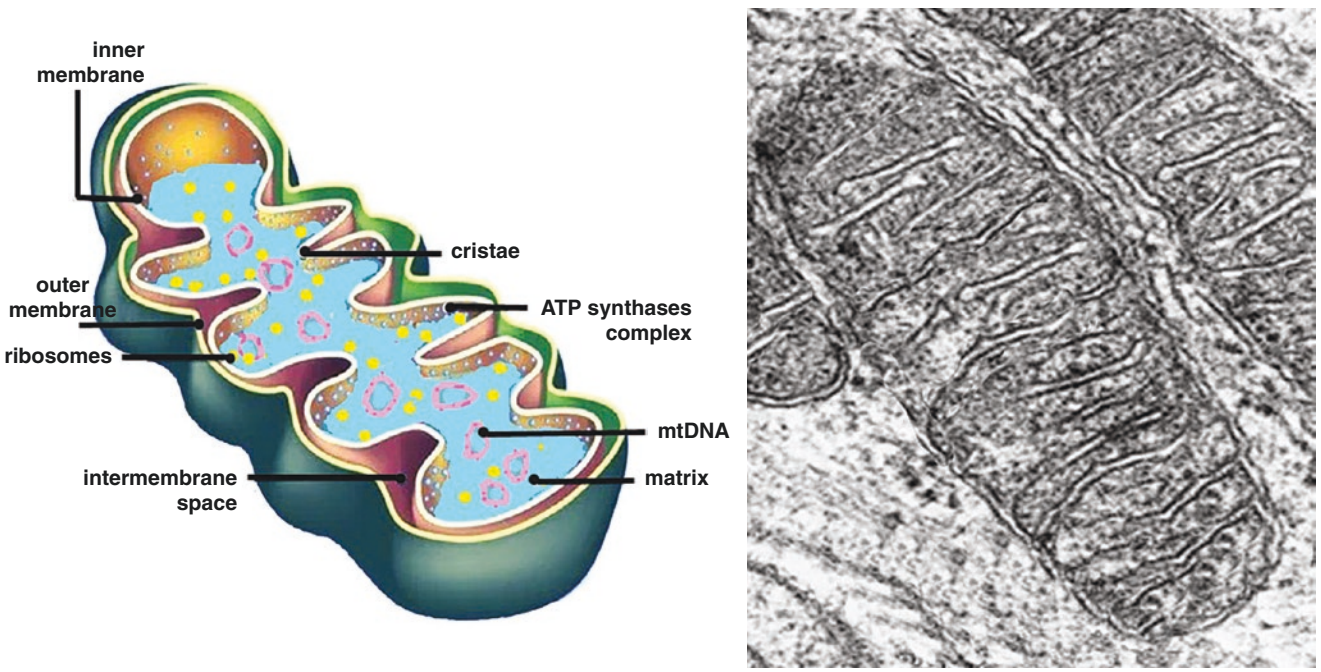

Fig. 2.10 Schematic structures and electron microscopic appearances of mitochondria

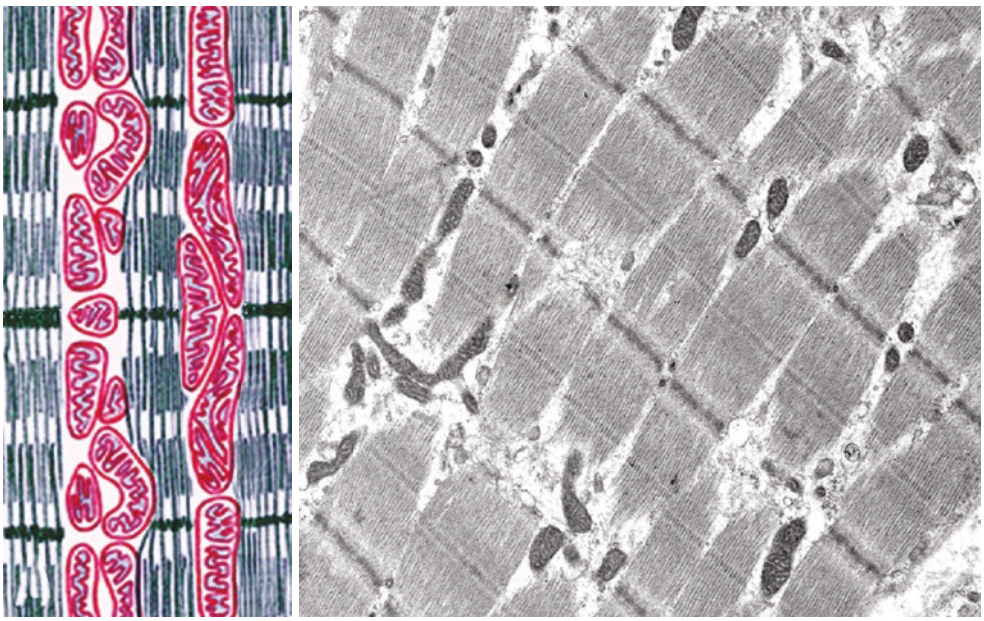

Fig. 2.11 Mitochondria are mostly located between myofibrils. The schematic appearance of striated muscle on the left, and the electron microscopic view on the right

are produced from foods. The number of mitochondria in a cell varies according to energy needs, and this number is between 1 macro mitochondrion and 2000. For example, they are very abundant in muscle, brain and liver cells. Mitochondria make up about 20% of liver cell weight. Their placement within the cell also differs. They are more densely located in the neck region of the sperm to support tail movement. However, in striated muscles, mitochondria are found between myofibrils and under the sarcolemma to provide contraction and relaxation (Fig. 2.11). With regard to human cells, only erythrocytes lack mitochondria similar to bacteria and blue-green algae. These tiny but powerful factories that produce life energy are partly controlled by the nucleus, which is the management unit of the cell. However, they also have their own genetic structure and can reproduce themselves by dividing. In other words, mitochondria are essentially autonomous entities. The most widely accepted evolutionary view is that the mitochondria and chloroplasts in green plants originate from a bacterium that was engulfed by an ancestral eukaryotic cell [50–52].

Mitochondria are the only organelles with genetic material other than the nucleus. Only 2% of the mitochondrial proteins required for the functioning of metabolism are synthesised by the mitochondrial genome. In addition, human mtDNA is a double-stranded circular molecule like bacterial DNA. mtDNA has an independent replication and transcription system. However, since most of the enzymes required for this replication and transcription are synthesised by nuclear DNA, it does not have complete autonomy. While there are two allelic genes belonging to the mother and father in a cell nucleus, each mitochondrion contains 2–10 mtDNA genomes. Mitochondria increase their number autonomously by dividing in case of energy shortage. The mitochondrial content of each cell and the mtDNA content of each mitochondrion are different [49–52].

The mitochondrial genome includes 37 genes. Of these, 13 encode structural proteins in the respiratory chain (nicotinamide adenine dinucleotide dehydrogenase, cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthetase), while 2 encode RNA and 22 tRNA genes. mtDNA differs significantly from nuclear DNA. Besides its core DNA of 3 million kilobases, mtDNA has a very small genome of only 16–17 kilobases. In addition, mtDNA has a compact structure and does not contain introns. The only noncoding region in mtDNA is the 100-base-pair-long D loop region. In addition, the four codons of mtDNA carry different messages from nuclear DNA. For example, AGA, playing the stop code role in nuclear DNA, encodes the amino acid arginine in mtDNA.

There are many causes of secondary mitochondrial dysfunction in old age including ischaemia, anoxia, malnutrition, maltreated/untreated diabetes, hypothyroidism, hormone/neurotransmitter defects, viral infections and alcohol use. Additionally, certain medications affect mitochondrial function, for example, antitumoral (doxorubicin), antibacterial (tetracycline, chloramphenicol) and anticonvulsant drugs (valproic acid). Mitochondrial respiratory chain dysfunction is considered one of the pathogeneses of late-onset neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's. It has been shown that mtDNA mutations occur in tissue with ageing. In addition, inherited nuclear DNA defects also cause mitochondrial diseases because 98% of mitochondrial proteins are encoded by the nuclear genome. However, mitochondrial diseases that

occur because of mtDNA mutations are primitively understood [53].

The incidence of mitochondrial diseases in those under 65 years of age is 1 in 8000. The minimum amount of mutant mtDNA that will cause dysfunction is called the threshold effect (Fig. 2.12). Clinical findings occur when the threshold value is exceeded. The mitochondria in sperm gather almost entirely in the neck to meet the movement energy requirements of the tail. Therefore, the mitochondria remain outside the egg during fertilisation, so mtDNA is almost always of maternal origin. Only 1/10,000 of the mtDNA in the zygote is thought to originate from sperm. Mitochondrial diseases due to mtDNA defects show a specific maternal inheritance (Fig. 2.13) that does not follow Mendelian rules [52, 53].

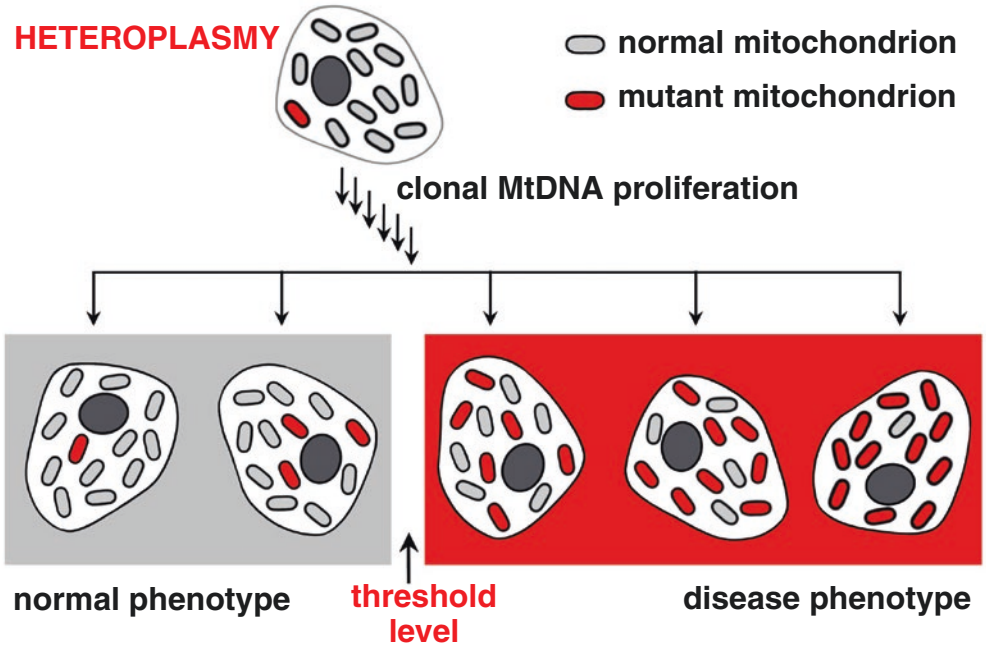

Fig. 2.12 The minimum amount of mutant MtDNA that will cause mitochondrial dysfunction is called the threshold effect

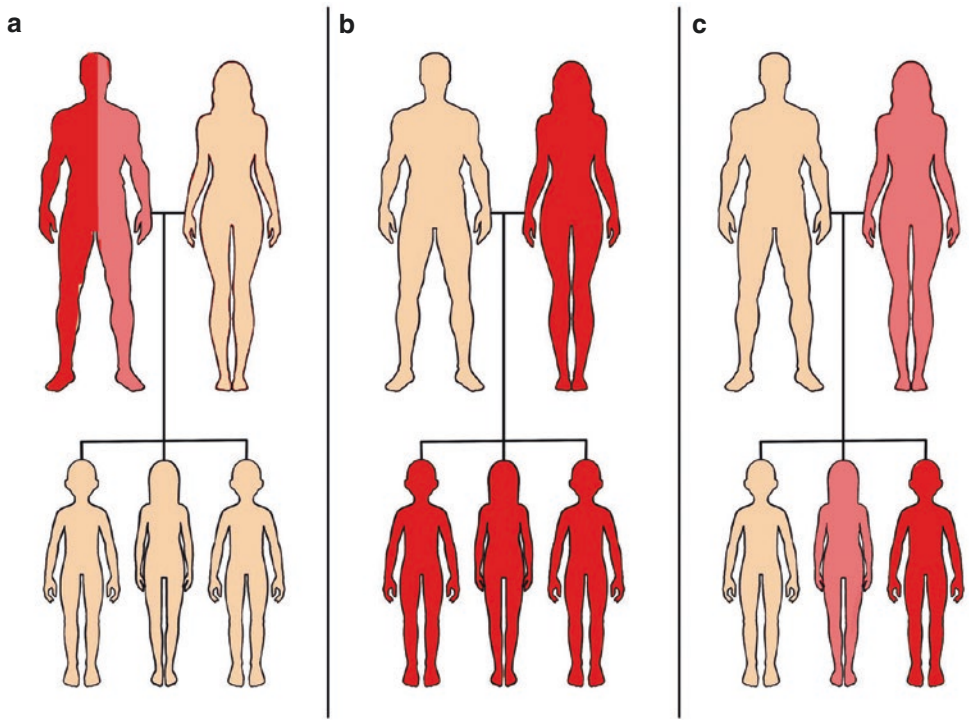

Fig. 2.13 Inheritance patterns of mitochondrial diseases: (**a**) Father with mitochondrial disease (mtDNA homoplasmic or heteroplasmic mutant), (**b**) mother with mitochondrial disease (mtDNA homoplasmic mutant) and (**c**) mother with mitochondrial disease (mtDNA heteroplasmic mutant)

Understanding the Cell's Energy Production

During eukaryotic cell metabolism, life energy is formed from the breakdown of substances from food or previously stored molecules. The free energy generated is stored as high-energy compounds called adenosine triphosphate (ATP): the battery or accumulator in cells. ATP carries out the energy transfer with the phosphate in its structure. The breakdown of nutrients is energy-releasing (exergonic), a catabolic reaction during which the nutrients undergo oxidation. The main goal of oxidation is to release energy. Up to 40% of the energy stored in ATP is used in vital functions. A very small part of the remaining energy is dissipated as heat while maintaining body temperature. Most ATP remains between the bonds of water and carbon dioxide molecules, which are end products of aerobic respiration. A substance called thermogenin is found in brown adipose tissue, which is detected temporarily in newborns and throughout life in some animals. This channel protein partially reduces the proton flow into the matrix and thus reduces the rate of ATP synthesis, allowing the energy produced in the respiratory chain to be kept in the form of more heat. All vital functions such as biosynthesis, active transport, neural transmission, contraction and transmission of genetic information are energy-dependent reactions. Adding a phosphate group to an organic molecule is called phosphorylation. The formation of ATP by adding phosphate to adenosine monophosphate (AMP) and ADP is a form of phosphorylation. There are three ways this can occur. Firstly, photophosphorylation with the help of sunlight takes place in green plants. Secondly, substrate level phosphorylation is a mechanism called anaerobic respiration or fermentation, and thirdly, oxidative phosphorylation is the synthesis of ATP when high-energy electrons pass through the electron transport system or electron transport chain (ETC) during aerobic respiration [49, 50].

An example of substrate-level phosphorylation is the glycolysis reaction where six-carbon glucose is broken down into simpler three-carbon pyruvic acid. This is a common pathway in both aerobic and anaerobic respiration. Two ATPs are used to activate glucose at the beginning of the reaction. At the end of the reaction, two molecules of pyruvate and four ATPs are formed. The net gain is two ATPs. The reaction takes place in the cytoplasm and oxygen is not required. During this process, the coenzyme nicotinamide adenine dinucleotide (NAD) is reduced by gaining two hydrogens. Pyruvic acid decomposes to lactic acid or ethyl alcohol under anaerobic conditions or to carbon dioxide and water in the presence of oxygen. The continuation of this process in which lactate or ethanol is produced is called fermentation. Wine, vinegar, kefir and yogurt making are also fermentation events carried out by microorganisms [49].

Lactic acid fermentation is observed when there is not enough oxygen in human muscle tissue. Here, pyruvate, the end product of glycolysis, is reduced to lactic acid by taking two hydrogens from $NADH^+$ H in the medium. Meanwhile, NADH is oxidised to NAD. The formation of oxidised NAD ensures the continuation of glycolysis. In this way, there is no pyruvate and excessive hydrogen accumulation in the environment. Four ATPs also are gained in fermentation; two of these molecules are net gain. Another molecule that functions in the muscles is creatine phosphate. Lactic acid is metabolised when enough oxygen reaches the muscles at rest which can synthesise some ATP. Creatine takes phosphate from ATP, which is then stored as creatine phosphate. A hydrogen is removed from the remaining lactic acid and converted to pyruvic acid. Pyruvate is then converted to glucose. Glycogen is synthesised from glucose and mainly stored in both the liver and striated muscles. The heart muscle does not destroy the excess lactic acid but uses it as energy. During contraction, phosphate separates from creatine phosphate and converts ADP to ATP. Synthesised ATP is used in contraction and creatine is released. In summary, the energy stored in the muscles are glycogen and creatine phosphate. During muscle contraction, while glycogen, oxygen, glucose, creatine phosphate and ATP decrease, carbon dioxide, lactic acid, inorganic phosphate, ADP and heat increase. All these reactions take place in the cytosol. The presence of a little lactic acid makes the muscle work better. This is the reason why warmup exercises are done before sports. The feeling of fatigue after sports is caused by the effect of lactic acid on the brain cells. Excessive lactic acid accumulation causes physiological tetany and cramps. In eukaryotic cells, the participation of pyruvic acid in aerobic respiration reactions takes place within the mitochondria because the enzymes involved in respiration are in the mitochondrial membranes and matrix (Fig. 2.14). The aerobic respiration reactions come in three forms: glycolysis, the Krebs cycle and oxidative phosphorylation. Glycolysis is a form of substrate-level phosphorylation and forms the first stage of aerobic respiration. The Krebs cycle (citric acid cycle) or tricarboxylic acid cycle is the breakdown of pyruvic acid to carbon dioxide and water under aerobic conditions. This cycle is the common carbon pathway in the respiratory breakdown of fats, proteins and carbohydrates. Because fatty acids and amino acids carry different numbers of carbon atoms, they cause different numbers of ATP to be produced. The resulting amount of water and carbon dioxide is also different. For example, fatty acids carry less oxygen and more hydrogen; therefore during respiration less carbon dioxide and more water are formed. Consequently, lipids are a good source of water storage in animals living in the desert. This reaction begins with the formation of 2C acetyl-CoA from pyruvic acid. The formation of acetyl-CoA indicates that there is oxygen in the environment. $NADH^+H^+$ is pro-

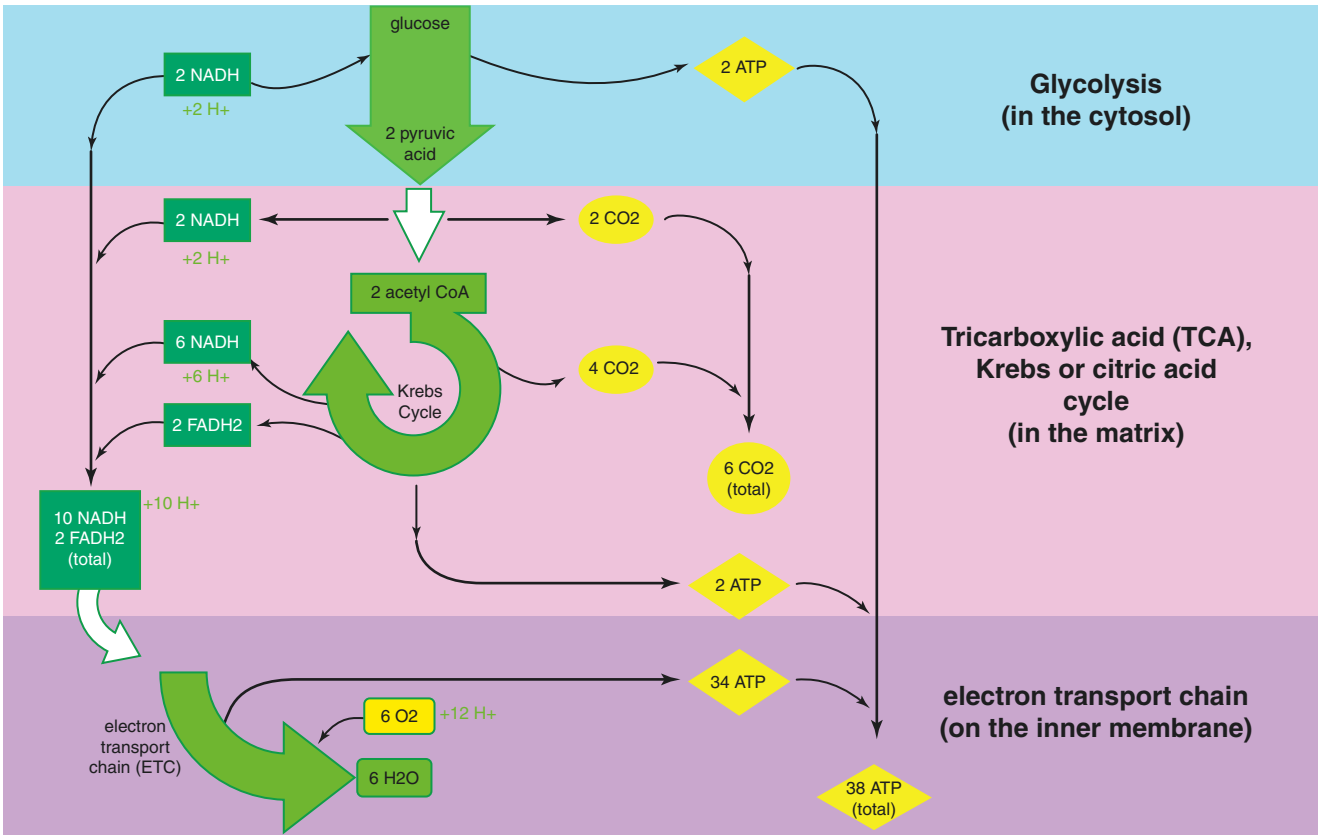

Fig. 2.14 Schematic pathways of energy production

duced during the formation of acetyl-CoA from pyruvic acid. Acetyl-CoA combines with oxaloacetic acid to form citric acid with six carbons. Oxaloacetic acid is then formed again from citrate as a result of sequential reactions. As a result of the breakdown of a glucose molecule, six $NADH^{+}H$ and two FADH2 are formed in the Krebs cycle reaction, and four carbon dioxide molecules are released [51, 52].

Since ATP is produced with the help of enzymes in the Krebs cycle (citrate cycle), it is actually an example of substrate-level phosphorylation. The Krebs cycle occurs in the mitochondrial matrix. Only succinate dehydrogenase, the enzyme involved in the formation of fumaric acid from succinic acid, is found in the inner mitochondrial membrane. This is because this enzyme is also involved in complex 2 of ETC. In the cytosol, the target of the hydrogens that are first removed by NAD and then released is the oxygen taken from the air by aerobic respiration. The aim is to release the energy, while these hydrogens are transported to the final acceptor, oxygen, and to store this released energy by forming ATP molecules. In summary, molecules to which hydrogen or electrons are donated to are oxygen compounds because molecular oxygen is an insatiable electron acceptor. This process is called the electron transport chain (ETC) or oxidative phosphorylation. In other words, an ETC is needed for the formation of more ATP. This ETC takes place in the inner membrane of mitochondria and the molecules in charge are cytochromes and oxygen. The proteins involved are arranged in an array on this membrane. In addition, each of these molecules are enzymes. The enzymes working in this circuit are arranged in the inner mitochondrial membrane from the highest energy level to the lowest, and this arrangement occurs according to the oxidation reduction potential. Each molecule takes electrons from the previous molecule, causing the molecule to be oxidised. Meanwhile, the electron-accepting molecule is reduced. Enzymes that make up the ETC are coenzyme Q > cytochrome b > cytochrome c > cytochrome a > cytochrome a3 > oxygen. Accordingly, the energy level of electrons in the Krebs cycle and ETC is highest in coenzyme Q and lowest in oxygen. The enzymes used in the oxidation of pyruvate and fatty acids and in the citric acid cycle are found in the matrix as well as important molecules such as NAD, FAD, ADP and P. The respiratory chain consists of five separate enzyme complexes in the inner mitochondrial membrane. Complexes I and IV are involved in electron transport, and complex V is involved in ATP synthesis. Enzymes carrying electrons are coenzyme Q and cytochrome A, B and C molecules. Coenzyme Q and cytochrome C are mobile, while other enzymes are attached to the inner membrane. Except for coenzyme Q, all are protein in nature. Several complementary substances are also

needed for these reactions to occur. Iron is found in complexes I–III and copper in IV. In addition, riboflavin (B2), which is a vitamin, is found in the prosthetic group of complex II. These carrier enzymes take electrons and donate them to the next complex. Electrons combine with oxygen and protons in these complexes and water is formed. That is why oxygen is needed and why most oxygen consumption in the body takes place here. While glucose, amino acids and fatty acids are metabolised, coenzymes called nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) take their electrons in the form of H^+, which is released as an intermediate product. If the hydrogen, which is separated from the nutrients through NAD, is transferred directly to molecular oxygen, the reaction will cause a huge amount of free energy to be released, destroying the cell. However, this biochemical reaction takes place in many intertwined stages, and these stages provide the gradual release of energy. Meanwhile, substances that capture hydrogen are reduced. As a result, energy-rich molecules in the form of $NADH^+$ and $FADH^+$ are formed. These molecules donate their electrons, that is, their hydrogens, to electron carrier molecules. For example, reduced NAD transfers the electron it has taken to a flavoprotein, so that it can be oxidised, allowing it to remove hydrogen from a second substrate. Transfer of electrons to carrier enzymes occurs in the form of hydrogen atoms transferring to coenzyme Q and electrons transferring to cytochromes. During each transfer, ADP and P combine to release ATP. This ATP formation takes place in complex V and in the mitochondrial matrix. In fact, most enzymes are attached to the inner membrane. The emitted electrons pass into the intermembranous space between the inner and outer membranes. From here, they pass through complex V and enter back into the matrix. Thanks to the ATP synthetase enzyme in the matrix, they are used to produce ATP with the ADP + P reaction. ADP, which will form ATP in the matrix, is transported from the cytoplasm of the cell to the mitochondrial matrix by an adenine nucleotide carrier. NADH cannot pass through the inner mitochondrial membrane. Therefore, it donates its electrons to the flavin mononucleotide (FMN), putting it into electron transport. The main enzyme of complex I is NADH dehydrogenase; for complex II it is succinate dehydrogenase; for complex III it is ubiquinone-cytochrome C oxidoreductase; for complex IV it is cytochrome oxidase; and for complex V it is ATP synthetase [49, 53, 54].

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The Procedure of Nerve Biopsy

3

Mustafa Barutçuoğlu, Murat Kaan Atalay, and Gulden Diniz

In this section, questions such as how the patients are prepared, which nerves can be selected for biopsy, how large the material should be, how it is sent to the pathology laboratory and what complications may occur in the biopsy area will be answered. Nerve biopsy taking will be explained with pictures and illustrations.

Nerve biopsy is commonly used in specialised centres to diagnose or support a diagnosis in various diseases with peripheral nerve involvement. For example, identifying cell infiltrations, amyloid deposits or determining the special pattern of axonal loss can sometimes be crucial in diagnosing systemic vasculitis and various other systemic or immune diseases [1, 2].

Immunohistochemistry and other advanced methods can clarify many doubts when diagnosing with nerve biopsy [1]. Specific pathological neural tissue findings such as outfolding of myelin in Charcot-Marie-Tooth 4B disease or the presence of very large axons in giant axonal neuropathy may be definitive when determining a final diagnosis [3–7].

The Peripheral Nerve Society guidelines have restricted the use of nerve biopsy for certain clinical situations. Examples include vasculitis involving neural tissue, chronic inflammatory/infectious neuropathies, primary/secondary neoplasms and various genetic diseases with distinctive lesions [1, 8].

The final step in diagnosing an unknown origin of neuropathy is usually to take a nerve biopsy. Detection of specific findings in the biopsy of the removed nerve tissue provides a high level of evidence for pathological diagnosis [8, 9].

The few available guidelines about the technical and evaluation procedures are generally based on the interpretation of nerve biopsies from short case series, which are limited to a small number of specialist pathologists and/or neurologists [8]. If there is still uncertainty in the diagnosis after all necessary laboratory investigations and neurophysiological examinations are complete, a nerve biopsy may be considered. With this procedure, the experience of the surgeon and the evaluation of the neural tissue by an experienced laboratory are of primary importance.

The sample should be taken from a nerve expected to be affected. The sural nerve is an easily accessible sensory nerve that runs superficially between the lateral malleolus and the Achilles tendon (Fig. 3.1). Therefore, it is frequently chosen for biopsy. It generally contains up to 10 nerve fascicles that comprise 3300–8000 myelinated and 10,500–45,500 unmyelinated fibres. It is not advisable to select the sural nerve when it is terminally depleted. In this situation, it can be less informative than a nerve with recently affected fibres. Sural nerve biopsy is widely used and is a useful tool for diagnosing peripheral neuropathy. The most common reasons for choosing the sural nerve in biopsies are the nerve can be easily located, it is purely sensory and therefore no motor deficit occurs after biopsy, and this nerve tends to be affected by neuropathy as it is the distal branch of a long nerve [9].

This surgical procedure has been confirmed to be safe, and serious adverse events are reported in less than 0.3% of cases [1]. Although the morbidity is low, the risk-benefit ratio of the procedure should be clearly explained to the patient, and their informed consent should be obtained. The surgical technique required to obtain a suitable and adequate specimen has been described in detail by some authors. Sural nerve biopsies are generally performed under local anaesthesia. However, sedation may be required in addition to local anaesthesia in childhood. Nerve biopsies should always be performed under sterile conditions by an experienced operator [2, 10].

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The patient is taken to the operating room table in the lateral decubitus position (Fig. 3.2). A surgical pillow is placed between the two knees. The upper knee is slightly bent, whilst the lower leg is extended straight. In this surgical procedure, the sural nerve biopsy is taken posterior to the lateral malleolus. A local anaesthetic, such as 1% lidocaine, is injected into the incision site. The skin incision is then extended 4–5 cm proximally behind the lateral malleolus, parallel to the Achilles tendon (Fig. 3.3).

Adequate exposure of the nerve is required, with an incision long enough to remove it without causing damage. Approximately 3–4 cm of nerve resection is sufficient in adults; less should be taken in childhood. The incision must be deepened carefully until the lesser saphenous vein can be identified. The light-yellow, nacre-like, bright sural nerve is defined medially below the lesser saphenous vein. This part is the most reliable anatomical landmark used to locate it. The sural nerve lies at this level just adjacent to or deep to the lesser saphenous vein. To view the nerve, the vessel must be withdrawn. Rarely, the lesser saphenous vein may be mistakenly identified as the nerve [2, 10].

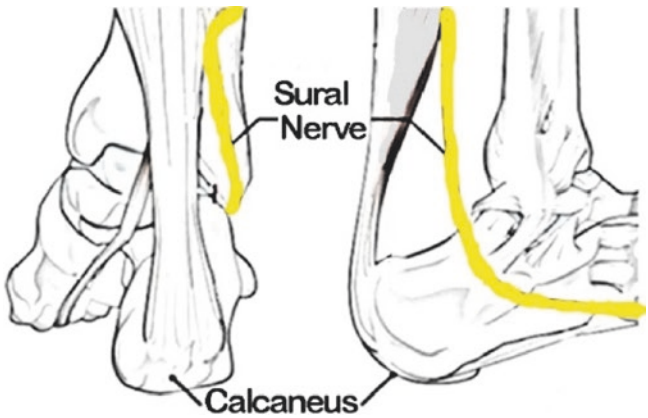

Fig. 3.1 Sural nerve pathway in the foot

It is important to avoid grasping, stretching or squeezing the nerve with forceps during removal. After a careful dissection, a 4/0 or 5/0 silk suture is passed with a right-angle clamp under the nerve which is easily released from the surrounding tissue. These silk sutures are tied proximally and distally without overtightening so that they do not cut the sural nerve (Fig. 3.4). When the sural nerve is identified and before cutting, a small amount of lidocaine can be injected a few millimetres proximal to the transection site with a dental needle to reduce pain. Some authors do not recommend this, as it can damage the integrity of nerve fibres. After cauterising the nerve from above and below with bipolar cautery, it must be sharply cut from the outer parts with straight or curved Metzenbaum scissors. Excessive cauterisation may damage the biopsy material. Less cauterisation may cause neuroma development from the proximal end. Silk sutures on the biopsy are left short proximally and long distally. This shows which side of the biopsy material is proximal and which side is distal after the biopsy is taken. Haemostasis is achieved and the surgical field is duly closed in layers. A single dose of antibiotic is administered during the surgery, and the patient is discharged with regular redressing advice. Stitches are usually removed after 7–10 days. Oral pain relief may be used for a few days. In the early period, contact of the wound with water is not recommended [2, 10].

Regarding other nerves, the superficial peroneal nerve may be preferred as simultaneous muscle biopsy can be taken from the peroneus brevis muscle during the same surgical procedure (Fig. 3.5). If symptoms are more prominent in the upper limbs, superficial radial nerve biopsy is often preferred (Fig. 3.6). On the other hand, if the neuropathy

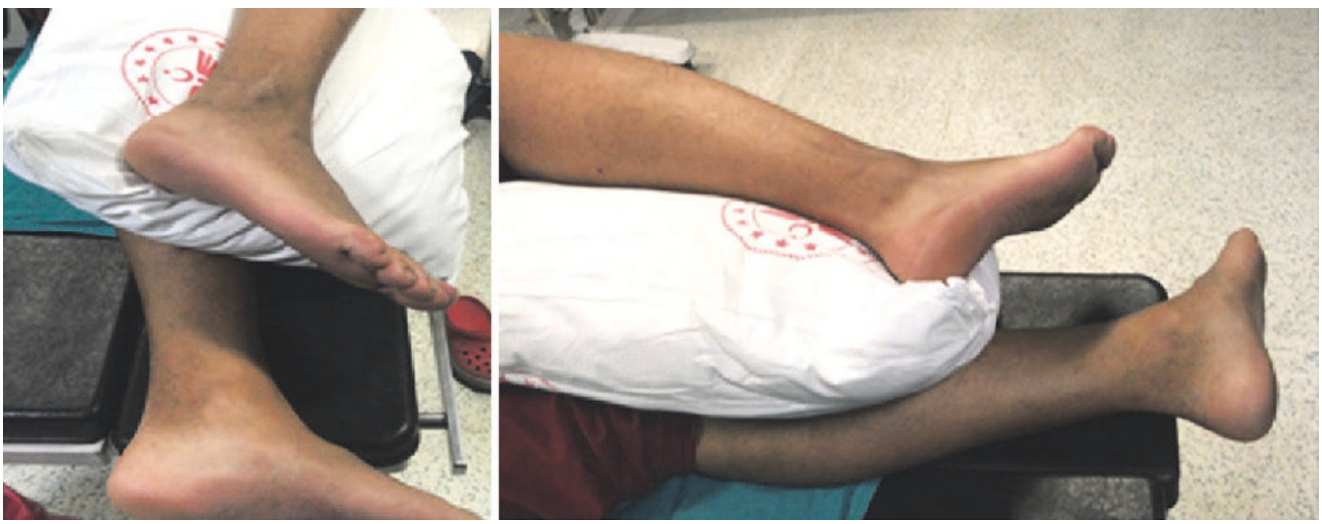

Fig. 3.2 The lateral decubitus position

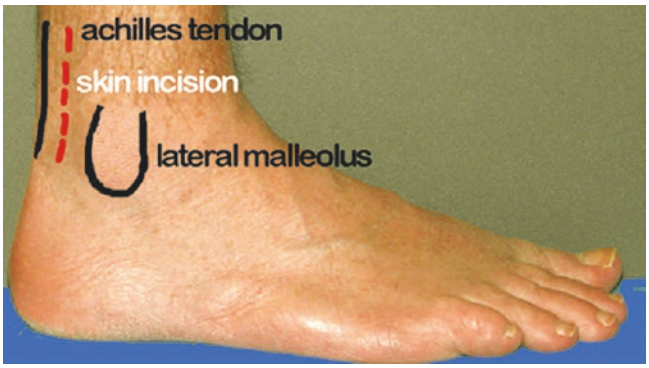

Fig. 3.3 The incision site used to take a biopsy from the sural nerve

entirely affects the motor neurons, obturator nerve biopsy can be very useful to differentiate lower motor neuron diseases and motor neuropathies (Fig. 3.7) [11–15].

It should be noted that biopsy material composed of just a few nerve fascicles does not limit complications such as sensory deficit, and it will be less diagnostic. Therefore, complete excision of the nerve which is approximately 4–5 cm in length is recommended. This is especially important for diseases causing multifocal skip lesions and inflammatory processes. Such foci are more likely to be detected if the entire nerve is examined. Although the morbidity of nerve biopsy is much greater than muscle biopsy, the handling of nerve biopsy is much simpler. In addition, serious artifacts do not

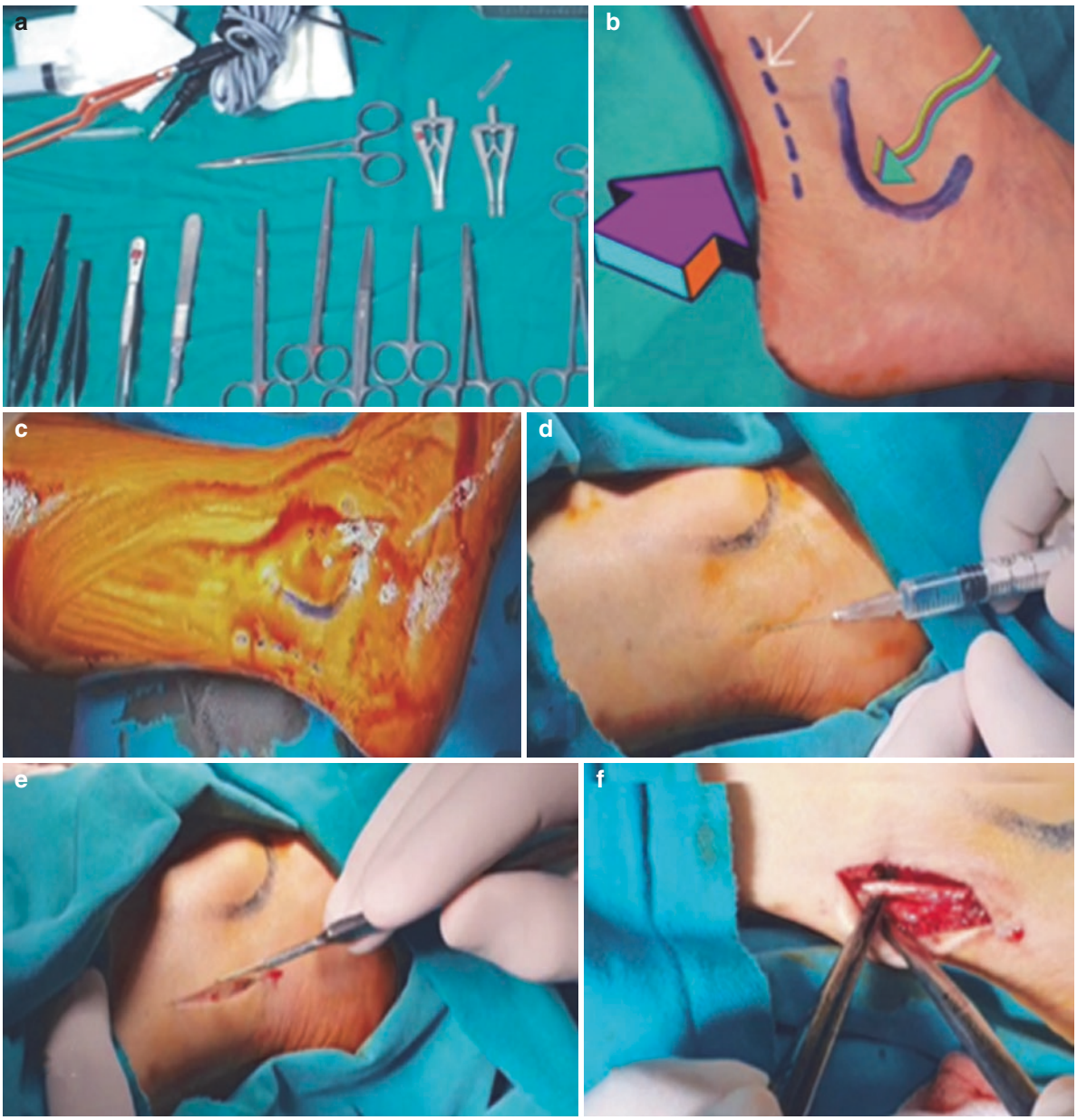

Fig. 3.4 Sural nerve harvesting. (**a**) Preparation stage for nerve biopsy, (**b**) marking of anatomical regions such as external malleolus, Achilles tendon, sural nerve (**c**) Sterilization of the biopsy area (**d**) Injection of the anesthetic drug into the biopsy site (**e**) skin incision, (**f**) searching of sural nerve (**g**) dissection of the sural nerve from the surrounding tissues (**h**) sural nerve biopsy specimen (Photograph taken by M. Fatih Sari, MD, neurosurgeon, Balikesir University Neurosurgery department)

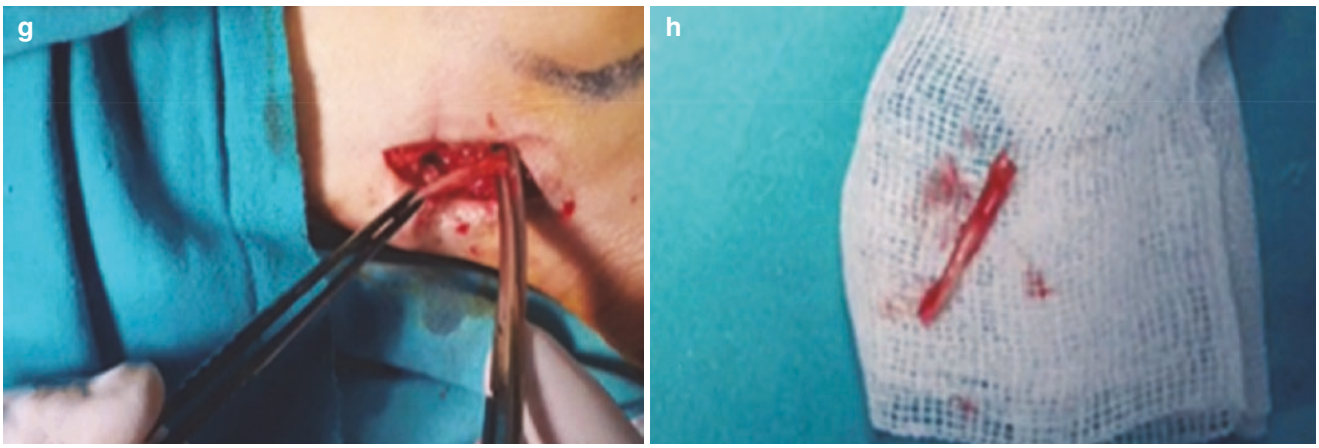

Fig. 3.4 (continued)

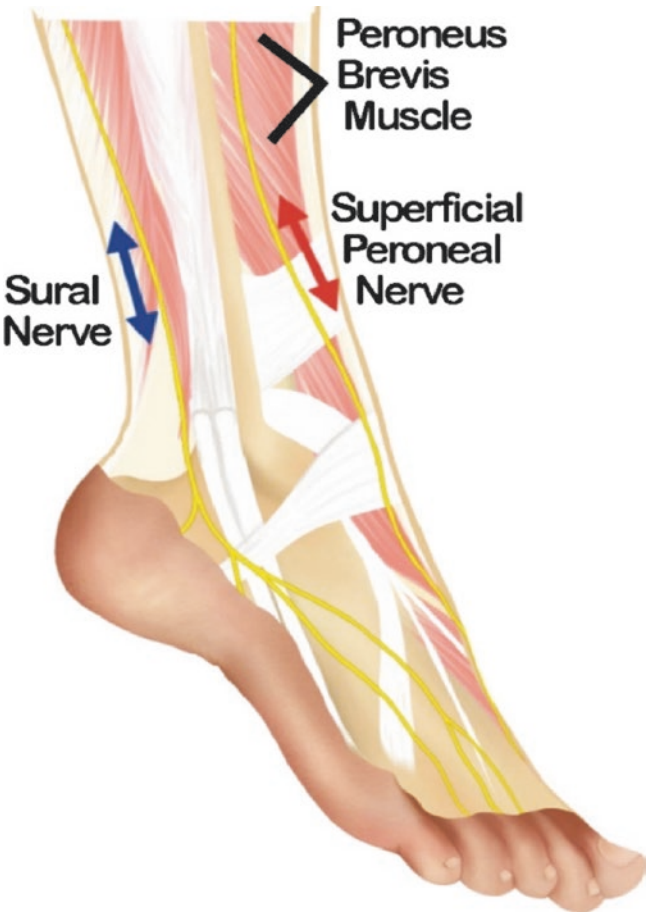

Fig. 3.5 Position of the sural and superficial peroneal nerves in the lateral ankle and dorsum of the foot

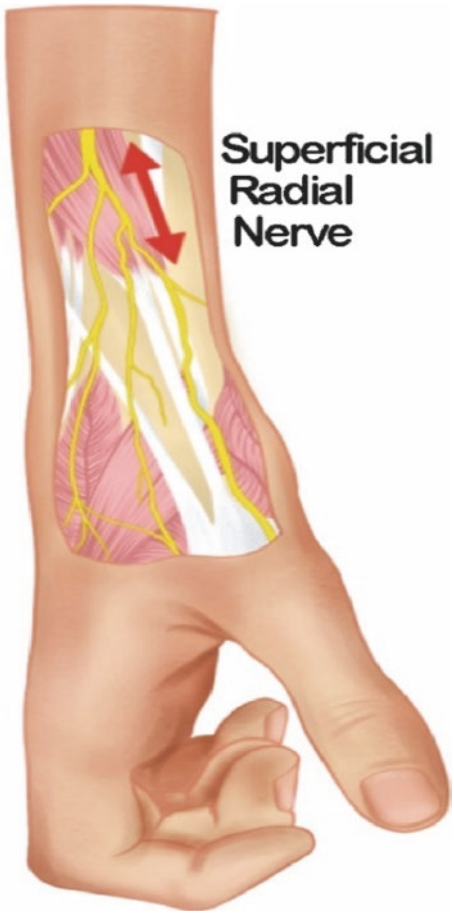

Fig. 3.6 Position of the superficial radial nerve in the radial aspect of the wrist

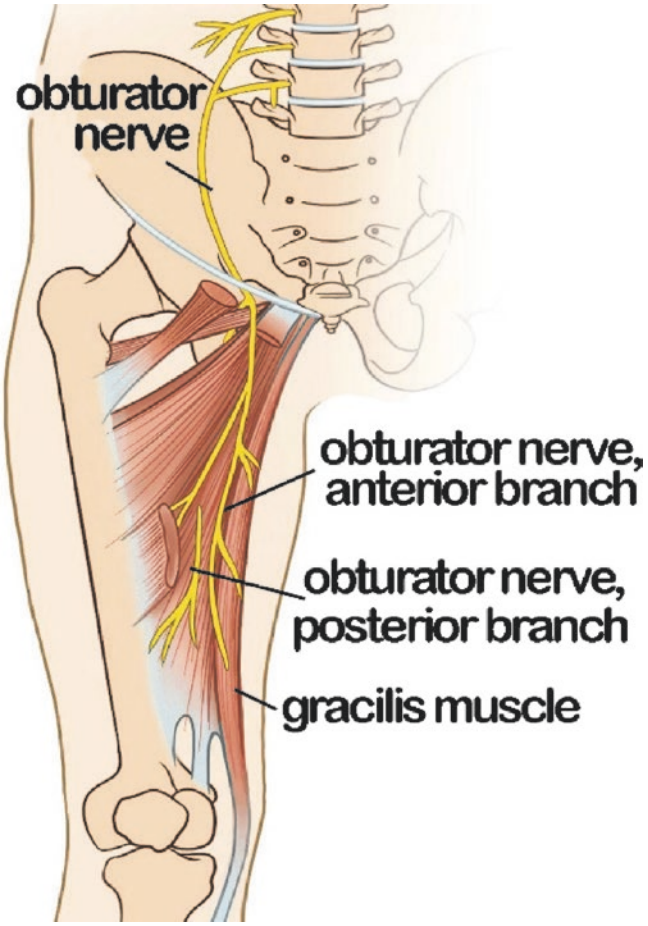

Fig. 3.7 Position of the obturator nerve in the anterior aspect of the hip and leg

occur during transport and sectioning of nerve specimens [11–15].

Theriault et al. published a study describing neurological deficits and complications after sural nerve biopsies in detail [16]. The most common problems were sensory symptoms; chronic pain; wound site complications, including wound infections; and impact on daily life. Permanent sensory deficit affecting biopsied areas was a common and expected complication following sural nerve biopsy (92.9%). Complete sensory recovery is extremely rare, and its rate of occurrence ranges from 0 to 10% in adults and 35% in children [16–18]. In general, this surgical procedure is considered safe.

Motor neuron disease (MND), like amyotrophic lateral sclerosis (ALS), is a neurological disorder characterised by the degeneration of motor neurons. ALS generally affects both lower motor neurons (LMN) and upper motor neurons (UMN). Classical clinical presentation of ALS is usually very diagnostic. Contrarily, motor neuropathy (MN) primarily affects the motor nerves. In most cases, nerve conduction studies differentiate between these two situations. But for some cases in the grey zone, the differential diagnosis may be challenging. As early differentiation between ALS and MN is important for prognosis and therapeutic approach, a motor nerve biopsy should be taken. In this situation, an obturator nerve biopsy with a muscle biopsy from the gracilis muscle should be considered as a potential differential diagnostic tool [19].

Each laboratory has a preferred method for evaluating the nerve specimen. Most laboratories will provide special containers for the biopsy to be placed in immediately. Some peripheral nerve centres have a nerve biopsy kit containing the necessary materials and provide the surgeon with all the materials he needs. For general nerve biopsy examination, frozen, methacrylate and paraffin sections are satisfactory. It is recommended to orient the tissue both transversely and longitudinally for sectioning. Several tissue blocks should preferably be prepared for both paraffin and plastic embedding. Sections should be stained with general purpose stains, and additional sections should be available for special examination. The most accurate approach is for the surgeon to deliver the material to the laboratory without letting it dry by using gauze moistened with saline or in a closed container instead of a fixation solution. Most often, the first step of histopathological examination is the staining of longitudinal and transverse frozen sections with haematoxylin-eosin [11, 12]. It is essential to obtain adequate tissue samples for all pathological examinations. An insufficient tissue sample, inappropriate manipulation and unsuitable specimen transportation will lead to inconclusive biopsy findings after completing this invasive, potentially morbid and costly intervention [11–15].

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The Procedure of Muscle Biopsy

4

Önder Kalenderer and Serkan Erkuş

In this section, questions such as how the patients are prepared, from which muscles the biopsy is taken, how large the material should be, how it is sent to the pathology laboratory, and what are the complications that may occur in the biopsy area will be answered. Muscle biopsy taking and transporting will be explained with pictures and illustrations.

Introduction

Muscle biopsy is an important interventional procedure used to detect underlying neuromuscular disorders in patients presenting with acute or progressive muscle weakness [1–4]. The cause of the clinical condition can be clarified by examining a small amount of muscle tissue, performed by a pathologist experienced in neuromuscular disease. Different findings related to metabolic, infectious, or inflammatory processes can be revealed by muscle biopsy. Consequently, rapid and effective treatment approaches can be prescribed by physicians. Currently, with developing medical technologies, the detection of diseases by using genetic analysis is becoming simpler and more widespread. However, in addition to the diagnostic capacity of muscle tissue histopathological examination, these examinations can also guide the approach of necessary advanced research methods.

Muscle diseases result from the disruption of the structure and function of muscle cells. Many of these, which can be seen at any age, are genetically inherited. Due to recent genetic studies, the differential diagnosis can be confirmed by identifying genetic defects [5, 6]. Genetic analyses performed with venous blood, taken in the amount of 10 cc, can be onerous to perform in terms of cost and logistics. Moreover, the diagnosis of specific diseases may not always be made with such studies. *Duchenne* and *Becker muscular dystrophy*, which are common among muscle diseases, are caused by a defect in the gene encoding the dystrophin protein. Genetic studies can detect a defect in the gene that encodes dystrophin. However, the disease can also be diagnosed by muscle biopsy, performed with a suitable method, as well as clinical information. The reverse is also acceptable [7]. Following the diagnosis of neuromuscular disease with muscle biopsy, the disease group and hereditary characteristics can be revealed by genetic studies. Thus, genetic counselling can be provided to protect subsequent generations by performing family screening. Both methods seem to be part of a complementary process.

Although muscle biopsy may seem simple as a method, it requires extensive planning to achieve an accurate result. This planning should not only incorporate the appropriate technique but also include processes such as material handling conditions, delivery to the pathology laboratory, preparation and examination of samples. Initially, the patient who is thought to have muscle disease is referred to the relevant branch surgeon by the clinician. The fresh specimen taken by the surgeon properly, without damaging the muscle, is immediately transported to the laboratory where the muscle can be examined within the appropriate shipping rules. Here, the pathological findings are evaluated by an experienced pathologist, and the accurate diagnosis is made in the light of clinical findings. Therefore, the entire planning process should be prepared and implemented in cooperation with an experienced physician, surgeon, pathologist, and technical team [7, 8]. In this section, these procedures for muscle biopsy will be described.

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Indications

Muscle diseases frequently appear in childhood or adolescence. Muscle weakness is a condition that affects normal daily functional life. The inability to acquire motor skills such as holding one's head up, sitting, walking, and standing or being lax (hypotonic) during infancy is probably due to causes of muscle weakness seen in early age groups. In older children, teenagers or adults, clinical reflections occur with the involvement of major muscle groups in the lower extremities. Difficulty in ordinary daily activities such as walking, running, going up and down stairs, and getting up from sitting are preliminary warning signs for muscle diseases. In some cases, the affected muscle groups are in the upper extremity. Hence, patients may have difficulty in performing simple functions such as combing their hair, attaching hairpins, and reaching for the shelf, which requires raising their arms. These routine movements, which we do not have difficulty in doing in daily life, cause great problems for patients with muscle diseases. Since muscle diseases are a rare group, there may be delay in recognition or negligence. It is important to observe the signs, especially in infants and young age groups. Gradually deteriorating functions may cause activity changes in children. With symptoms such as frequent fatigue and weakness, children may turn to play activities where they can make less effort. Families may consider this behavior of their children as spoiled, lazy, and attitudinal, and therefore there may be delays in seeking a physician. In muscle diseases, besides weakness and functional disorders, cramps and pains may also present as complaints. In such functional losses, muscle biopsy can be used as a diagnostic procedure if myopathy is likely according to clinical and laboratory examinations.

Muscle diseases can be metabolic, inflammatory, and infectious in nature or affect the neuromuscular junction. These distinctions can be made with specific examinations performed during muscle biopsy, providing identification of the disease or disease group [9, 10]. In *Duchenne muscular dystrophy* and *Becker muscular dystrophy*, which are among the most common muscle diseases, absence or low levels of dystrophin protein in the biopsy will be the pathological finding leading to the diagnosis [11, 12]. Inflammatory myositis such as *polymyositis*, which is limited to muscle tissue, and *dermatomyositis*, which is a disease of collagen tissue and involves skin, muscle, and subcutaneous tissue, are conditions that can present with muscle weakness. Detection of pathological findings such as inflammatory cells and perifascicular atrophy in muscle biopsies performed in these patients support the diagnosis [13–15]. *Inclusion body myositis*, another type of inflammatory myositis, can be distinguished histopathologically from a neuropathy-like condition by the presence of rimmed vacuoles [16]. Differential diagnoses can be made with a muscle biopsy performed in a patient with a suspected prediagnosis of *amyotrophic lateral sclerosis (ALS)*, which is a progressive muscle disease with motor neuron damage [17, 18]. In addition, muscle biopsy can define parasitic diseases such as *trichinosis* and *toxoplasma* histologically. Muscle biopsy can also be performed in some autoimmune diseases such as *myasthenia gravis*, which can involve voluntary muscle of the body, especially those that control the eyes [19]. In these patients, the diagnosis can be confirmed by a muscle biopsy from the eyelid performed by oculoplastic and orbital surgeons.

Biopsy Procedure

Muscle biopsy is a procedure that requires meticulous attention and should be performed by a competent surgeon. It can be performed under regional anesthesia and less frequently under general anesthesia, depending on the patient's age, compliance, and disease involvement. General anesthesia is often preferred for young patients who cannot be oriented and cooperative during the procedure or in patients who cannot be immobile.

Muscle biopsy can be performed using two methods: (1) open biopsy and (2) needle biopsy. During both methods, the muscle tissue should be excised from the relevant muscle tissue without damaging the specimen, and it should be immediately delivered to the pathology laboratory appropriately. Regardless of the method used in muscle biopsy, an equipment assessment should be completed before the procedure, and the pathology laboratory conditions should be discussed with the authorized personnel.

Open Biopsy

Incisional biopsy is the more preferred method. It is performed by directly seeing the muscle following local anesthesia applied to the skin and subcutaneous tissues. Before an open biopsy, the biopsy location should be evaluated, particularly the regional infection risk, circulation of the extremity, and palpability of the muscle tissue.

It is crucial that the procedure room where the biopsy will be performed is compliant with the local sterilization rules and that the surgical team obeys the rules of asepsis. The patient is initially taken to the theatre/procedure room, and the preparation process begins by appropriately positioning the patient for access to the biopsy site. Then cleaning of the skin area over the biopsy site is performed with an antiseptic solution such as betadine or chlorhexidine. The

sterile area is covered and prepared for the procedure. The surgeon injects a local anesthetic in the skin and subcutaneous tissues in the area where the muscle tissue can be palpated. During this time, contamination with medication and damage to the muscle tissue should be avoided. Generally, 5–20 mL of 1% lidocaine is sufficient for anesthesia. Since the use of epinephrine (1:100,000) in addition to lidocaine can reduce regional bleeding, it can be applied in combination. After the local anesthesia procedure, there will be no response to painful stimuli in the relevant area within 2–3 min. Then a small skin incision is made with the scalpel. The thickness of the soft tissue between the skin and muscle tissue varies according to the biopsy location and the physical structure of the patient. In the proximal thigh area where there is a thicker fat layer, the skin incision may be larger than in the upper extremity or distal lower extremity incisions. After the skin incision, the subcutaneous soft tissue is dissected bluntly, and the muscle tissue covered with fascia is made visible with the help of an automatic retractor. The fascia tissue is cut with the scissors and the muscle body is exposed. The area to be taken for the fresh specimen must be from muscle body where the muscle fibers are dense. In the musculotendinous areas, since there are more connective tissues than the muscle fibers, the evaluation of the samples obtained from this region will be insufficient. Before the muscle tissue is excised with small sharp scissors, the muscle can be marked with a thin suture, which can be safely removed after excision. The amount of muscle tissue needed may differ according to the laboratory examinations to be made. Generally, a sample measuring $1 \times 0.5 \times 0.5$ cm with a long axis parallel to the muscle fibers is sufficient for general examinations (Fig. 4.1). It is crucial to avoid bruising, crushing, or traumatizing the muscle tissue during excision, as this may disrupt the examinations. The sample taken is prepared and sent to the laboratory via carrier personnel as soon as possible, without compromising sterility.

After biopsy, the surgical area is evaluated in terms of bleeding. After bleeding control (hemostasis) is achieved, first the fascia and then the subcutaneous and skin tissues are repaired, and a sterile bandage or dressing is applied. Methods such as epinephrine, pressure application, cauterization, and ligation can be used for bleeding control. It may be preferable to wrap a bandage with moderate pressure for a few hours after dressing.

Needle Biopsy

Needle biopsy is performed by inserting a special needle into the tissue to be sampled. Since the amount of tissue taken at one time will be small, more than one sample may be required depending on the examination (Fig. 4.2). A smaller incision is made in the surgical area, which is prepared similarly to an open biopsy. After the fascia is passed, sampling is done with the help of a 5-mm-diameter Bergström needle or similar, such as Weil-Blakesley conchotome. Since the material obtained is relatively small, vacuum-assisted methods have started to become more popular over time to gain larger amounts [20–22].

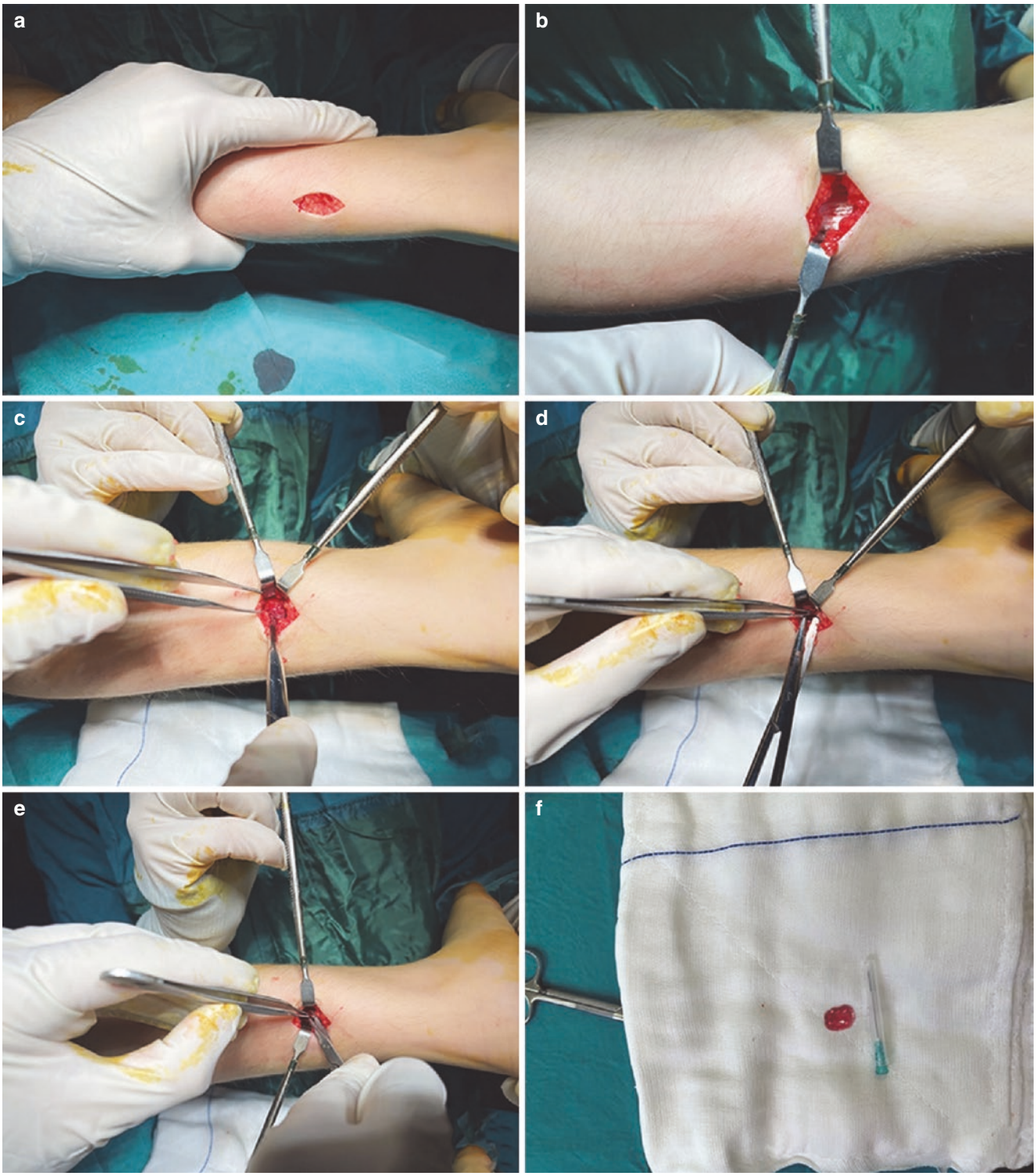

Fig. 4.1 Steps of open biopsy technique for gastrocnemius muscle (**a**–**f**). Following local anesthesia, a small skin incision is made (**a**). After the skin incision, the subcutaneous soft tissue is separated, and the fascia is made visible (**b**). The fascia tissue is cut with the scissors, and then the muscle body is reached (**c**). The muscle tissue is excised with small, sharp scissors (**d**, **e**). The fresh specimen is placed on saline-soaked gauze (**f**). Fascia is sutured and the skin is closed

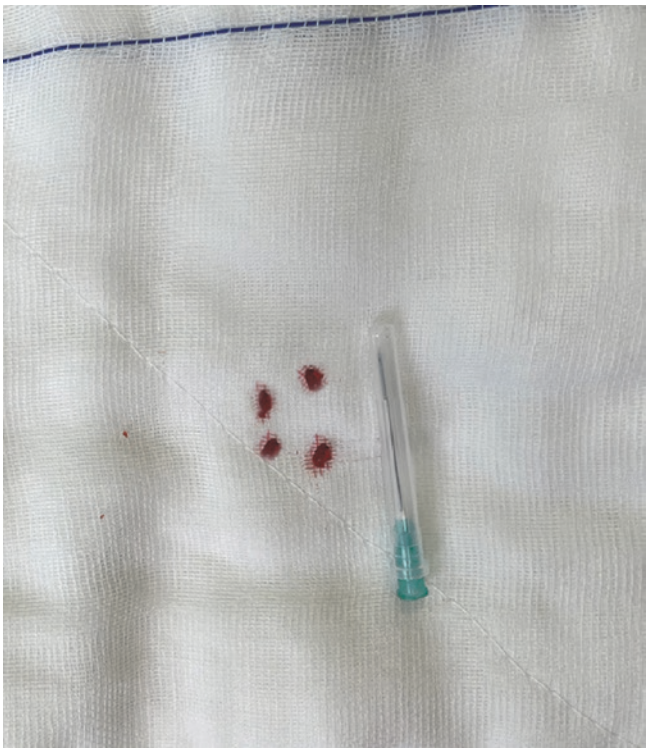

Fig. 4.2 Specimens obtained by using a needle biopsy device

Patient Preparation Before the Procedure

The general health of the patient who will undergo muscle biopsy should be evaluated. Before the procedure, an informative conversation should take place between the patient and the physician. The discussion should include how the muscle biopsy will be performed, what she/he may feel during the procedure, and complications that may occur after the procedure. Afterward, the informed consent form should be read and signed by the patient. The biopsy is usually performed as an outpatient procedure, and pain is prevented by regional anesthesia. For those receiving local anesthesia, it would be appropriate to prepare any medications regularly taken before the procedure, in consultation with the patients' usual physicians. If general anesthesia will be used, the preparation processes of the patients will be longer and more detailed. Which method will be utilized is determined according to the discussions and evaluations between the patient and the physician.

Although the area is numbed using local anesthesia, mild discomfort feelings such as detraction can be perceived by the patient, while muscle tissue is excised. The patient should be informed beforehand that this is a normal process.

Muscle biopsy is generally taken from major proximal muscle groups such as the deltoids, quadriceps, biceps, and triceps. However, it is important to know the extent of the disease when determining the area where muscle biopsy will be performed. In the presence of some distal myopathies, tibialis anterior, forearm extensor, or flexor muscles may be preferred. At this point, evaluations made using electromyography (EMG) and/or muscle strength tests can help to identify the muscle area where disease involvement is the most prevalent, while also assessing for the area which is most surgically accessible with a low risk of complications. Biopsy samples obtained from symptomatic muscle (power of 4/5 on the Medical Research Council scale used to grade strength) gain significance [23]. Furthermore, in metabolic-induced muscle diseases, biopsy materials obtained from the most involved and/or least involved muscle tissues may become crucial.

Biopsies of patients referred for muscle biopsy should be performed before starting immunosuppressive therapy, especially in inflammatory myopathies. It should be kept in mind that immunosuppressive agents may disrupt muscle tissue structure and cause false evaluations. Additionally, a 6-week waiting period is recommended between procedures, since EMG examinations can also cause changes in muscle tissue.

Shipping of the Specimen

Muscle biopsy can be considered as a relatively simple and minor surgical intervention. However, in order to optimize the benefits obtained from the procedure for the patient, each step should be performed carefully and duly in accordance with other procedures. There are still only a few centers where histopathological examinations of muscle tissue are performed. Performing the biopsy without the cooperation of an experienced laboratory, and instead sending it to a routine pathology laboratory, will bring about limitations in the process and repetition of interventional procedures may be required.

Whether it is an open biopsy or a needle biopsy, the obtained fresh specimen should never be placed in a protective liquid. The muscle tissue is instead placed between gauze which is moistened with the help of physiological saline so that it does not dry out. The material should be shipped to the laboratory quickly in a sterile and airtight pathology examination container. If the transport process will exceed 3–4 h, the sample container should be placed in a thermos containing a water-ice mixture. If the biopsy material is soaked in saline, or gauze pads are sodden, the muscle tissue may absorb the fluid. These liquids can turn into ice crystals during storage. Technical mistakes which may occur during these controllable steps can make investigations inconvenient. The most appropriate transportation and storage conditions should be made in cooperation with

the pathology laboratory where the sample will be examined.

It is important to convey the patient's information with the muscle pathologist as well as the technical transport details of the biopsy material. The patient's age, clinical findings, pathological biochemical tests, physiological status, and preliminary diagnoses should be shared with the pathologist. The pathologist may send referrals for other laboratory investigations that may be necessary in the muscle tissue histopathological examination. To ensure accurate management of this process and to optimally benefit the patient, technical applications of the processes must be carried out properly, and the communication of key information within the team must be complete.

Technical Issues

Muscle biopsy is a technical procedure with some considerable details. It is important to pay attention to these technical details to diagnose the disease or disease group correctly. Sample material taken from a muscle group affected by the disease is the most critical step that will lead to the correct diagnosis. However, it should be kept in mind that in muscle diseases, the involvement may not be diffuse but may instead by irregular. Therefore, it should be noted that the sample taken during biopsy can be reported as having "no pathological findings" in disease with irregular involvement such as polymyositis. Moreover, samples from weakened or atrophic muscle tissue may not be histologically recognized as "skeletal muscle tissue." If this occurs, the pathological condition causing the disease may not be detected.

It is important to convey the muscle tissue information to the pathology laboratory to aid histological examination. Although skeletal muscle tissue has a similar structure in each region, there are three different muscle fibers and myofibrillar distributions of different densities. For example, slow oxidative fibers (type 1) are more localized in the limb and back muscles associated with walking and maintaining posture. These muscle fibers are smaller in diameter than others, and they contain high levels of myoglobin and mitochondria. Fast glycolytic fibers (type 2b), which have properties opposite to type 1 fibers, are dense in the finger and eye extraocular muscles, which contract quickly and allow for the performance of fine motor skills. Rapid oxidative-glycolytic fibers (type 2a) are like a histological combination of both muscle fibers (type 1 and type 2b). They contain high glycogen and myoglobin. Some neurogenic disorders can affect the distribution of these muscle fiber types [4, 24–26]. In line with this basic information, if the muscle tissue type taken during biopsy is not stated to the pathologist, it may be required to repeat the biopsy due to misdiagnosis or inconclusive examination.

Muscle biopsy is a procedure that does not often need to be repeated throughout the course of disease. Examinations made at the right time and from the appropriate muscle tissue are sufficient. However, in cases such as centronuclear myopathy, there are differences in biopsy examinations performed in early childhood and in the following years. Diagnosis is supported with these changes. But, in cases such as *Duchenne muscular dystrophy*, the disease can be detected by the initial biopsy procedure, and subsequent investigations will only show that the involvement is progressing. Furthermore, properly stored specimens can be later re-evaluated by another experienced pathologist using different methods.

During muscle biopsy, care should be taken to remove the muscle tissue without trauma. It should be noted that the use of electrocautery during the procedure may disrupt the histological structure of the sample and render it unrecognizable. It is crucial to remove the muscle tissue using the appropriate surgical instruments without crushing or fragmenting it. Designed for this purpose, auxiliary tools such as the Rayport clamp can be used, or the muscle tissue can be excised by hanging it with suture materials.

With the needle biopsy method, a smaller sample is obtained compared to the open biopsy procedure. In some labs it may not be possible to process these small samples. For this reason, it is important to coordinate with the laboratory before a chosen method is applied. Since any muscle tissue can be pathological in a diffusely distributed disease, needle biopsy becomes more relevant. When a small amount of muscle tissue is required for a biochemical examination in such diseases, needle biopsy is a useful alternative.

Complications

If the biopsy was performed under local anesthesia, the patient may be permitted to go home after a short period of clinical observation. Resting for a day or two, keeping the biopsy area clean and dry, and dressing at appropriate intervals are sufficient to ensure recovery. After the applied lidocaine effect time is completed, pain may be felt in the procedure area. Rarely, signs of infection such as pain, redness, fever, swelling, discharge, and undesirable conditions such as bleeding may occur in the surgical area.

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Preparation of Muscle and Nerve Biopsy

5

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In this section, the freezing, cutting and staining processes of muscle and nerve biopsies in the pathology laboratory will be explained in detail. In addition, the staining principles of commonly used histochemical, enzyme histochemical and immune histochemical stains specific to muscle and nerve biopsies; the preparation of dye solutions and the staining method will be explained in detail. Electron microscopic procedures will be also explained in this section.

Preparation of Muscle Biopsy

Changes in any tissues due to injury can be evaluated as a spectrum which differs according to tissue types. This spectrum is very narrow in some tissues such as peripheral nerves. We will also explain in detail in the nerve biopsy section that loss of myelin, the presence of axonal degeneration and inflammation are the three main changes we observe in peripheral nerve biopsies, and we can only grade these differences [1]. However, skeletal muscle shows a wide spectrum of histopathological changes when exposed to injuring agents. In addition, if the necessary conditions are not met during the biopsy section and follow-up procedures, the skeletal muscle cells show changes that mimic diseases due to the special myofibrillar arrangement, the rich mitochondrial content and the original network of T-tubules [1, 2]. For example, in a muscle biopsy sample, if we do routine follow-up and take sections from paraffin blocks, the changes we can see in paraffin sections are minimal. Moreover, enzyme stains and most immunohistochemical stains do not work. The muscle biopsy must be examined in frozen sections. In addition, no matter how advanced the frozen device is, in sections taken without liquid nitrogen, muscle cells look like a sieve, and the biopsy becomes unusable because of the crystallisation of sarcoplasmic fluid (Fig. 5.1). Muscle biopsy is generally ordered for diagnostic purposes, used by clinicians who evaluate patients with weakness suspected to be caused by muscle disease [1–5].

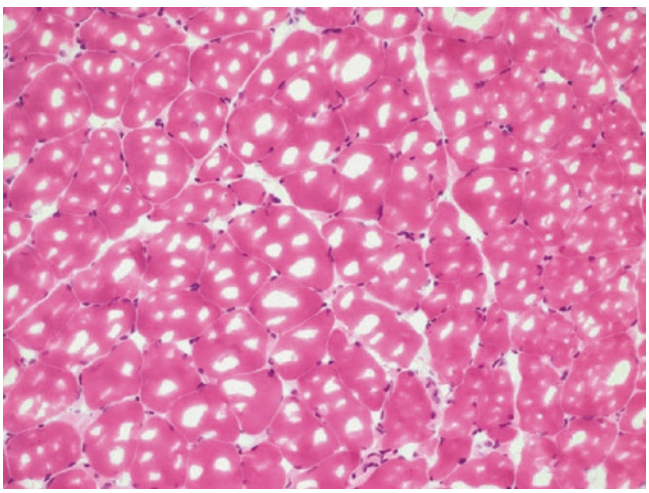

Fig. 5.1 The vacuolar appearance due to slower freezing without liquid nitrogen (H&E × 100)

Clinical information is essential when evaluating a muscle biopsy, and clinicopathologic correlation is critical for accurate interpretation. The clinician must provide the patient's clinical data. Personal communication between the pathologist and clinician is crucial. Therefore, it is claimed that the phone is almost as useful as the microscope in interpreting muscle biopsies. Generally, an open biopsy procedure to obtain muscle is preferred. Open biopsy is especially indicated for disorders with patchy involvement like inflammatory myopathies [2].

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The muscle biopsies should ideally be performed by surgeons qualified in muscle biopsy techniques and who know specimen submission procedures. The clinician, who is familiar with the patient, must ensure that an appropriate muscle is sampled. For example, if the symptoms occur in the arms rather than the legs, a specimen from the lower extremity cannot reflect the disease correctly. When choosing the location of the biopsy, the most important step is to choose a muscle that is affected by the disease. Therefore, the specimen must be obtained from a muscle which is actively involved in the disease. However, a specimen from a seriously affected muscle, in which marked atrophy is present, is generally representative of end-stage histopathology that is difficult to evaluate (Fig. 5.2). In animal experiments, it has been shown that type 1 and type 2 myofibers can physiologically transform into each other. Some muscles may consist only of type 1 or type 2 myofibers, just like in birds. Therefore, the familiar muscles should be sampled for muscle biopsy evaluation. Muscles generally chosen for biopsy include the deltoid and biceps of the upper extremity and the quadriceps and gastrocnemius of the lower extremity. These muscles have repeatedly been examined for comparison according to several features such as the percentages of fiber type and size of muscle fiber. If there is also an indication for nerve biopsy, the peroneus brevis muscle, next to the superficial peroneal nerve, is chosen for the muscle biopsy site [2, 6–9]. Electromyography (EMG) can help to identify the affected muscle; however, one must be careful to harvest the biopsy from a distant area to trauma. The best way is to limit EMG study to a single side of the body and perform muscle biopsy on the opposite side. If the pathological process looks nonhomogeneous or multifocal, magnetic resonance imaging (MRI) or ultrasound can be used to select an affected muscle. In addition, the biopsy must be obtained from the belly of the muscle and distant to the tendon. This will reduce the possibility of collecting a myotendinous connection that can be confused with fibrosis due to myopathy or muscular dystrophy (Fig. 5.3). Sampling from the sites of trauma or pin sticks that occurred due to EMG, injection, immunisation or another reason must be avoided because these areas may show different pathological appearances such as pseudo-vasculitis (Fig. 5.4), focal inflammation and even muscle necrosis [2, 4].

The sampled muscle tissue should be of at least 0.5 cm in diameter to permit adequate observation of the pathological process. Biopsies must be transported to the laboratory as fresh tissue wrapped in saline-moistened gauze. During transportation, the biopsy must not be immersed in saline as

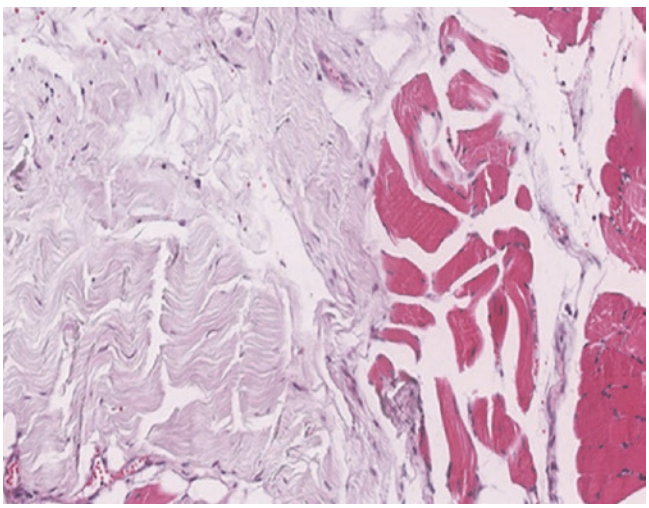

Fig. 5.3 A muscle biopsy specimen sampled from the myotendinous junction (H&E × 40)

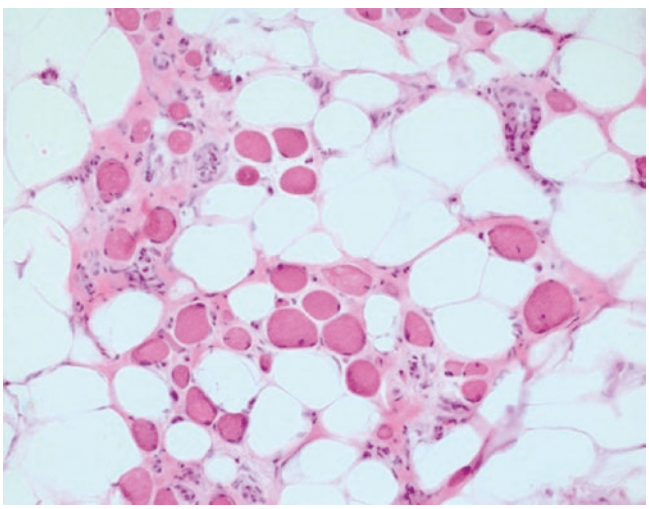

Fig. 5.2 The muscle tissue consists entirely of connective tissue in routine staining (H&E × 100)

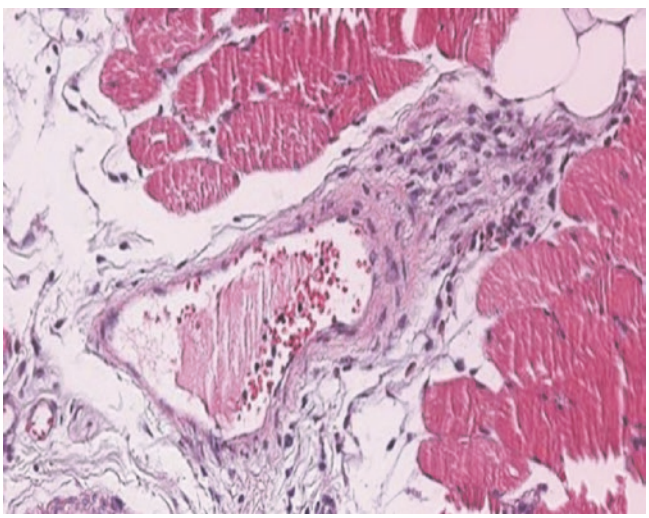

Fig. 5.4 A roughly handled muscle biopsy sample, which can mimic an appearance like vasculitis in the perimysium. Neutrophils and macrophages are beginning to exit out of the vessel walls (H&E × 100)

it can cause artefacts such as intercellular oedema, shrinkage of muscle cells, the occurrence of sarcoplasmic holes and even an appearance like mini-/multicore disease when the muscle is frozen (Fig. 5.5). Similarly, if the tissue is pulled too much during surgical incision or pathological procedures, a deceptive appearance called contraction artifact, appears (Fig. 5.6). As this appearance can also be observed in myopathies, it can create a handicap for reaching a differential diagnosis. Similarly, if the sections do not adhere well to the slide and are treated too coarsely during staining, curling may be observed at the edges of the muscle fibers. This occurs due to the separation of the section from the slide (Fig. 5.7). The muscle spindles are specialised structures located between the muscle fascicles in the perimysium and serve as sensory organs (Fig. 5.8). The muscle spindles are associated with the coordination of muscle activity, stretch and the maintenance of muscle tone. Each muscle spindle is surrounded by fibrous connective tissue containing myelinated nerve fibers. They consist of 8–10 specialised muscle fibers called intrafusal fibers which are innervated by multiple nerves and named, according to the arrangement of their nuclei, as a nuclear bag or nuclear chain fibers. Intrafusal muscle fibers are 7–8 mm long in humans and are oriented parallel to the surrounding muscle fibers. If the muscle is sent in fixation solution like formaldehyde or alcohol, the biopsy is almost always nondiagnostic. This is because most muscle-specific primary antibodies used in immunohistochemical staining only work on frozen sections, and therefore, no special staining can be performed. In summary, muscle and nerve specimens should not be steeped in saline, fixative or any liquids [1–5].

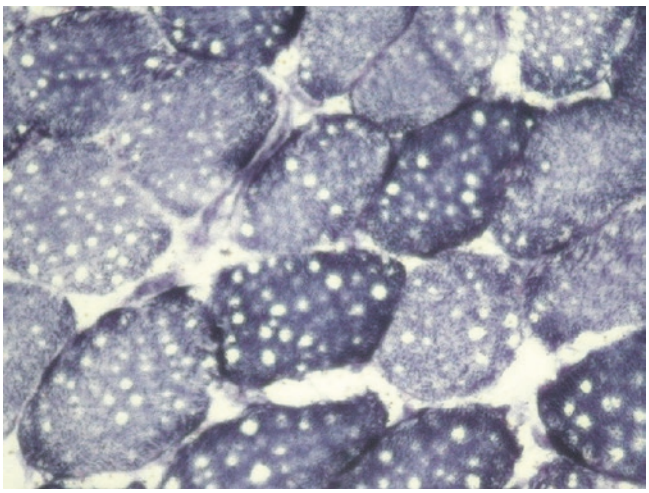

Fig. 5.5 The appearance of mini multicore disease due to transportation of the biopsy in saline solution (NADH-TR × 400)

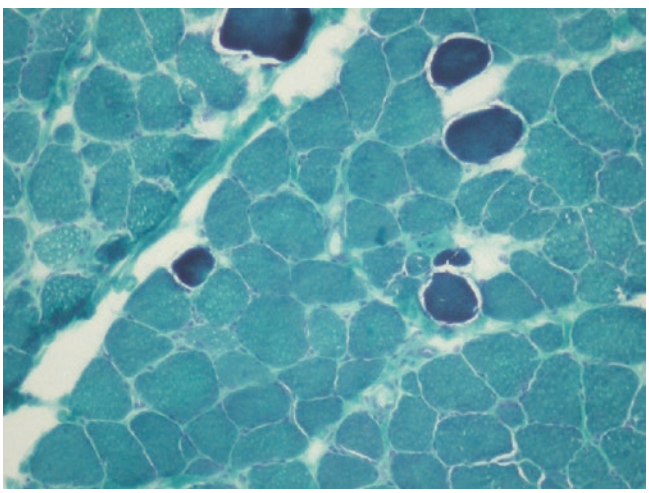

Fig. 5.6 Contracted fiber (modified trichrome × 100)

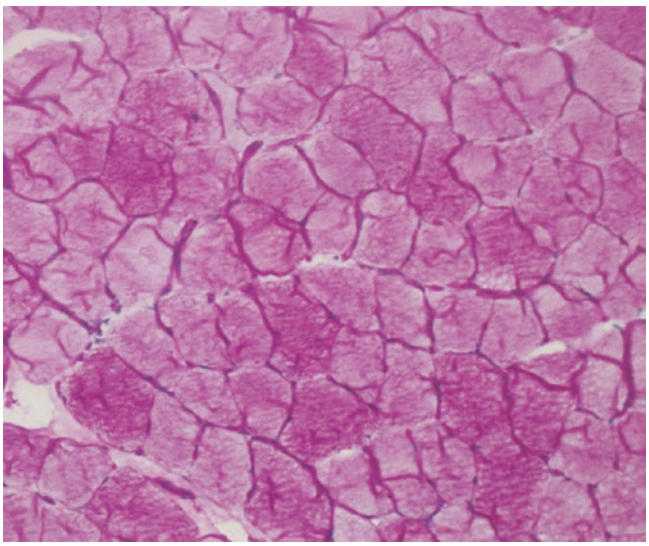

Fig. 5.7 Artefacts caused by the section lifting off the slide during staining (PAS × 200)

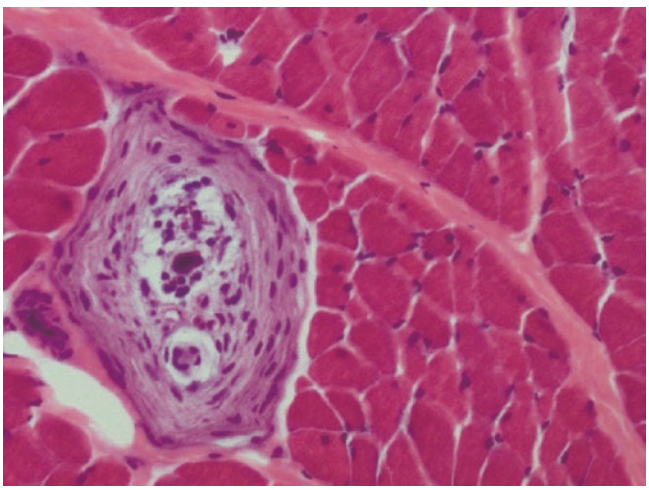

Fig. 5.8 Muscle spindles (H&E × 200)

Fig. 5.9 Different liquid nitrogen containers: (**a**) 60-L capacity, (**b**) 5-L and 1-L capacity

There is a wide variety of liquid nitrogen transport and storage containers available commercially (Fig. 5.9). There are even tanks in large centres that can store hundreds of litres of nitrogen. However, those used for muscle biopsy examinations in smaller peripheral laboratories are those that store 35 L of liquid nitrogen, have a tap and require a refill every 3 months. However, as mentioned before, the dimensions of the nitrogen tank are not so important. It is possible to also freeze the muscle biopsy in containers with a capacity of 10 L, or even in containers with a capacity of 1 L produced for transportation purposes (Fig. 5.9) The disadvantage of smaller-sized nitrogen containers is that they require more frequent filling. For example, the 10 L one needs filling once a month. If storing liquid nitrogen in a 1-L liquid nitrogen container, it will last for an average of 5 days. To keep the liquefied gases in a liquid state, it is necessary to store them under pressure or at lower temperatures. In the early years when I started studying muscle biopsy, I only had a 1-L nitrogen container, and every Saturday it was sent to the liquid gas filling facility. At first, I was running out of liquid nitrogen for the biopsies that came on Friday, but then I was able to delay the nitrogen run-out time by 1 more day by keeping this small container in the deep freezer.

In the laboratory, muscle tissue is dissected for optimal pathological evaluation. Clinical diagnosis and the amount of tissue sampled can guide decisions regarding material separation. However, the standard approach is to divide the material into three parts to prepare: (1) a frozen section; (2) an epon resin block, for possible electron microscopic examination; and (3) a paraffin block. Frozen tissue should be stored after freezing sections to be used in future histopathological, biochemical and molecular studies. Since the sections taken from the paraffin block are not suitable for examining the morphological and enzyme histochemical features of the muscle cell, the tissue may not be separated for paraffin blocking if the material sent is too small. Similarly, epon blocking may be left out if the biopsy sample is too small, since electron microscopy is a must for differentially diagnosing only a few specific diseases. On the other hand, even if your centre does not have an electron microscope, if the material is sufficient, do not forget to fix a piece of tissue with a diameter of 1 mm in glutaraldehyde. The material can be stored for up to 6 months at +4°C in 2.5–4% pH 7.4 glutaraldehyde solution. As fixed tissues accumulate, you can prepare the epon blocks for your archive. Thus, if a situation for any patient develops in the future where an elec-

tron microscopic feature is diagnostic, the material may be evaluated at a centre that performs electron microscopic examination, or an agreement can be made between your laboratory and a centre in which electron microscopic examination can be enabled [10]. Remember that archival blocks have played a key role in understanding the molecular properties of oncological pathology and neoplasms. Retrospective examination of materials, stored under appropriate conditions in advanced centres for decades, has revealed the common points of histopathologically dissimilar tumours. For example, it would not be possible to accurately diagnose and treat nervous system tumours without knowing the molecular features. For this reason, it is essential to archive the materials under appropriate conditions, considering what can be done in the future. Similarly, frozen fresh tissue should ideally be stored at −80°C in the freezer. However, if you are committed to muscle biopsy examination and cordially want to apply it, it is also possible to store the tissues in an ordinary freezer at −26°C, and despite losing enzymes that denature the fastest at −26°C, enzyme dyes are not too much of a problem since you apply them immediately. Sarcolemmal proteins begin to denature in muscle tissue you keep for several years. In addition, high-quality DNA and RNA may not be extracted in molecular studies. However, if you really want to work on muscle biopsy examination, not having a −80°C freezer is not an obstacle. During the first few weeks when the patient's initial evaluations are made and new sections of the biopsy are likely to be requested, you can keep the tissue in a normal freezer, then you can send it to a −80°C freezer in your hospital [1–5].

After the muscle reaches the laboratory, it is separated for different examinations, and the tissue is frozen by implanting according to the direction of the muscle fibers. The direction of the muscle fibers can be identified with a special dissection microscope or simply with a magnifying glass and a young keen eye. Our aim is to orient the muscle so that it can be observed in transverse sections. To maintain this position, the tissue should adhere to a flat cork floor with the special water-based cryostat embedding medium used during freezing. Then the biopsy is immersed in a volatile liquid precooled to −160°C in liquid nitrogen in a special sealed container (Fig. 5.10). You can also do this by directly immersing the tissue and holding it for a few minutes in liquid nitrogen that you have taken from the nitrogen tank in a special container. Just as the size of the nitrogen tank will not affect the quality of your quick-freezing process, you can freeze the tissue in liquid

Fig. 5.10 Freezing processes for muscle biopsy. (**a**) Preparation of the muscle biopsy for freezing, (**b**) Attachment of the muscle biopsy material to the cork in the transverse position of the muscle fibers, (**c**–**e**) Preparation of liguid nitrogen, (**f**) Immersion of the material in chilled methyl butane, then freezing in liquid nitrogen

nitrogen with the help of a simple holder you have prepared yourself, without special carriers and holders. For this purpose, you can use conical centrifuge Falcon tubes with a 50-mL capacity, which are used in molecular examinations and are resistant to long-term −80 to +112°C, with a simple hanger that you will prepare with thick copper wire. By hanging the Falcon tube in the tank and filling the tube halfway, muscle tissue can be frozen in liquid nitrogen. However, it is stated that freezing the material directly in liquid nitrogen may cause artefacts by accumulations of gaseous nitrogen around the tissue which slows the freezing of the tissue. Therefore, it is recommended to freeze the tissue in volatile liquids such as isopentane, 2-methyl butane or propane, which have a very-low-freezing temperature, cooled in liquid nitrogen. With this method, to freeze the biopsy in isopentane, the tube is filled halfway with isopentane (2-methyl butane) and cooled in liquid nitrogen for a few minutes. Even after the tissue is placed, the tube can be closed again and kept in the nitrogen tank for 3–4 min (Fig. 5.9). Even without these volatile liquids, the muscle on the cork placed in the empty falcon tube can be frozen in the liquid nitrogen tank with the tube closed. However, freezing in the air can also cause artefacts due to slower freezing [11–13].

The cryostat temperature suitable for taking sections from the frozen muscle tissue is between −21 and −23°C. If the muscle has a lot of adipose tissue, a lower temperature may be required. Very thin sections are not necessary for muscle biopsy as the other tissues. For enzyme histochemical studies, 8–10-micron-thick sections are suitable. It is recommended that tissue thickness should not exceed 5–6 microns for automatic immunohistochemical staining in the device. For histochemical evaluation, the slides can be left to dry at room temperature overnight. However, if enzyme histochemical staining will not be done immediately, the sections should be kept in the cryostat or freezer without dry wrapping in clingfilm. In addition, fresh staining of the modified trichrome from histochemical staining is recommended because, when stained in the following days, the staining feature may appear as ordinary trichrome stains. Furthermore, the use of specific slides with increased adhesion properties during frozen sectioning is recommended for preventing spillage and artefacts during staining. After the cryosections are finished, the frozen tissue should be stored together with the mushroom under it, wrapped in an airtight foil, in an Eppendorf or special box. Most laboratories use a −80° freezer to store the remaining fresh muscle tissue [11].

Histochemical Stains and Reactions of Muscle Biopsy

For routine examination of muscle biopsy material, the haematoxylin and eosin (H&E) stain is used. This dye is the most used staining method in histopathology. Its popularity is based on its capacity to clearly demonstrate a great number of different tissue structures, its extensive applicability to different tissues, its diverse preparation in several ways and its relative simplicity. Haematoxylin has been extracted from the tree *Haematoxylon campechianum* which originated in the Mexican state of Campeche but is now mainly cultivated in the West Indies. In fact, haematoxylin itself is not a dye, but hematein (the oxidation product of haematoxylin) is a natural dye. Hematein can be naturally produced from haematoxylin via natural oxidation by exposure to light and air, but this process takes several months. Chemical oxidation using different oxidants quickly converts the haematoxylin to hematein. Different oxidants are used in different haematoxylins. For example, sodium iodate is used in Mayer's haematoxylin, and mercuric oxide is used in Harris's haematoxylin. However, the product formed by chemical oxidation has a shorter useful lifetime because the natural oxidation process, caused by effects of air and light, continues and eventually destroys the hematein, converting it to a colourless compound [11].

Another disadvantage of hematein is its own poor affinity for tissues. The nuclear staining of hematein without the help of a mordant is not adequate. To increase its nuclear affinity, the salts of several metals such as aluminium, iron and tungsten are commonly used. Erlich's haematoxylin, Delafield's haematoxylin, Mayer's haematoxylin, Harris's haematoxylin, Cole's haematoxylin and Carazzi's haematoxylin are the haematoxylin dyes which use aluminium salts as mordant. Different staining times between 5 and 45 min are required according to the used haematoxylin. Mayer's haematoxylin and Carazzi's haematoxylin are preferred due to a short nuclear staining time of frozen sections. The major disadvantage of aluminium-mordanted haematoxylin is its sensitivity to any subsequently applied acid staining solutions. For example, picric acid used in the Gomori's trichrome stain removes most of the haematoxylin so that the nuclei are not discernible. In such situations, iron-mordanted haematoxylin should be used. Weigert's haematoxylin, Heidenhain's haematoxylin and Verhoeff's haematoxylin are commonly used iron-mordanted haematoxylin stain samples. There is only one widely used tungsten-mordanted haematoxylin, originally known as Mallory PTAH stain, developed as a combination of haematoxylin with 1% aqueous phosphotungstic acid. The PTAH stain can highlight many tissue structures such as muscle striation, glial fiber and myelin. Therefore, it is used as a general central nervous system (CNS) stain [11, 12].

Eosin is the most suitable stain to combine with haematoxylin for routine histopathological examination, and it is also used as the counterstain. Standard haematoxylin and eosin (H&E) staining for paraffin sections includes deparaffinisation and rehydration of sections, staining with haematoxylin for a longer period, blueing and differentiation, staining with eosin, dehydration, clearing and finally

mounting. This technique lasts up to an hour and differs according to the chosen haematoxylin. In addition, there are both progressive and regressive techniques for H&E stains. Progressive stain does not include the differentiation stage with acid solution and instead uses the autostainer. Regressive stain is not used in automatised staining devices, as the acid solution used for differentiation after haematoxylin staining injures the machine chambers. For muscle or nerve biopsy, the *quick progressive H&E stain* is performed as follows [11, 12]:

1. Place the muscle tissue on the cork onto a chuck.
2. Cut the tissue into 5–6-micron sections.
3. Fix section in 96% alcohol for 8–10 s.
4. Stain the slide into Harris's haematoxylin for 1 min.
5. Wash the slide in tap water for 10–20 s until the section turns blue.
6. Stain in 1% aqueous eosin for 10–15 s.
7. Rinse in tap water.
8. Dehydrate in ascending ethyl alcohol series.
9. Clear with xylene, and mount with a mounting medium like the Entellan, Canada balsam and so on.

In fact, these products are trademarks which include synthetic resin or different polymers made of mixed acrylates dissolved in toluene or other solvents. They are used for the permanent mounting and storage of microscopic slides.

As a result of H&E staining, the nuclei look blue-black and cytoplasm, as well as soft tissues, are seen in varying shades of pink.

Several techniques are developed for the identification of distinct components of the connective tissues, and some of them are called trichrome stains. The term 'trichrome' means the ability to stain distinct tissue components in different colours due to the use of different molecular sizes of dyes together. The structure and density of the protein network directly relate to the staining of the different tissue components. For example, the smaller molecules in trichrome stains can penetrate and dye all tissues. The colour of the smallest molecules in trichrome stains is generally red. Therefore, almost all tissues are coloured red by trichrome stains. Contrarily, the larger dye molecules can penetrate only collagen, which has the least dense protein network and is seemingly quite porous, staining it blue or green according to the type of trichrome stain. The general rule of trichrome staining is that whenever a larger molecule penetrates a tissue component, the smaller one changes its place with the larger molecule. Therefore, the smaller molecule cannot stain that tissue. But in practice, the rules are less rigid and staining processes are changeable. For example, acid fuchsin can stain collagen when used as a combination with picric acid in van Gieson's elastic stain. However, when used with light green in Gomori's trichrome, it can stain the muscles.

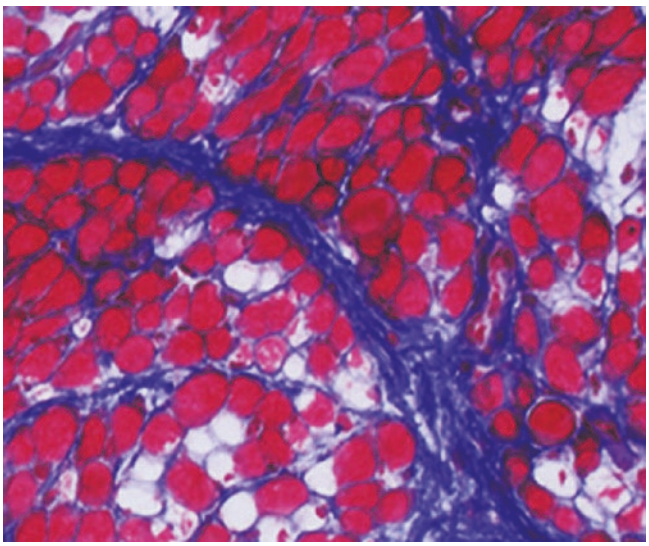

Fig. 5.11 Increased intercellular fibrosis (Masson's trichrome × 100)

Similarly, the colour of collagen is different in each trichrome stain according to the type of large molecule. For example, collagen is Saxe blue in Masson's trichrome with methyl blue (Fig. 5.11), and it is Nile green in Gomori's trichrome with light green (Fig. 5.12).

Formaldehyde-fixed tissues cannot produce optimal results with the trichrome stains because the fixation is a negatively impacts the tissue penetrance of dyes. Therefore, the treatment of the tissue with heat and acidic solutions like picric acid is to be required. Low pH between 1.5 and 3 and heat has been shown to help the penetrance of larger molecules and increase the staining quality. One of the three dyes in the trichrome stains is used for nuclear staining and generally; it is a kind of haematoxylin. Iron-mordanted haematoxylin is preferred because it is more resistant to acid solutions than aluminium-mordanted haematoxylin.

In our laboratory, we use the Gomori's trichrome method in manual staining as it is easier to apply than the Masson's trichrome method in autostainer. Gomori's trichrome is a one-step trichrome staining procedure including a smaller molecule red stain (chromotrope 2R) and a larger molecule blue-green connective fiber stain (light green) in a mixture of phosphotungstic acid and glacial acetic acid. A special modification of Gomori's trichrome is known as Engel Cunningham modification, and it is also used in muscle and nerve biopsy examinations. In other specific methods of trichrome staining including the Gomori's trichrome, muscle tissue is stained red because it only allows the penetration of smaller dye molecules, while in the modified trichrome staining, muscle cells are stained blue (Fig. 5.13). Red-purple-stained components of cells are nuclei, mitochondria and certain special structures such as nemaline rods. Similarly, the nerve plexus is entirely red in all types of trichrome stain, while only myelin is red in the modified tri-

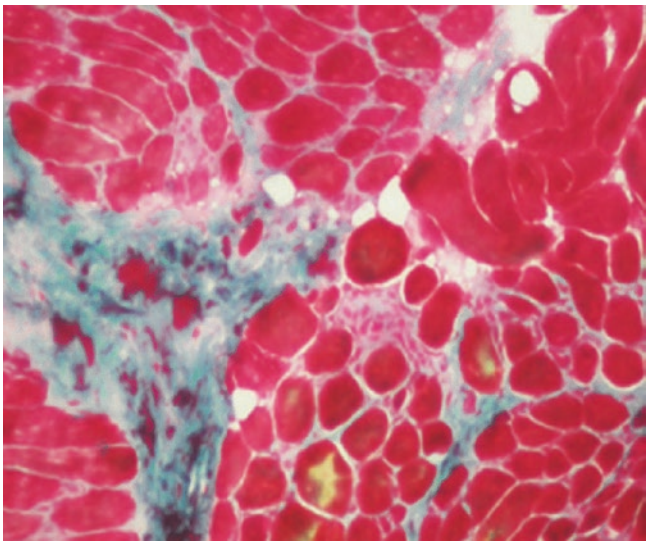

Fig. 5.12 Increased intracellular fibrosis (Gomori's trichrome × 200)

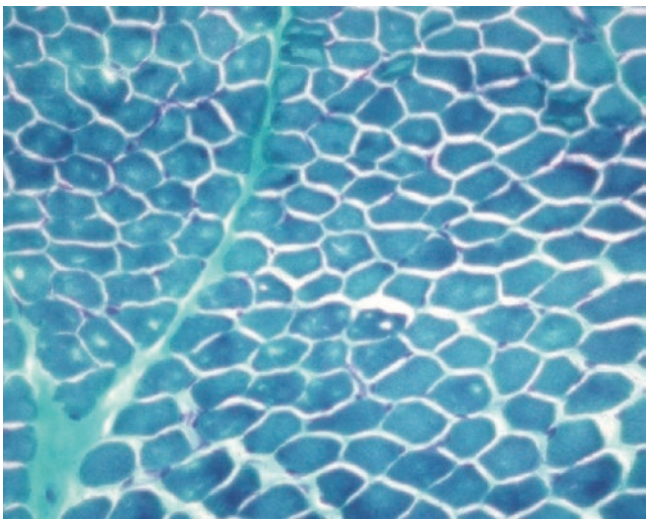

Fig. 5.13 A muscle biopsy sample (modified Gomori's trichrome ×40)

chrome stain (Fig. 5.14). If this staining, which is very important in the differential diagnosis, cannot be performed, it would not be wrong to say that a special muscle and nerve examination should not be performed. The only difference between the modified trichrome from the Gomori's trichrome is the use of fast green instead of light green. However, due to the problem I have experienced in the supply of fast green, I have been using the same mixture for both dyes for years, and if the sections from paraffin blocks are stained after picric acid treatment, the muscles are painted red, and if the frozen section is painted daily, the muscles are painted blue-green. However, muscles in red can be observed, especially in frozen sections that could not be stained daily, or the biopsy was not transmitted to the laboratory immediately [11, 12].

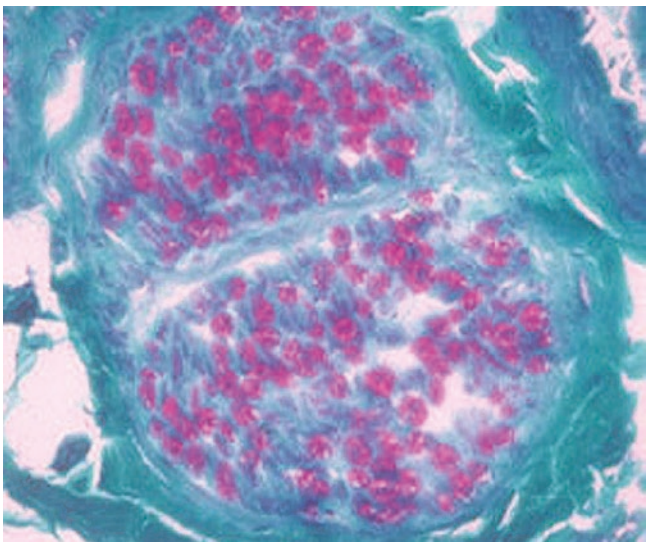

Fig. 5.14 A nerve biopsy sample (modified Gomori's trichrome ×100)

For muscle or nerve biopsy, ingredients of the Gomori's and modified Gomori's trichrome stains and their performances are as follows [11, 12]:

Gomori's (modified gomori's) mixture	
Cromotrope 2R	0.6 g
Light green (for modification fast green)	0.3 g
Phosphotungstic acid	0.6 g
Glacial acetic acid	1.0 mL
Distilled water	100 mL

1. Place the deparaffinised slides in the Bouin solution (mixture of 75 mL of saturated aqueous solution of picric acid and 25 mL of 40% aqueous solution of formaldehyde to give 100 mL total volume), and incubate at 58–60°C for an hour.
2. Wash the slide in tap water for 3–5 min until the yellowish appearance of the tissue sections is decolourised.

   The first two steps are absent in modified trichrome technique.
3. Stain the slide or frozen sections in Weigert's haematoxylin for 5 min.
4. Rinse in tap water.
5. Rinse in distilled water.
6. Stain in Gomori's trichrome mixture for 10–20 min (until green).
7. Rinse in tap water. If sections appear too red, differentiate in 0.2% acetic acid for a few minutes, and then rinse in tap water.
8. If sections appear to be dark green, immerse in tap water and wait several minutes.
9. Dehydrate rapidly in ascending ethyl alcohol series.
10. Clear with xylene and mount with a permanent mounting medium.

As a result of Gomori's trichrome staining, the nuclei look blue-black, all tissues other than connective tissue are seen in varying colours of red and reddish brown; collagen and fibrous tissues are seen in Nile green.

As a result of modified Gomori's trichrome stain, almost all tissues including muscle and nerve cells are seen in blue, connective tissue is seen in Nile green, and specific structures such as nemaline rod and inclusions, mitochondria, nuclei and myelin of nerves are seen in varying colours of red and reddish purple.

Masson's trichrome is other commonly used trichrome stain. This technique is more complex and is performed with several steps in which phosphomolybdic acid, glacial acetic acid, acid fuchsin and methyl blue are used. For nuclear staining, Weigert's haematoxylin is preferred. In most of the ready-to-use Masson's trichrome kits, methyl blue is used for collagen staining, and connective tissue is seen in Saxe blue colour. However, there is light green as fiber stain in some kits. In this situation, collagen is seen in Nile green colour similar to Gomori's trichrome [11, 12].

Another stain commonly used in muscle biopsy evaluation is the periodic acid- Schiff (PAS) stain. This technique has a wide range of applications. The PAS reaction is a helpful indicator of the presence of tissue carbohydrates. If an enzyme digestion stage is added, the storage of glycogen can be also identified. The PAS reaction is an oxidative process. The periodic acid brings about oxidative cleavage of carbon-to-carbon bonds to form dialdehydes. These aldehydes react with fuchsin-sulphurous acid and basic pararosaniline in the medium to form magenta-coloured compounds which are alkyl sulphonate in structure. Other substances that may show PAS positivity are the extracellular mucins containing a reactive hexose component such as neutral mucins, chondroitin sulphates, heparan sulphate and both epithelial and connective tissue mucins such as hyaluronic acid. Some mucoproteins of basement membranes, thyroid colloid and the glycolipids which comprise cerebrosides and gangliosides found in nervous tissues show positive reactivity with PAS staining. The Schiff's solution used in PAS staining is perhaps the most difficult to prepare as it is the most rapidly deteriorating reagent among all histochemical dyes. To prepare the Schiff's reagent, 1 g of basic fuchsin is dissolved in 200 mL of boiling distilled water. The flask of water should be removed from the heater just before adding the basic fuchsin to avoid the premature colouring of the reagent. Then the solution is allowed to cool to 45°C, and 2 g of sodium bisulphite ($NaHSO_3$) or sodium metabisulphite (NaS_2O_5) is added with mixing. The solution is cooled to room temperature, and 2 mL of concentrated hydrochloric acid is added. This current solution is stored for 24 h in the dark at room temperature. The next day 3 g of activated charcoal is added and filtered through a No. 1 Whatman paper until the solution becomes either clear or very pale yellow. The prepared reagent should be stored in a dark container at 4°C. Unfortunately, it will lose its activity over time, even under optimal conditions.

For muscle or nerve biopsy, the *periodic acid-Schiff* (*PAS*) *stain* is performed as follows [11, 12]:

Deparaffinised or frozen sections are brought to distilled water. Another slide should be treated with 1% diastase solution for 1 h at 37°C to break down the glycogen. The glycogen can be also broken by the amylase in saliva.

1. Treat with 1% periodic acid solution for 5 min.
2. Wash well with several changes of distilled water.
3. Cover sections with Schiff solution for 15 min.
4. Wash well in running tap water 5–10 min.
5. Stain nuclei with Harris's haematoxylin for 2 min.
6. Rinse in tap water.
7. Dehydrate in ascending ethyl alcohol series.
8. Clear with xylene and mount with a permanent mounting medium.

As a result of PAS staining, the nuclei look blue-black; glycogen and other periodate-reactive carbohydrates are seen in magenta (Fig. 5.15). *In diastase-treated sections, glycogen is not stained* (Fig. 5.16).

Several techniques have been developed for the identification of glycogen and lipid accumulations in muscle tissue that can occur during some metabolic diseases [12, 13]. Glycogen accumulation can be perfectly demonstrated with magenta colouring after PAS staining. In addition, the presence of glycogen can be evidenced by the loss of staining after diastase treatment when compared to the untreated sections.

For the identification of lipid storage, Sudan black and oil red O stains are commonly used [12–15]. Chemically

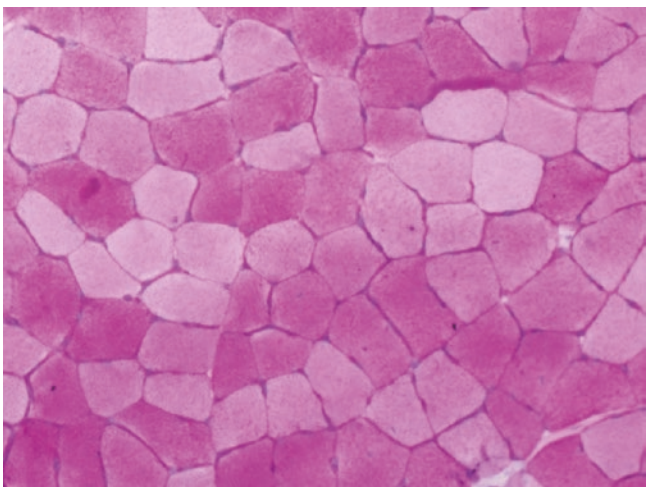

Fig. 5.15 A normal muscle biopsy sample (PAS ×100)

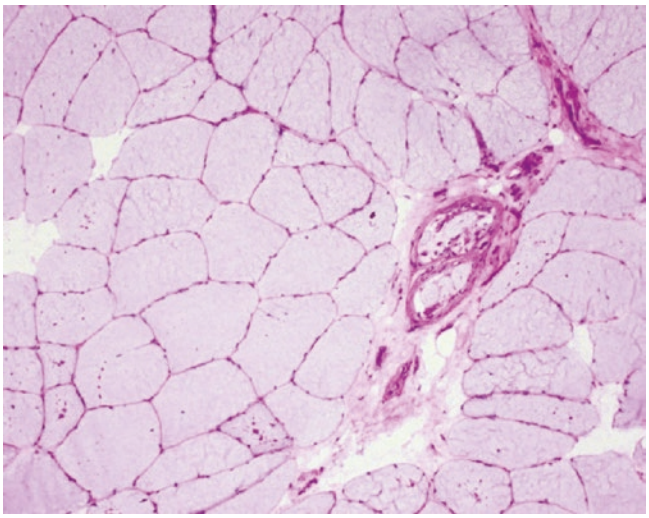

Fig.5.16 A normal muscle biopsy sample (dPAS ×100)

distinct lipids cannot be distinguished by any of these methods. These dyes only demonstrate the presence or absence of lipids. All lipid dyes should be performed on frozen sections because the tissue lipids are dissolved during long tissue processing with xylene. A 5-min fixation is also recommended for frozen sections, and ordinary neutral-buffered formalin is a satisfactory fixative. Similarly, after lipid staining, an aqueous mounting medium like glycerine is preferred when mounting the stained slides because the organic solvents present in the ordinary mounting media can dissolve the dye-lipid blocks in the tissues. However, I mount the air-dried slides with Entellan. Since the organic solvents in Entellan take a long time to dissolve the dye-lipid complexes in the tissue and I take microscopic photographs of the oil red O-stained slides right after mounting, I do not have any problems. In addition, while the slides already mounted with glycerine cannot be archived, I do archive slides mounted with a permanent mounting medium.

The Sudan black technique used for muscle biopsy is known as the modified Chiffelle and Putt's Sudan Black stain. 700 mg Sudan black dye is dissolved in 100 mL of the lipid solvent propylene glycol, and sections are treated with this solution. Propylene glycol is a dye solvent so it will not dissolve any of the tissue lipids. The Sudan black is more soluble in the lipids of the tissue compared to the solvent. Therefore, the dye moves out of the solvent and stains the tissue lipids. This physical process should last for a minimum of 2 h, and overnight incubation with Sudan black dye solution is preferred. Boundary-surface adsorption plays an essential role in this process.

In my own practice, I prefer the oil red O lipid staining technique as it is simpler and quicker. For muscle or nerve biopsy, the oil red O stain is performed as follows [11, 12]:

Oil red O stock mixture	
Oil red	0.5 g
Absolute isopropyl alcohol	100 mL

1. Frozen sections are rapidly immersed in 60% isopropyl alcohol.
2. Slide is transferred to diluted oil red O solution (mixture of 0.6 mL of stock solution with 0.4 mL of distilled water) for 15 min.
3. Slide is differentiated in 60% propyl alcohol for 5 min.
4. Wash in distilled water.
5. Stain nuclei with Harris's haematoxylin for 2 min.
6. Rinse in tap water for blueing.
7. Dehydrate in ascending ethyl alcohol series.
8. Air-dried slide is mounted with a permanent mounting medium.

As a result of oil red O staining, the nuclei look blue-black, lipids are seen in red (Fig. 5.17), *while with Sudan black staining lipids are seen in black* (Fig. 5.18).

Amyloid is an abnormal, extracellular, proteinaceous fibrillar deposit found in organs and tissues. Amyloid fibrils are formed by soluble proteins, which assemble to form insoluble fibers that are resistant to degradation. To date, more than 20 plasma proteins have been identified that form amyloid. Despite the distinction in amino acid sequences and native structure, these peptides are composed predominantly of β-sheet structures which make up their characteristic polypeptide backbone. This characteristic architecture of amyloid is responsible for the fibrillar conformation, its resistance to proteolysis and its insoluble characteristics [16]. Visual detection of amyloid accumulation is required for muscle and nerve biopsies especially in patients with amyloidosis. To achieve this, the Congo red dye technique is commonly used. When stained with Congo red, amyloid deposits exhibit apple-green

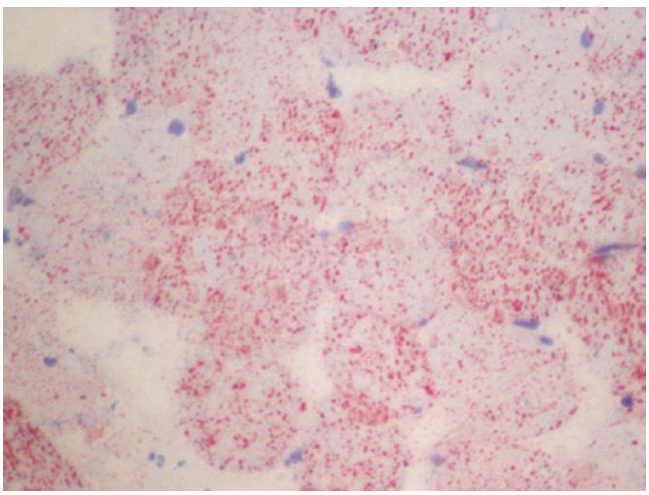

Fig. 5.17 Lipid storage in a muscle sample (oil red O ×200)

birefringence under polarised light. The staining of amyloid is similar to the staining of cotton in the textile sector. Most dyes used for the identification of amyloid were developed by the textile industry. For example, Congo red stain was the first direct cotton dye and was invented in 1884. The main disadvantage of Congo red stain is that, like most amyloid dyes, there is short duration of colourisation. If tissues are left standing in fixative for a long time, the staining becomes less sensitive and intense. The structural linearity of amyloid permits binding of carbohydrate hydroxyl groups with the azo and amine groups of the Congo red stain. The staining solution contains a high percentage of alcohol and free alkali which break native internal hydrogen bonds between polysaccharide chains, providing more potential sites for the binding of the dye.

For muscle and nerve biopsy, *Congo red staining* is performed as follows [11, 12]:

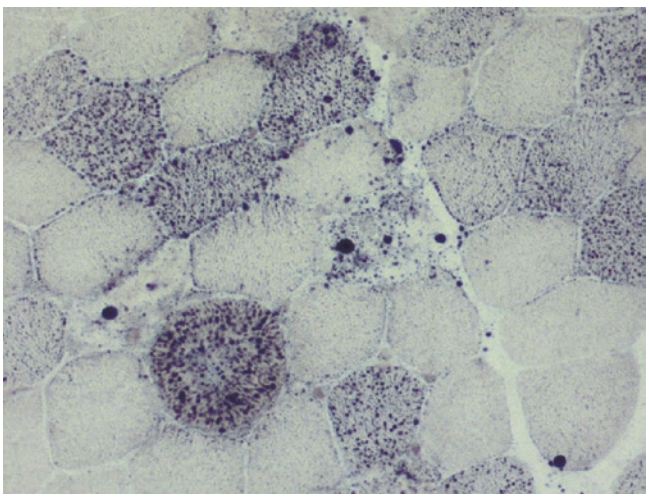

Fig. 5.18 Lipid storage in a muscle sample (Sudan black ×200)

Congo red mixture	
Congo red	0.2 g
80% ethanol with saturated NaCl	100 mL
Adjusted to pH 10.5–11 with NaOH	

1. Stain nuclei with Mayer's haematoxylin for 2 min.
2. Immerse sections in saturated NaCl in 80% ethanol for 30 min.
3. Transfer to Congo red solution without washing and incubate for 30 min.
4. Wash with ethanol.
5. Dehydrate in ascending ethyl alcohol series.
6. Clear with xylene and mount with a permanent mounting medium.

As a result of Congo red staining, the nuclei look blue-black, and amyloid looks red with green birefringence under polarised light (Fig. 5.19).

In my own practice, I prefer the Lieb's crystal violet staining method for amyloid because this technique is simpler and quicker. It is the oldest staining method for amyloid, but it is far more specific and sensitive. Its staining mechanism remains unexplained. For muscle and nerve biopsy, crystal violet staining is performed as follows [11, 12]:

1. Frozen sections are stained with 0.5% crystal violet solution (a mixture of 0.5 g of crystal violet, 100 mL of distilled water, 3 mL of alcohol 95% and 0.3 mL of concentrated HCL) for 5 min.
2. Wash in tap water for 5 min, and then wash in distilled water.
3. Air-dried slide is mounted with a permanent mounting medium. Like oil red O staining, the use of solvent-based mounting medium is not recommended.

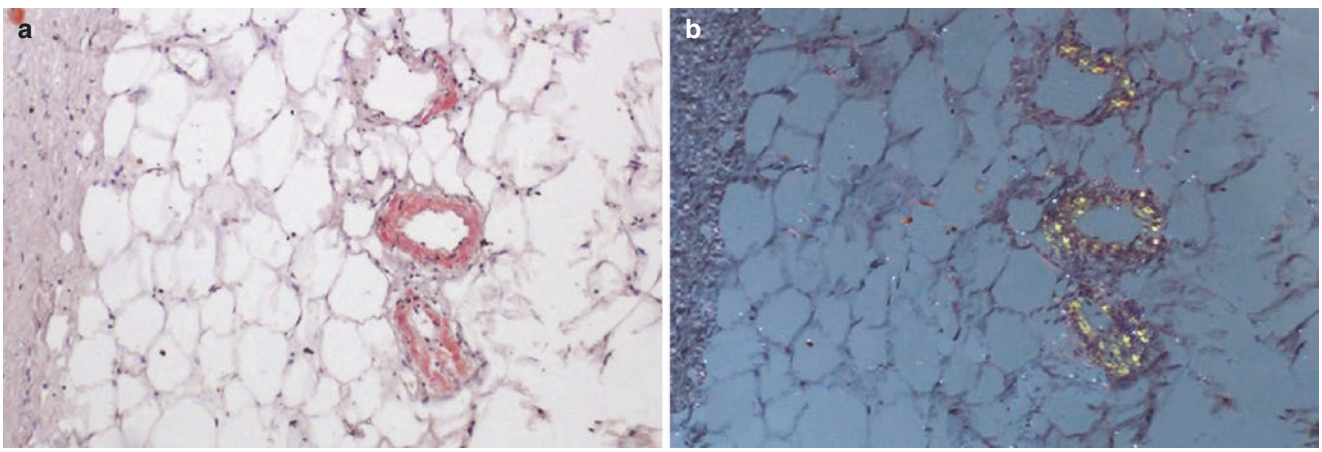

Fig. 5.19 Amyloid deposits in the vessels: (**a**) with conventional light microscopic examination and (**b**) with polarised microscopic examination (Congo red O ×200)

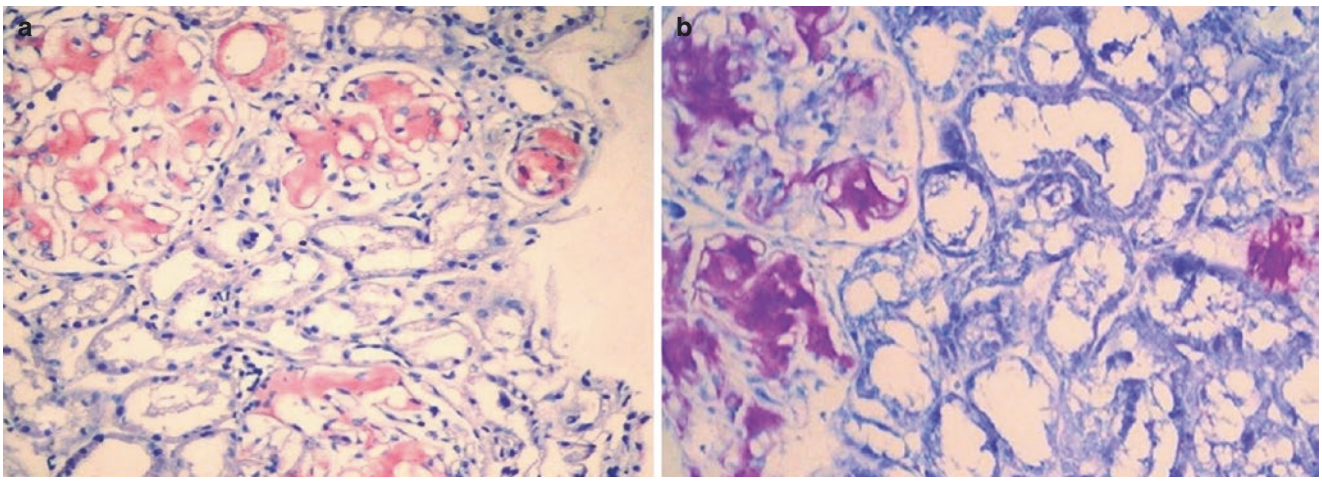

Fig. 5.20 Amyloid deposits in the glomeruli and vessels: (**a**) with Congo red stain and (**b**) with crystal violet stain

As a result of crystal violet staining, amyloid looks pink-purple; background looks blue.

This crystal violet method does not stain all types of amyloid, but generally, it more clearly identifies amyloid in cardiac muscle than the Congo red method (Fig. 5.20).

Modified Russel-Movat stain is a pentachrome stain developed by Movat in 1955 and modified by Russell in 1972 to highlight the various constituents of connective tissue, especially within cardiovascular tissue, with five colours in a single stained slide. It can be used to diagnose vascular, heart and lung diseases. It highlights many tissue components in a single histological slide. This stain is obtained from a mixture of five stains. These five different stains include alcian blue, Verhoeff haematoxylin and crocein scarlet combined with both acidic fuchsine and saffron. At pH 2.5, alcian blue is fixed by electrostatic binding with the acidic mucopolysaccharides. The Verhoeff haematoxylin has a high affinity for the negative-charged nuclei and elastin fibers. The combination of crocein scarlet with acidic fuchsine stains acidophilic tissue components red. The collagen and reticulin fibers are dyed yellow by saffron [17–19].

Staining solutions	
1% alcian blue solution	
Alcian blue	1 g
Distilled water	100 mL
Glacial acetic acid	1 mL
Alkaline alcohol	
Ammonium hydroxide	10 mL
95% alcohol	90 mL
Haematoxylin solution	
5% absolute alcoholic haematoxylin	
Ferric chloride, 10% aqueous solution	
Iodine solution	
Iodine	2 g
Potassium iodide	4 g
Distilled water	100 mL
Sodium thiosulfate (5%)	
Sodium thiosulfate	5 g
Distilled water	100 mL
Crocein scarlet (stock solution)	
Crocein scarlet	1 g
Distilled water	99.5 mL
Glacial acetic acid	0.5 mL
Acid fuchsin (stock solution)	
Acid fuchsin	1 g
Distilled water	99.5 mL
Glacial acetic acid	0.5 mL
Crocein scarlet-acid fuchsin (working solution)	
Mix eight parts stock Crocein scarlet solution with two parts stock acid fuchsin solutions.	
Alcoholic safran solution	
Saffron	6 g
Absolute alcohol	100 mL
Keep tightly closed to prevent hydration	

Staining procedure

1. Deparaffinise and hydrate in distilled water.
2. Stain in alcian blue for 20 min.
3. Wash in running tap water for 5 min.
4. Place slides in alkaline alcohol for exactly 1 h.
5. Wash in running tap water for 10 min.
6. Rinse in distilled water.
7. Stain in haematoxylin solution for 12–15 min.
8. Rinse in several changes of distilled water.
9. Differentiate each slide, by dipping 2–3 times in 2% aqueous ferric chloride; rinse in distilled water and check under microscope.
10. Place slides in sodium thiosulfate for 1 min.
11. Wash in running tap water for 5 min; rinse in distilled water.
12. Stain in crocein scarlet-acid fuchsin for 1–3 min, depending on desired redness.

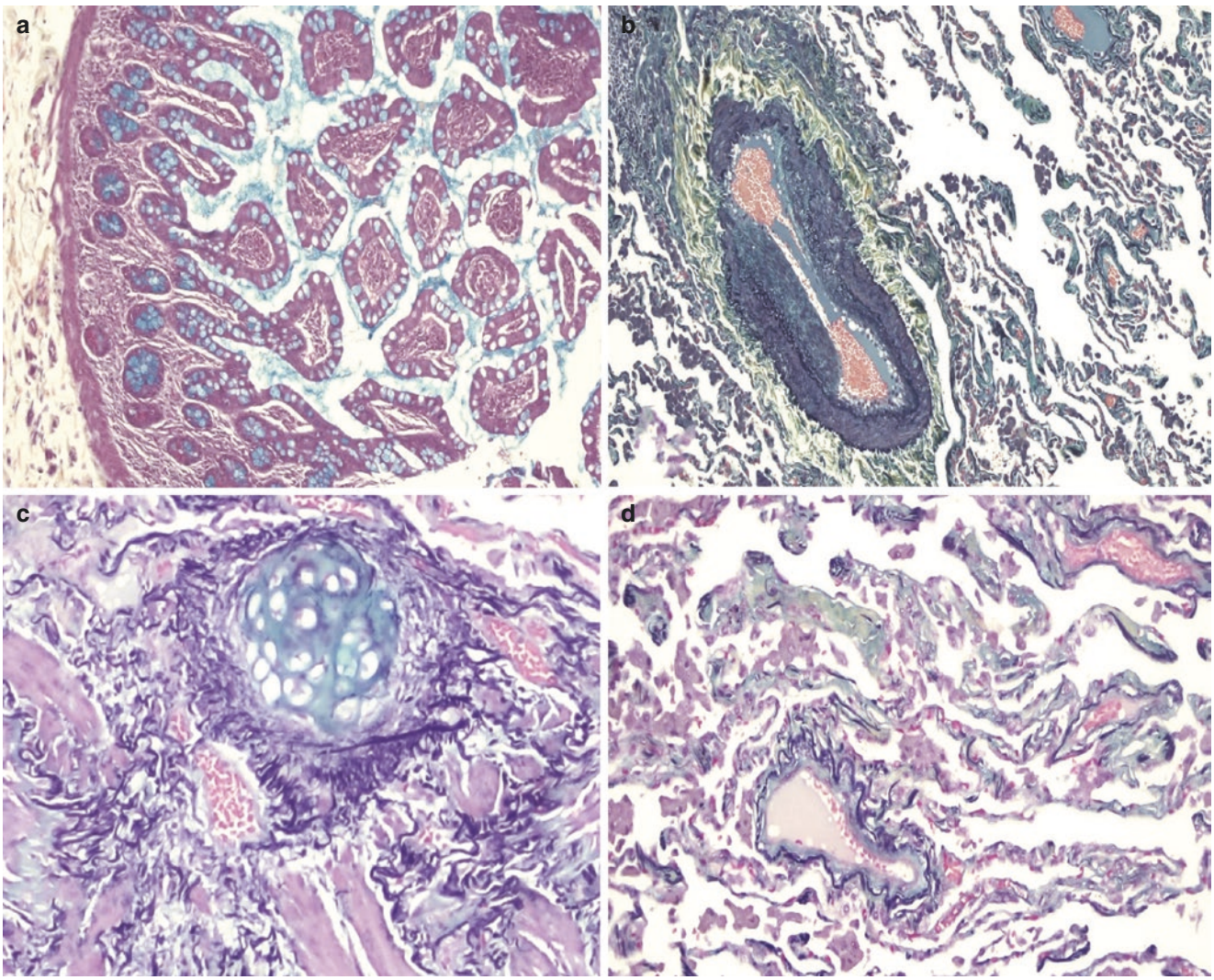

Fig. 5.21 Modified Russell-Movat pentachrome staining of small intestine (**a**) and lung (**b–d**). (This photograph is from the archive of Phillip Ruiz, MD, PhD, Professor of Surgery and Pathology, Director of Immunology and Histocompatibility Laboratory of Miami University)

13. Rinse in several changes of distilled water.
14. Rinse in 5% acetic acid water.
15. Place slides in 5% aqueous phosphotungstic acid, two changes of 5 min each.
16. Rinse in 5% acetic acid water.
17. Rinse in three changes of absolute alcohol.
18. Stain in safran for 15 min.
19. Rinse in three changes of fresh absolute alcohol and two changes of xylene, and mount in mounting medium.

Differentiation of the elastics stain should be accomplished quickly until the elastic fibers remain black and the background becomes relatively clear. Skin biopsy can be used as a control tissue during staining. The most important step of staining is the complete removal of alkaline alcohol with running water because the presence of remnant alkaline alcohol inhibits the stains that follow. Russell-Movat stain can only be used with paraffin sections.

As result of modified Russell-Movat staining, nuclei and elastic fibers look black, collagen including the reticular fibers look yellow, ground substance and mucin look blue, and fibrin looks bright red and muscles look red (Fig. 5.21).

Enzyme Stains and Reactions of Muscle Biopsy

Enzyme stains are widely used in muscle biopsy examination for two key reasons. One of them is the ability to discriminate myofiber types because the distribution and changes in fiber types are key features for differentially diagnosing neurogenic myopathies and various other neuromus-

cular disorders. The other reason for using enzyme stains is to evaluate mitochondrial dysfunction which is essential for diagnosing mitochondrial diseases. The calcium method for myofibrillary adenosine triphosphate (ATPase) identification, traditionally employing solutions of different pH values, is mainly used to distinguish muscle fiber types. ATPase enzyme staining was developed nearly 70 years ago and has important disadvantages which should be noted. At first, it is a time-consuming analysis and should only be applied to frozen sections. Moreover, permanent staining is not achieved. The ATPase enzyme reaction is critically dependent on pH, temperature and time of incubation. During ATPase staining, altered pH levels of different incubation solutions inactivate the myosin-ATPase enzyme of specific fiber types. The active enzyme of other fiber types is attached to a calcium atom replaced by cobalt. Finally cobalt and ammonium sulphide form black insoluble precipitants (Fig. 5.22). These different points require attention to achieve a satisfactory fiber type distinction in this complicated staining method. If pH adjustments of all solutions and timing is not well regulated, the enzyme reaction does not happen. For example, sodium hydroxide must be fresh (under 2 months old), and stock ammonium sulphide must be yellow in colour. When ammonium sulphide becomes old or it oxidises, it becomes progressively red. If its colour has turned to red, it should not be used.

In fact, the six different fiber types, type 1, 1C, 2C, 2A, 2AB and 2B, can be distinguished based on their staining features with myofibrillar adenosine triphosphatase (ATPase) performed using preincubation pH values of 4.3, 4.6 and 10.4 [5]. However, this fine distinction is not so important in practice, and muscle fibers may be broadly categorised in two types. Type 1 fibers are also known as slow, red or oxidative myofibers, while type 2 fibers are known as fast, white or glycolytic myofibers. Type 2 muscle fibers are further subdivided into 2A (glycolytic), 2B (glycolytic/oxidative) and immature 2C which are changing types due to reinnervation. As ATPase enzyme staining requires a separate skill, I use the immunohistochemical evaluation of myosin heavy-chain isoforms for myofiber discrimination (Fig. 5.23). There are commercial primary antibodies of slow myosin, fast myosin and neonatal myosin which discriminate type 2C and regenerated fibers (Fig. 5.24). Human skeletal muscles, regardless of their minimal variation, are composed of cells with type 1 and type 2 features, like a checkerboard pattern. In most muscles, 1/3 of the skeletal muscle is composed of type 1 myofibers and 2/3 is composed of type 2 myofibers [20].

It is often possible to distinguish muscle fiber type with mitochondrial enzyme staining. Even with oil red O and PAS staining, due to the usual lipid and glycogen presence in muscle cells, fiber types can be discriminated because type 1 myofibers are rich in mitochondria and lipids, whereas their glycogen content is low. Type 2 myofibers, on the other hand, are rich in glycogen and have less mitochondria.

The mitochondria are energy producers of the cells, and an electron transport chain is required for the formation of more ATP. This electron transport chain is in the inner membrane of mitochondria, and the molecules in charge are cytochromes and oxygen. The proteins involved are arranged in an array on this membrane. In addition, each of these molecules is an enzyme. The enzymes working in this circuit are arranged in the inner mitochondrial membrane from the highest energy level to the lowest, and this arrangement occurs according to the oxidation reduction potential. Each molecule takes electrons from the previous molecule, causing the molecule to become oxidised. Meanwhile, the electron-accepting molecule is reduced. Accordingly, the energy level of electrons in the Krebs cycle and electron transport chain is highest in coenzyme Q and lowest in oxygen. The enzymes used in the oxidation of pyruvate and fatty acids, and in the citric acid cycle, are found in the matrix. For example, nicotinamide adenine dinucleotide (NAD), flavin

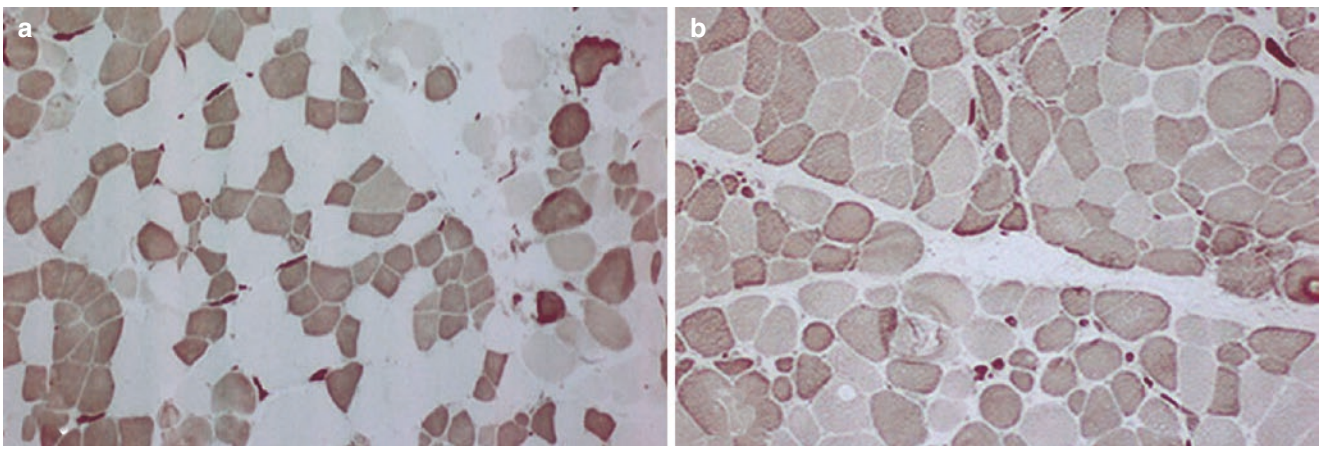

Fig. 5.22 (**a**) ATPase reaction of type 1 fibers in pH 4.3. (**b**) ATPase reaction of type 2 fibers in pH 10.4 (ATPase ×100)

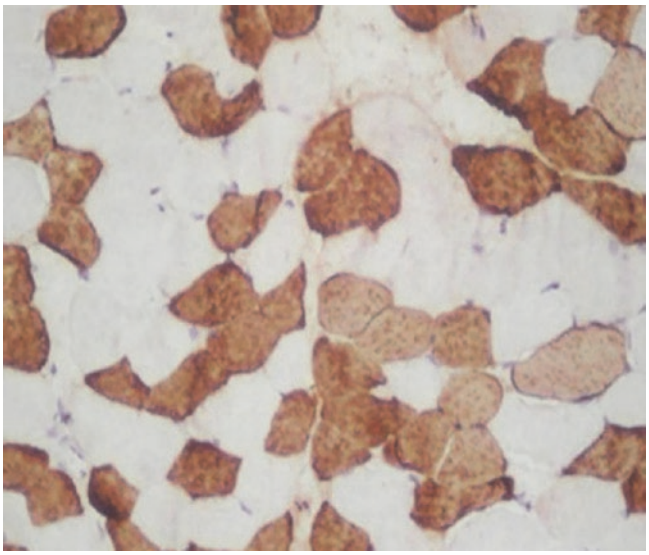

Fig. 5.23 Fast myosin expression of type 2 fibers (DAB ×200)

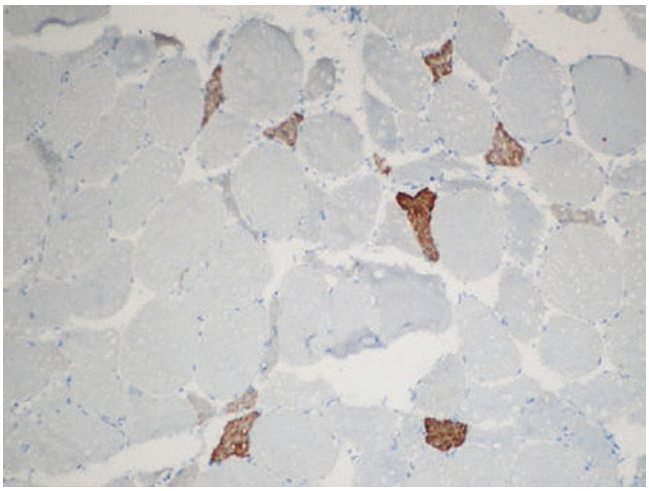

Fig. 5.24 Neonatal myosin expression of immature fibers (DAB ×200)

adenine dinucleotide (FAD), adenosine diphosphate (ADP) and phosphate (P) are some of these important molecules. The respiratory chain consists of five separate enzyme complexes in the inner mitochondrial membrane. Complexes I, II, III and IV are involved in electron transport, and complex V is involved in ATP synthesis. Of these stages, only the enzyme subgroups of complex II are encoded solely by nuclear DNA, because these enzyme subgroups are also involved in the Krebs cycle that takes place in the mitochondrial matrix (Fig. 5.25). Subgroups of enzymes in other steps are encoded by both nuclear and mitochondrial DNA. The NADH dehydrogenase of complex I, succinate dehydrogenase of complex II and cytochrome oxidase of complex IV are used enzymes which we can show the function of mitochondria by the special enzyme stains such as NADH-TR, COX, SDH and combined COX-SDH. The complex II

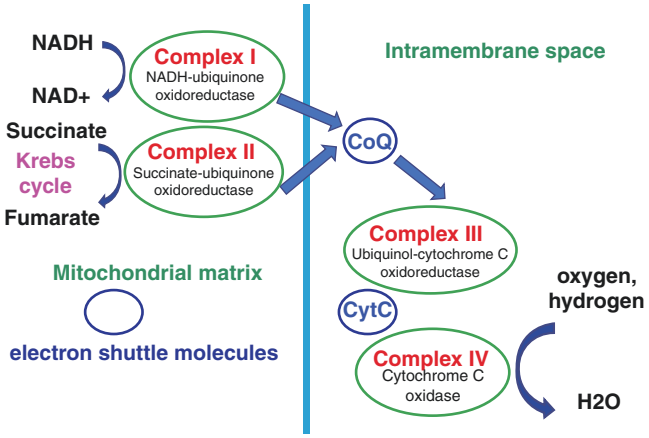

Fig. 5.25 Schematic of the electron transfer chain

enzyme, which is completely encoded by nuclear DNA, is also found in cases of mitochondrial dysfunction. Therefore, by making a combined COX-SDH stain, functional and non-functioning mitochondria can be distinguished [21].

In muscle biopsy, three different oxidative enzyme staining methods can be performed. In all enzyme staining, type 1 myofibers are stained darker because they are richer in mitochondria. One of them is NADH-TR enzyme staining. Diaphorase is a term given to various flavoprotein enzymes which have the property of transferring hydrogen from reduced NADH to various dyes. In the NADH-TR enzyme stain, nitroblue tetrazolium (NBT) dye is generally used as the hydrogen acceptor, and the product of the reduction is the water-insoluble formazan pigment. Enzymatic activity releases hydrogen from the substrate, and this free hydrogen is transferred to the tetrazolium. At the final stage of the reaction, the NBT is converted to dark-blue formazan pigment, highlighting the site of enzyme activity. An important point to remember is that all enzyme dyeing should be incubated at 37°C.

The second enzyme dye, succinic acid dehydrogenase (SDH), is a soluble iron flavoprotein that catalyses the reversible oxidation of succinic acid to fumaric acid. The histochemical demonstration of the activity of this enzyme is identified by incubating frozen sections with a mixture of succinate substrate and NBT. Under appropriate conditions, tetrazoliums are reduced to formazans which are water-insoluble, dark, blue-coloured compounds.

Cytochrome oxidase enzyme dye is the third and most difficult to apply enzyme dye, but it is the best indicator of the dysfunction of mitochondria. Cytochrome oxidase (COX) is the general name for Complex IV of the oxidative respiratory chain of enzymes located in mitochondria. The use of diaminobenzidine (DAB) results in a brown insoluble compound at the site of cytochrome oxidase activity.

These three-enzyme dyes I have listed are also available in commercial ready-to-use kits. In our laboratory, I prepare the reagents for all enzyme staining myself. I freeze and

store the dyeing solutions that I prepare in batches by dividing them as small quantities into special, tiny, capped tubes. This storage method provides great convenience during dyeing, and it is more cost-effective. Moreover, chemicals sold separately have a very long service life. For enzyme dyes, apart from these reagents, pH meters and precision balances are also required. I also use phosphate buffer solution for all enzyme staining.

For muscle, NADH-TR and SDH enzyme staining is performed as follows [11, 12]:

Stock solution for NADH	
0.2 M of phosphate buffer, pH 7.4 solution 10%.	5 mL
NADH.	8 mg
Mix it well and share to approximately 40–50 Eppendorf microtubes, and store at −25°C.	
Stock solution for SDH	
0.2 M of phosphate buffer, pH 7.4 solution 10%.	5 mL
Sodium succinate.	135 mg
Mix it well and share to approximately 40–50 Eppendorf microtubes, and store at −25°C.	
Stock solution for NBT	
0.2 M of phosphate buffer, pH 7.4 solution 10%.	10 mL
NBT.	20 mg
Mix it well and share to approximately 40–50 Eppendorf microtubes.	
And store at −25°C.	

Store fresh frozen sections in the frozen cabinet until enzyme staining begins. Take one tube of each frozen reagent that you have separated into smaller tubes from the freezer, and wait for them to thaw. Once the NBT solution has dissolved, divide it equally into Eppendorf of NADH and SDH stock solutions. Take the slides on wet paper in the paint container to be painted, and surround the sections with a special pen so that the paint does not scatter. Incubate a slide with the NBT-added NADH solution for 20 min and the NBT-added SDH solution for 1 h at 37°C. At the end of the incubation, wash with distilled water. Dehydrate in ascending ethyl alcohol series. Clear with xylene and mount with a permanent mounting medium. Store a few SDH and NBT mixtures in the refrigerator for use in the combined COX-SDH stain without freezing.

As a result of both NADH-TR and SDH enzyme staining, areas of enzyme activity look dark blue purple (Fig. 5.26).

For muscle, COX enzyme staining is performed as follows [11, 12]:

Stock solution	
0.2 M of phosphate buffer, pH 7.4 solution 10%	5 mL
DAB	140 µL
Cytochrome C	5 mg
Catalase	25 µL

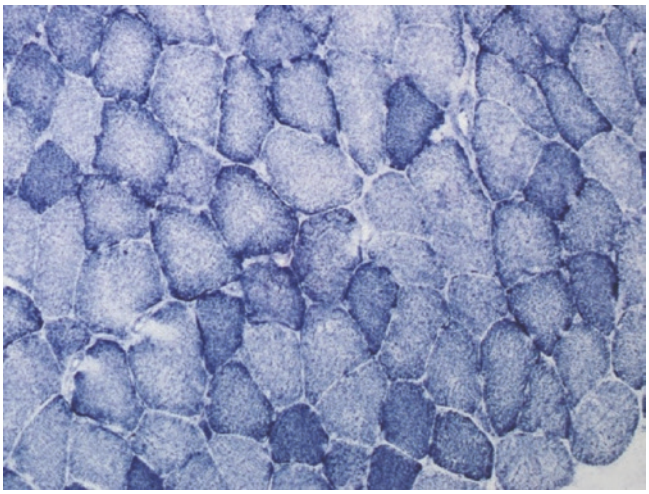

Fig. 5.26 Presence of mitochondria (SDH x 200)

Mix it well and adjust to pH 7.4 with NaOH. It is very important that the working solution is at a neutral pH; otherwise the reactions will not occur.

Share to approximately 40–50 Eppendorf microtubes, and store at -25°C.

Store fresh frozen sections in the frozen cabinet until enzyme staining begins. Take one tube from the COX stock reagents that you have separated into smaller tubes from the freezer, and wait for it to thaw. Take two slides on wet paper to the paint container to be painted, and surround the sections with a special pen so that the paint does not scatter. Incubate the slides with the melting COX reagent for an hour. At the end of the incubation, one slide stained with COX is incubated with the remaining SDH and NBT mixture for an hour, too. Wash both slides with distilled water after incubation. Dehydrate in ascending ethyl alcohol series. Clear with xylene and mount with a permanent mounting medium.

As a result of COX enzyme staining, areas of enzyme activity look brown (Fig. 5.27). *If there is a myofiber with mitochondrial dysfunction, it looks blue; this appearance highlights the presence of mitochondria which cannot complete oxidative phosphorylation* (Fig. 5.28).

Acid phosphatase, a hydrolytic enzyme, is one of the relatively frequently used enzyme stains. After staining, an increase in enzyme activity is observed in vacuoles which occurs in various glycogen storage diseases, as well as in inflammatory processes. When the naphthol acid phosphate in the staining solution is hydrolysed by tissue acid phosphatases, some naphthol derivatives form. These naphthol derivatives produce a red azo dye with the unstable diazonium salt to highlight the areas of enzyme activity (Fig. 5.29). Two percent methyl green is used for counterstaining slides which

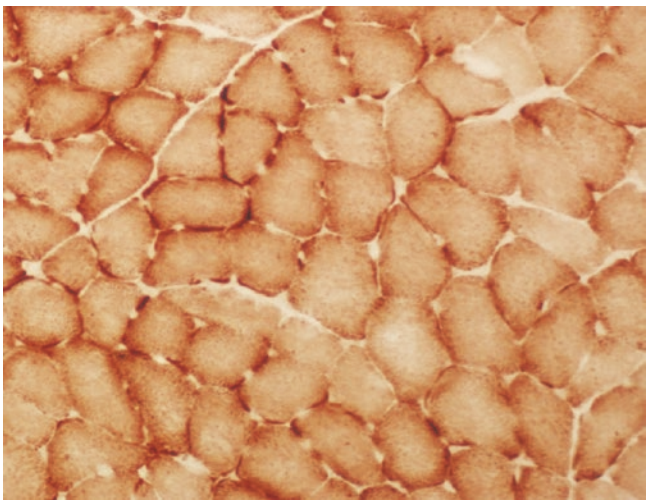

Fig. 5.27 Normal mitochondrial function (COX ×200)

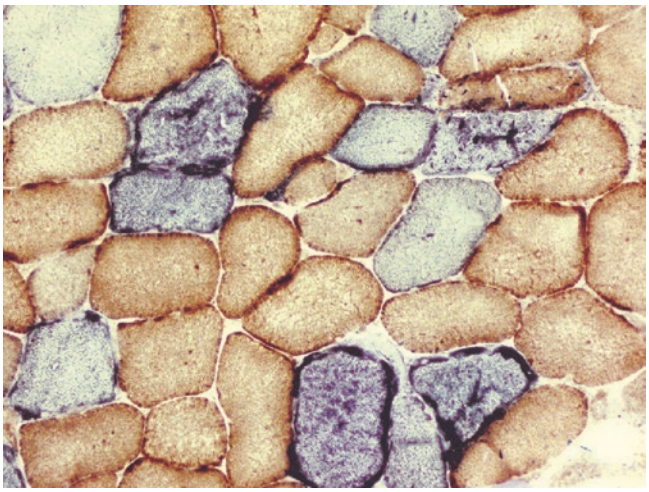

Fig. 5.28 The presence of pathological blue fibers (combined COX-SDH ×200)

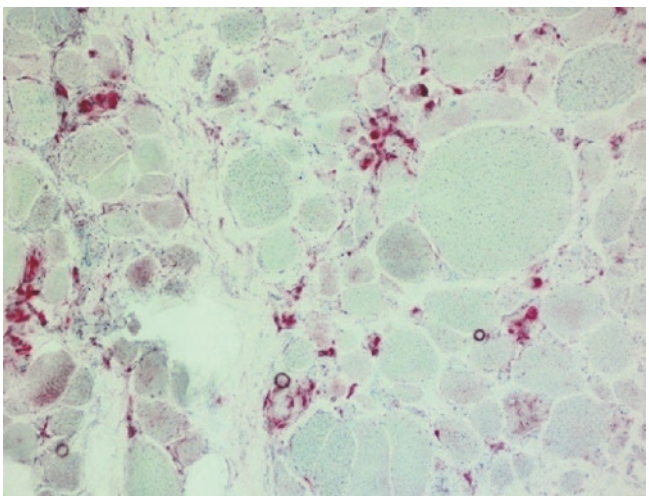

Fig. 5.29 Areas of enzyme activity are seen as red (acid phosphatase ×200)

are finally mounted with synthetic resin. This enzyme staining is not routinely performed on every muscle biopsy sample in our laboratory because it is a time-consuming analysis and the reaction takes place in a narrow pH range (pH 4.7–5). Moreover, there are simpler methods to show inflammation [3].

Myophosphorylase enzyme staining is a very useful analysis for differentially diagnosing McArdle disease which is glycogen storage disease type V. In this staining, phosphorylase affects the glucose-1-phosphate in the working solution, and forms, in the presence of a glycogen primer, polysaccharides composed of alpha-1,4-glycosyl units. Adenosine-5′-monophosphate functions as an activator in this analysis. While more complex polysaccharides are formed in this in vitro reaction, glycogen is broken down into smaller sugar molecules in the in vivo reaction. This is because the concentration of the substrate is high, while the inorganic phosphate concentrations are lower [22].

For muscle, muscle phosphorylase enzyme staining is performed as follows [11, 12]:

Stock sodium acetate solution	
Distilled water	100 mL
Sodium acetate	1.37 g
Mix it well and store at 4°C.	
Phosphorylase mixture	
Distilled water	5 mL
Glucose-1-phosphate	25 mg
Adenosine monophosphate	5 mg
Glycogen	2 mg
Stock sodium acetate solution 3.6 mL	
With 0.4 mL 5% acetic acid	4 mL
Ethyl alcohol (95%)	1 mL
Adjust pH to 5.6, mix it well, share to approximately 40–50 Eppendorf microtubes, and store at -25°C.	
Lugol solution	
Distilled water	100 mL
Iodine	1 g
Potassium iodide	2 g
Mix it well and store in a dark coloured bottle at room temperature.	

Incubation time must be a minimum of 2 h, and overnight incubation is preferred. Exposure of the sections to the Lugol iodine solution after incubation results in a varied colour formation and the newly formed polysaccharide A negative reaction is yellow. Unbranched short chains also give a negative reaction (Fig. 5.30). Polysaccharides of 8–12 units give a reddish colour, followed by a dark-brown colour as the length of the chain increases. Chain lengths of 30–35 units give a dark-blue colour (Fig. 5.31). The reason the colour is blue rather than red-brown is that the polysaccharide formed by phosphorylase action is not normal glycogen, which is coloured red-brown if the branching enzyme can work. However, alcohol in the incubation medium eliminates the action of the branching enzyme.

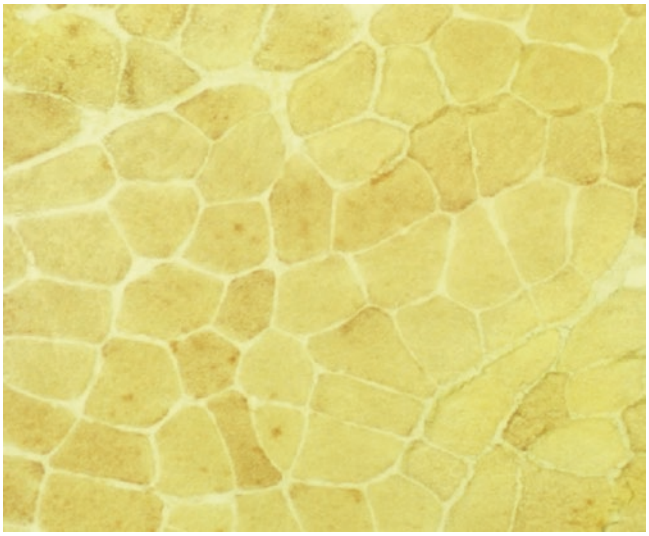

Fig. 5.30 Absence of phosphorylase activity in muscle of a patient with McArdle disease (phosphorylase ×100)

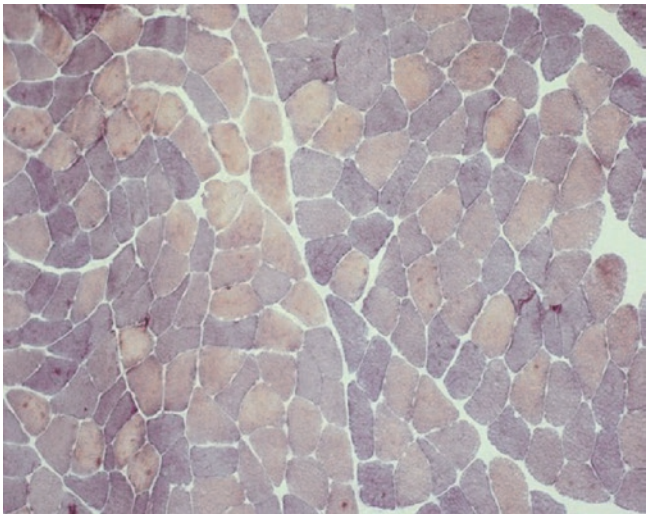

Fig. 5.31 Normal muscle phosphorylase activity (phosphorylase ×100)

Immune-Based Analyses Used for Muscle Biopsy

Immunohistochemical tests are diagnostic tests using antigen and antibody (Ab) reactions. These tests combine cell or tissue antigens with Abs developed against them, labelled with different substances, which aim to make the antigens visible microscopically. Some dyes were used at first to make antigens visible under light microscopy. However, most of them affect the immune reactivity and impair the sensitivity. Fluorochromes such as fluorescein and rhodamine are used in direct immunofluorescence (DIF) examination. The most widely used markers today are enzymes [21].

To produce polyclonal Abs, an Ab is injected into a mouse, and after a waiting period of 2–3 weeks for Ab formation, immune globulin-rich serum is separated from the blood taken from the animal. Although this method is easy, it is not preferred because it requires a lot of serum. Additionally, cross-reactions occur frequently due to different antigens encountered by the animal. Antigens can be injected into a mouse to obtain monoclonal Abs, too. At the end of the time required for Ab formation, B-lymphocytes are separated from the blood taken. Hybrid cells are obtained by fusing B-lymphocytes into myeloma cells in the hypoxanthine/aminopterin/thymidine (HAT) medium. Myeloma cells are immortal. However, B lymphocytes provide the hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme necessary for them to survive in the HAT environment. Thus, immortal hybrid cells that will produce the desired monoclonal Ab are obtained (Fig. 5.32).

The reaction of the body against foreign substances (antigens = Ag) in its hereditary structure is called the immune response or immunity. The basic Ab of immunohistochemical reactions, immunoglobulin G (IgG), has a structure like the letter Y and can bind two antigens. During binding, each arm of the letter Y binds individual antigens instead of wrapping them around a single antigen. Thus, three-dimensional complex structures are formed during antigen-antibody reactions. Immunohistochemical staining methods can be direct or indirect. In the direct method, the monoclonal Ab developed against the sought antigen is incubated with the tissue. It is then stained with a chromogen, which will be activated by the enzyme bound to the antibody. If there is no antigen in the tissue, the antibody will not be able to bind, and there will be no staining as it is removed from the tissue during washing. Although direct methods are easy to implement, they are expensive because it is necessary to label the antibody developed against each antigen separately [11, 23–25].

In the indirect method, the cost is reduced because here each Ab is not individually labelled. First, monoclonal Ab is produced as in the direct method, but this time, one more step is added before adding chromogen. In this step, antibodies are made against the mouse that produces specific antibodies from another animal, such as a rabbit or goat, and only these secondary antibodies are labelled. Therefore, when defining secondary antibodies, host and target animal species are declared such as rabbit anti-mouse Abs or goat anti-rabbit Abs. If an experimental study is to be carried out in mice, care should be taken that the secondary antibody to be used should not be anti-mouse to avoid cross and aberrant staining. Due to phylogenetic similarity, primary antibodies, usually anti-mouse or anti-rat, are not used in mouse or rat experiments. These labelled secondary antibodies in the indirect method can be used to detect all kinds of antigens [21].

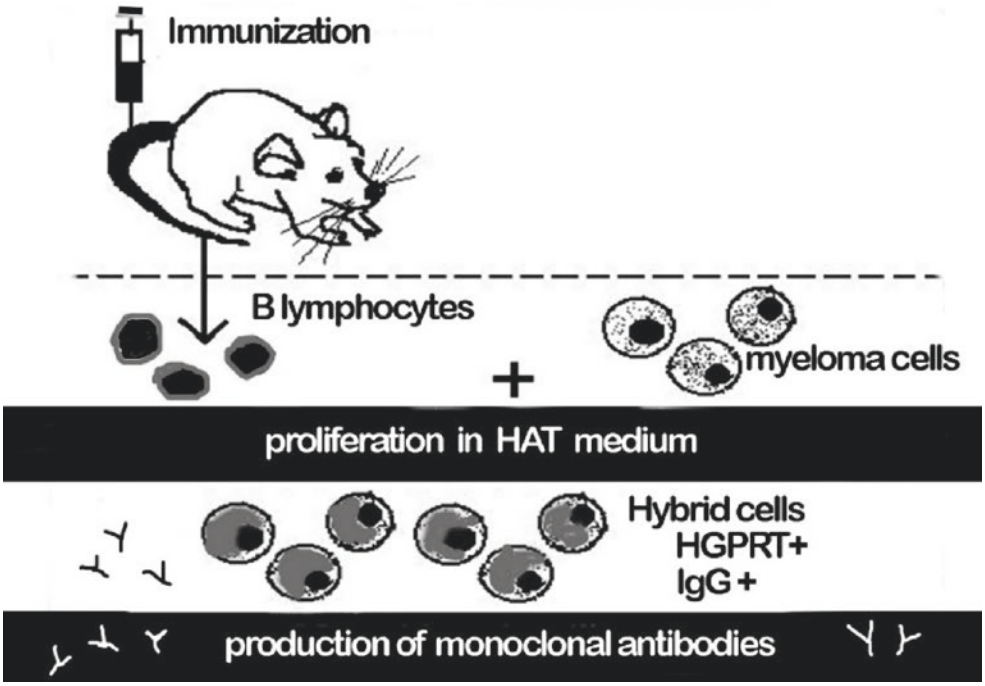

Fig. 5.32 Production of antibodies

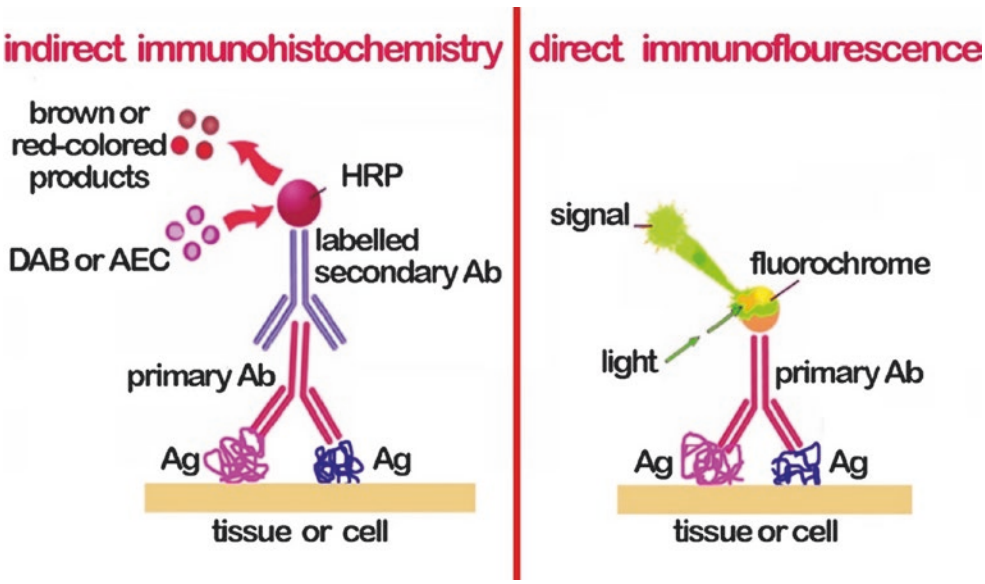

Fig. 5.33 Illustration of indirect and direct immunohistochemistry and immunofluorescence techniques

Protein A obtained from *Staphylococcus aureus* can also be used instead of a secondary antibody. This substance is both easily labelled and has a high affinity for antibodies. The avidin-biotin complex (ABC) system was developed based on the affinity of avidin, a glycoprotein in egg white, for the vitamin biotin. An avidin-like molecule is also obtained from the bacterium *Streptomyces avidinii*. Today, more sensitive and specific secondary binding reagents and techniques for indirect immunohistochemical stains have been developed. In the indirect methods, staining artefacts have also been reduced. The principle of most immunofluorescent staining techniques is like direct immunohistochemical analysis, with the primary antibody being labelled with fluorochromes. In summary, although different methods are applied, the principles of them are the same (Fig. 5.33). Horseradish peroxidase (HRP) is the most used enzyme to activate chromogen in both direct and indirect methods. The most used chromogenic substrate for peroxidase is 3,3-diamino-benzidine (DAB), and the staining is brown. 3-amino 9-ethylcarbazole (AEC) is a red-coloured, less toxic substance that can also be used as a peroxidase substrate. However, AEC is not preferred as it fades in a much quicker than DAB. In the event of needing to perform a double immunohistochemical stain on a single slide, DAB is used

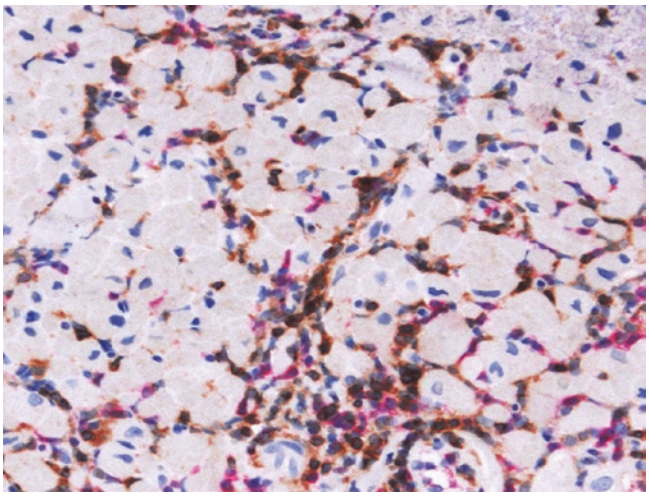

Fig. 5.34 Severe rejection in a heart transplant biopsy. CD4(+) T cells are in brown with DAB, and CD8(+) T cells are in red with AEC. This photograph is from the archive of Phillip Ruiz, MD, PhD, Professor of Surgery and Pathology, Director of Immunology and Histocompatibility Laboratory of Miami University

for one antigen and AEC is used for the other (Fig. 5.34). Another chromogenic substrate used for immunohistochemical analyses is fast red. But it can be used as only an alkaline phosphatase substrate. When reacted with alkaline phosphatase, fast red produces an insoluble red precipitate at the reaction site on tissues and cells.

If immunohistochemical examinations are to be applied to paraffin sections, they must be pre-treated because during the time spent in tissue follow-up and paraffin embedding, antibody binding epitopes of antigens in the tissues become immunologically ambiguous. To strengthen antigenicity, the tissue should be treated with alkaline or acidic solutions and high heat, although it varies according to the antibody. For this purpose, pH 6.0 citric acid or pH 8.0 ethylene diamine tetra acetic acid (EDTA) solutions are commonly used. The slides in the solutions are boiled in the microwave, in special closed automatic immunohistochemistry devices or simply in a pressure cooker for 20 min. Similarly, after this pre-treatment step, incubation with 3% hydrogen peroxide and the primary blocker in the kit for 5–10 min is essential to reduce the endogenous tissue peroxide reaction that will lead to activation of chromogen. On the other hand, almost all commercial primary antibodies used in muscle biopsy are only to be used on frozen sections. Therefore, staining procedures starting directly from primary blocking of endogenous peroxides and pre-treating for antigen retrieval is not required [11, 12, 21, 23–25].

Today, there are many commercial primary antibodies of a wide variety of brands that react with a significant proportion of striated muscle proteins. They may differ in expression location and intensity according to their clones. For example, while collagen 6 expression with an Ab is entirely extracellularly localised, cytoplasmic expression can also be observed with another Ab. Again, as many of these antibodies are not widely used, they are very expensive. For example, a 100-test keratin antibody can be sold for less than 10 euros, while a 100-test antibody used in muscle biopsy can cost 1000 euros. Moreover, these antibodies have not yet been validated and cannot be used for diagnostic purposes. These antibodies are generally sold with the phrase that 'they are suitable for research purposes only'. For the reasons I have listed, I think that only antibodies showing the causative agent of neuromuscular diseases with a high incidence should be used in a pathology laboratory where special muscle and nerve biopsies are evaluated. For example, I have been staining slides for perinuclear emerin expression for a long time, and the rate of patients in whom I have detected a loss of emerin expression is very low. Similarly, detecting the presence of anti-titin antibody may be not required. In such rare muscle diseases, it would be much more appropriate to screen these patients for rare muscle diseases with multiple genetic testing panels, after distinguishing whether the pathology is muscular dystrophy using muscle biopsy. Similarly, the dystrophin antibody should be taken from the polyclonal one to reduce cost because there are three different dystrophin antibodies on the market that recognise the N-terminal, C-terminal and rod part of dystrophin. The company selling it markets it as a trio and proposes that triple staining is required for all biopsies. I have performed triple immunohistochemical analyses separately for years, and I have not come across a single biopsy where dystrophin expression is completely normal with one type of antibody, and the others are missing. I now believe that staining dystrophin with a polyclonal antibody is safer and more cost-effective [1, 2, 12].

On the other hand, I think that muscle biopsy should not be performed without dystrophin, sarcoglycans (especially alpha, beta and gamma) and neonatal myosin antibodies. This is because deletion analyses are only performed for screening purposes in dystrophinopathies, and since it is the most common muscular dystrophy, if a dystrophin defect is detected in muscle biopsy, sequencing should also be done. Moreover, genetic change cannot distinguish the two types of disease, which have very different severities. The focal presence of dystrophin in biopsy leads to the diagnosis of Becker muscular dystrophy, especially in young children whose clinical findings are not yet well established, whereas the diagnosis of Duchenne muscular dystrophy is made by detecting diffuse expression loss. Sarcoglycanopathies are a group of one of most common muscular dystrophies, and if in one of them an expression defect is present, focal defects generally occur in the other sarcoglycans. If they are examined together, the type of sarcoglycanopathy can be determined. However, it is kept in mind that focal sarcoglycan defects may be seen in dystrophinopathies. Finally, neonatal

myosin is the most essential antibody for muscle biopsy examination because it highlights the presence of immature myofibers, regenerated myofibers, or whether myopathy is present [1].

Preparation of Nerve Biopsy

Changes in peripheral nerves due to injury can be evaluated in a more limited spectrum. These variations can mainly be classified as loss of myelin, the presence of axonal degeneration and inflammation. These three main changes observed in peripheral nerve biopsies also can be sorted by their severity [1]. Today, nerve biopsy continues to be a useful diagnostic tool in some diseases including amyloid neuropathy, specific inflammatory disorders, atypical chronic inflammatory demyelinating polyneuropathy (CIDP) and localised nerve vasculitis, where nerve biopsy remains the 'gold standard' investigation. However, nerve biopsy is almost never completed only to confirm the presence of a nerve disease. The patient's history and findings of physical examination and electrodiagnostic studies can almost always carry that out. The nerve biopsy may be required for diagnosing a few rare diseases with a characteristic histologic hallmark, such as vasculitis, granulomatous disease and amyloidosis. It may also be useful for differentially diagnosing progressive polyneuropathies in which there remains no diagnosis despite a complete workup. In such cases, histologic evidence of inflammation or demyelination can be determined in the nerve biopsy. It was previously claimed that a combined muscle and nerve biopsy has increased the detection of vasculitis, but recent studies have not confirmed this suggestion. Even now it is reported that muscle biopsy alone is diagnostically more useful than nerve biopsy [8, 9, 12, 26–30].

The specimen must be obtained from an affected nerve. Most neuropathies show distal placement. The sural nerve is almost always comprised of sensory fibers, or it may contain only a few motor fibers. The sural nerve is an easily accessible nerve that lies superficially between the lateral malleolus and Achilles tendon. Therefore, it is frequently chosen for biopsy. It usually contains up to 10 nerve fascicles that comprise 3300–8000 myelinated and 10,500–45,500 unmyelinated fibers. It isn't advisable to select the sural nerve when it is terminally depleted. In this situation, it can be less informative than a nerve with recently affected fibers. Among other nerves, the superficial peroneal nerve may be preferred as concomitant muscle biopsy from the adjacent peroneus brevis muscle may be taken during the same surgical procedure. The radial nerve may be preferred when symptoms predominate in the upper limbs. If the neuropathy entirely affects the motor neurons, obturator nerve biopsy is very useful to differentiate motor neuropathies and lower motor neuron diseases [30–32].

It should be noted that harvesting a sample composed of just a few nerve fascicles does not cause fewer complications like sensory deficits, and it will be less diagnostic. Removal of a shorter segment can also leave a severe sensory deficit. Therefore, complete excision of the nerve, which is approximately 5 cm in length, is recommended. This is especially important when looking for multifocal skip lesions including inflammatory processes. Such foci are more likely to be detected if the entire nerve is examined. Although the morbidity of nerve biopsy is much higher than that of muscle biopsy, the handling of nerve biopsy is much simpler. In addition, serious artefacts do not occur during transportation and sectioning of nerve specimens [27, 30].

Sometimes the tissue has been immersed in formaldehyde solution due to the lack of clinicopathological correlation. In this situation, frozen sections cannot be made and several special stains like modified trichrome, oil red O and most immunofluorescence examinations cannot be performed. Also, sending the material in fixation solution is an essential step to avoid for muscle biopsy, since most of the muscle-specific primary antibodies used in immunohistochemical staining work only on frozen sections and so almost no special staining could be performed. Therefore, muscle tissue in a paraffin block is absolutely nondiagnostic. However, specific immunohistochemical stains applied in nerve biopsies are adapted to paraffin sections, and fixed material can be used for some of the diagnostic analyses [1, 33].

At first, the fresh nerve biopsy material is divided into three parts, each of them used for evaluation in a frozen section, in a paraffin section and in electron microscopic examinations. For frozen section, one piece of nerve is sliced along its length at 2–4-mm intervals. Some pieces of the nerve should be placed in the frozen holder so that it can be seen transversely in some of the sections and longitudinally in others. This is because on the longitudinal sections, detection of segmental demyelination is relatively easier. Although sections frozen in liquid nitrogen are recommended for nerve biopsies in the guidelines, frozen sections without liquid nitrogen may not create severe frozen artefacts which cause nondiagnostic appearances because there is no complex intracellular arrangement in peripheral nerves like striated muscles and there is a limited spectrum of histopathological changes. Frozen sections with a thickness of 6–8 microns should be taken as required for special examinations. While taking more frozen sections, the first slide to be stained with haematoxylin-eosin stain should be examined by an experienced pathologist. This initial examination is essential for the framing of the diagnosis and for deciding on any additional investigations that may be required. One of the most important findings to be detected in this first examination is whether the biopsy was taken from the nerve. An inexperienced surgeon can sample the adjacent, similar tissue like a vein or tendon instead of the nerve. As the tissue

to be removed during the surgical procedure is usually tied at both ends, there may be no noticeable bleeding, even if it is a vessel. In the laboratory, it can be identified that the material sent is a vessel, as the material has a lumen in it noticeable during the dissection. Although there is no necessity for a quick diagnosis for most neuropathies, it is important to inform the clinician immediately in the case of vasculitis and ischemia due to microvasculitis so that the treatment can be planned early. The frozen sections have the advantage of being able to provide an immediate diagnosis for some diseases such as vasculitis, and it is useful to banking frozen tissue which can be used in future molecular and biochemical studies. In addition, the lipid dyes and immunofluorescence analyses can be used in frozen sections. In fact, almost all analyses except electron microscopic examination can be performed on frozen tissue, and even if freezing artefacts cause problems, electron microscopic examination can be attempted on frozen tissues when necessary. However, the most important disadvantage of frozen sections is that they provide much more inferior histopathological detail [9–12, 33].

Although the fiber teasing procedure has been routinely performed at many nerves biopsy laboratories over many years, it is not widely respected as a diagnostic tool for differentially diagnosing neuropathies. If fiber teasing is required, a 1.2-cm nerve segment, taken immediately after biopsy, is fixed in glutaraldehyde or formalin, post fixed in osmium tetroxide in phosphate buffer solution (PBS) at pH 7.4 for 3–5 h at room temperature and passed through graded glycerin from 30 to 100%. It may be stored for several weeks in the glycerin before dissection. During analysis, the material is placed in a pool of glycerin on a glass slide on the stage of a dissection microscope, and the proximal ends of the material are grasped. Then, connective tissue is stripped off, bundles of fascicules are subdivided, and fascicles are pulled apart with the help of a special fork. Finally single-teased fibers are separated, and approximately five single-teased fibers are placed on a second slide covered with glycerin to be microscopically examined. Teased fiber analysis generally visualises abnormalities such as fiber degeneration, axonal atrophy, axonal swellings and tomacula formations. Even if it is claimed that teased fiber examination is the best method for differentiating axonal neuropathy from demyelinating neuropathy, it has been proven that these changes may also occur in controls [11, 34].

For general nerve biopsy examination, frozen, methacrylate and paraffin sections are satisfactory. As nerve biopsy is invasive, costly and associated with possible morbidity, it is recommended that all parts of the sural nerve should be taken instead of a few fibers so that multiple blocks of tissue can be embedded. Tissue is recommended to be oriented both transversely and longitudinally when sectioning. Preferably, several blocks of tissue for both paraffin and plastic embedding should be prepared. Multiple sections should be stained with general purpose stains, and additional sections should be available for special purposes. Frozen sections with haematoxylin and eosin (H&E) are used to make a quick evaluation of the nerve. Although modified trichrome and oil red O stains are performed on frozen sections, Congo red, trichrome, crystal violet, Holmes axon stain and Weil's myelin stains are used on paraffin sections. The basic stain used for semithin plastic-embedded sections is toluidine blue. As histological details on the paraffin section are more satisfactory, all IHC analyses of nerve biopsy are performed on paraffin sections.

Histochemical and Immunohistochemical Stains of Nerve Biopsy

For routine examination of frozen and paraffine sections of nerve biopsy material, the haematoxylin and eosin (H&E) stain is used as previously mentioned in the muscle biopsy processes. Similarly, Gomori's trichrome, Modified Gomori's trichrome, PAS, dPAS, oil red O, Congo red and crystal violet stains are also performed for nerve biopsies. When identifying demyelination, the modified trichrome stain is the most useful histochemical dye. However, the enzyme stains, except for alkaline and acid phosphatases, are not used for nerve biopsies. The toluidine blue stain is specifically used for nerve biopsies, and it is one of the basic histochemical stains used for the evaluation of semithin sections of resin blocks. However, toluidine blue can stain resin-embedded tissues, if performed at high alkaline pH and with heating [31–34].

For frozen or paraffin-embedded tissue sections of nerve biopsy, *toluidine blue staining* is traditionally performed as follows [11, 12, 32]:

Staining solution	
Toluidine blue	1 g
Distilled water	100 mL

Adjusted to pH 2.3–2.5 with NaOH and/or HCL (pH > 2.7 decreased the metachromasia)

1. Deparaffinised or frozen sections are brought to distilled water.
2. Immerse sections in staining solution for 2–3 min.
3. Wash well with distilled water.
4. Dehydrate quickly in ascending ethyl alcohol series (ten dips each since stain fades quickly in alcohol).
5. Clear with xylene and mount with a resinous mounting medium.

As a result, toluidine blue staining the paraffin or frozen sections, granules of mast cells (Fig. 5.35) *and myelin looks magenta; axons look blue.*

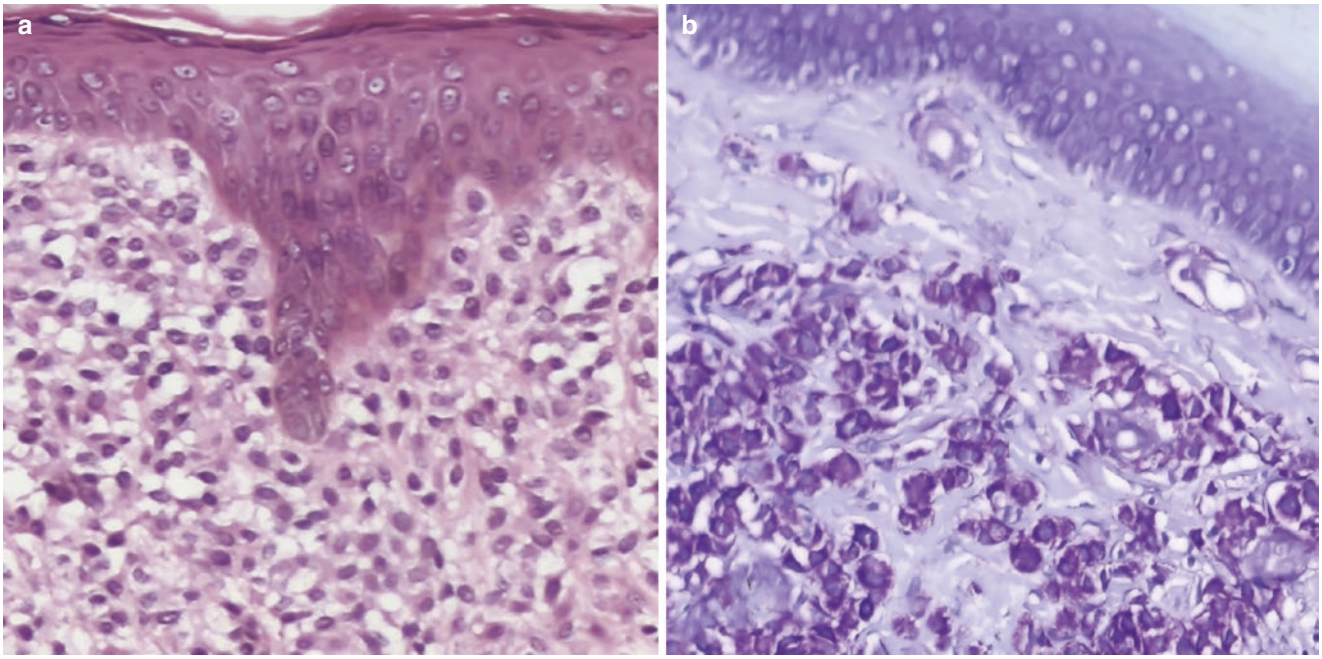

Fig. 5.35 Mast cells in a skin biopsy. (**a**) H&E x 400, (**b**) Toluidin blue stain x 400

For semithin sections of nerve biopsy, *toluidine blue staining* is performed as follows [11, 12, 32]:

Staining solution	
Toluidine blue	1 g
Sodium tetraborate (borax)	1 g
Distilled water	100 mL

Dissolving of borax increases the pH of the final staining solution to about pH 11. This alkaline pH value is necessary for staining of resin-embedded sections.

1. Semithin sections are covered with a few drops of staining solution and then placed on a hot plate at 65°C.
2. After heating for 3–5 min, excess stain is washed off in running hot water.
3. Blotted dry or air-dried if preferred.
4. Mount with a resinous mounting medium.

As a result of adequate toluidine blue staining of the resin-embedded sections, tissue appears deep blue, and resin remnants must not be stained. Unfortunately, staining fades with time, and stained slides must be stored in a light-proof box.

The *toluidine blue and basic fuchsin stain (paragon multiple stain)* is also a basic histochemical stain used for the evaluation of semithin sections of resin blocks [30].

For nerve biopsy, *paragon multiple staining* is performed as follows [11, 12, 32, 33]:

Staining solution	
Toluidine blue	1 g
Basic fuchsin	0.4 g
50% ethanol	150 mL

Add all ingredients together, mix it well and filter before use. The solution can be stored at room temperature.

1. Semithin sections are covered with a few drops of staining solution and then placed on a hot plate at 65°C.
2. Sprinkle sodium borate lightly over staining solution.
3. After heating for 3–5 min, a green sheen cover appears on the staining surface.
4. Currently, staining is complete, and excess stain is washed off in running warm water.
5. Blotted dry or air-dried if preferred.
6. Mount with a resinous mounting medium.

As a result of adequate paragon multiple staining of the resin-embedded sections, collagen appears magenta to pink, cytoplasm appears blue, nuclei appear deep blue, and myelin appears very dark blue to black. Fading of the stain with time is also inevitable (Fig. 5.36).

During vasculitis, peripheral nerve involvement, fibrinoid necrosis, thrombosis and both haemorrhage on the vessel walls and perivascular areas occur due to involvement of capillaries and postcapillary venules of the nerves. In chronic vasculitis, the perivascular epineurial haemosiderin deposits can be identified with Perl's Prussian blue stain. The hydrolysis of dilute mineral acids can release ferric ions from protein-bound tissue deposits. However, all ferrous ions do not produce a coloured reaction product. For colourisation, deposits must include ferrocyanide ions as haemosiderin. When deposits consist of haemosiderin, they precipitate as a blue-coloured and water-insoluble complex, potassium ferric ferrocyanide known as Prussian blue. For nerve biopsy, **Prussian blue staining** is performed as follows [11, 32, 35]:

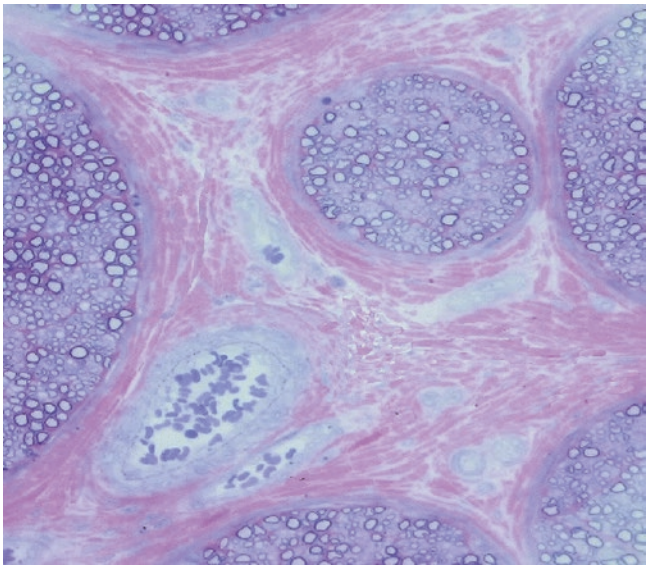

Fig. 5.36 Sural nerve (toluidine blue and basic fuchsin stain ×40)

Staining solutions 1	
20% hydrochloric acid (HCL)	20 mL
Distilled water	80 mL
Staining solutions 2	
Potassium ferrocyanide (K4FeCN6)	10 mL
Distilled wate	90 mL
Mix these two solutions equally just before the process.	
Neutral red stain	
Neutral red	1.0 g
Distilled water	100.0 mL
Glacial acetic acid	1.0 mL

Dissolve the dye in the distilled water. Add the acid. Mix well. Filter into the reagent bottle.

For this reaction, neutral buffered formalin should be used. Other fixatives, especially acidic dichromate fixatives and acidic decalcification fluids should be avoided. These reagents can cause hydrolytic loss of ferric ions from tissues.

Neutral red stain is used for counterstaining and for colourisation of the background. Therefore, any dyes with red colour, even eosin dye, can be used to achieve this.

1. Deparaffinised or frozen sections are brought to distilled water.
2. Immerse sections in mixture solution (solution 1 and 2) for 20 min.
3. Wash well with distilled water.
4. Counterstain with filtered neutral red stain for 5 min.
5. Clear with xylene and mount with a resinous mounting medium.

*As a result of Prussian blue staining of the paraffin or frozen sections, deposits of haemosiderin look deep blue; background looks red (*Fig. 5.37*). With longer incubation, asbestos bodies can be identified in blue-black.*

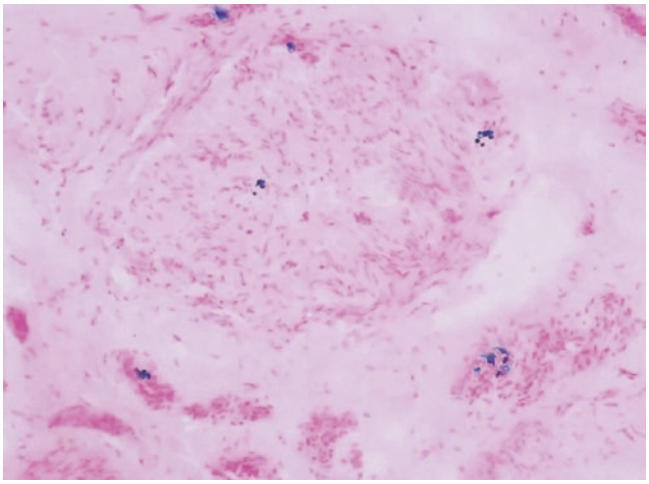

Fig. 5.37 Deposits of haemosiderin in a sural nerve biopsy (Prussian blue ×100)

The combined Van Gieson and Verhoeff's staining methods can be used for nerve biopsies. This dye can demonstrate elastic fibers, as well as several connective tissue elements [11].

For nerve biopsy, elastic Van Gieson staining is performed as follows:

Solution A	
Haematoxylin	5 g
70% ethanol	100 mL
Solution B	
Ferric chloride	10 g
Distilled water	100 mL
Solution C (Lugol's Iodine Solution)	
Iodine	1 g
Potassium iodide	2 g
Distilled water	100 mL
Verhoeff's staining solution (usable Life up to 48 h)	
Solution A	2.5 mL
Solution B	1 mL
Solution C	1 mL
Add in the above order, mixing between additions.	
Van gieson's solution	
Saturated aqueous picric acid solution	50 mL
1% aqueous acid fuchsin solution	9 mL
Distilled water	50 mL

1. Deparaffinised or frozen sections are brought to distilled water.
2. Immerse sections in Verhoeff's staining solution for 15–30 min.
3. Wash with tap water.
4. Differentiate in 2% aqueous ferric chloride (mix 2 mL of distilled water and 0.5 mL of solution B) until elastic

tissue fibers appear black on a grey background. This lasts approximately 30 s.
5. Wash with water.
6. Rinse in 95% alcohol to remove any staining due to iodine alone.
7. Immerse sections in Van Gieson's staining solution for 3 min.
8. Only blot, because washing in water after Van Gieson's solution impairs the colours.
9. Dehydrate quickly in ascending ethyl alcohol series.
10. Clear with xylene and mount with a resinous mounting medium.

As a result of elastic Verhoeff-Van Gieson staining of the paraffin or frozen sections, collagen looks red, other tissues look yellow, and elastic fibers look black (Fig. 5.38).

By using the combined Van Gieson and Verhoeff's staining technique to evaluate the distribution of elastic fibers within the peripheral nerve, it has been shown that elastin is in all three connective layers of the peripheral nerve. Thick elastic fibers, consisting of both amorphous elastin and microfibrils, are in the perineurium and in the epineurium. The endoneurium contains small collections of elastic fibers distributed between the axons. However, the overall presence of elastic fibers is lesser than collagen fibers. Therefore, the flexibility of the peripheral nerve is mainly provided by collagen. During electron microscopic evaluation, the distribution of these fibers can be shown in detail. However, in the frozen sections, the presence of elastic fibers can only be roughly demonstrated. In neuropathies, the exact location and number of elastic fibers cannot be discriminated with elastic dyes due to proliferation of elastin precursors, formerly known as oxytalan fibers [36, 37].

In cases when inflammation is not clearly visible with routine stains, immunohistochemical leucocyte common antibody (LCA, CD45) is used to detect small collections of leukocytes. A small number of epineurial or endoneurial lymphocytes is within normal limits (<20/mm^2), and a few lymphocytes may normally be found around epineurial vessels giving the impression of slight cuffing. However, endoneurial capillary lymphocytic cuffing is certainly abnormal, and more than two leukocytes in a nerve fascicle should be regarded as an indicator of inconspicuous inflammation. In chronic inflammatory demyelinating polyneuropathies (CIDPs), the CD4+/CD8+ lymphocyte ratio is mildly reduced. Similarly, IgM, IgG and complement deposits in the walls of endoneurial blood vessels and on the surface of Schwann cells can be detected using direct immunofluorescence. The vascular deposits seen as granular patterns are immune complexes, and the Schwann cell deposits in the linear patterns are antigens that bind to the cell membrane. All of these deposits can rarely be found in nonimmune-mediated neuropathy or normal controls [21, 23, 24].

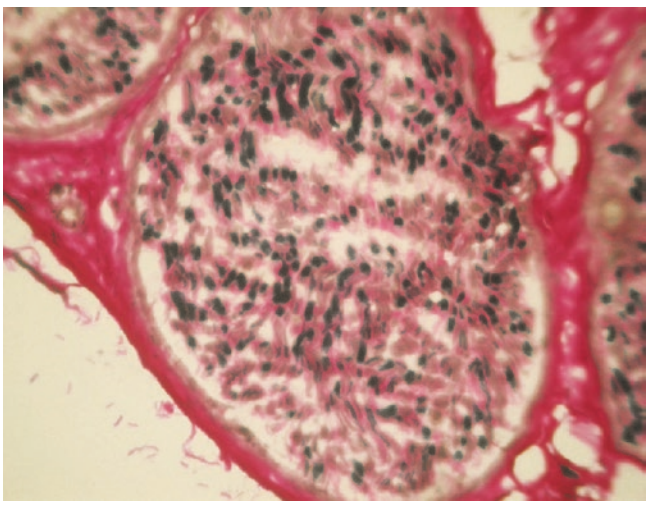

Fig. 5.38 Sural nerve (Verhoeff's Von Gieson stain ×100)

The predominant inflammatory cell in any vasculitis is the T lymphocyte, as well as activated macrophages. The number of B cells is insignificant. Almost all of the lymphocytes are helper (CD4+) and cytotoxic (CD8+) T cells. In inflammatory neuropathies, plasmacytes are not seen among the inflammatory cells. The observation of plasmacytes may suggest the presence of a circulating paraprotein. Depending on the duration of the inflammatory process, a variable number of neutrophils and eosinophils may also be present. In any case, the presence of neutrophils should lead one to think of a diagnosis of vasculitis. Direct immunofluorescence may reveal accumulations of immunoglobulin, complement or fibrinogen in epineurial vessel walls, in most nerve biopsies, in patients with vasculitic and diabetic neuropathy. Therefore, epineurial and perimysial vascular deposits of immunoglobulins or C3 are seen as a specific marker of vasculitic neuropathy [28].

Neuropathy is generally considered inflammatory in the presence of more than two lymphocytes per each nerve plexus. However, such a small number of inflammatory cells may not be distinguishable with routine staining. Therefore, immunohistochemistry and immunofluorescence examinations are required in nerve biopsies. LCA, CD20, CD3, CD4, CD8 and CD68 immunohistochemical staining should be routinely applied to each case, enabling the ability to distinguish between T and B lymphocytes, T-lymphocyte subtypes and macrophages. In addition, neurofilament and myelin basic protein immune staining should be performed to distinguish axon and myelin, respectively, in nerve biopsies.

In addition, two histochemical dyes that have been used for many years to stain axon and myelin can also be applied. The Holmes method for nerve cells and fibers can be used to aid identification of axons in the peripheral nerves.

For nerve biopsy, **Holmes axon staining** is performed as follows [11]:

Impregnation solution	
1.5% boric acid solution	2.75 mL
2% borax solution	2.25 mL
20% pyridine solution	0.25 mL
Distilled water	19.75 mL
Reducing solutions	
Hydroquinone	10 mg
Sodium (di) sulphite	100 mg
Distilled water	1 mL
Prepare just before using the reducing solution and don't store.	

1. Deparaffinised sections are brought to distilled water.
2. Immerse sections in silver nitrate, 20% for 2 h at room temperature.
3. Place in impregnating solution without washing overnight at 37°C in a covered vessel.
4. Remove slides, drain excess solution, and place in reducing solution for 10–15 min.
5. Wash in running water for 3 min, and then rinse in distilled water.
6. Tone in gold chloride, 0.2% for 3 min.
7. Rinse briefly in distilled water.
8. Develop in oxalic acid; 2% under the microscope until axons are thoroughly blue-black.
9. Rinse in distilled water.
10. Place in sodium thiosulfate, 5% for 5 min.
11. Wash in running water for 5 min.
12. Dehydrate through graded ethanol baths 13. Clear with xylene and mount with a resinous mounting medium.

As a result of Holmes staining of the paraffin sections, axons look deep blue-black; background looks grey to pale pink (Fig. 5.39).

Weil's stain is used for the identification of myelin in paraffin sections. The underlying principle of Weil's method involves the reduction of chrome salt to chromium dioxide by myelin. Then, the chromium acts as a mordant for the haematoxylin, strengthening the stain. This procedure is generally conducted on sections from formalin-fixed, paraffin-embedded tissue that are cut at a thickness of at least 8–10 μm [11].

Staining solution-A	
Ferric ammonium sulphate	4 g
Distilled water	100 mL
Staining solution-B	
Haematoxylin	1 g
Absolute ethanol	100 mL
Just prior to staining, make a **working solution** by mixing equal volumes of solution-A and solution-B.	

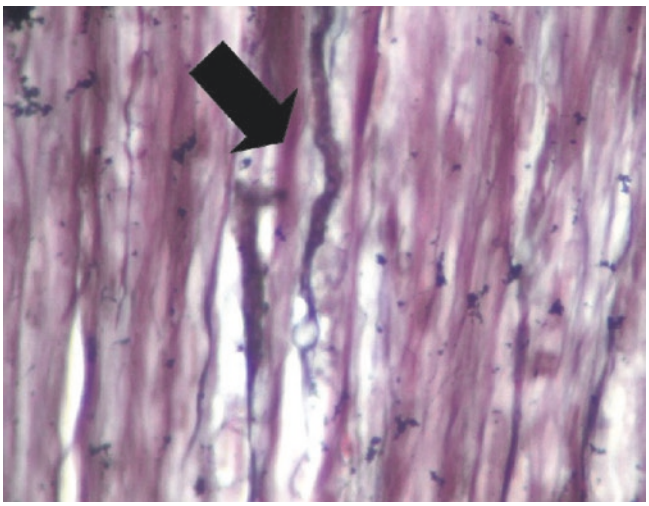

Fig. 5.39 A giant axon (black arrow) in a sural nerve (Holmes stain ×400)

Weigert's differentiator solution	
Borax (sodium tetraborate)	1 g
Potassium ferricyanide	1.25 g
Distilled water	100 mL

1. Deparaffinised sections are brought to distilled water.
2. Put slides in freshly prepared mixed staining solution at 50–55°C for an hour.
3. Wash slides well in tap water.
4. Partially differentiate in solution-A until distinguishable blueish-black myelin sheaths form a pale grey background, for approximately 8 min.
5. Check the sections under the microscope.
6. Wash well in tap water.
7. Complete differentiation in Weigert's differentiator solution until distinguishable deep-blue myelin sheaths from a clear background, for 2 min.
8. Check the sections under the microscope.
9. Wash well in tap water.
10. Dehydrate through graded ethanol baths.
11. Clear with xylene and mount with a resinous mounting medium.

As a result of Weil's myelin staining of the paraffin sections, myelin-containing structures will be stained black, nuclei look blue, and background looks colourless to pale yellow (Fig. 5.40).

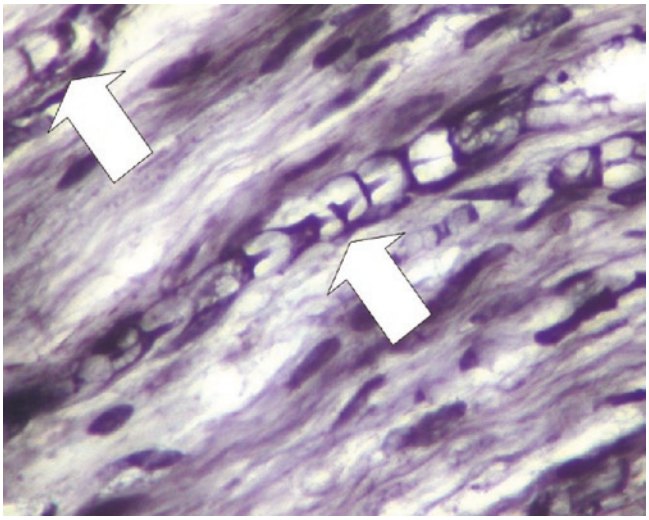

Fig. 5.40 Rare myelin sheets (write arrows) in a sural nerve biopsy (Weil's stain ×400)

Diagnostic Usefulness of Electron Microscopy for Muscle and Nerve Biopsies

The first electron microscope was built in 1932 by Max Knoll and Ernst Ruska. Optical microscopes use light to obtain the image, while electron microscopes use electrons instead of light. Since the wavelength of the electron is several thousand times smaller than that of light, it is possible to obtain more detailed images with this microscope. With some electron microscopes, the 0.2-nm object can be visualised, while the best optical microscopes can only show an object of 250 nm. Electron microscopes can be divided into two in terms of working principles. With the scanning electron microscopy (SEM) type, the image is obtained by making use of reflected electron beams, while in the other type, the rays passing through the object form the image. Electrons were formerly known as cathode rays. These were obtained in vacuum tubes and accelerated in electric fields. These rays, deflected by electric and magnetic fields, would be made visible on a screen. It was possible to focus the cathode rays to a small area by using the coil carrying electricity. It was theoretically shown that a magnetic coil can collect electrons at a focus, just as an optical lens collects light at one focus. Transmission electron microscopy (TEM) has a working system like optical microscopy. The only difference is that an electron beam is used instead of a light beam. Although the physical working system is completely different, electron lenses are used here instead of optical lenses. The image is acquired on a screen or photographic plate. The whole process and acquisition of the image is carried out entirely in a vacuum as the electrons can very easily deviate from their path. Electrons are obtained from an electrically heated electron gun with an incandescent filament from tungsten. A potential difference of 100,000 volts occurs between the anode and the filament. Electrons emitted from the filament are accelerated and ensured to be of sufficient power to obtain an adequate image on the screen. It is only possible to obtain 1000 times larger images with optical microscopy, while an image up to 500,000 times larger can be obtained by electron microscopy. As with an optical microscope, the irradiation can be concentrated in a specific area. However, too much condensation can cause damage to materials such as polymers and biological materials. The electron lens, which is closest to the object, is the most important part of the instrument. This lens achieves an intermediate magnification of 50–100. The projector system easily processes this enlarged image, as the angular width of the incoming beam trials is reduced in the magnification process. Almost all electron microscopes have two or three lenses. These provide a magnification of 250–500,000. The scanning microscope, on the other hand, is more like a closed-circuit television system than an optical microscope. The first part of the microscope is focused on the object by an electron lens, like a television camera. Electrons from where it hits are collected, and their power is increased. The second part of the microscope is like a television receiver, in which there is a cathode ray tube. Thus, an image like a high-quality television picture is obtained [11, 34, 38, 39].

Electron microscopic evaluations of muscle and nerve biopsies generally use TEM. For TEM examination, a 1-diameter tissue sample is dissected and rinsed in 2.5% glutaraldehyde solution pH 7.4 which is used for the first fixation of tissues. Osmium tetroxide solution is used for the second fixation of tissues [38].

The solutions are used for tissue processing for TEM as follows:

Phosphate buffer solution (PBS)	
0.2 M sodium phosphate monobasic solution	90 mL
Sodium phosphate dibasic solution	410 mL
1% $CaCl_2$	2.5 mL
Distilled water	497.5 mL
Mix well and adjust pH to 7.4 with HCl.	
Glutaraldehyde solution	
Glutaraldehyde	2.5 mL
PBS	97.5 mL
Osmium tetroxide solution	
Break an ampule containing solid osmium tetroxide	1 g
PBS	100 mL

The 1% osmium tetroxide solution should be prepared at least 2 days before staining because osmium tetroxide hardly dissolves. Both fixatives should be stored in a refrigerator. They are stable for several months at 4–8°C [11, 12, 30–34, 36, 37].

First fixation: The fresh tissue is initially fixed in 2.5% glutaraldehyde solution for 24 h in the refrigerator. If the TEM examination will be performed right away, 2 h of fixa-

tion at room temperature is enough. If the examination will be performed later in another laboratory, the tissues can be stored in 2.5% glutaraldehyde at 4–8°C up to 6 months. After the first fixation, the tissues are washed for three times 5 min with PBS.

Second fixation: The slides are secondarily fixed in 1% osmium tetroxide solution for 2 h. The slides are transferred to the PBS after second fixation and washed in distilled water for 10 min.

After fixation and washing, the water in the tissues should be extracted because the presence of water creates severe artefacts by collapsing under vacuum in the TEM, so water should be replaced with an organic solvent. Acetone is the most used solvent in TEM. This organic solvent stage may create some differences in the cells' molecular structures. Dehydration of the specimen is usually done using a graded series of acetone from 40–100%. The tissues are incubated in each of the grades of acetone for at least 10 min. The dehydrating processes should be adjusted to the size and kind of tissue. These incubations with acetone can be repeated up to three times [11, 31, 36].

After dehydration, the tissues are infiltrated by another intermediary solvent which is replaced with the dehydration solution. These infiltration processes are made with graded epon-acetone solutions. For example, the tissues are infiltrated with 1:1 epon-acetone for 30 min, then infiltrated with 3:1 epon-acetone for 45–60 min, and finally immersed in fresh 100% epon twice for 2 h [30–34, 38].

For EM, most used embedding material is resin. Polymerisation of the resin monomers, which are known as epon, forms a hard plastic. This process is done by polymerizing resin in a high temperature. After incubation in a 70°C oven for 2 days, epoxy resin blocks are ready for sectioning. Once the epoxy resin has polymerised, it is insoluble and inert. The samples should be sectioned at 50–500 nm thickness for TEM. These ultrathin sections can only be cut by ultramicrotome with a diamond knife, and they are placed on metal grids as opposed to glass slides because the electron beam cannot penetrate glass slides. The ultrathin sections are transferred to grids which are 3 mm in diameter and are copper specimen carrier coated with a carbon film 0.1 micron thick. The stain using 1% uranyl acetate and alkaline lead citrate together is commonly used for TEM evaluation. Tissues are seen in classic black and white two-dimensional images [11, 38].

The semithin sections, with 1 micron thickness, are also prepared from resin blocks with an ultramicrotome and are stained with toluidine blue dye. In these sections, the fine detail of nerve fibers such as fiber type, diameter and myelinisation status is superiorly identified [11, 34, 38].

The construction of the electron microscope has made a huge revolution in biology by making it possible to examine internal structures of cells and biological specimens at the atomic level. Up until today, several types of electron microscope have been designed, and the 50-nm resolution used in the 1930s was improved to the 0.14-nm resolution used at the end of the twentieth century. With electron microscopy, important information can be gained in certain clinical conditions. Contrarily, electron microscopy is not necessary if the diagnosis has already been established. In such cases, it may be used to confirm the diagnosis [11, 34, 38, 39].

EM evaluation of nerve biopsy is useful for two reasons. One of them is to identify unmyelinated nerve fibers, which are not adequately visualised by light microscopy, and the characterisation of their ultrastructural features such as widely spaced myelin, focally folded myelin and so on. The second aim is to detect axonal dystrophy and other axonal changes in unmyelinated axons. In addition, mitochondrial neuropathies and inflammatory cells can be diagnosed with the help of EM (Fig. 5.41). In most neuropathology laboratories where specific muscle biopsy evaluations can be performed, EM evaluation of muscle biopsy is also made. However, diagnostic yields from muscle biopsy are less than that of nerve biopsy mainly due to the scarcity of muscle fibers examined [40, 41].

In the second half of the last century, when electron microscopy became increasingly popular, it was assumed that electron microscopy would be very important in the differential diagnosis of several diseases and even most tumours, with the detection of certain pathognomonic small structures. The two disease groups in which electron microscopy is most important when examining muscle biopsies are lysosomal storage diseases and mitochondrial myopathies. However, in lysosomal storage diseases, these accumulations may not be observed diffusely and equally in every muscle fiber, and so they may be overlooked if only present in a

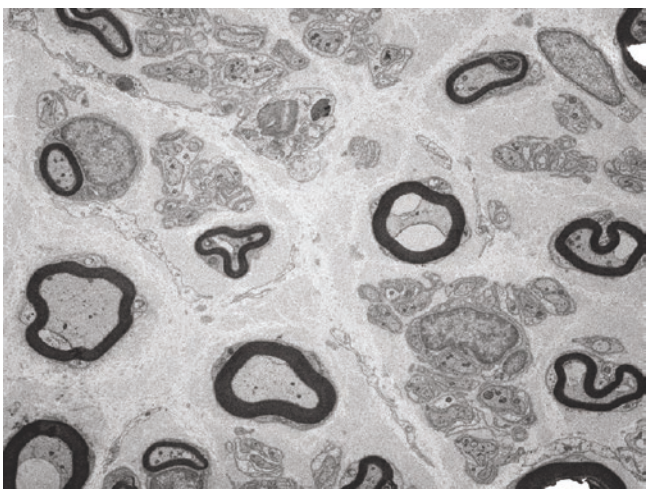

Fig. 5.41 Normal nerve (TEM 1000). (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

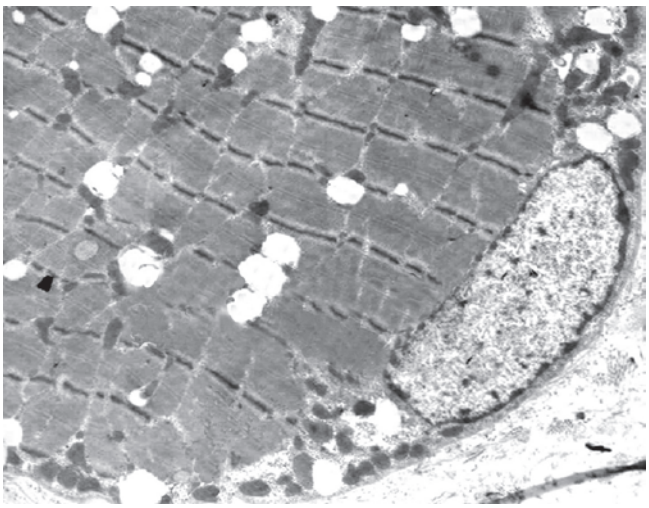

Fig. 5.42 Normal muscle (TEM ×4000)

small number of cells within a limited area (Fig. 5.42). Similarly, in mitochondrial diseases, defective mitochondria divide spontaneously and accumulate in the subsarcolemmal area due to the inability to meet the energy requirement of the cell. This accumulation of nonfunctional mitochondria can be easily observed in electron microscopy. However, the utility of electron microscopic examination in mitochondrial diseases is still limited because nonfunctional defective mitochondria can accumulate due to ageing and oxidative stress. It has been reported that the percentage of muscle fibers with defective mitochondrial deposits in advanced age can be around 5%. Therefore, the increase in the ratio of these fibers needs to be studied in larger areas. On the other hand, subsarcolemmal mitochondrial accumulation may be more exaggerated in children than in adults, and it is extremely difficult to decide whether these are nonfunctioning defective mitochondria during electron microscopic examination [40, 41].

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How to Read a Muscle or a Nerve Biopsy?

6

Gulden Diniz and Bahattin Erdogan

In this section, the normal structure of muscle and nerve cells will be examined, and changes that are within normal limits but can be interpreted as pathological will be discussed. Pathological findings and changes that may be diagnostic for some diseases will be explained with an abundance of histopathological pictures.

How to Read a Muscle Biopsy?

Clinical information is essential to evaluate a muscle biopsy, and clinicopathologic correlation is critical for the correct interpretation of histopathological differences. Without clinical information about the patient, the final diagnosis of a muscle biopsy should never be considered definitive. Paradoxically, any muscle biopsy should always be evaluated blindly at first to prevent clinical information about the patient from influencing judgments. As soon as the muscle tissue is delivered to the laboratory, it should be frozen in liquid nitrogen, as mentioned in the previous section. Frozen sections with a thickness of 6–8 μm should then be taken for special examinations as required. In these slides, it is aimed to observe the muscle cells in a transverse position. While taking frozen sections, the first slide to be stained with haematoxylin and eosin stain should be examined by an experienced pathologist. This initial examination is essential for the shaping of diagnosis and for deciding on any additional investigations that may be required. In this first examination, very useful information can be obtained, such as whether the sampling was made from the correct muscle, the adequacy of the material, whether the material was transmitted to the laboratory correctly and whether the muscle was placed in the desired position. Although there is no necessity for an early diagnosis for most muscle and neuromuscular diseases, it is important to inform the clinician immediately in cases of vasculitis and rhabdomyolysis so that the treatment can be planned early. One benefit of immediately examining the first prepared haematoxylin and eosin (H&E)-stained slide is to recognise such situations that require early intervention [1–4].

Sometimes the tissue can dry out due to a lack of moisture from the saline gauze that the muscle is wrapped in or due to prolonged transport. In this case, instead of taking a section immediately, I would suggest that a few drops of saline are dropped into the biopsy container, and the biopsy is left for at least 1 h in the refrigerator. In the previous section, we talked about ideal biopsy procedures. In most textbooks, it is stated that for enzyme staining, if biopsy transport takes more than a few hours, the material should be stored in dry ice (−70°C) because the ice battery will not last. However, I have come across muscle biopsy materials sent by couriers from distant cities and found that all enzyme dyes work, except for ATPase staining, in biopsies stored at +4–8°C for up to 3–4 days. For discrimination of fiber types, I have used immunohistochemical evaluations with the myosin heavy chain type 1 and 2 antibodies; therefore, I now recommend refrigerating rather than freezing biopsy material of patients from distant centre, whose biopsy has been taken recently, and delivering it to our laboratory during working hours the next day [3, 4].

In haematoxylin and eosin (H&E) staining of muscle biopsy cross-sections, normal muscle cells must be polygonal in shape, and similar in size. Each muscle fiber is surrounded by a very thin connective tissue called endomysium, while the larger bundles are surrounded by the perimysium. Endomysium cannot be identified without special connective tissue staining like trichrome. Myofiber diameters increase with age, for instance, each fiber is 12–15 μm in diameter at birth and around 30 μm at 6–7 years of age. In adult males, myofibers are larger, with a diameter of around 50–60 μm.

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However, it should not be forgotten that all muscle fibers must be uniform in size and shape (Fig. 6.1). After repetitive injuries due to muscle diseases, the main pathological appearance is that myofibers have different shapes and sizes (Fig. 6.2). While the muscle fiber diameter of an active sportsman is 60 μm, the muscle fibers of a sedentary elderly woman may be 40 μm, like those of a child, but this size should not vary within the individual. In addition, the size of different muscle fiber types varies according to gender. For example, type 2 myofibers in men are larger than type 1 myofibers, while type 1 and type 2 myofibers in women are roughly the same size. Therefore, from a normal muscle biopsy, the age and gender of patients can be estimated. In summary, the one of findings to be noted during muscle biopsy examination is the difference in myofiber size and shape [1–4].

Regular exercise usually causes the hypertrophy of only Type II muscle fibers, and a sedentary life cause's atrophy. Selective Type I fiber hypertrophy leads to a suspicion of spinal muscular atrophy (SMA). Hypertrophy of mixed fiber types occurs in chronic denervation and in muscular dystrophy. If atrophy is determined, and the shape of atrophic fibers is round, the presence of chronic myopathy should be suspected. In this situation, the small, rounded fibers are generally Type I in nature (Fig. 6.3). If there are small angular fibers with type II features, a diagnosis of neuropathy should be considered (Fig. 6.4). It should be kept in mind that hereditary inclusion body myopathies can appear like neuropathies in muscle biopsies, with small angular fibres type II in nature [3, 4].

During histopathological examination, the human skeletal muscles, regardless of their minimal variation, are composed of cells with type 1 and type 2 features in a checkerboard-like pattern. In birds that evolutionary located before mammals, every muscle was homogeneously composed of the same type of cells. Contrarily in humans, approximately a third of all skeletal muscles are composed of type 1 myofibers (Fig. 6.5) and two thirds of type 2 myofibers (Fig. 6.6). Physiologically, type 2 fibers (especially type 2B) are fast-twitch, glycolytic, anaerobic and fatigable. Type 1 fibers are slow-twitch, oxidative and fatigue-resistant fibers. In addition, a group of type 2 fibers known as type 2A has partly oxidative and aerobic features; therefore, they are also fatigue resistant. Muscle cell types can be easily distinguished with immunohistochemical examinations. This is usually achieved using antibodies that are myosin heavy-chain slow (slow myosin) and myosin heavy-chain fast (fast myosin) [1]. Traditionally ATPase staining, applied at different pH values, can also be used. Other enzyme staining such as NADH (Fig. 6.7), COX (Fig. 6.8) and SDH (Fig. 6.9) and histochemical dyes such as PAS (Fig. 6.10) and oil red O (Fig. 6.11) can

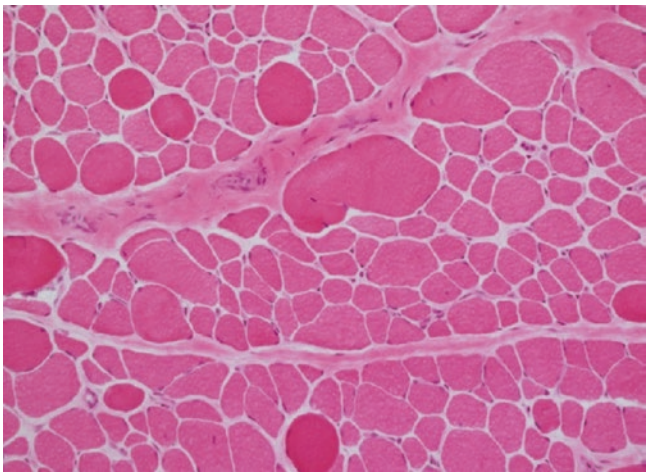

Fig. 6.2 Differences in the size and shape of myofibers in a muscle disease. Note the dark pink contracted fibers (H&E ×100)

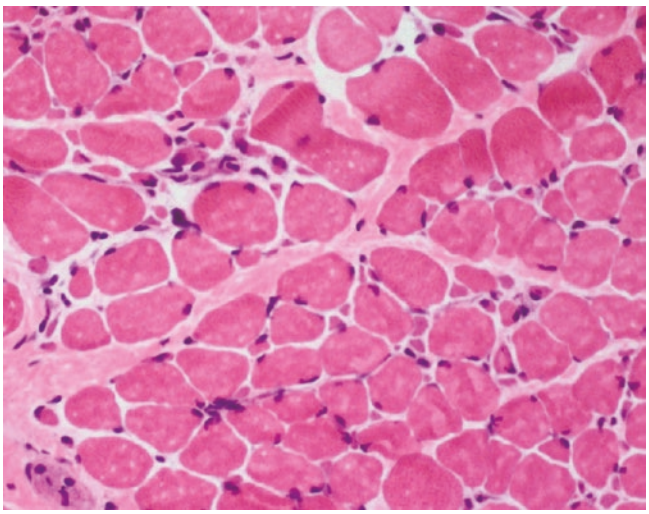

Fig. 6.3 Small round atrophic myofibers in a chronic myopathy (H&E ×200)

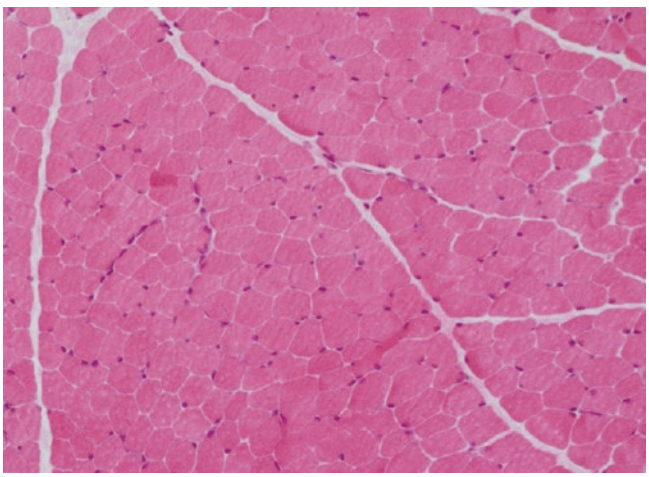

Fig. 6.1 Uniform appearance of myofibers in a normal muscle (H&E ×100)

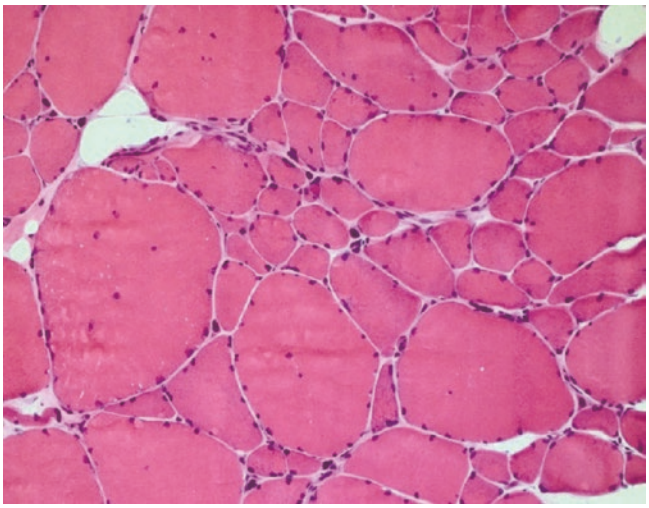

Fig. 6.4 Small angular atrophic myofibers in a chronic neuropathy (H&E ×200)

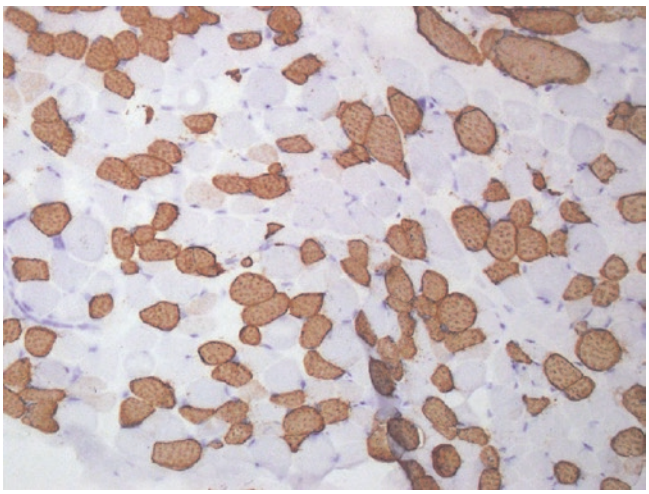

Fig. 6.5 A third of skeletal muscle showing slow myosin expressed Type I myofibers (DAB ×100)

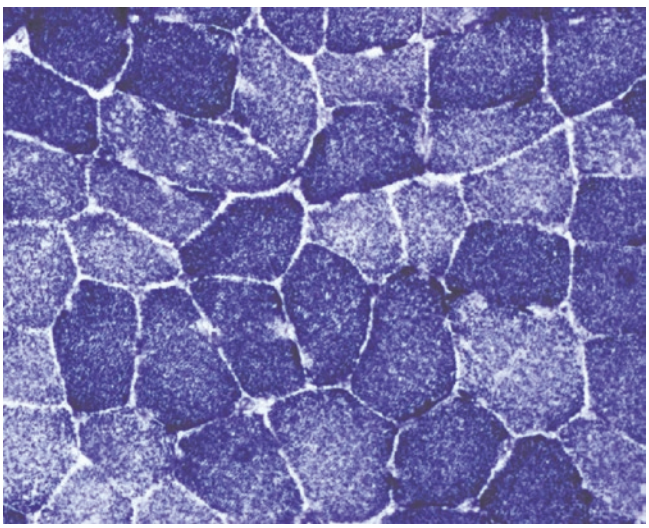

Fig. 6.7 Normal checkerboard distribution of muscle fiber types (NADH-TR ×400)

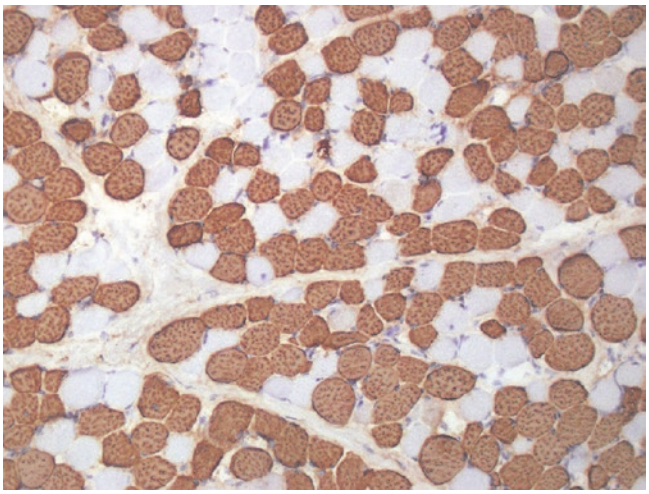

Fig. 6.6 Two thirds of skeletal muscle showing fast myosin expressed Type II myofibers (DAB ×100)

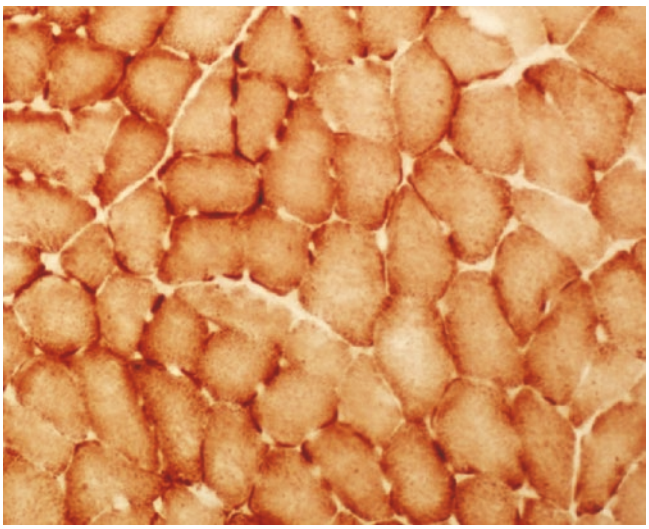

Fig. 6.8 Normal distribution of muscle fiber types (COX ×200)

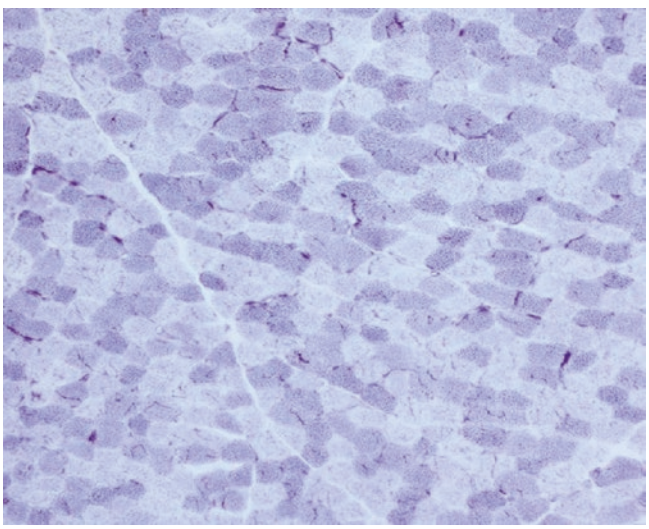

Fig. 6.9 Normal distribution of muscle fiber types (SDH ×40)

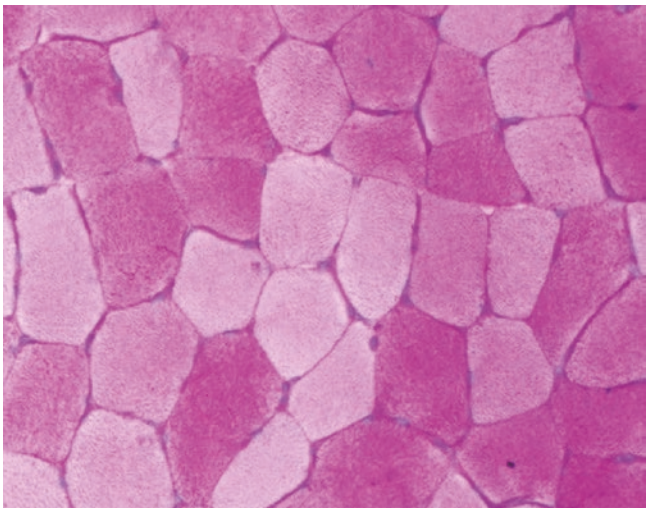

Fig. 6.10 Normal distribution of muscle fiber types (PAS ×200)

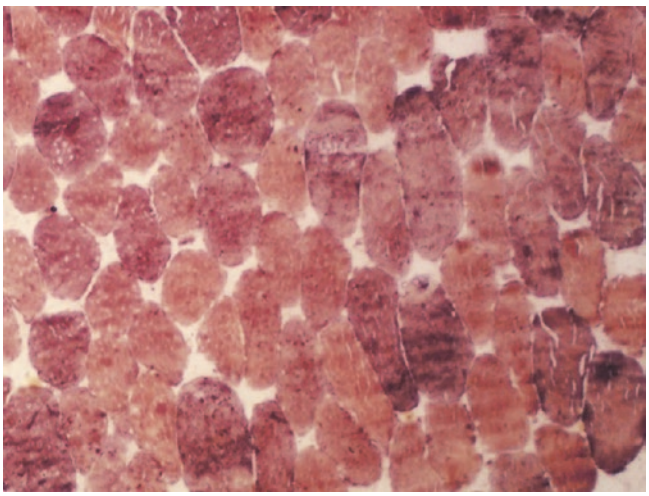

Fig. 6.12 Normal distribution of muscle fiber types (phosphorylase ×100)

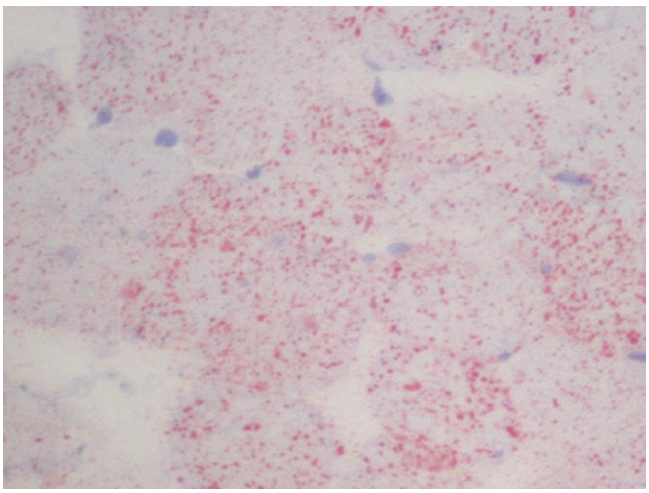

Fig. 6.11 Normal distribution of muscle fiber types (oil red O ×400)

also discriminate the fiber types because different fiber types include distinct cellular contents. For example, type I fibers include more mitochondria, lipids and oxidative enzymes like the leg muscles of birds. Type II fibers include more glycogen and phosphorylase enzymes (Fig. 6.12) and fewer lipids like the breast muscles of birds [1–4].

Evaluation of the size, distribution and proportions of type 1 and 2 myofibers in muscle biopsy provides very useful clues to aid the differential diagnosis of neuromuscular disorders. However, biopsies should always be taken from the same muscles because compositions of fiber types may be different in some muscles. Biopsies should be taken from the quadriceps or gastrocnemius muscles when investigating diseases involving the lower extremities. For diseases affecting the upper extremities, the deltoid muscle is suitable. In animal experiments, it has been shown that type1 and type 2 myofibers can physiologically transform into each other and that some muscles may consist only of type1 or type 2 myofibers. For example, a switch occurs in some fast muscles of mice, including the tibialis anterior and plantaris, which at birth contain a small proportion of type 1 fibers that disappear completely during postnatal development. A similar switch occurs in rats as the soleus muscle, which is a slow muscle, shows a transformation from type 2 fibres into type 1 during postnatal development [5, 6]. It should be kept in mind that the fiber phenotype can change in response to hormonal or neural effects, and nerve activity is a basic determinant of the fiber type in the human [6].

Grouped fiber types are seen generally in neuropathies associated with denervation and re-innervation. During these consequent innervation cycles, types of fibers may be changed (Fig. 6.13). The presence of multiple small groups with the same type of fibers is most associated with ALS. In chronic denervation and motor neuron diseases, large groups of the same fibers may be seen (Fig. 6.14). Perifascicular atrophy is a special type of atrophy that is generally seen in dermatomyositis. In this situation, atrophic fibers lie next to perimysium (Fig. 6.15). However, it should not be forgotten that atrophic myofibers may be found in small groups in muscular dystrophies, especially in dystrophinopathies (Fig. 6.16). On the other hand, the atrophic fibers of both type 1 and type 2 can be seen scattered individually in most myopathies (Fig. 6.17). Staining with immunohistochemical neonatal myosin antibodies is very useful to demonstrate atrophic myofibers because injured, atrophic, necrotic and even regenerated fibers express neonatal myosin. Regenerated muscle fibers are usually of Type 2C morphology; therefore, they can also be demonstrated with neonatal myosin.

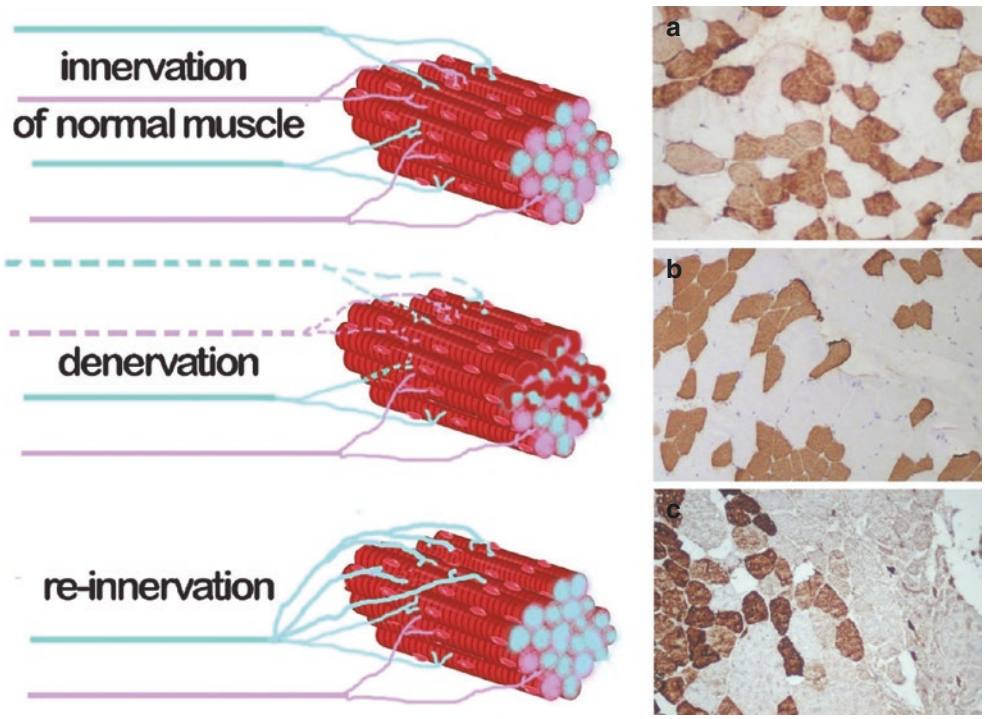

Fig. 6.13 Innervation cycles leading to grouped of fiber types: Fast myosin immunohistochemical staining shows (**a**) normal checkerboard dispersion of muscle fibers, (**b**) reduction in type 2 fiber ratio, and mild grouping (**c**) pronounced group atrophy

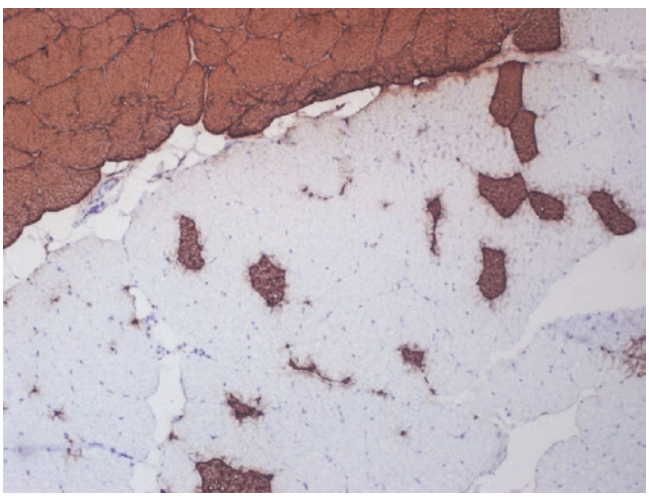

Fig. 6.14 Prominent group atrophy with immunohistochemical fast myosin staining (DAB ×100)

Normally, myofibers with type 2C morphology are rare in healthy muscles, except during the intrauterine or new-born period. An increase in type 2C muscle fibers (>5%) should be accepted as an important pathological finding, especially if it is determined in a patient over the age of 12 months. Additional reasons for an increase in type 2C fibers include immaturity, regeneration and coenzyme Q10 deficiency [7–9].

The disorders of fiber-type composition are very important for the differential diagnosis of neuromuscular disorders. The thick filament of a sarcomere is largely constituted of myosin, which includes both heavy and light chains. Heavy and light chains of myosin also have different isomers, which have been determined to cause some NMDs. The two main myosin heavy chains (MHCs) are type 1 (slow myosin) and type 2 (fast myosin). These MHCs and other less common types are composed of different amounts of MHC isomers (MYH). MYH7 is the most common isomer of slow fibers. Type 2A fibers are composed of MYH2 and MYH7, and type 2B fibers are composed of MYH1, MYH2 and MYH7. On the other hand, foetal fibers are almost entirely composed of MYH4, and in adults, it can be only expressed in regenerated fibers. Embryonic and neonatal fibers are also pathological fibers that are rarely seen after birth and are usually composed of MYH3 and MYH8, respectively. The most common myofibril isomer is MYH 2, and it is also known as conventional myosin of striated muscle [7, 8, 10].

Skeletal muscle fibers are mainly composed of red muscle fibers, intermediate muscle fibers and white muscle fibers. Red muscle fibers, known as type 1 muscle fibers, are slow-twitch oxidative fibers. They contain many mitochondria and myoglobulins. They are smaller in diameter than type 2 fibers and are fatigue-resistant fibers. The myosin-ATPase

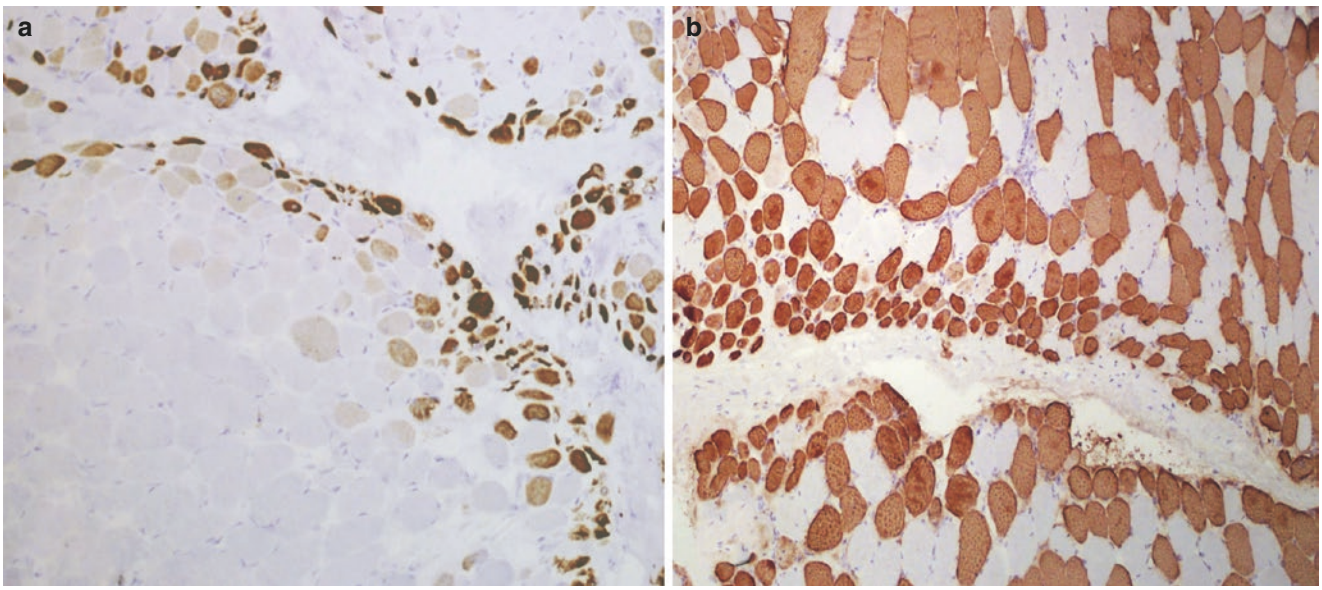

Fig. 6.15 Perifascicular atrophy is highlighted by (**a**) neonatal myosin and (**b**) fast myosin staining (DAB ×100)

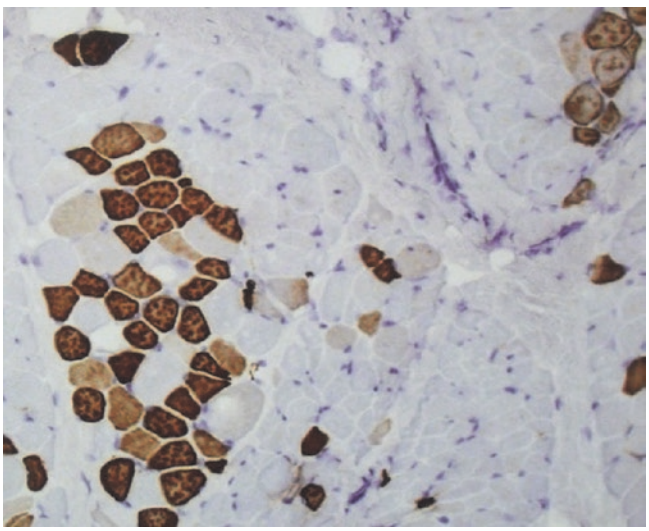

Fig. 6.16 Small group atrophy is highlighted by neonatal myosin staining in a case with DMD (DAB ×100)

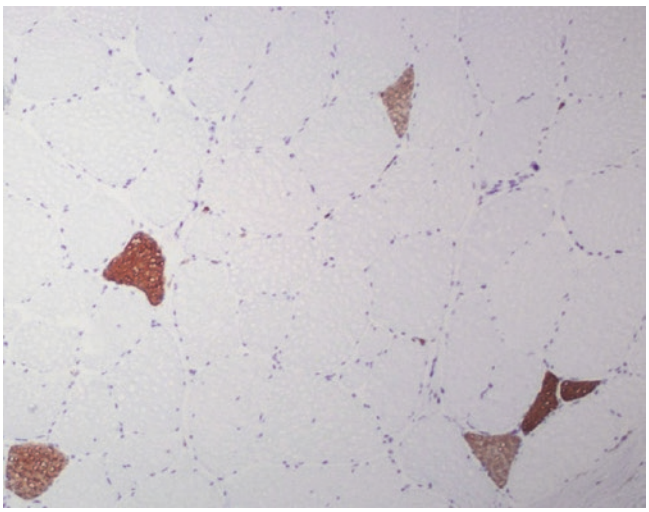

Fig. 6.17 Single-fiber atrophy is highlighted by neonatal myosin staining (DAB ×100)

reaction rate (twitch rate) is slow. These fibers are often found in the leg and back muscles because they are related to gait, posture and endurance. It is well developed in marathon runners. Type 2A fibers are intermediate fibers with both oxidative and glycolytic properties. They contain lots of mitochondria and have high myoglobin content, as well as plenty of glycogen. Therefore, they also have the ability of anaerobic glycolysis. Type 2A fibers consist of fast-twitch but also fatigue-resistant motor units. Therefore, they develop well in 400–800-m sprinters, middle-distance swimmers and hockey players. Type 2B fast glycolytic fibers are larger-diameter white fibers that contain less myoglobin and mitochondria. Their oxidative enzyme levels are low, and their anaerobic enzyme capacity is high. They store high levels of glycogen, contract quickly (fast-twitch) and get tired quickly. The myosin-ATPase activity, that is, the reaction rate is very high. Fatigue develops due to lactic acid production. They contract quickly and adapt to fine movements. Therefore, they are found in high concentrations in the fingers and extraocular muscles for fine motor skills. Additionally, they are more abundant in the neuromuscular junction than Type I fibers and are well-developed in sprinters and weightlifters. Muscle fiber sizes vary according to gender. For example, type 2 myofibers in men are mildly larger than type 1 myofibers, while type 1 and type 2 myofibers in women are roughly the

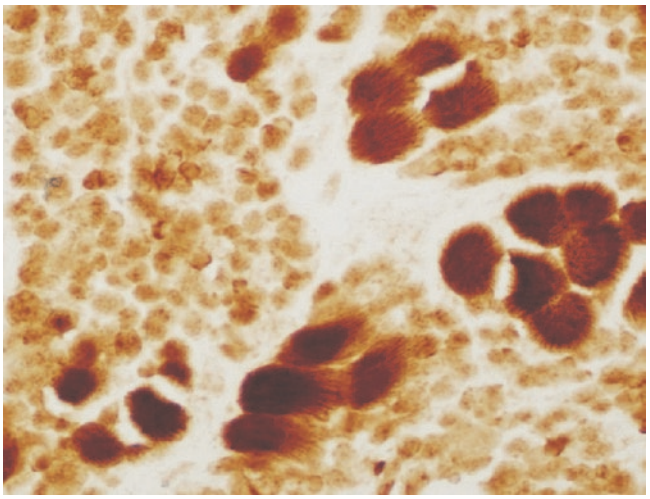

Fig. 6.18 Hypertrophic type 1 fibers in a case with SMA (COX ×100)

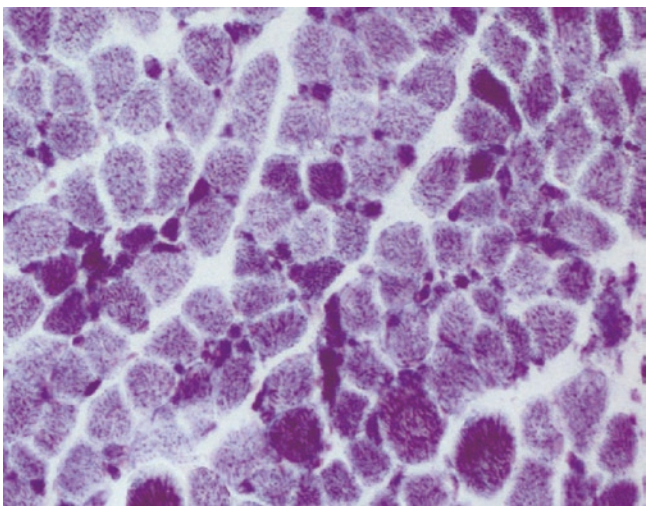

Fig. 6.19 Hypertrophy and atrophy of mixed fiber types (NADH-TR ×200)

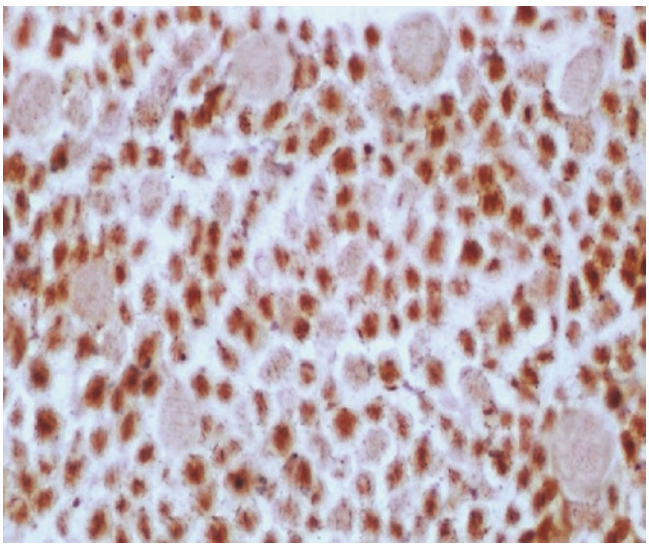

Fig. 6.20 Smaller type 1 fibers in a case with centronuclear myopathy (COX-SDH ×100)

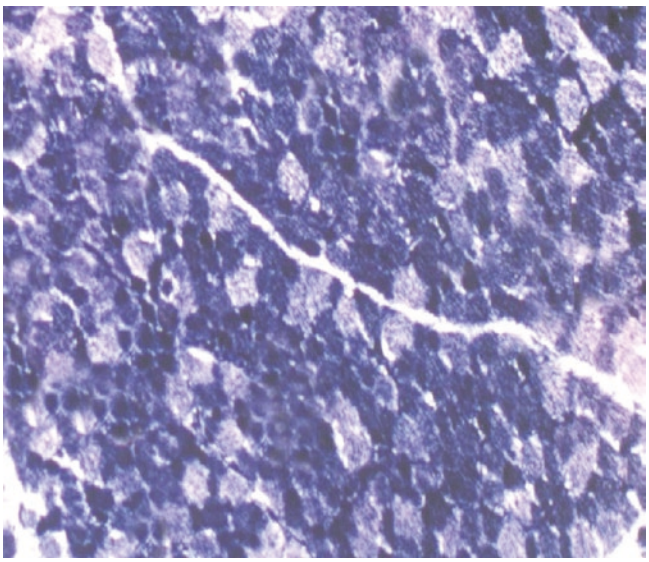

Fig. 6.21 Type 1 fiber predominance (NADH-TR ×100)

same size. In summary, the presence of different pathological fibers and differences in the proportion of normal fiber types can help to diagnose NMDs and learn the patient's individual sporting habits [10–13].

Hypertrophy of type I fibers is seen in spinal muscular atrophy (Fig. 6.18), while hypertrophy of type II muscle fibers is usually seen in regular exercise. Hypertrophy of mixed fiber types (Fig. 6.19) is associated with chronic denervation, chronic myopathies, muscular dystrophies and occasionally inflammatory myopathies, except for inclusion body myopathy (IBM). In hereditary myopathies, type 1 fibers are unusually small (Fig. 6.20), while small type 2 fibers are seen in acquired disorders; in congenital myasthenia gravis, type 2 fibers are usually smaller than type 1 fibers. In some muscle diseases including myopathy with RYR1 mutations, central core myopathy, bulbospinal muscular atrophy and LGMD-1A, there is a predominance of type 1 fibers in skeletal muscles (Fig. 6.21). Atrophy of type 2 fibers is generally a nonspecific finding that can be associated with a wide range of situations. Reasons for smaller and fewer type 2 fibers can be aging, disuse, weight loss, systemic disease and paraneoplastic syndromes. On the other hand, the significance of type 2 predominance in adult muscles is uncertain [1, 2, 11–13].

There are also various electron microscopic criteria for fiber type identification that have been proposed mainly for animal muscles. For human muscles they are less reliable in estimating fiber types, during electron microscopic examina-

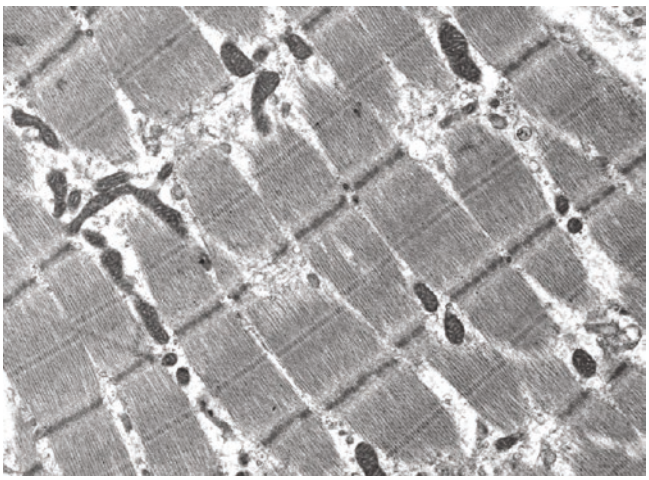

Fig. 6.22 In a normal type 1 myofiber, there are many mitochondria located between myofibrils. (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

tion. These criteria for type 1 fibers are smaller diameter, more numerous and larger mitochondria, abundant lipid globules and wider Z-discs (Fig. 6.22). Changes observed in nuclei of skeletal muscle cells are also very helpful in the differential diagnosis of neuromuscular disorders. Nuclei are initially internally located during the embryonic and foetal developmental stages and then move peripherally until birth. However, in muscle cells that regenerate in the event of disease or damage, the nuclei are located internally. Therefore, the number of cells with internal nuclei increases in various muscle diseases (Fig. 6.23). In chronic neuropathic and myopathic processes, the proportion of fibers with internal nuclei is 10–30%. Myotonic dystrophy type 2 is well known for the high presence (30%) of internal nuclei, mostly of type 2 fibers. The presence of a single, centrally placed nucleus in almost all fibers is pathognomonic for centronuclear myopathy (Fig. 6.24). Especially in neurological disorders, the nuclei of denervated atrophic muscle cells are packed together. This specific appearance is called nuclear clumping (Fig. 6.25). Pyknotic clumps are mostly seen in chronic denervation without reinnervation, myotonic dystrophy type 2 and chronic untreated myasthenia gravis. The nuclei of regenerated fibers are also different. They are centrally placed with a vesicular appearance, and they can also have a nucleolus (Fig. 6.26) [11–15].

Skeletal muscle fibers contain hundreds to thousands of nuclei situated below the plasma membrane and distributed along the fibers, except for small clusters of specialised nuclei, which sit at the neuromuscular junction. The nuclear position of all cell types is dynamically controlled in space and time. The cytoskeleton, which transmits pushing or pulling forces onto the nuclear membrane, mediates nuclear motions. Microtubules have long been recognised as crucial

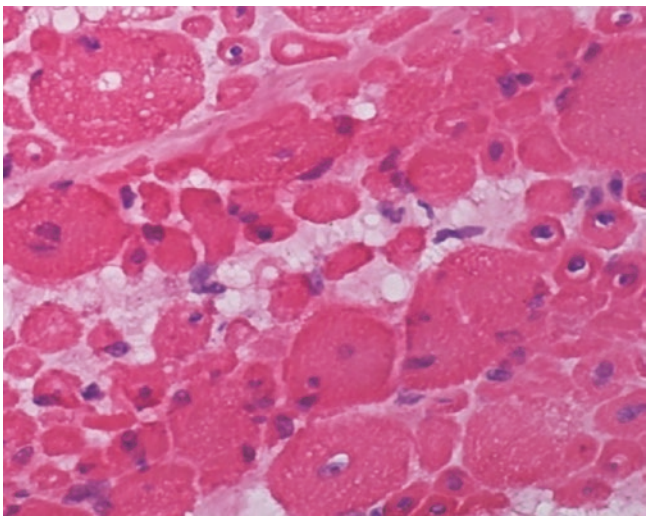

Fig. 6.23 Increasing rate of central nuclei in a myopathy (H&E ×200)

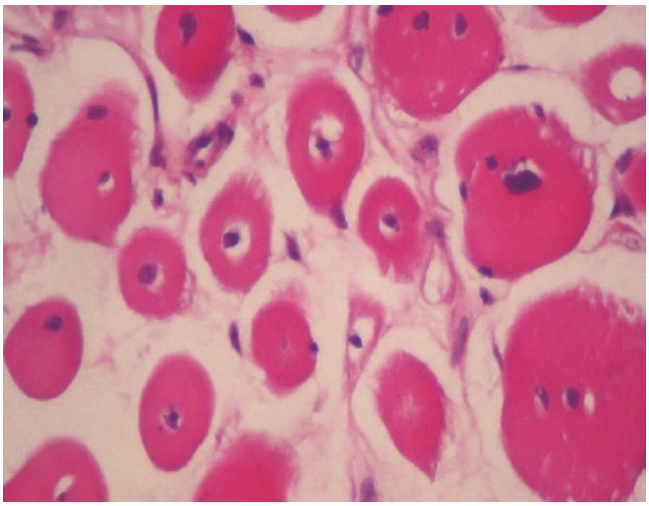

Fig. 6.24 A case of centronuclear myopathy (H&E ×200)

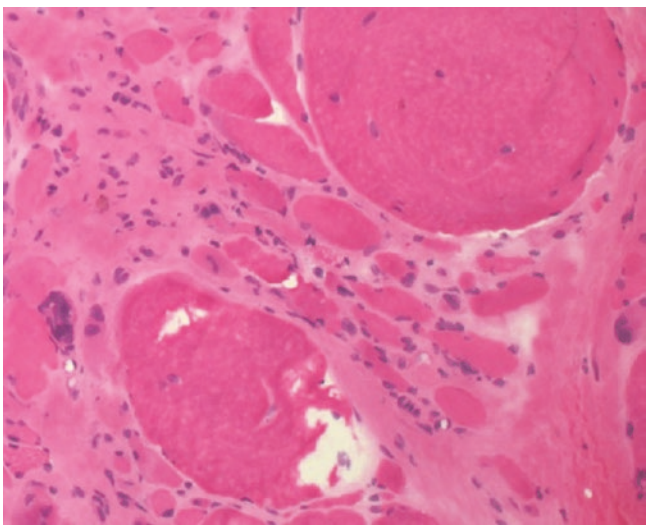

Fig. 6.25 Nuclear clumping in a neurogenic myopathy (H&E ×200)

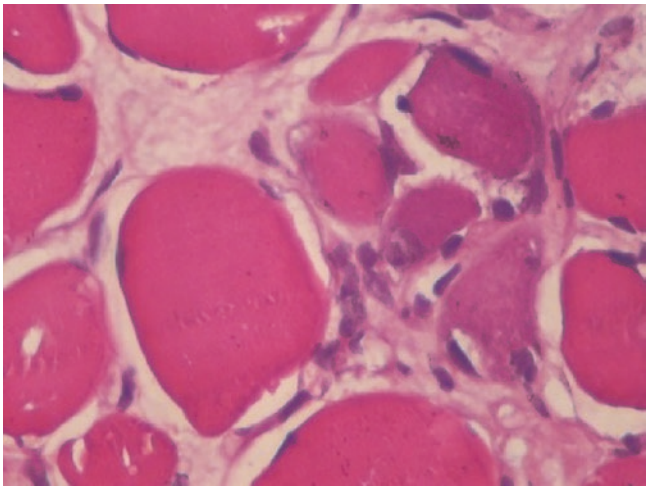

Fig. 6.26 Regenerated fibers (H&E ×400)

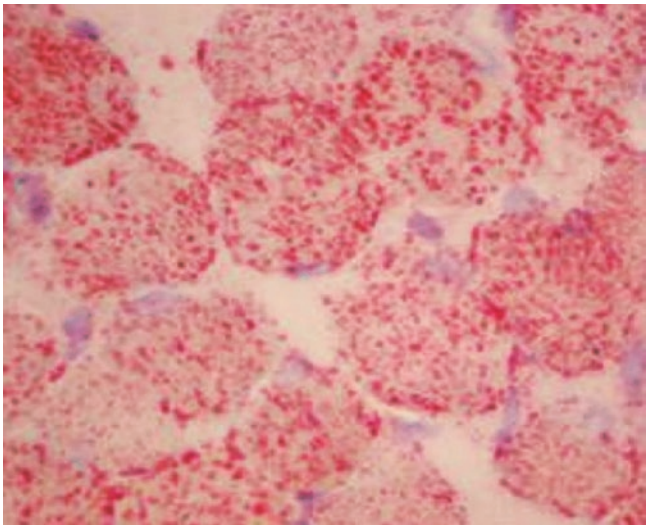

Fig. 6.27 Lipid storages (oil red O ×200)

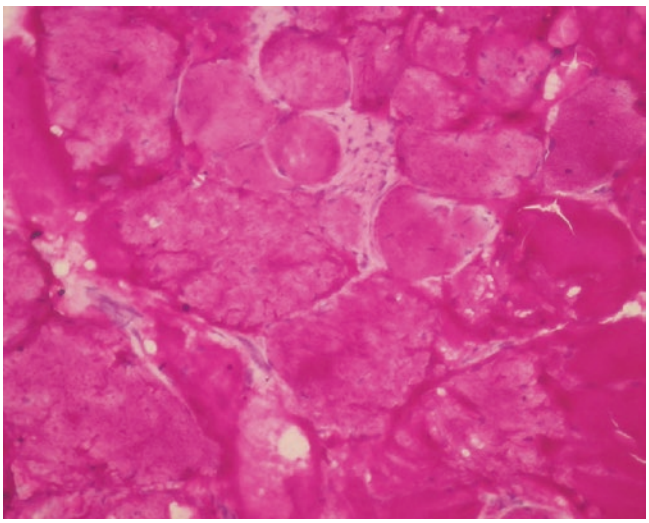

Fig. 6.28 Glycogen storages (PAS ×200)

players controlling nuclear positioning. Recent research has provided insight into the mechanisms controlling nuclear location within cells [16]. The actin and cytoplasmic intermediate filament cytoskeletons have been implicated in this function more recently, and different molecular links between the nuclear membrane and cytoplasmic elements have been identified for different cells. It is not yet understood exactly how nuclei arrive at positions along fibers for skeletal muscle; however, nuclei have been shown to preferentially localise near blood vessels, especially in slow-twitch oxidised fibers. In addition, it has been determined that desmin deficiency significantly impacts the distribution of nuclei along fibers but does not prevent their close association with vessels [15–17].

In the differential diagnosis of metabolic diseases, muscle biopsy is very useful because any stored substances caused by a metabolic disturbance that are not normally present can be easily determined. Histochemical PAS, dPAS and oil red O dyes (Fig. 6.27), routinely applied to the biopsy, can distinguish whether the accumulated material is glycogen or lipid. The greatest difficulty in the differential diagnosis is to interpret whether irregular vacuoles are due to freezing artefacts or material deposition (Fig. 6.28). In lipid storage diseases, accumulation is generally in type 1 myofibers, whereas in glycogen storage diseases, accumulation is predominantly in type 2 myofibers. For this reason, fibers with stored material often show a checkerboard distribution, and this appearance is helpful for discrimination from artefactual vacuoles (Fig. 6.29). Isolated vacuoles can be observed in glycogen storage disease-type V, also known as McArdle disease. Crescent-shaped positive stains, shown using PAS, are due to subsarcolemmal glycogen accumulation, which is pathognomonic for this disease (Fig. 6.30). Small, isolated vacuoles may be observed in some myopathies, as well as myositis. Some of these vacuoles are surrounded by a rim (rimmed vacuoles), especially with modified trichrome staining, and are referred to as inclusions (Fig. 6.31). Inclusions can be both intranuclear and intracytoplasmic (Fig. 6.32). Some neuromuscular diseases progress with vacuolar changes, and the characteristics of these vacuoles are considered in their differential diagnosis [12].

Mitochondrial diseases are multisystem disorders, and they are among diseases that are difficult to diagnose because their phenotypes can change over time. Moreover, if the problem is in the mitochondrial DNA, a genetic examination may not be useful from a peripheral blood sample, like other muscle diseases. For this reason, it may be much easier to diagnose with special examinations using a muscle biopsy. Mitochondria are capable of autonomous division when there is an energy crisis in the cell. Then dysfunctional mito-

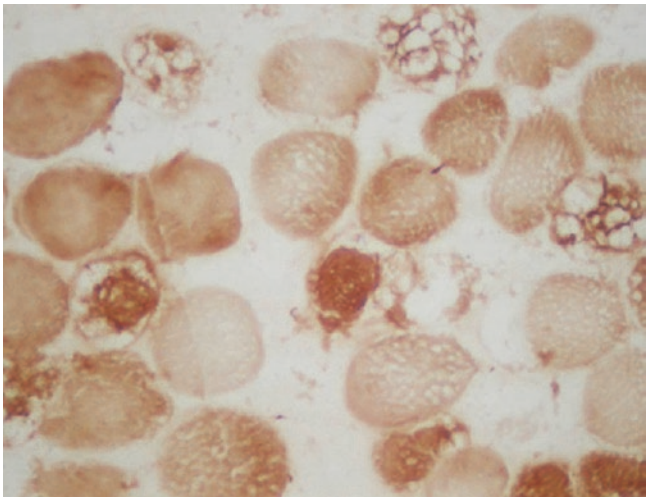

Fig. 6.29 Vacuoles are mainly in type 1 fibers (COX ×100)

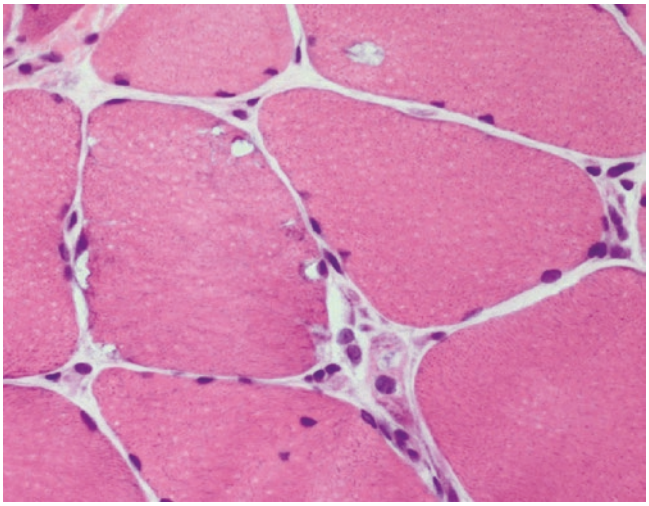

Fig. 6.32 Intranuclear and intracytoplasmic inclusions (H&E ×400)

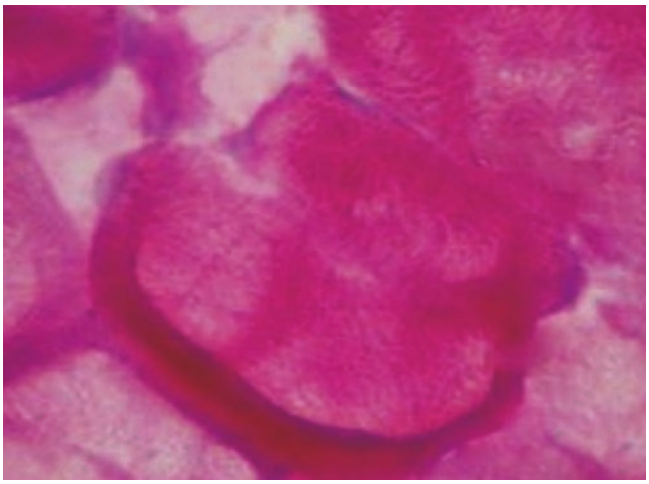

Fig. 6.30 Crescent-shaped glycogen storages (PAS ×400)

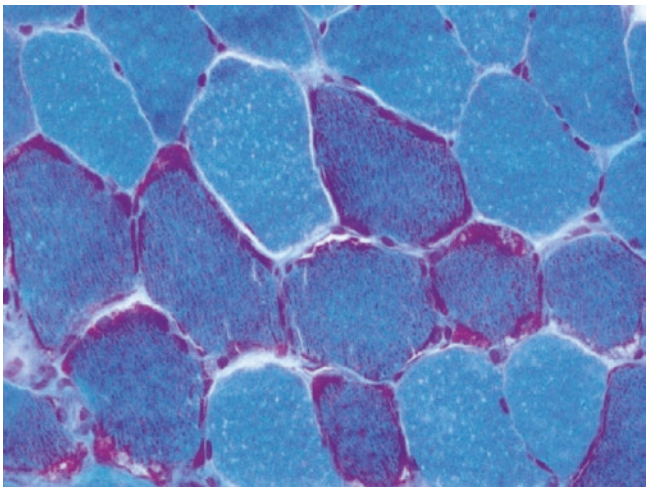

Fig. 6.33 Ragged red fibers (modified trichrome ×200)

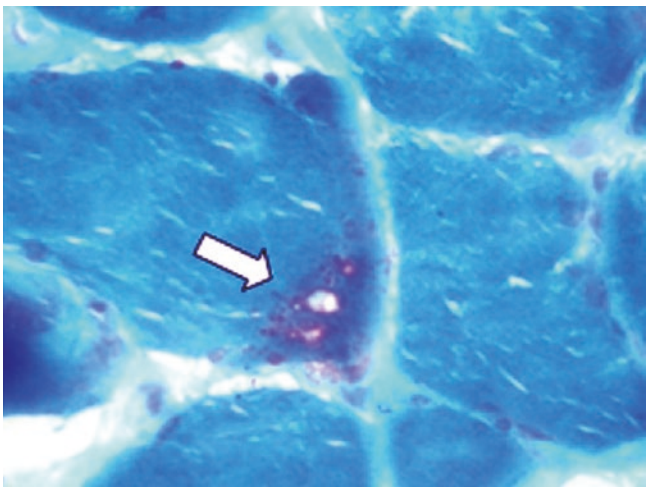

Fig. 6.31 Inclusion (arrow) in a myopathy (modified trichrome ×400)

chondria proliferate as irregular accumulations under the sarcolemma. These accumulations are stained in red magenta with the modified trichrome and are called ragged red fibers (Fig. 6.33). The same accumulation leads to the formation of ragged blue fibers in SDH enzyme staining (Fig. 6.34). In every mitochondrial myopathy, there may not be enough mitochondrial proliferation to create a ragged red fiber appearance, especially in infancy and early childhood. However, it can be proved that mitochondria exist but do not function by combined COX-SDH staining (Fig. 6.35) [18–20].

Oxidative enzyme reactions such as NADH-TR and succinate dehydrogenase not only distinguish the types of fibers but also highlight cell organelles and myofibrillary arrangement. Oxidative enzyme activity is predominantly located in the mitochondria, which are visible as small darkly stained

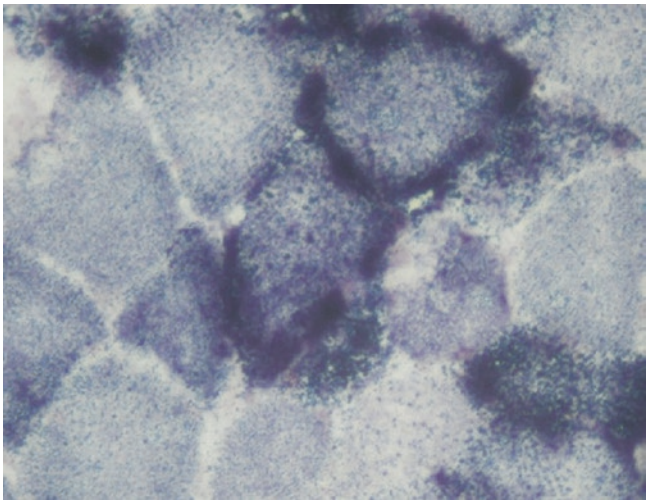

Fig. 6.34 Ragged blue fibers (SDH ×400)

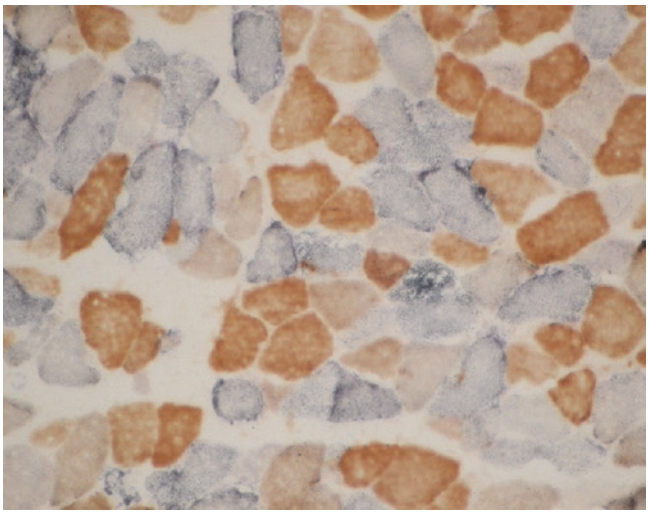

Fig. 6.35 Pathological blue fibers (combined COX-SDH ×200)

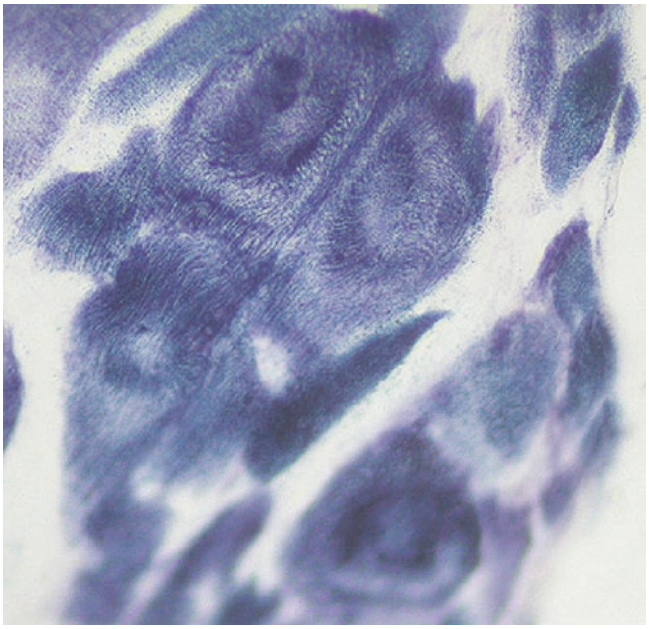

Fig. 6.36 Target fibers (NADH-TR ×400)

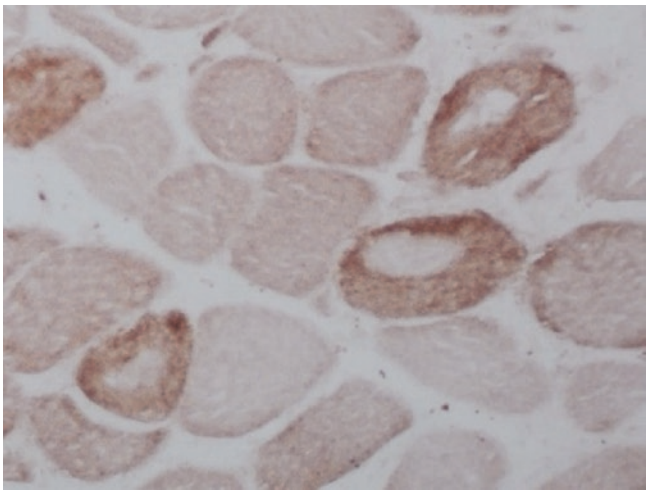

Fig. 6.37 Central cores in three type 1 myofibers (combined COX-SDH ×200)

dots at high magnification. The intermyofibrillar network, but not the myofibrils, is stained with oxidative enzymes, and the orderly cytoplasmic architecture of normal fibers are revealed. In the transvers sections, the sarcoplasmic myofibrillary arrangement has a mildly granular appearance. Abnormal internal fiber architecture can be determined in different neuromuscular disorders with NADH staining. In addition, they can be pathognomonic to some diseases. For example, processes of denervation and reinnervation in neuropathies cause the target fibers (Fig. 6.36). Cores shown by oxidative enzyme staining are pathognomonic for central core myopathy (Fig. 6.37). Trabecular or lobulated fibers caused by the lace-like arrangement of myofilaments are specific findings for some diseases, including LGMD-2A, Ullrich CMD and Bethlem myopathy (Fig. 6.38). Whorled fibers are commonly seen in different muscular dystrophies (Fig. 6.39). Similarly, based on myofibrillar arrangement patterns, muscle fibers with different appearances are called ring fibers (Fig. 6.40); moth-eaten fibers; hyaline fibers, which are pale stained; and contracted fibers (Fig. 6.41). Split fibers (Fig. 6.42) are also commonly found, especially in muscular dystrophies and some chronic neuropathies [1–3, 18, 21–26].

In some myopathies, prominent small rods have been observed in the sarcoplasm using modified trichrome, and they have been called nemaline bodies, which means string in ancient Greek. These myopathies are known as nemaline rod myopathies (Fig. 6.43). However, it should be kept in mind that very different patterns may occur in muscle dis-

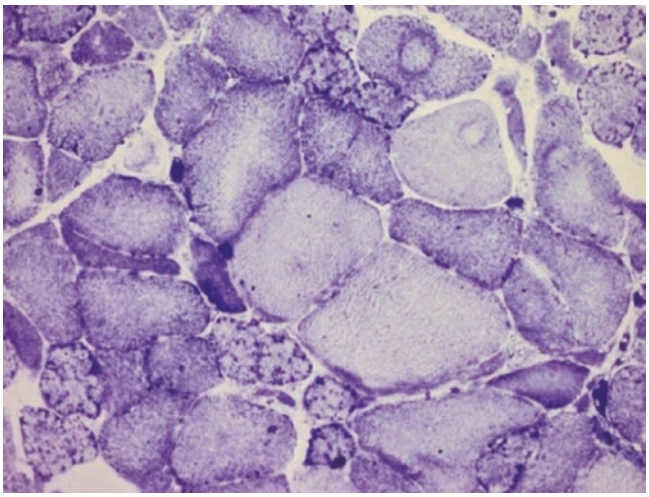

Fig. 6.38 Trabecular (lobulated) fibers (NADH-TR ×100)

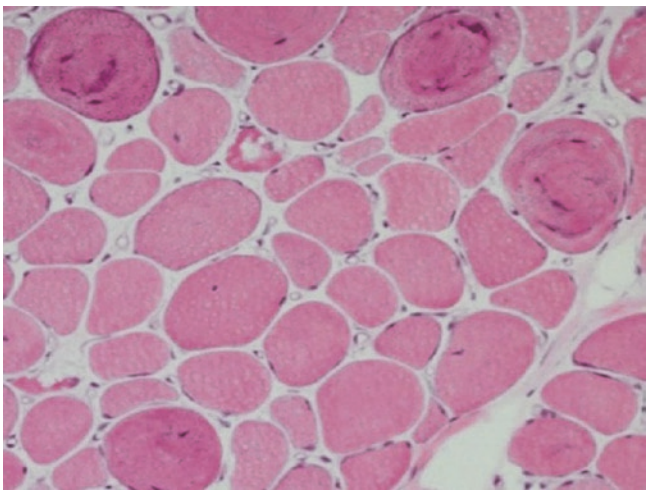

Fig. 6.39 Whorled fibers (H&E ×100)

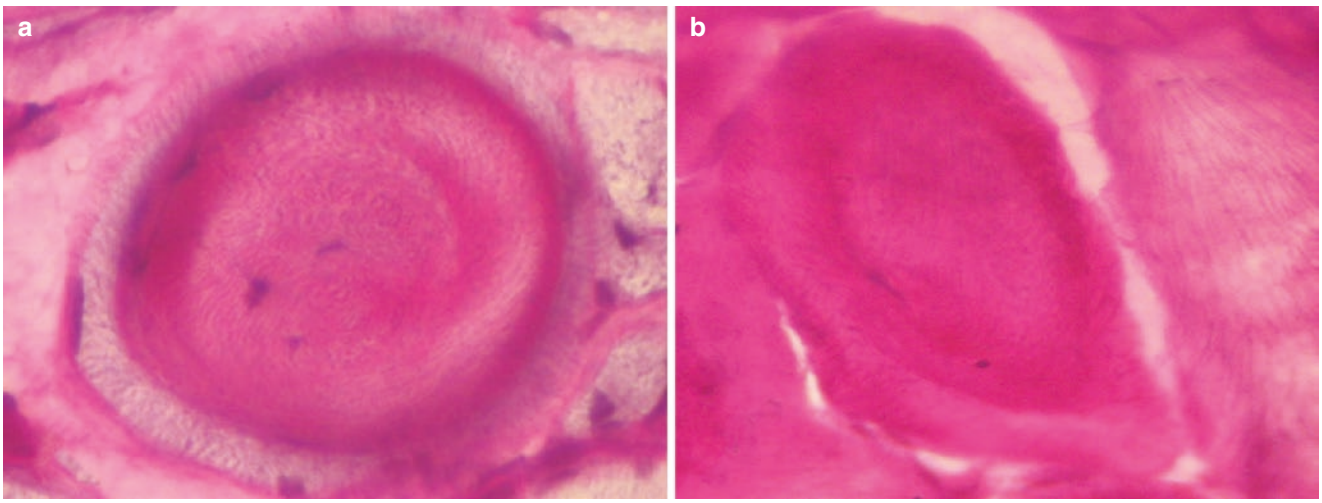

Fig. 6.40 (**a**) Ring fiber in an 8-year-old boy with MD (H&E ×1000). (**b**) Ring fiber in a 14-year old boy with LGMD-2B (H&E ×1000)

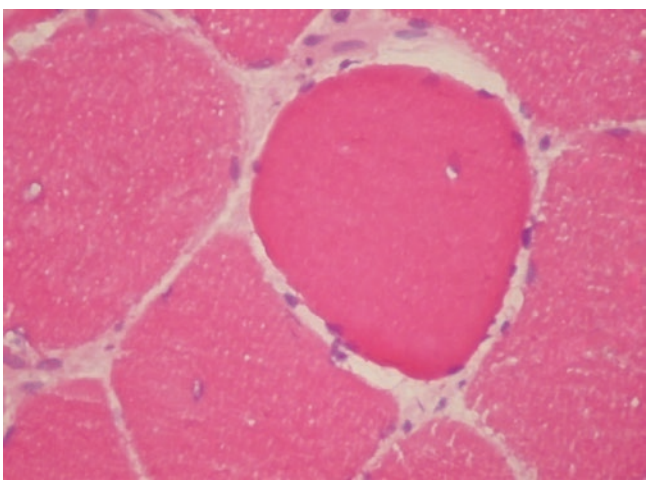

Fig. 6.41 Contracted fibers (H&E ×400)

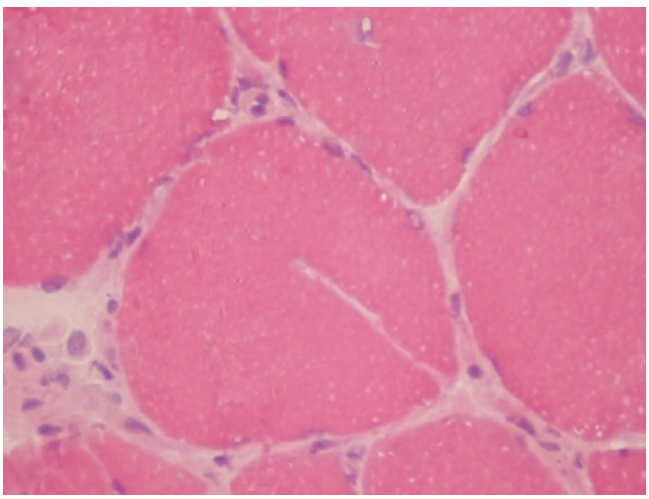

Fig. 6.42 Split fibers (H&E ×400)

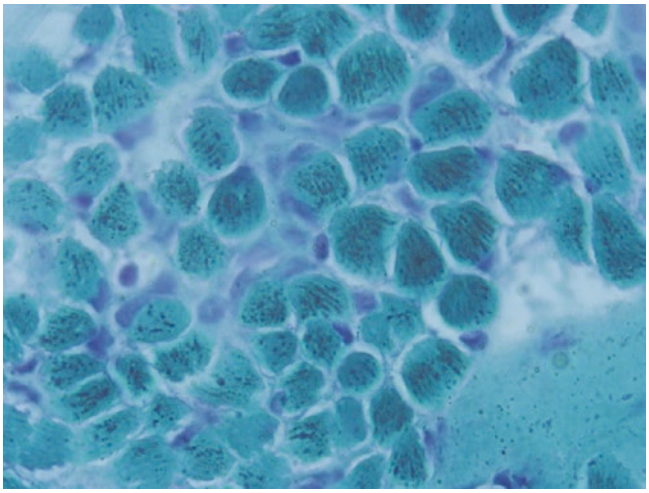

Fig. 6.43 Nemalin rod myopathy (modified trichrome ×200)

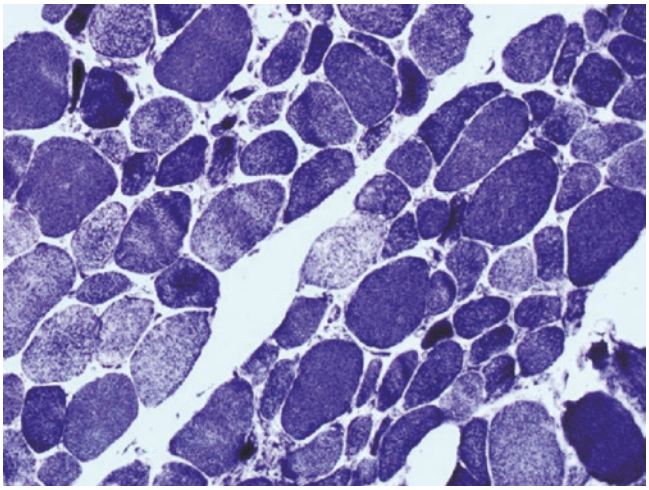

Fig. 6.44 Cytoskeletal disorganisation (NADH-TR ×100)

eases due to cytoskeletal irregularities, especially in NADH-TR stains (Fig. 6.44). Even in a muscular dystrophy patient, some fibers may show COX negative properties. This situation should not be described as a mitochondrial disease [1–3, 21–26].

Necrosis of single cells or small groups of cells is common in muscular dystrophies and inflammatory myopathies. In both, regenerated fibers are seen, which proves the presence of necrosis (Fig. 6.45). However, inflammatory infiltration of lymphocytes is only observed in inflammatory myopathies, whereas macrophages play the leading role in muscular dystrophies and rapidly remove dead cell remnants from the environment. Rhabdomyolysis is necrosis involving numerous muscle cells and may often accompany metabolic, toxic or infectious diseases (Fig. 6.46). Lymphocytes play the leading role in inflammatory diseases. The predominance of T or B lymphocytes among the inflammatory cells and the localisation of the inflammation are important for the differential diagnosis of inflammatory myopathies. The humoral immune mechanism is prominent in dermatomyositis, and the infiltration is localised in the perimysium (Fig. 6.47). Due to this localisation, the cells adjacent to the perimysium are atrophied (perifascicular atrophy). Conversely, cellular immunity plays a dominant role in polymyositis, and T lymphocytes are mostly located in the endomysium (Fig. 6.48). Therefore, it is very important to determine the inflammatory cell profile by immunohistochemical examinations for differential diagnosis in inflammatory myopathies [1, 12–19, 24–28].

Muscle tissue shows many pathological changes due to disease or trauma, and these changes can be seen with many different histochemical, immune histochemical and enzyme histochemical staining, especially on frozen sections. For example, rare myofibers that show mitochondrial dysfunc-

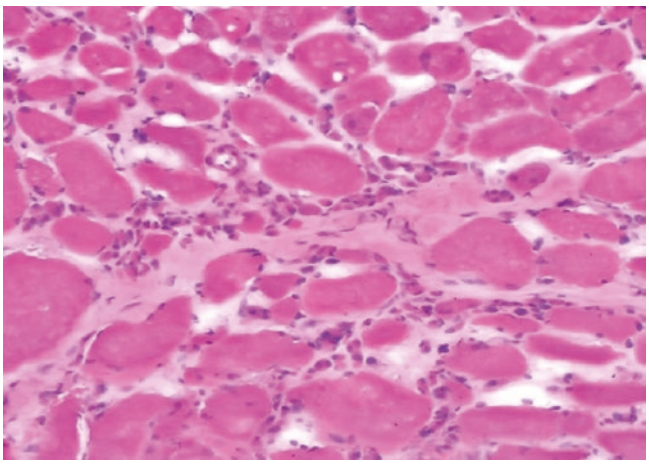

Fig. 6.45 A small group of regenerated fibers (H&E ×100)

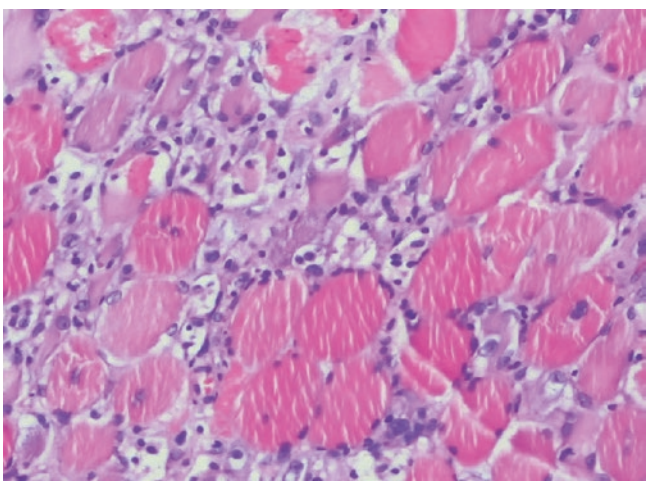

Fig. 6.46 Severe regeneration after rhabdomyolysis (H&E ×100)

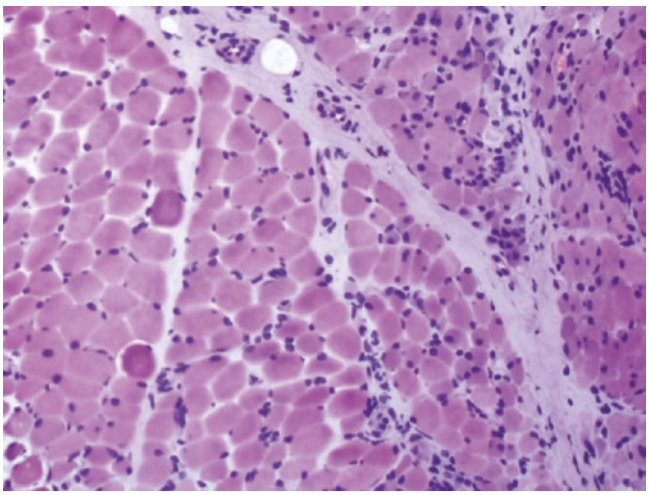

Fig. 6.47 Dermatomyositis with perifascicular atrophy (H&E ×100)

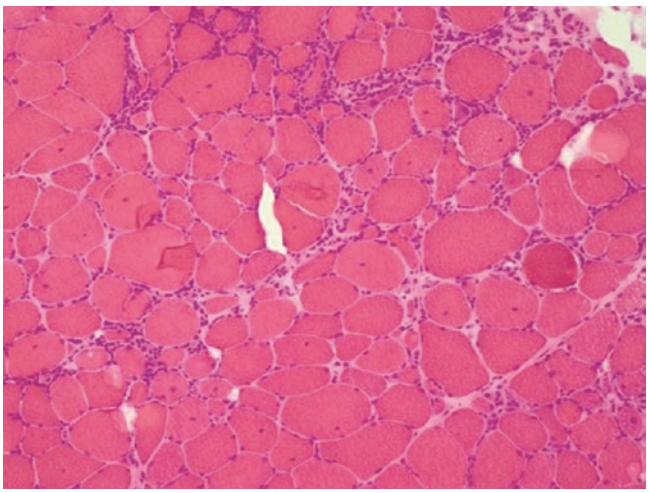

Fig. 6.48 Polymyositis (H&E ×100)

tion can be found in various diseases, even in healthy patients as their number increases with aging. This condition should not be interpreted as mitochondrial myopathy. Electron microscopic examination may be useful in distinguishing the exact location of these changes and the organelles affected. However, observing a very small area in electron microscopy carries risks to the interpretation as pathological findings can be artefacts due to defects during tissue follow-up or nonspecific changes. The small tissue sample allocated to electron microscopy may not adequately represent the entire muscle tissue, but when rare fibers showing mitochondrial dysfunction are seen in the biopsy area, a diagnosis of mitochondrial myopathy can be identified. Similarly, insignificant isolated peculiar structures can be evaluated as pathological (Fig. 6.49) [12, 29, 30].

The longitudinally oriented fibers provide more ultrastructural information in electron microscopic evaluation (Fig. 6.50). Contrary to nerve biopsy, evaluation of semithin sections is not widely used in the muscle biopsy. It must be kept in mind that a definitive diagnosis is very difficult by electron microscopy alone. Accurate diagnosis is only possible if all clinical, electrophysiological, biochemical, genetic, histopathological and electron microscopic findings are evaluated together.

The developments of molecular analyses have diminished the diagnostic role of electron microscopy. Electron microscopic evaluation of muscle biopsy can be used to search for

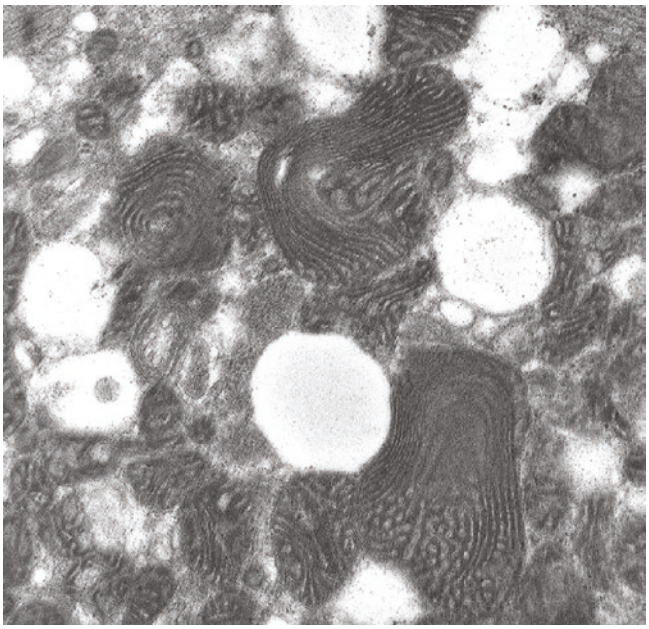

Fig. 6.49 Irregular clusters of peculiar mitochondria in a patient with muscular dystrophy (EM ×30,000)

subtle changes to help decide whether the muscle is normal or abnormal. In addition, the nature of structures observed during light microscopic evaluation can be clarified, and structures only visible at the ultrastructural level can be identified by electron microscopic evaluation [12, 30].

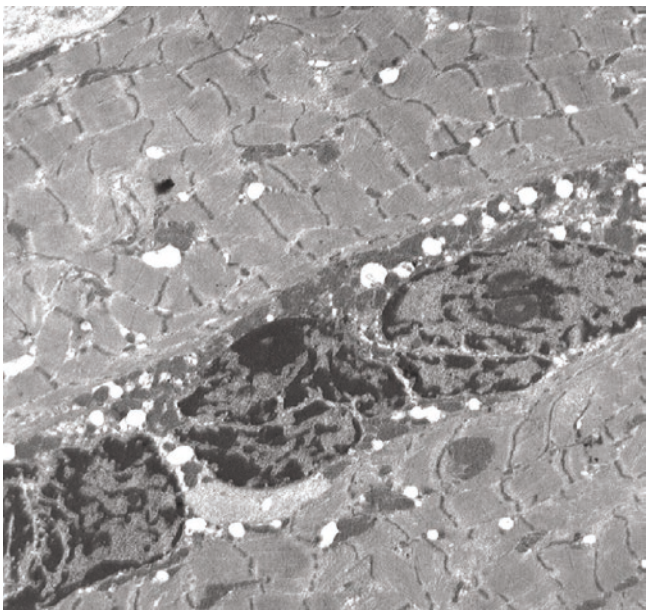

Fig. 6.50 Nonspecific myofibrillary and nuclear disorganisation in a patient with noninflammatory myopathy (EM ×10,000)

How to Read a Nerve Biopsy?

Clinical information is also essential to evaluate a nerve biopsy like a muscle biopsy, and clinicopathologic correlation is critical for correct interpretation of histopathological differences. Without clinical information about the patient, the final diagnosis of nerve biopsy should not be decided. Paradoxically, any nerve biopsy should always be evaluated blindly at first, to prevent clinical information about the patient from influencing judgments. After the nerve biopsy is delivered to the laboratory, it can be frozen in liquid nitrogen, as detailed in the previous section, and frozen sections with a thickness of 6–8 μm should be taken as required for special examinations. Although freezing sections in liquid nitrogen is recommended for nerve biopsies in textbooks, liquid nitrogen is not essential for nerve biopsy procedures because there is no complex intracellular arrangement in peripheral nerves, unlike striated muscles, and therefore there is a limited spectrum of histopathological changes [31–33].

When investigating frozen sections, the first slide should be stained with H&E stain and examined by an experienced pathologist. This initial examination is essential for the framing of possible diagnoses and for deciding on any additional investigations that may be required. One of the most important findings to be detected in this first examination is whether the biopsy was taken from a nerve. An inexperienced surgeon could have sampled the adjacent vein instead of the nerve. The tissue removed during the surgical procedure is usually tied at both ends, and therefore there may not be any noticeable bleeding. In the laboratory, it can be assessed whether the material sent is a blood vessel by checking for a lumen during dissection (Fig. 6.51) and the presence of a plexiform architecture, which would not be observed in a vessel during microscopic evaluation. The incidence of sampling a vessel instead of a nerve has been reported as up to 4% in the previous study [31]. In the advanced stages of demyelinating neuropathies, nerve bundles may display a similar appearance to smooth muscle bundles on the walls of large vessels due to increased fibrosis. However, the typical appearance of nerve fascicles, which are surrounded by the perimysium in the peripheral nerve, indicates that the tissue is nerve tissue, not a vessel, even if the lumen is not visible (Fig. 6.52). Although there is no need for an early diagnosis for the most neuropathies, it is important to inform the clinician immediately in the case of vasculitis and ischemia due to microvasculitis so that the treatment can be planned early [31].

Sometimes the tissue may be immersed in a formaldehyde solution due to a lack of the clinicopathological correlation. In this situation, frozen sections and a few special analyses such as modified trichrome and oil red O cannot be performed. Placing the material in a fixation solution prevents examination of muscle biopsies because most of the muscle-specific primary antibodies used in immunohistochemical staining work only on frozen sections. Therefore, no special staining can be performed, and muscle tissue preserved in a paraffin block is absolutely nondiagnostic. However, immunohistochemical stains applied in nerve biopsies are adapted to paraffin sections. Moreover, even if the material is sent fresh and unfixed, as we explained in more detail in the previous section, the nerve is divided three parts; each of them is used for evaluation in frozen section, in paraffin section and in electron microscopic examinations [4, 34, 35].

The nerve biopsy sample should include the whole nerve rather than a few fascicles for the evaluation of larger vessels and epineurial connective tissue. In this way, the presence of skip inflammations can be determined, and comparative evaluation of different fascicles can be made. As a more invasive and morbid examination, nerve biopsy evaluation is not proposed concomitantly with every muscle biopsy. But if it is necessary, muscle tissue can be sampled during nerve biopsy harvesting. It has been reported that when a muscle biopsy is performed at the same time as a nerve biopsy, the rate of diagnosis of immunologic, vascular and amyloid disorders is increased [31, 36–39].

If the reason for neuropathy is determined by clinical examinations and laboratory analysis, a nerve biopsy is unnecessary. In some patients with renal insufficiency, diabetes mellitus and alcohol abuse, a nerve biopsy can be performed to evaluate the basic pathophysiology and severity of neuropathy. Nerve biopsy is indicated in two groups of

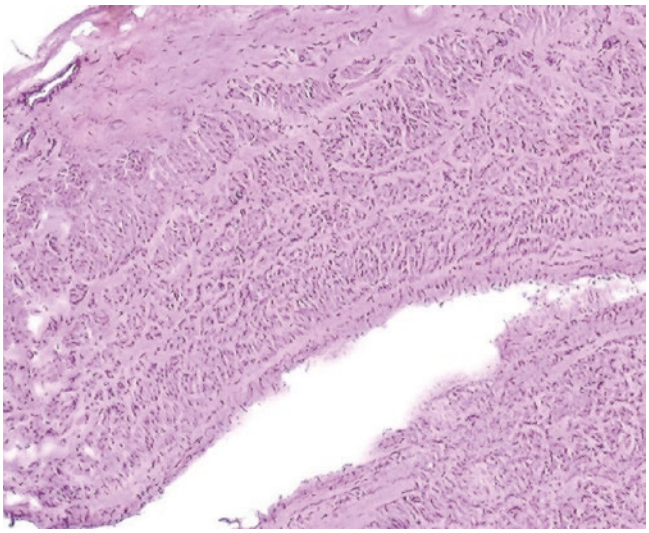

Fig. 6.51 A large vessel harvested instead of a nerve (HE ×100)

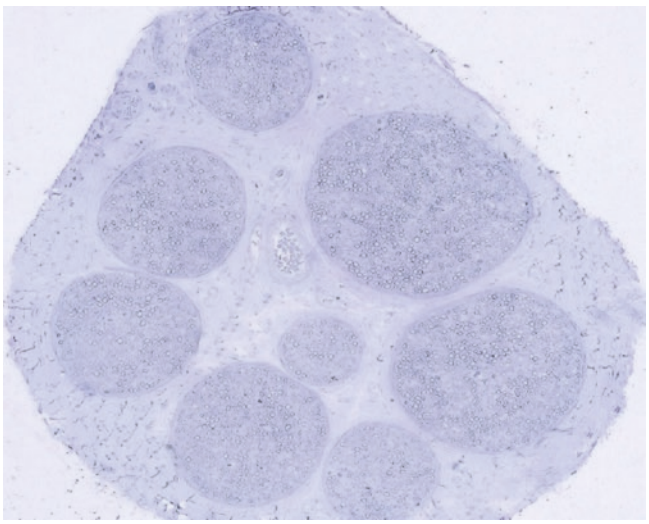

Fig. 6.53 Semithin section of a sural nerve biopsy (toluidine blue ×40)

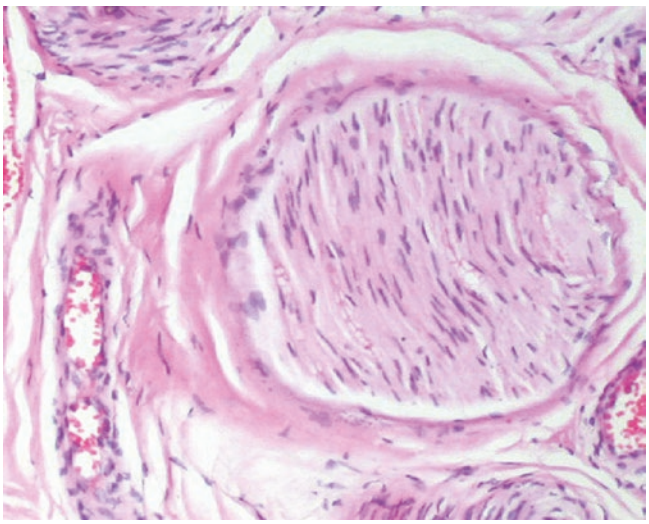

Fig. 6.52 A nerve fascicle covered by perimysium (HE ×400)

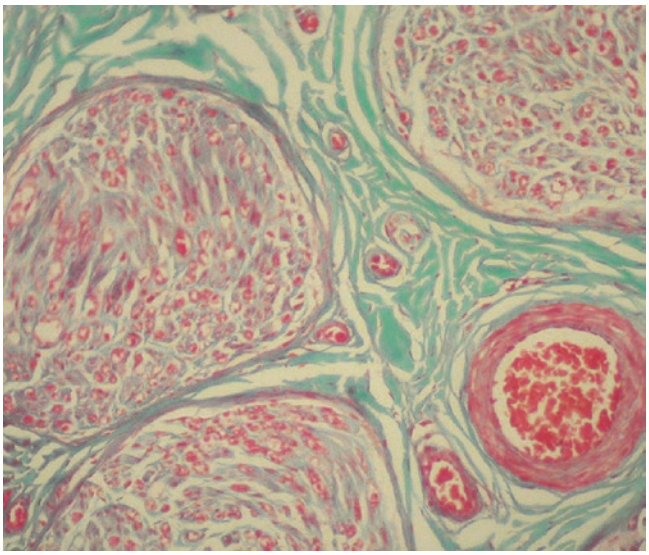

Fig. 6.54 Connective tissue layers of peripheral nerve (Gomori's trichrome ×200)

patients, patients suspected of vasculitis and patients with clinically significant neuropathy without known cause [31, 33, 34, 38].

When nerve biopsy examination is required, biopsy is most often harvested from the sural nerve. The sural nerve is a 2–3-mm-diameter pearly-white cord that generally includes 5–15 nerve fascicles (Fig. 6.53). All the nerve fibers of the fascicles are wrapped by endoneurium. Epineurium is the outermost layer and interstitial tissue that wraps all nerve fascicles, as well as whole nerve (Fig. 6.54). The epineurium makes up half of the cross-sectional area of the sural nerve and includes several vessels that are most often involved in vasculitic neuropathy. A few mononuclear inflammatory cells can be identified around the arterioles and venules in the epineurium of normal nerves. In addition, several fibroblasts and mast cells are present in the epineurium. But it is not very easy to decide whether the degree of all these cells is abnormal. The perineurium separates the endoneurium that wraps all single-nerve fibers from the epineurium. The endoneurium also contains Schwann cells, capillary vessels, fibroblasts, rare mast cells and longitudinally oriented collagen fibers. The capillary vessels in the endoneurium differ from counterparts in other tissues. Their endothelial cells are non-fenestrated and display tight junctions. These capillaries, like vessels in the brain, are impermeable to most substances and constitute the blood-nerve barrier (BNB). It has been proven that many peripheral neuropathies such as diabetic neuropathy, Guillain-Barré syndrome, paraproteinemic neuropathy, leprosy and lead exposure alter the BNB. Many studies

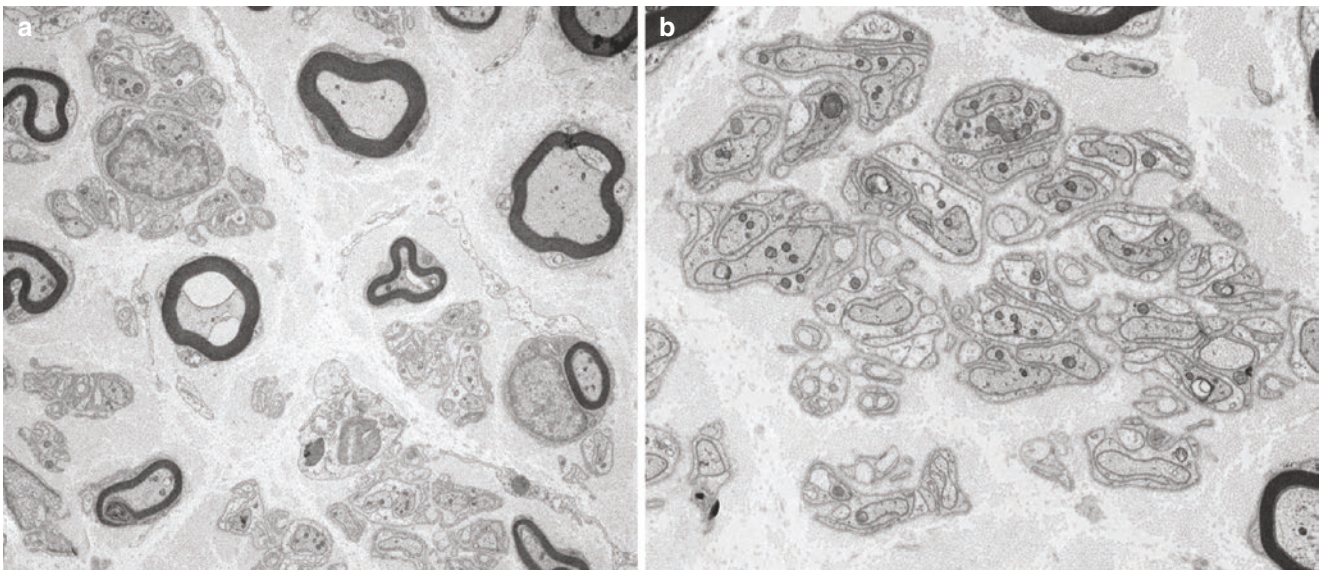

Fig. 6.55 Normal sural nerve showing fields with normal appearance: (**a**) Mix of large and small myelinated and unmyelinated fibres (TEM ×1000) and (**b**) small unmyelinated fibers (TEM ×2500). (These photographs are from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

have indicated that BNB abnormalities can play an essential role in the pathogenesis of neuropathies [36].

Almost all nuclei in the endoneurium belong to the Schwann cells, and up to 10% of nuclei of endoneurium belong to the fibroblasts or endothelial cells. Nonmyelinated fibers are four times more prevalent than myelinated fibers in the sural nerve, and they range from 0.5 to 3 μm in diameter. These small unmyelinated nerve fibers can be reliably demonstrated by electron microscopy (Fig. 6.55). Myelinated fibers range from 3 to 17 μm in diameter. The conduction velocity of axons ranges from 35 to 75 M/s in myelinated axons and 0.1–5 M/s in unmyelinated axons. The larger myelinated axons conduct at faster velocities than smaller myelinated axons. Axon atrophy reduces the rate of axonal conduction. Thickly myelinated fibers conduct a depolarisation more quickly because the myelin layers insulate the axon and prevent the diversion of the signal. Depolarisation only occurs at nodes of Ranvier. Conduction velocities are 50% of adult values at birth; then it increases to 75% of adult values at 1 year of age. From 3- to 5-year-old, the conduction velocities reach to the same values as adulthood. For adults, it decreases slightly with age, especially over the age of 60. The rate of decreasing conduction is 0.5–4 M/s per decade, and aging affects sensory axons more than motor axons [34–36].

The changes observed in peripheral nerves due to aging are not limited to a decrease in conduction velocity. Several structural changes also occur in the peripheral nerves with age. For instance, all three connective tissue layers, especially the outer layers, thicken with age. Myelinisation begins at 18 weeks of gestation; all fibers are myelinated at

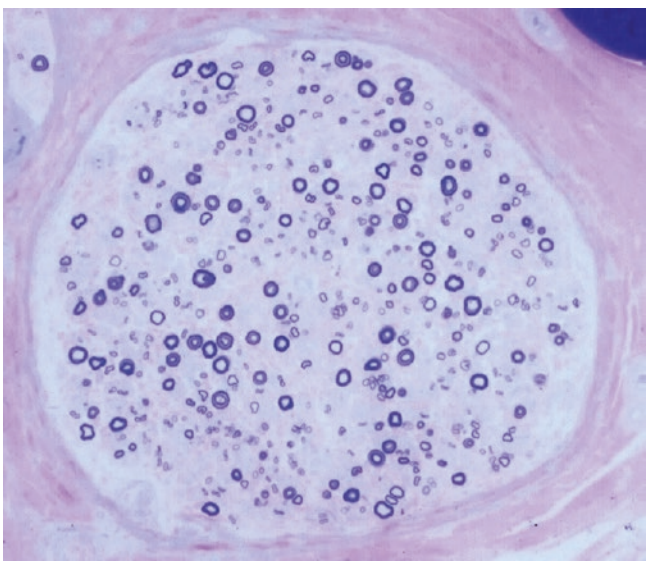

Fig. 6.56 Sural nerve (toluidine blue and basic fuchsin stain ×100)

birth and increase in thickness throughout childhood. The absolute thickness of nerve fibers is relative to axon size. The ratio of the diameter of an axon without myelin compared to the diameter of a completely myelinated axon is normally around 0.6, and this ratio is known as *G*-ratio. The *G*-ratio can be calculated by electron microscopic evaluation and in semithin sections (Fig. 6.56), but as the axons are not usually visible, the *G*-ratio cannot be reliably calculated in paraffin or frozen sections [34–36].

The diameter of axons increases up to 5 years of age. Concomitantly, the endoneurial area of the sural nerve

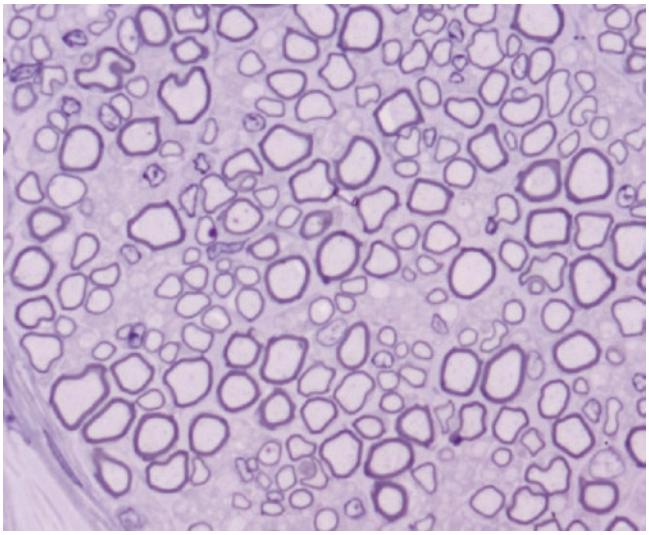

Fig. 6.57 The three nerve types of the sural nerve in the semithin section (toluidine blue ×1000)

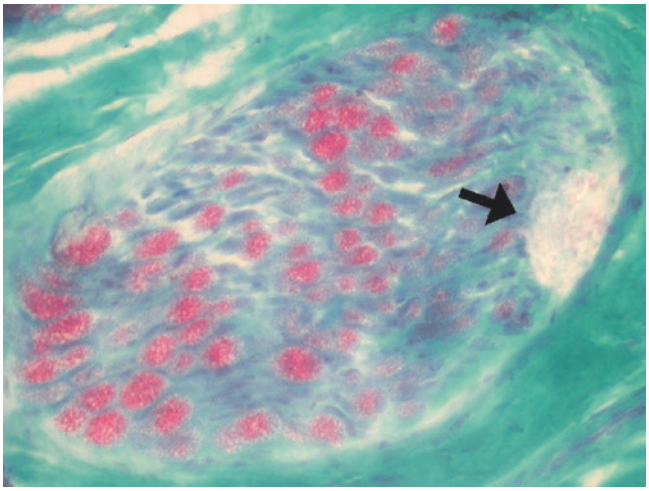

Fig. 6.58 Black arrow points to Renaut body (modified trichrome ×100)

expands with age and reaches to full maturity at 10 years old. The internodal length of myelinated fibers between the Ranvier clefts is 200–300 μm at birth, and 200–1800 μm in adults. The total length of axons increases in parallel with somatic growth. In contrast, the density of axons reduces with increasing age. The density of myelinated nerve fibers decreases continuously throughout adult life by approximately 50% between the ages of 20 and 80. Intra-axonal PAS-positive structures analogous to corpora amylacea can be incidentally found within myelinated nerve fibers in nerve biopsies of older people. In addition, a small number of regenerating axons are normally seen in aging [34–36].

The different types of nerve fibers can be discriminated in each fascicle of peripheral nerves (Fig. 6.57). These are large myelinated, small myelinated and very small nonmyelinated fibers. Normal axons, 3 μm in diameter, have a myelin sheath. Therefore, if an axon is over 3 μm in diameter but does not have myelin sheath, it must be evaluated as a denuded (demyelinated) axon. Nonmyelinated small C fiber axons are gathered into bundles called Remak bundles. These take place when a non-myelinating Schwann cell surrounds the axons and bundles them tightly. By squeezing its cytoplasm in between the axons, the Schwann cell prevents them from encountering one another. Remak bundles' condition changes over time. Similarly, the location affects how many C fiber axons are present in each Remak bundle. For instance, in the L5 dorsal root ganglion of rats, there are large bundles with more than 20 axons, whereas smaller bundles are present in distal nerve segments. All axons can only be seen in plastic resin semithin sections or electron microscopic examination. In the frozen and paraffin sections, myelinated fibers fill the entire area of the nerve fascicle, and small unmyelinated fibers or Remak bundles cannot be identified. Axons can occasionally be seen in the centre of some fibers. Vasculitis is the most common diagnosis made based on nerve biopsy. Clinically, vasculitis can be systemic and involve other organs or be localised to peripheral nerves. In systemic vasculitis, the diagnosis can be based on biopsy findings of any affected organ, but in nonsystemic vasculitic neuropathy, the diagnosis is only made based on nerve and muscle biopsy [36, 38–41].

Cylindrical hyaline structures called Renaut bodies, attached to the inner layer of the perineurium, can be seen in both normal and pathological nerves. They are not seen during foetal life and early childhood. It has been proven that they are composed of fibroblasts and perineurial cells mixed with extracellular matrix. This matrix contains randomly oriented collagen fibers as well as the elastin precursors, formerly called oxytalan filaments. There are no axons or Schwann cells in this matrix. Renaut bodies are also associated with thickened perineurial capillaries, and their number increase during age. As they are mainly seen at sites of nerve compression, it is possible that they act as protective cushions for endoneurial components (Fig. 6.58). In addition, it is thought that they may be formed as a secondary reaction to trauma. Renaut bodies immunohistochemically express similar markers to perineurial cells such as epithelial membrane antigen (EMA), Glut-1 and claudin-1. Therefore, it has been suggested that these bodies have a perineurial origin rather than an endoneurial [34]. There can be lipid granules in normal Schwann cells of myelinated fibers known as Reich or Pi granules. They are seen in the cytoplasm of demyelinated fibers, but they are not found in Remak fibers. Pi granules can be found as alone or in clusters. These clusters may be mistaken as abnormal lipid accumulation from different storage diseases. Pi granules are generally regarded as lysosomes because they can be identified by the acid phosphatase staining [34–36].

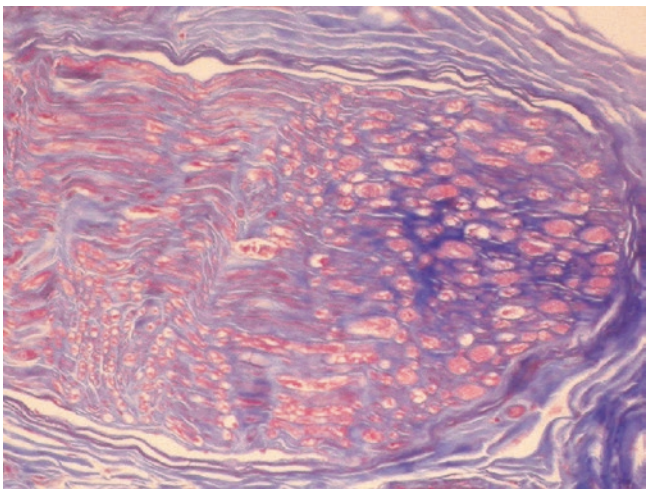

Fig. 6.59 Decreased myelin and increased endoneurial fibrosis in a nerve plexus (Masson's trichrome ×200)

The three cardinal findings that can be evaluated from nerve biopsies due to peripheral neuropathies are axonal changes, loss of myelin and presence of inflammation (Fig. 6.59). Toluidine blue staining of semithin section and electron microscopic examination is essential for both detailed examinations of nerve fibers and evaluation of the first two changes. Immunohistochemical and immunofluorescent stains are used to prove the presence of inflammation, loss of myelin or axons, and to differentiate cell phenotypes. These examinations can all be applied to frozen or paraffin sections. Therefore, even in cases where electron microscopic examination cannot be performed, findings that will contribute to the clinical differential diagnosis can be obtained. In addition, if a small piece is separated from the biopsy for electron microscopic examination, embedded in a plastic block and archived, it can be sent to a centre where electron microscopy can be performed if needed [4, 34–36].

Axonal degeneration is a common finding encountered in nerve biopsies, and it can be the result of injury to either the axon or the body of neuron. They can both cause Wallerian degeneration in which clusters of regenerated and regenerating fibers are usually seen. The lack of Wallerian degeneration suggests a more proximal or somatic defect. When damage occurs in a neuronal body or the trunk of nerves, a degenerative process called Wallerian degeneration starts in the axon distal to the injury's site (Fig. 6.60). This process induces the secondary breakdown of myelin throughout the distal segment of the nerve after transection. The onset of Wallerian degeneration is 3–5 days in the axon of motor nerves and 6–10 days in the axon of sensory nerves. Fragmentation of distal axon and the proliferation and activation of Schwann cells along with macrophages occurs at the site of injury. Then Schwann cells line up along the degenerated axonal trace and newly formed axonal sprouts embed in these

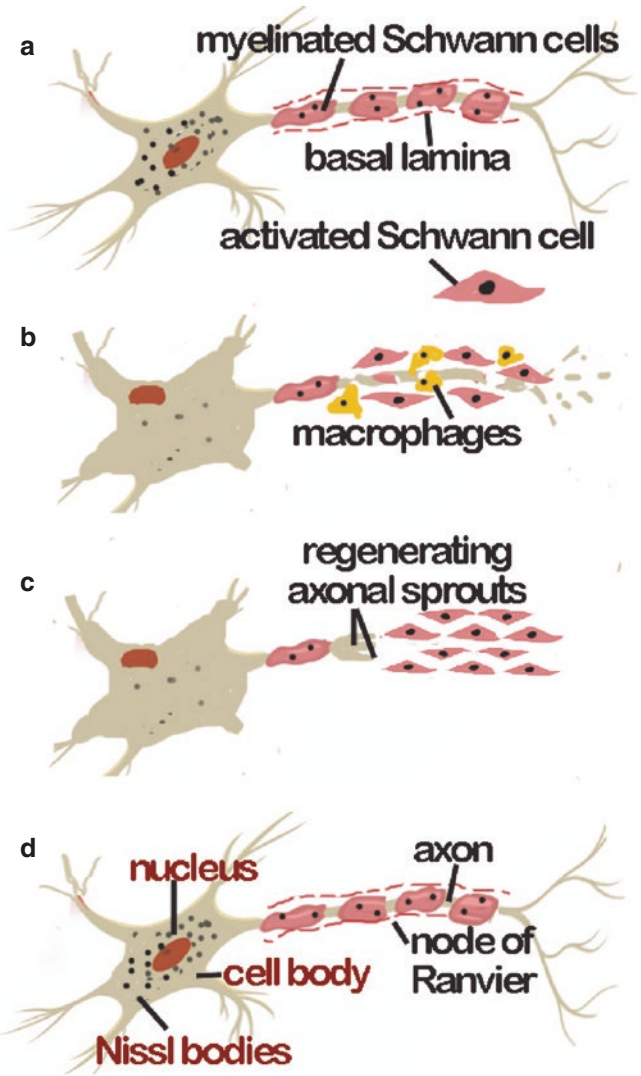

Fig. 6.60 Wallerian degeneration

Schwann cells' columns in various directions. The regeneration process may be completely successful depending on the intensity of the injury and may take several months. Regeneration is normally seen 3–4 days following an injury and the regenerating axonal sprouts grow at 2–3 mm per day. Some neurotrophic growth factors and chemotactic factors which are expressed by activated Schwann cells fibroblasts and macrophages help the regeneration process. This process is also accompanied by several changes in the neuron body and proximal part of the injured axon. These changes are central chromatolysis in the neuronal perikaryon and remyelination by Schwann cells. In addition, the injured axon proximal to the lesion retracts and decreases in diameter; subsequent sprouting and attempts of functional regeneration will occur. An injury close to the cell body can cause irreversible degeneration and even cell death. If axonal regeneration and remyelination are successful, the reinnerva-

tion of the target organs can occur. However, some factors including ischemia, cytotoxic drugs, hematoma and cicatrisation can prevent reinnervation due to inhibition of nerve growth and disruption of perineurial continuity. When the injury is too wide to bridge, a benign neoplasm called a traumatic neuroma may form from exuberant and disorganised proliferation of nerve fibers, Schwann cells and other microenvironmental components [35, 36].

Chemical neurotoxin exposure, trauma, impaired perfusion and inherited neuropathic disorders can cause axonal degeneration. Regardless of the causative event, degeneration in the peripheral and central nervous systems evolves via a common mechanism. Most studies indicate that Ca^{2+} accumulation in injured axons has a significant initiator effect, and the cumulative effects of increasing intra-axonal Na^{+}, decreasing K^{+} and axolemmal depolarisation favour a reverse Na^{+}/Ca^{2+} exchange operation which promotes Ca^{2+}/-mediated axon degeneration. In the past, Wallerian degeneration was understood to be a passive disintegration of axons when they were severely injured. But recent studies have shown that Wallerian degeneration is an active process mediated by several genes. Genes associated with Wallerian degeneration are the slow Wallerian degeneration (WldS) gene, the nicotinamide mononucleotide adenylyl transferase 2 (NMNAT2) gene and the sterile-α and Toll/interleukin 1 receptor (TIR) motif-containing protein 1 (SARM1) gene [40].

Following the axonal changes, the myelin sheath also degenerates. In the early stages of axonal degeneration, myelin sheaths are normal. Then several changes like fragmentation, autophagy and the occurrence of myelin ovoids (Fig. 6.61) can be identified. The remaining basal lamina and Schwann cells together form the denervated Schwann cell bands called Büngner's bands. These structures are proliferating Schwann cell columns within the basal lamina in the distal part of nerve that are awaiting regrowing axons from the proximal nerve. Axon and myelin debris are cleared by the Schwann cells or macrophages. The degeneration of unmyelinated nerve fibers leaves the basal lamina unsheathed and flattened stacks of Schwann cells behind. After losing their target axons, Schwann cells tend to form collagen pockets by unsheathing bundles of collagen fibers, which are like small axons in diameter. Collagen pockets usually occur due to the loss of small axons, increased age and absence of axon regeneration. Otherwise, Schwann cells wrap the smaller regenerating axons, and this process looks like the sprouting of axons. In summary, histological findings of axonal degeneration are the clumping of macrophages, sprouting axons, occurrence of Büngner's bands, collagen pockets, myelin fragmentation and myelin ovoids (Fig. 6.62). All these findings can be comprehensively evaluated by electron microscopic examination [41–44].

The most common pathological reaction of the myelin sheath is segmental demyelination. In primary demyelinating diseases, the damage to the myelin sheath originates from the abnormal function of the Schwann cells, while secondary demyelination typically follows axonal degeneration. The myelin sheath produced by subsequent remyelination cannot reach either the thickness or the length of the original sheath. Redundant basal laminae and multiple loose basal lamina loops around these axons are also a frequent occurrence. Repeated cycles of de- and remyelination form pathognomonic onion bulbs where the proliferated Schwann cells circularly surround the axon within the scaffold of the basal lamina. Onion bulbs are observed with both semithin

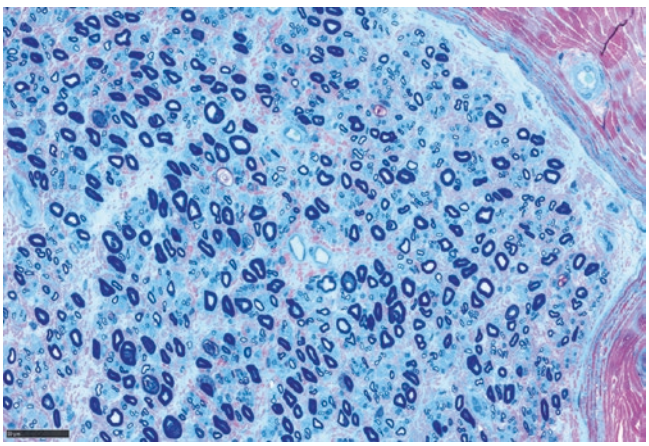

Fig. 6.61 Left superficial peroneal nerve biopsy: acute axonal degeneration and myelin ovoids (toluidine blue and basic fuchsin stain ×1000). (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

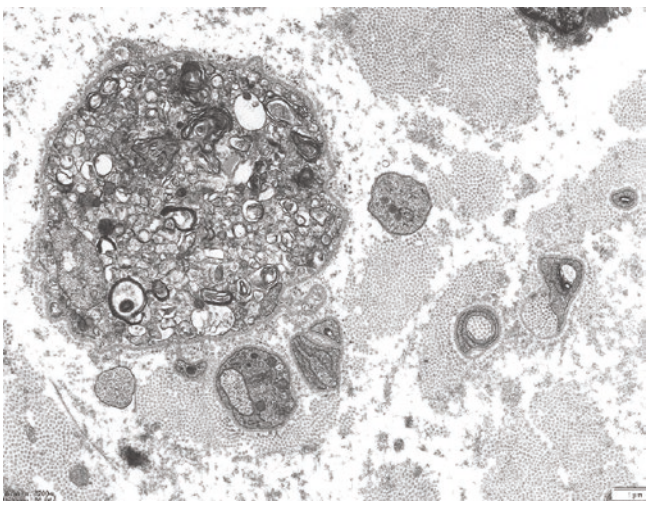

Fig. 6.62 Digestion of myelin and axonal debris, within an intact original Schwann cell basal lamina (TEM ×10,000). (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

(Fig. 6.63) and EM examinations (Fig. 6.64). Older onion bulbs lack a central axon. Congenital demyelinating neuropathies show basal lamina onion bulbs which lack vital Schwann cells and only consist of the remnants of basal lamina sheets around the axon [36, 38–41, 45–49].

In cases when inflammation is not obvious with routine stains, the immunohistochemical leucocyte common antibody (LCA, CD45) is used to detect small collections of leukocytes. A small number of epineurial or endoneurial lymphocytes is normal within limits (<20/mm^2), and a few lymphocytes may be normally found around epineurial vessels giving the impression of slight cuffing.

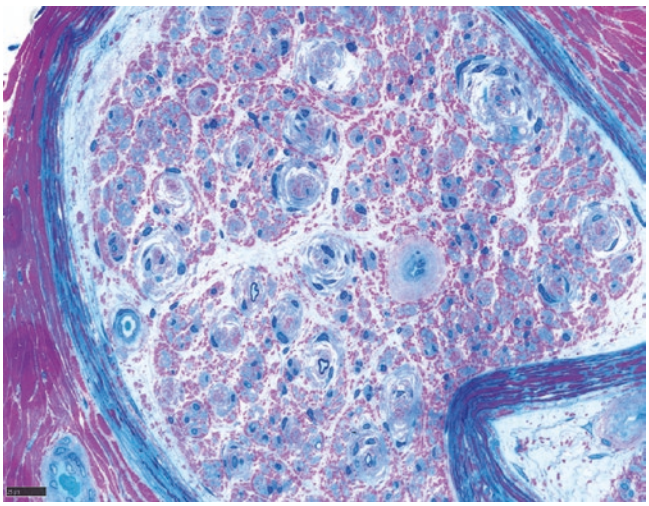

Fig. 6.63 Onion bulbs (toluidine blue and basic fuchsin stain ×1000). (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

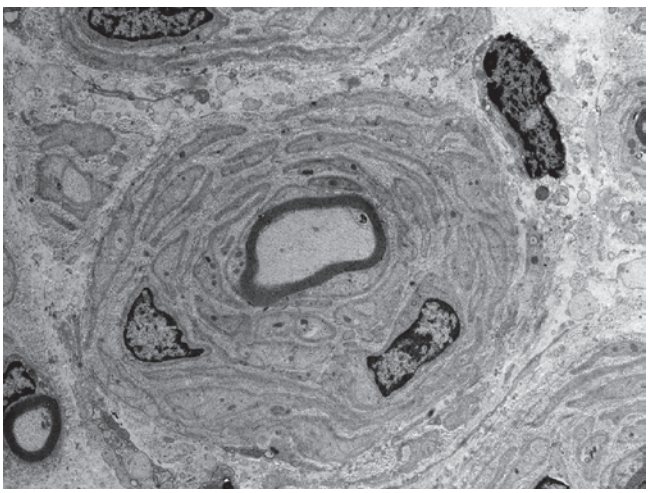

Fig. 6.64 Onion bulbs (TEM ×10,000). (This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London)

However, endoneurial capillary lymphocytic cuffing is certainly abnormal, and more than two leukocytes in a nerve fascicle should be regarded as an indicator of inflammation [32]. In other words, three or more endoneurial cells labelled with LCA per fascicle on the cross-section is an abnormal finding [31]. In chronic inflammatory demyelinating polyneuropathies (CIDPs), the CD4+/CD8+ lymphocyte ratio is mildly decreased. Similarly, IgM, IgG and complement deposits in the walls of endoneurial blood vessels and on the surface of Schwann cells can be detected by direct immunofluorescence. The vascular deposits in the granular patterns are immune complexes. Antigens that bind to the cell membrane of the Schwann cell are seen in linear patterns. All these deposits are rarely found in nonimmune-mediated neuropathy or normal biopsies [35, 36, 38].

The predominant inflammatory cells in any vasculitis are lymphocytes and macrophages. Almost all the lymphocytes are a helper (CD4+) and cytotoxic (CD8+) T cells. Depending on the duration of the process, a variable number of neutrophils and eosinophils may also be present. Direct immunofluorescence may reveal accumulations of immunoglobulin, complement or fibrinogen in epineurial vessel walls in most nerve biopsies in patients with vasculitic and diabetic neuropathy. Epineurial and perimysial vascular deposits of immunoglobulins or C3 are a specific marker of vasculitic neuropathy [35].

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Molecular Diagnostic Procedures and Genetics in Neuromuscular Disorders

7

Berk Ozyilmaz and Gulden Diniz

Genetic Basis of Diseases

The basis of heredity in all living things is a chemical structure called deoxyribonucleic acid (DNA). Nucleotides, which are the building blocks of DNA, are named according to their chemical components which are called bases. These are the nucleotides: adenine (A), thymine (T), cytosine (C), and guanine (G). Three-nucleotide units form *codons* in DNA. Each codon encodes an amino acid. Nucleotide sequences that can produce a functional product are called a *gene*. The information encoded in DNA is used to synthesize proteins in ribosomes using ribonucleic acid (RNA). The parts of the gene that participate in protein synthesis are called *exons*, while the parts that do not participate but carry the tools that regulate the gene's function are called *introns*. When a protein is to be synthesized, pre-mRNA is produced from DNA and it consists of both exons and introns. Then *splicing* occurs and introns are removed, creating mRNA which carries only exons (Fig. 7.1). With the human genome project, the normal DNA sequence of all genes has been revealed, so that the DNA data of patients can be compared with the normal DNA sequence in genetic tests. The study also revealed that although human DNA contains around 20,000 genes, much greater numbers of proteins are produced. This is due to the *alternative splicing* mechanism which underlies protein diversity. Accordingly, structural splicing can produce a protein by using all exons of a gene, while alternative splicing can produce a different protein isoform by using different exon clusters [1].

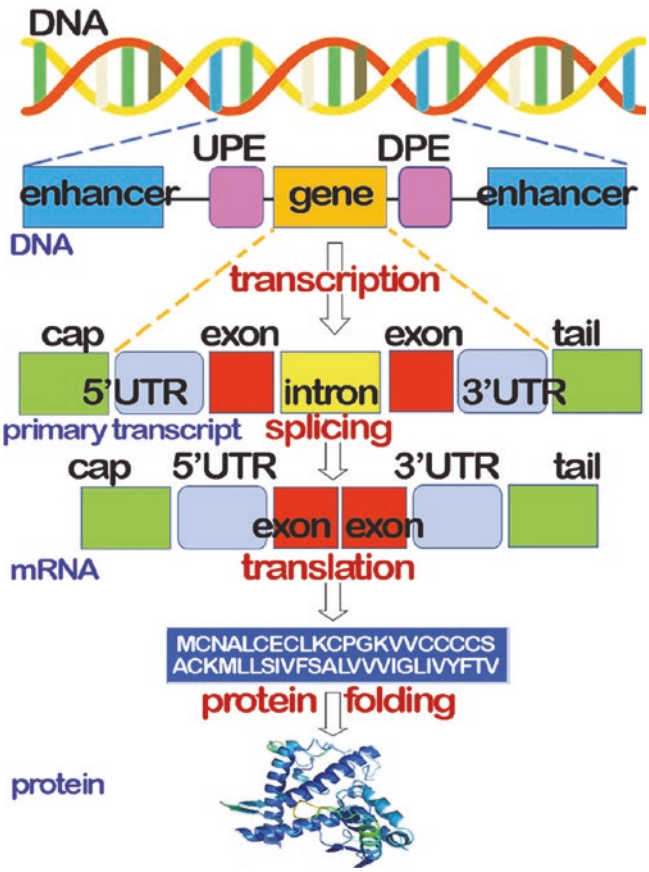

Fig. 7.1 Mechanism of protein synthesis. *CAP* catabolite activator protein, *UPE* upstream promoter element, *DPE* downstream promoter element, *UTR* untranslated region, *mRNA* messenger ribonucleic acid

All of a human's DNA, named the *genome*, is packaged into protein-nucleotide complexes called *chromosomes*. While human cells carry (23 pairs) 46 chromosomes, reproductive cells such as eggs and sperms carry 23 chromosomes. Thus, the 23 chromosomes from egg and sperm combine to form an embryo with 46 chromosomes, receiving the genetic characteristics of both its mother and father. The presence of 23 pairs of chromosomes in the nuclei of

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our cells causes all DNA to be stored in duplicate. Each copy of a gene on both (homologous) chromosomes is called an *allele*. A disease is passed on via *autosomal recessive* inheritance if it occurs when both alleles of the gene associated with that disease lose function. For a disease inherited in an autosomal recessive manner, if one of the two copies of the gene is defective and the other is intact, the person is defined as a *carrier*. Carriers classically do not show signs of disease, but they can pass the defective gene to their offspring. If the loss of function of one allele is sufficient to induce the disease, the disease is inherited with *autosomal dominant* inheritance. There is no carrier status in autosomal dominant inheritance. Since women have two X chromosomes and men have one X chromosome, recessive and dominant inherited diseases of genes on the X chromosome (*X-linked recessive* and *X-linked dominant*) show a clinically distinctive pattern of inheritance. In X-linked recessive diseases, women are often carriers, and their sons have a 50% chance of getting the disease [1].

Genetic diseases are often associated with changes in the DNA found in the cell nucleus. However, changes in *mitochondrial DNA* (mtDNA or mDNA) in the mitochondria of the cell cytoplasm can also lead to genetic diseases. Mitochondrial diseases associated with mitochondrial DNA, which contains 37 genes, are inherited in a completely different way because mitochondrial DNA comes entirely from the mother. A woman with mitochondrial disease can pass the disease on to her children of both sexes, but an affected boy cannot pass the disease on to his own children [2].

Molecular Genetic Diagnostic Methods and Bioinformatics Approaches

Genetic diseases occur when the function of a gene is disrupted. Different molecular genetic testing approaches are used to identify this loss of function. The first step in molecular genetic tests is to obtain DNA from the samples to be studied. Whether it is peripheral blood or tissue samples taken by biopsy, the obtained cells are subjected to various processes, and the DNA in their nuclei is extracted. Today, conventional manual methods for DNA isolation (such as phenol-chloroform or salting-out) have been replaced with kit-based optimized methods. These modern methods can extract DNA from very small samples. Furthermore, even circulating cell free DNA (cell-free DNA or cfDNA) can be used for some molecular genetic tests. Regardless of which molecular genetic diagnosis method will be used in the next step, polymerase chain reaction (PCR) is used to amplify the DNA region or regions of interest. The PCR products (amplicons) obtained are read with specific methods (Sanger, next-generation sequencing, etc.) regarding to the test to be applied [3, 4].

Changes in the DNA sequence, called *variants*, can cause DNA to be misread (missense and frameshift variants), incompletely read or not read at all (nonsense variants). In this case, the protein, which is the product of the gene or genes, is produced incorrectly, incompletely, or not produced at all. If a variant in the DNA is found in both alleles, it is called *homozygous*, and if it is found in only one allele, it is called *heterozygous*. If the variant is in a gene on an X chromosome in a male patient, it is called *hemizygous* (e.g., DMD) [1].

Apart from sequence variants, *copy number changes/variants* (CNVs), which can lead to loss (deletion) or gain (duplication) of a part of DNA, can also impair the functions of genes by changing their dosage. The expanse of these copy number changes vary widely. A copy number change can include a single nucleotide, some exons of a gene, all sequences of a gene, or several genes.

When performing molecular genetic analysis of a genetic disease, it is critical to apply testing appropriate to the aetiologic mechanism specific to that disease because different genetic disorders require different genetic testing approaches. Moreover, the changes that each molecular genetic test method can detect are different from each other.

Rare genetic diseases are mainly caused by *sequence variants*, *copy number changes*, and *triple nucleotide repeat alterations*.

In the detection of *sequence variants*, the DNA sequence of the region of interest is obtained by various molecular genetic methods, and this data is compared with the normal (reference) sequence. The traditional method of obtaining the DNA sequence, the use of which is gradually decreasing, is the Sanger sequence method. With Sanger sequencing, DNA regions in the 100–1000 base-pair range can be sequenced. Each DNA segment is analyzed separately, and it is not suitable for the analysis of large genes. It is, however, suitable for the analysis of a small number of DNA regions, known mutation analysis, and validation studies. *Next-generation sequencing* (NGS) is the current and popular sequencing method. It produces much more data at a much cheaper cost than previous methods. It has the advantage of being able to analyze multiple DNA segments (amplicons) simultaneously. Therefore, NGS can be used for the analysis of a targeted gene, a gene panel consisting of many genes, all coding regions in the human genome (whole exome sequencing, WES) or the entire human genome (whole genome sequencing, WGS). In addition, NGS-based methods can be used in cfDNA and mtDNA analysis [5].

The size of the targeted region is important in the analysis of *copy number changes*. Deletions and duplications up to a certain size can be detected by sequencing methods, but the gold standard diagnosis method of copy number changes at the molecular level is multiplex ligation-dependent probe

amplification (MLPA). The MLPA method is used as the first step test in investigating the etiology of diseases in which intragenic copy number changes are frequent, such as spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), and Charcot-Marie-Tooth-1A (CMT1A) [6].

Triple nucleotide repeat alterations cause an increase in tri-nucleotide repeats, which are normally found in certain and limited numbers, and lead to the deterioration of the stabilization and function of genes. As a result of the mechanism called *anticipation*, the number of repeats may increase from generation to generation; thus the clinical findings become evident early, and the diseases become more severe in future generations. Increasing the number of tri-nucleotide repeats in a gene (e.g., increasing the number of CTG repeats from 35 to 3000 in the DMPK gene, in myotonic dystrophy type 1 disease) impairs the function of the gene, as well as the PCR method used in the diagnosis of the disease. Therefore, special approaches such as triplet-primed PCR (TP-PCR) are used in the diagnosis of these diseases [7].

Regardless of the method used for diagnosis, bioinformatic analysis is critical to correlate the data obtained with the clinic. Today, the increase in data obtained with the widespread use of NGS method has also revealed difficulties in the interpretation of this data. Reliable databases such as the Human Gene Mutation Database (HGMD) and Clinvar are the first resources used for the interpretation of sequence variants detected by NGS. Demonstrating the variant as pathogenic or likely pathogenic can provide a definitive diagnosis of the disease taking into account the inheritance pattern of the disease (such as homozygous for autosomal recessive, heterozygous for autosomal dominant). When interpreting variants defined as the *variant of unknown clinical significance* (VUS), the aim is to reach an interpretation by using various rules and criteria with the recommendations of reliable institutions such as the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP). ACMG recommendations provide an interpretation, based on the population frequency of the variant, in silico analysis data (computational predictive programs) and functional study data. This interpretation, together with family study and clinical compliance assessment, can provide a diagnosis [8].

Another challenge with molecular genetic analysis is the correct interpretation of mtDNA analysis data. In the case of mtDNA-specific heteroplasmy, variants may be present in some of the DNA molecules and not in others. The low ratio of mtDNA variants may lead them to become undetectable by molecular genetic methods (a false-negative result). In addition, mtDNA analysis should be performed on the affected tissue if possible (e.g., in mitochondrial myopathies, it should be performed from DNA extracted from the affected muscle tissue). Many mtDNA variants are undetectable in blood (a false-negative result) samples because the high replication rate of normal cells in the blood causes normal cells to proliferate at an increased rate compared to cells with the pathogenic mtDNA variants [9].

Molecular Genetic Basis of Neuromuscular Diseases

The diagnosis of neuromuscular disorders (NMDs) has been dependent on molecular genetic methods as a result of the development of advanced technology laboratory methods in recent years. However, without the guidance of the clinician and histopathology, it is difficult to determine the candidate genes for analysis and the methods to be used. Therefore, loss of time, resources, and workforce may occur. For this reason, the diagnosis of NMDs should be made with a full multidisciplinary approach. In addition, both the etiopathogenic mechanisms and clinical features of the diseases in the NMD group are different from each other. For these reasons, the diagnostic approaches of each are disease-specific (Fig. 7.2) [10, 11].

Dystrophinopathies

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are two diseases of different severity, resulting from the same etiology as a result of mutations in the dystrophin gene. Dystrophinopathies occur due to mutations in the DMD gene located on the X chromosome. The dystrophin gene is 3000 kb in size and consists of 79 exons. It is associated with a deletion of one or more exons in more than 65% of patients. The "hot spot" where deletions are more common is between exons 45 and 53. Disease severity in dystrophinopathies is not correlated with deletion size. According to the *open reading frame* (ORF) rule, if a mutation preserves the ORF (does not shift the reading frame or generate a stop codon), a short but functional protein and a milder phenotype (BMD) is observed. To confirm this information, immunohistochemical analyses found that the dystrophin protein was completely absent in children with DMD and partially present (10–40%) in BMD patients. Deletions and duplications in 65–80% and point mutations in 20–35% play a role in the etiology of DMD. For this reason, MLPA is applied as the first-line test in dystrophinopathies. DMD gene sequence analysis is recommended in patients without deletion/duplication [12].

Commonly, the mother of an affected boy is a carrier. According to the X-linked inheritance rules, 50% of the boys are affected, and 50% of the girls are carriers in each pregnancy of this mother. In cases where the mother is not a car-

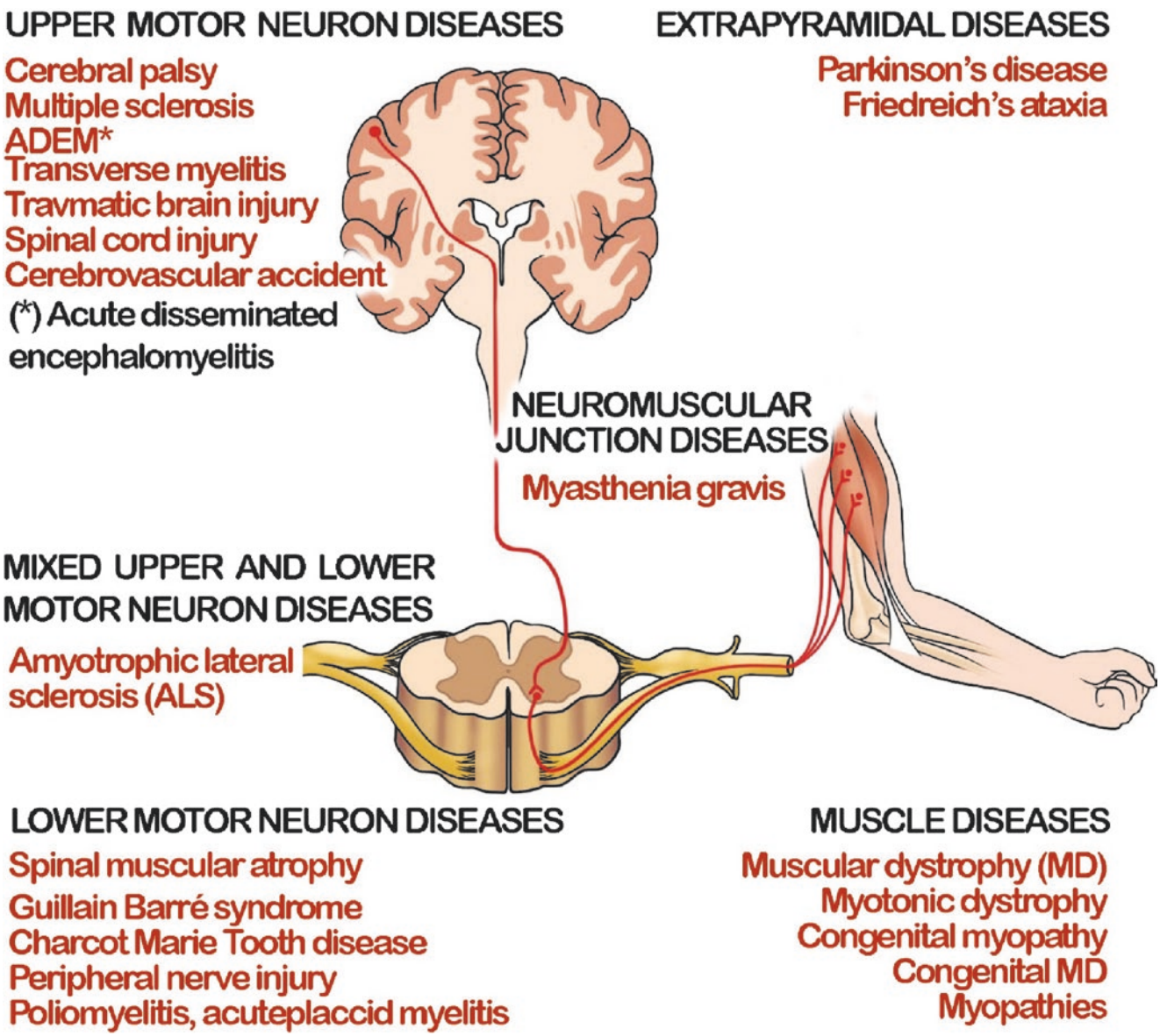

Fig. 7.2 Genetic basis of NMDs

rier, the disease may have appeared in that boy for the first time in the family. Studies have shown that de novo mutations are responsible for approximately 17% of affected male patients. Rarely, there may be an undetectable *germline mosaicis*m in maternal peripheral leukocytes. In this case, although the mother is not a carrier, there is up to 20% risk of recurrence of the disease. Preimplantation genetic diagnosis (PGD) or prenatal diagnosis (PT) is strongly recommended for subsequent pregnancies of carrier or *germline mosaic* mothers due to the significantly increased risk of recurrence. In addition, carrier pregnant women should be closely monitored due to cardiomyopathy-related complications [13].

Myotonic Dystrophy Type 1 (DM1)

Myotonic dystrophy (dystrophic myotonia, DM) is the most common form of muscular dystrophy that begins in adulthood. It consists of two main types, Type 1 (DM1) and Type 2 (DM2). Both types of myotonic dystrophy are inherited in an autosomal dominant fashion. DM1 is caused by mutations in the DMPK gene with a CTG repeat expansion, while DM2 is caused by a CCTG repeat expansion in the CNBP gene. DM1 results from a repeat expansion of CTG triplet nucleotides in the 3′ untranslated region (UTR) of the DMPK gene. The European Molecular Genetics Quality Network (EMQN)

classifies CTG repeat expansions in the DMPK gene into 4 groups according to their clinical effects: from 5 to 35 repeats = normal (NL), from 36 to 50 = premutation (PM), from 51 to 150 = full penetrant (FP), >150 = full penetrant (FP) (severe clinical-juvenile/childhood form), and >1000 repeats = congenital form [14].

DNA regions rich in GC nucleotides are difficult to amplify in PCR. Moreover, conventional PCR can only detect alleles consisting of up to 85 CTG repeats. Southern blot is required to detect alleles with more than this number of repeats. Southern blot analysis is not suitable for routine analysis and screening approaches due to implementation difficulties. Therefore, a strategy method called triplet repeat primed PCR (TP-PCR) has been defined. For DM1, molecular genetic evaluation is recommended. Conventional PCR is used to find if there are two detectable (normal number) alleles or if there is a single detectable allele (the other allele is considered to be expanded). To determine allele sizes, triplet repeat Primed PCR (TP-PCR) is recommended [14].

A genetic phenomenon called *anticipation* is frequently seen in DM1 disease. Anticipation leads to an increase in the number of CTG repeats in the next generation. Children typically have increased CTG repeat counts relative to their parents. This results in a lower age of onset and worsening of the disease severity in each generation. The risk of anticipation in DM1 is higher in fully penetrating individuals compared to premutation carriers. The risk of anticipation should be emphasized in genetic counseling while discussing a prenatal diagnosis [14].

Facioscapulohumeral Muscular Dystrophy (FSHD)

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common type of muscular dystrophy. It is a slowly progressive muscle disease that often causes weakness of the facial, scapular, upper arm, lower leg, and hip girdle muscles. The genetic etiology of the disease is quite complex. The main etiopathogenic factor underlying the two main variants, FSHD1 and FSHD2, is the disruption of the regulation of the transcription factor DUX4. D4Z4 contraction-type mutations in FSHD1 and pathogenic variants in the SMCHD1 gene in FSHD2 change the regulation of the DUX4 transcription factor, abolishing its inactivation (*derepression*) and thus, DUX4 transcription, causing the effects and clinical findings of FSHD. Approximately 95% of FSHD patients have a *contraction-type* mutation in the D4Z4 macrosatellite sequence in the subtelomeric region of 4q35, causing pathological shortening of the D4Z4 repeat sequence. This group of patients is defined as FSHD1 (Landouzy-Dejerine muscular dystrophy), and the disease is inherited in an autosomal dominant manner. In addition, both the D4Z4 *contraction-type* mutation (1–10 repeat unit) and a specific haplotype (4A) are required for FSHD1 clinical manifestation. Five percent of patients have FSHD2, which is associated with the SMCHD1 gene. Both a pathogenic variant in the SMCHD1 gene and the 4A haplotype are required for FSHD2 disease to occur. Molecular genetic analysis in FSHD patients is used to determine whether they have 1–10 repeat units in the D4Z4 allele, by using the Southern blot method, and to search for the presence of a 4A/4B haplotype [15].

Limb-Girdle Muscular Dystrophy (LGMD)

The Limb-Girdle muscular dystrophy (LGMD) group of diseases includes a series of muscle diseases that differ in genetic etiology, age of onset, severity, and phenotype, predominantly affecting the proximal muscles. This dystrophy was evaluated in 28 different clinical types according to the classification made by the European Neuromuscular Center (ENMC) in 2018. DNAJB6, DES, TNPO3, HNRNPDL, CAPN3, CAPN3, DYSF, SGCG, SGCA, SGCB, SGCD, TCAP, TRIM32, FKRP, TTN, POMT1, ANO5, FKTN, POMT2, POMGNT1, DAG1, PLEC1, DES, TRAPPC11, GMPPB, ISPD, GAA, LIMS2, POPDC1, TOR1AIP1, POGLUT1, COL6A1/2/3, LAMA2, and POMGNT2 genes play a role in the etiology of LGMD with autosomal recessive and autosomal dominant inheritance patterns [10, 16].

In the past, the diagnosis of LGMD patients was made using the Sanger sequencing method, analyzing a single gene at each step. However, due to the high phenotypic variability in the disease group and the large sizes of most of the genes involved, this method has recently been replaced with next-generation sequencing (NGS) which can analyze multiple gene panels. NGS offers a fast, cost-effective, less labor-intensive, and more economical approach. With the use of NGS multigene panels, variant detection rates have increased from 3% to 33% in LGMD cases. It is recommended that patients who cannot be diagnosed with this method should be evaluated by methods such as clinical exome, exome, or whole-genome analysis [10].

Congenital Myopathies (CM)

The term congenital myopathy refers to a group of clinically, genetically, and histologically heterogeneous diseases that mainly affect muscle tissue, resulting from genetic defects in the structural proteins of muscle tissue [17]. When the diseases in the congenital myopathy group have histopathological specific changes, the diagnosis can be reached with the use of selected gene or multiple gene panels consisting of several genes. They are examined in five main groups:

Nemaline Myopathy

It has an incidence of approximately 1:50,000 live births. The clinical spectrum of the disease is quite wide. About one in six patients are affected by the severe form, and these babies have severe hypotonia and sucking and swallowing difficulties as soon as they are born. Many genes responsible for this group of diseases have been identified: NEB, ACTA1, TPM3, TPM2, CFL2, LMOD3, KBTBD13, KLHL40, KLHL41, etc. The most common among these is NEB gene-associated nemaline myopathy type 2 (NEM2). The disease is inherited in an autosomal recessive fashion, and it is associated with mild/moderate clinical manifestations.

Core Myopathy (Central Core and Multiminicore/Multicore)

Core diseases (central core and multiminicore/multicore) are the most common diseases of the congenital myopathy group. Like the other diseases of the group, hypotonia and muscle weakness are in the foreground, although in this group there is more proximal involvement. RYR1 and SEPN1 genes are responsible for these diseases. Central core myopathy is associated with the RYR1 gene and can be inherited in an autosomal dominant or autosomal recessive manner. Autosomal recessive forms are more severe and may cause fetal akinesia and congenital arthrogryposis syndromes. Multiminicore myopathy is associated with the RYR1 and SEPN1 genes and can be inherited via an autosomal dominant or autosomal recessive manner. Hypotonia, proximal muscle weakness, scoliosis, and arthrogryposis are common findings.

Centronuclear Myopathy (Myotubular Myopathy/Autosomal Centronuclear Myopathy)

While myotubular myopathy is X-linked, the expression, *centronuclear myopathy*, is used for autosomal inherited types. DNM2, MTM1, RYR1, BIN1, and TTN genes are responsible for the diseases. X-linked inherited myotubular myopathy may present with polyhydramnios and decreased fetal movements in the antenatal period in affected boys. The MTM1 gene is responsible for the disease. After birth, hypotonia, arthrogryposis, and respiratory distress occur. It is often fatal.

Congenital Fiber-Type Disproportionate Myopathy

Infants with congenital fiber-type disproportion have hypotonia and trunk/extremity/neck muscle weakness. TPM3, ACTA1, SEPN1, RYR1, and MAP3K20 genes are responsible for the diseases.

Myosin Deposition Myopathy

It is a rare disease. The MYH7 gene is responsible and is inherited in an autosomal dominant and recessive manner. It can begin in childhood and early adulthood. In addition to muscle weakness, calf hypertrophy and scapular weakness can also be seen. Diagnosis is difficult because the disease is subtle and heterogeneous.

Congenital Muscular Dystrophy (CMD)

The main difference between congenital muscular dystrophies (CMD) and other muscle diseases is that muscle weakness can be accompanied by structural and developmental brain anomalies. Since the diseases in the CMD group have clinically specific changes (such as ocular anomalies and brain development anomalies), the diagnosis can be reached with the use of a selected gene (such as POMT1 for Walker-Warburg syndrome) or gene panels consisting of several genes [18].

Many different methods are used in the classification of CMD, and in this section, classification according to the cellular localization of the gene with the defect will be used:

Structural Protein Defects

(a) Laminin Alpha-2 (LAMA2) (Merosin) Deficiency

Hypotonia, delay in motor developmental stages, and white matter anomalies (demyelination) affecting the cerebellum/corpus callosum/inner capsule are seen in MDC1A. Muscle weakness is either absent or very slowly progressive. Respiratory distress is common. It is accompanied by epilepsy and mental retardation. It is inherited in an autosomal recessive manner.

(b) CMD with Collagen VI (COL6A1/A2/A3) Deficiency

Ullrich CMD/Bethlem myopathy occurs with mutations in the COL6A1/A2/A3 genes. COL6A (1/2/3) defects are inherited in both an autosomal recessive and autosomal dominant manner. Generally, severe and homozygous mutations are associated with Ullrich CMD, while milder and heterozygous mutations are associated with Bethlem myopathy. Congenital hypotonia, proximal muscle weakness, and spinal stiffness can be seen in Ullrich CMD. Hyperlaxity of the distal joints is typical. In Bethlem myopathy, there is proximal muscle weakness and contractures in the elbows and wrists.

Glycosylation Defects

(a) *Dystroglycanopathies*: There are different phenotypes caused by the genes associated with dystroglycanopathies (POMT1, POMT2, FKTN, FKRP, LARGE1, POMGNT1 and ISPD).

(b) *Walker-Warburg Syndrome (WWS)*

Cerebro-ocular dysplasia or Walker-Warburg syndrome (WWS) is a type of CMD in which ocular dysplasia, hydrocephalus, and cerebral malformations can be seen. Corneal anomalies in the eye (microcornea,

microphthalmia, cataract), optic nerve problems (hypoplasia, complete absence), retinal anomalies (retinal detachment, dysplasia), and iris anomalies (hypoplasia, malformation) may be seen. In the brain, structural anomalies (hydrocephalus, brain stem hypoplasia, cerebellar cysts), neuronal migration anomalies (lissencephaly, polymicrogyria), and white matter changes can be seen. The Walker-Warburg phenotype can be caused by mutations in the POMT1, POMT2, FKTN, FKRP, POMGNT1, LARGE1, ISPD, GTDC2, and DAG1 genes.

(c) *Muscle-Eye-Brain (MEB) Disease*

Muscle-eye-brain (MEB) disease has a milder clinical manifestation of disease compared to WWS, typically presenting with hypotonia, progressive myopia, and mental retardation. Retinal degeneration, contractures, and spasticity may be seen in later ages. Neuroimaging findings are similar to those in WWS but milder. The MEB phenotype can be caused by mutations in the POMGNT1, FKRP, POMT2, POMT1, FKTN, and LARGE genes.

Defects of Endoplasmic Reticulum Proteins

(a) *Selenone (SEPN1)-associated myopathy*: This autosomal recessive muscle disease causes spinal stiffness associated with early motor developmental delays, thoracic lordosis, and scoliosis.

Defects of Nuclear Envelope Proteins

(a) *Lamin A/C (LMNA)-associated myopathy*: This autosomal dominant inherited CMD subtype, resulting from heterozygous mutations in the LMNA gene, typically causes neck muscle weakness and loss of head control. In the following periods, hypotonia and weakness of the trunk muscles and proximal and distal weakness in the extremities are seen. Restrictive lung disease resulting in respiratory failure occurs as muscle weakness progresses.

Spinal Muscular Atrophy (SMA)

Spinal muscular atrophy (SMA) is a genetic disease that causes muscle weakness due to motor neuron degeneration in the brain stem and spinal cord. While the incidence of the disease is 1/10,000, the frequency of carriage is 1/40–60. Hypotonia and proximal muscle involvement are the primary findings. Its clinical severity is variable. The most severe form, Type 0, may present in the prenatal period; Type I may present in the neonatal period; Type II and III may present in childhood; and Type IV may present in the adult period. Patients often die before 6 months in Type 0 and before 24 months in Type I.

The disease is inherited in an autosomal recessive manner. The responsible gene is SMN1 and SMN2 gene copy number changes are clinically relevant.

The SMN1 gene produces the survival motor neuron protein, which is critical for lower motor neuron development. Loss of function of the SMN1 gene occurs in 95–98% of the deletions and duplications of the SMN1 gene; 2–5% may also be the result of point mutations of the SMN1 gene. Homozygous deletion/duplications or the coexistence of deletion/duplication in one allele and a point mutation in the other allele are required for the disease to occur. The SMN2 gene is very similar in structure to the SMN1 gene, but it is unstable; thus it can only produce very low amounts of fully functional protein compared to SMN1. It has prognostic importance in the disease as an increase in the SMN2 copy number may alleviate the disease severity by partially compensating for the lack of SMN1 function. However, having two copies of SMN2 is often associated with SMA Type 1. In the case of having 3–4 copies of SMN2, clinical SMA may result in Type 2, Type 3, and Type 4. Apart from this, some single nucleotide changes such as c.859G > C in the SMN2 gene may alleviate the disease severity by causing more fully functional protein production from the SMN2 gene [19].

Although the MLPA method is accepted as the gold standard method for diagnosing SMA, point mutations cannot be detected with this method. In addition, the MLPA method cannot distinguish (2 + 0) silent carriers (5–8%) with two copies in one allele and zero copies in the other allele, from normal individuals [1 + 1]. Prepregnancy carrier screening is recommended by the ACMG and the American College of Obstetricians and Gynecologists (ACOG) since there is a high carrier frequency. However, it should be kept in mind that about 10% of false-negative results can be obtained due to the reasons mentioned above. This information should be emphasized both in carrier screening and genetic counseling in prenatal diagnosis planning [20–22].

Charcot-Marie-Tooth Disease (CMT)

Charcot-Marie-Tooth (CMT) is an inherited disease that progresses with chronic motor and sensory neuropathy. There is distal muscle weakness, sensory loss, suppressed deep tendon reflexes, and a high roof of the foot. The incidence of the disease is 1/3300. There are autosomal dominant, autosomal recessive, and X-linked types.

The first-line testing approach in all patients with suspected CMT should be a deletion/duplication analysis of the PMP22 gene with MLPA. Duplications involving the PMP22 gene are associated with CMT1A and comprise approximately 50% of all CMT cases. Multiple gene analysis with NGS (including at least common genes such as GDAP1,

GJB1, HINT1, MFN2, MPZ, PMP22, SH3CT2, and SORD) is recommended in patients who cannot be diagnosed with MLPA analysis [23].

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8 Radiological Procedures Used in the Differential Diagnosis of Neuromuscular Disorders

Dilek Öncel, Atilla Hikmet Çilengir, and Berna Dirim Mete

Introduction

Neuromuscular diseases are a large and heterogeneous group of diseases, both genetically inherited and acquired. They can be classified into four main groups, depending on the site of the pathology: neuron diseases (neuropathies), peripheral nerve diseases (neuropathies), neuromuscular junction diseases and muscle diseases (myopathies) [1, 2].

Most neuromuscular diseases do not have specific radiological findings. Depending on the stage of involvement (acute, subacute and chronic) in both nerves and muscles, oedema or atrophy may be observed. The main goals of radiological imaging should be to prove neuromuscular disorder involvement noninvasively, to determine the appropriate area for biopsy, to evaluate the response during treatment and follow-up and to establish the differential diagnosis. Therefore, neuromuscular imaging should provide high spatial and contrast resolution, especially for soft tissues [3–5]. In this section, radiological modalities that can be used when diagnosing neuromuscular diseases and relatively common muscular and neuronal pathologies, will be discussed.

Radiological Modalities in Neuromuscular Imaging

The radiological modalities used in neuromuscular imaging are diverse and have developed over the years alongside technological advancements. Among the most basic methods, radiography can only detect bone changes and soft tissue calcifications, and therefore, it has no place in the diagnostic algorithm. However, it can be used in the detection and follow-up of deformities during the course of certain diseases [4].

Ultrasonography

Ultrasonography is one of the more basic imaging modalities used to aid the differential diagnosis of suspected muscle disease. It is cheap, easily accessible and fast which are important advantages. It reveals morphological changes in muscles and can also show fat and connective tissue. It is typically used to detect muscle thickness, echogenicity changes and possibly atrophy, hypertrophy, fatty infiltration and fibrous replacement [3, 4, 6].

Ultrasonography is used to guide biopsy by identifying the most affected muscle groups. It is preferred, especially in children because it does not contain ionising radiation [6, 7]. However, its sensitivity in detecting dystrophic changes may vary significantly according to the disease. Due to insufficient tissue penetration of sound waves, ultrasonography cannot clearly define deep-located muscle tissues. Therefore it is only effective in evaluating superficial muscle groups. Another limitation is that it is an operator-dependent form of imaging [6, 7].

As well as imaging superficial muscles groups, it can be used in the evaluation of superficial and relatively thick peripheral nerves. In daily practice, the sciatic, median and ulnar nerves are structures that can be identified and evaluated by ultrasonography. In the acute and subacute stages of neuromuscular disorders, there is thickening and increased echogenicity of involved nerve tissue, whereas the nerve becomes thinner in the chronic period.

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Computed Tomography

Computed tomography (CT) provides better spatial resolution than ultrasonography, and it is also used to evaluate muscle morphology, identify fatty infiltration and muscle atrophy and determine the affected muscle groups. Unlike ultrasonography, it is not operator dependent. However, the soft tissue contrast is low and inflammatory changes cannot be detected. Therefore, it cannot be used to show muscle oedema preceding muscular degeneration. Significant attenuation differences between bone and soft tissues can create linear beam-hardening artefacts at the interfaces, which may cause problems with density measurements or visual evaluation. Most importantly, this form of imaging is not preferred because it emits ionising radiation [3, 5].

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) has high spatial resolution and is the modality with the highest contrast resolution, especially for soft tissues. Multiplanar imaging is another important advantage. This type of imaging clearly identifies the shape, volume and morphological features of muscles. Since muscle and muscle groups are separated by septa containing fat, they can be identified separately by MRI. Thus, specific involvement patterns can be revealed. Apart from findings such as fatty infiltration and loss of volume due to atrophy in advanced stages of disease, MRI may show muscle oedema due to inflammatory changes, which is the earliest finding. Additionally, it does not contain ionising radiation. For these reasons, it is the preferred method of neuromuscular imaging [3–5, 8].

The basic imaging technique MRI utilises is spin-echo sequences. Depending on the different relaxation times of the tissues, cortical bone (no signal), connective tissue (hypointense), fat (hyperintense) and muscle (intermediate intensity) can be distinguished. The examination must contain T1-weighted and fluid-sensitive sequences. The most preferred pulse sequences for fluid-sensitive imaging are fat-suppressed T2-weighted, proton-density images and short tau inversion recovery (STIR) sequences. Normal muscle tissue has an intermediate signal intensity on T1-weighted images, while it has a low signal intensity on T2-weighted images. T1-weighted images are used to identify fatty infiltration and muscle atrophy. In these images, fatty infiltration is observed as hyperintense areas in the muscle. Fluid-sensitive sequences are used to identify muscle oedema which is observed as a hyperintense signal within the muscle (Fig. 8.1). MRI is significantly superior to CT in identifying both dystrophic changes and oedema. Contrast use may increase the effectiveness of demonstrating the presence and extent of muscle involvement. However, it is not routinely used. MRI is very valuable in terms of identifying early muscle involvement, especially in cases where laboratory data is not particularly indicative [8–10]. MRI also provides high-contrast resolution of the nervous tissue. Neuropathic changes are frequently seen on T2-weighted images. In pathological nerve tissue, there is an increase in thickness and T2 signal intensity.

In some neuropathy cases, enhancement of the nerve tissue can be seen in postcontrast T1-weighted images. This

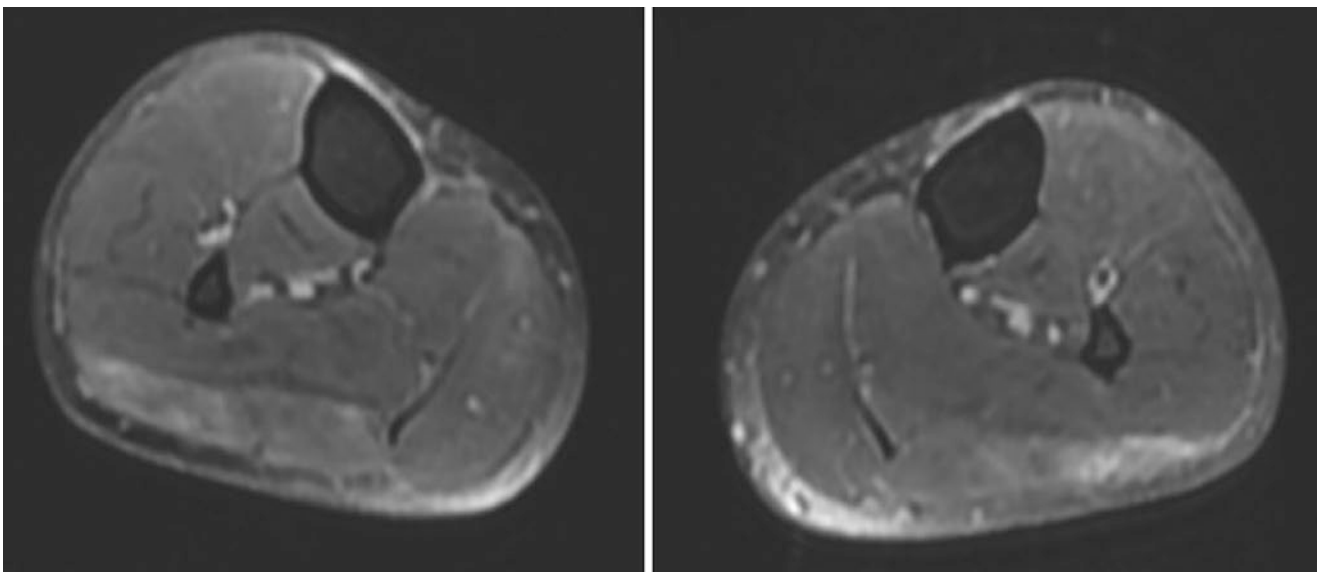

Fig. 8.1 Magnetic resonance imaging of a patient diagnosed with inflammatory myopathy. There is symmetrical oedema (bright areas) in the bilateral gastrocnemius muscles on the axial fat-suppressed T2-weighted image

supports the fact that pathology is occurring in the nerve tissue, but it cannot be used to make a specific diagnosis.

MRI can also be used in the follow-up of disease progression by quantitatively grading fatty infiltration. Grading is made according to the severity, ranging from normal muscle appearance to total fatty infiltration. The involvement of each muscle group can be evaluated and monitored separately using these standardised scales [8–10].

Magnetic resonance (MR) examinations are usually performed on the muscle groups that are thought to be affected and can be more extensively used for imaging the extremities. On the other hand, whole-body MRI can be used to show the involvement of other muscle groups other than the extremities in congenital and metabolic myopathies [11]. MRI can reveal specific patterns of involvement by identifying varying degrees of involvement in certain muscle groups, thereby narrowing the differential diagnosis list, in turn, guiding the search for specific gene defects. It can also be used to screen asymptomatic patients whose relatives have a genetic disease [9, 10, 12].

Apart from standard protocols, advanced MR techniques have been used in recent years, but they are still experimental and not used for routine examinations. T2 relaxation time measurement, fat quantification with the three-point Dixon method, MR spectroscopy and MR perfusion studies can be used to further analyse muscle changes. Contrast-enhanced dynamic T1-weighted series, arterial spin labelling and blood-oxygen-level-dependent imaging (BOLD) can differentiate inflammatory changes from degeneration and dystrophy by identifying increased microvascular perfusion [12, 13].

In summary, the preferred methods for neuromuscular imaging are ultrasonography and MRI. Ultrasonography can show atrophic changes and fatty degeneration, and it is suitable for biopsy guidance, but imaging is limited to the superficial tissues. MRI, on the other hand, can show both early and late findings in neuromuscular tissue and is suitable for detailed morphological evaluation and whole-body imaging.

Radiology in Muscular Pathologies

Muscle diseases represent a large and heterogeneous group, both acquired and genetically inherited. This group includes inflammatory, toxic, metabolic, endocrine-induced myopathies and muscular dystrophies [1, 2].

Different diseases may present with similar symptoms, although clinically, the pattern of involvement is useful in basic classification of diseases. Myopathic changes do not often occur randomly but develop in a specific pattern. This may be characteristic of an underlying protein alteration or even active gene defects. Therefore, determining the specific pattern of muscle involvement can save time and money by limiting the gene groups to be investigated and genetic testing to be performed [2–5]. In addition, another function of muscular imaging is to guide the biopsy by identifying the most affected muscles. This guidance is even more valuable in diseases with heterogeneous involvement [2–5].

Radiology in Muscular Dystrophy

Muscular dystrophies are a group of diseases with extensive variability, including more than 30 genetically inherited diseases, and they are generally characterised by progressive degeneration and muscle weakness [1–3]. The term ‘dystrophy’ describes fibrosis and fat replacement, which are pathologically determinative, although mostly seen in the later stages of disease (Fig. 8.2). Most muscular dystrophies are detected in childhood; however, some forms may remain silent until adolescence or even adulthood, and histopathological changes are mild. Nevertheless, the common feature of all dystrophies is progressive muscle weakness, although the severity may vary.

In the early stages of **Duchenne muscular dystrophy** (DMD), MRI can detect disease involvement in the gastrocnemius muscles. Pseudohypertrophy is observed in the gastrocnemius muscles, which become harder and rubbery as the muscle fibres are replaced by fat and connective tissue. Pseudohypertrophy can be identified by both ultrasonography and MRI and can be differentiated from true muscle hypertrophy [2–4]. In advanced stages, abnormal signal changes in the gluteus maximus and adductor magnus muscles, followed by signal changes in the quadriceps, rectus femoris and biceps femoris muscles, are observed on MRI. The sartorius, gracilis, semitendinosus and semimembranosus muscles are selectively spared. Involvement is observed as fatty infiltration and muscle atrophy, with inflammatory changes being either absent or minimal.

Muscle involvement in **Becker muscular dystrophy** is radiologically similar to DMD. Progression is slower and clinical signs are milder. Complications occur less and later. The ability to walk and survival last longer. It differs from DMD by being less common [3–5, 14].

Facioscapulohumeral muscular dystrophy is the third most common disease among the muscular dystrophies. Fatty infiltration and atrophy are observed in patients, especially in the upper girdle muscles. The most frequently and most intensely affected muscles are the trapezius, teres major and serratus anterior muscles, with the involvement commonly being asymmetrical. Although the involvement is more prominent on the face and upper extremities, it may also affect the lower extremities. The first finding on MRI is an increased T2 signal showing inflammatory-oedematous changes, followed by fatty infiltration. MRI-guided biopsy

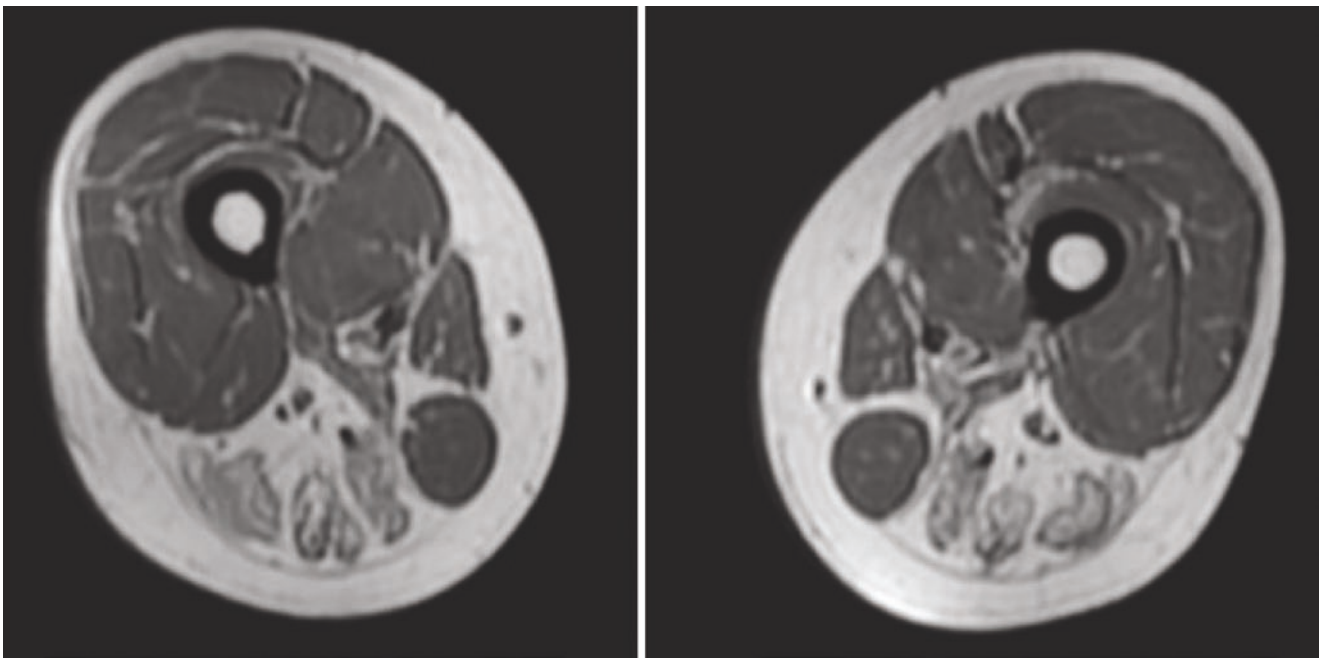

Fig. 8.2 Magnetic resonance image of a patient with muscular dystrophy. The axial T1-weighted image shows symmetrical atrophy and fatty infiltration in the bilateral biceps femoris muscles. While other muscle groups are normal in volume with normal intermediate signal intensity, there is an increase in T1 signal that supports fatty infiltration and a volumetric decrease in the biceps femoris muscles

taken from high-signal areas on the fat-suppressed T2-weighted series, in the early period, improves diagnostic accuracy [3–5, 14].

Limb-Girdle muscular dystrophies are a heterogeneous group with autosomal dominant and recessive inheritance. They usually occur in adolescence or adulthood. The clinical course may also differ. Fatty infiltration is observed, mostly in the gluteus maximus and posterior muscle groups of the calf and leg. However, the gracilis muscle is preserved [3–5, 14].

Congenital muscular dystrophy (CMD) refers to the most common group of autosomal recessive neuromuscular diseases. It is a clinically and genetically heterogeneous group of diseases characterised by early-onset progressive muscle weakness. In addition to muscle involvement, the brain and eyes are frequently affected. Central nervous system findings can be described in detail with cranial MRI examinations. Therefore, cranial MRI examination is indicated in cases with CMD. Muscle involvement varies according to the subgroups of the disease. For example, diffuse involvement of the posterior and lateral thigh muscles is observed in Ullrich CMD cases, while the gracilis, sartorius, adductor magnus and sometimes the rectus femoris muscles are preserved [3–5, 14].

Myotonic dystrophies occur in older children and adults. Decreased grey matter volume, decreased fractional anisotropy secondary to loss of white matter integrity and patchy hyperintense signal changes on T2-weighted series, especially in the frontal lobes and basal ganglia, can be detected in brain MRI examinations. Functional imaging may show increased activity in secondary motor areas. Muscle involvement can be detected as oedema on MRI in the early period [3–5, 14].

Radiology in Inflammatory Myopathies

Inflammatory myopathies are a heterogeneous group of diseases classified according to clinical, histopathological and laboratory findings. Sporadic inclusion body myositis, dermatomyositis, polymyositis, immune-mediated necrotising myopathy and overlap myositis are the most common ones.

In polymyositis, symmetrical diffuse oedema is observed in the pelvic girdle and thigh muscles. The intensity of oedema usually correlates with the severity of the disease [11]. The pattern of muscle involvement in dermatomyositis is like that of polymyositis. However, muscle oedema may be patchy and appear as a honeycomb pattern. Oedema may occur in the muscle fascia and subcutaneous tissue, and subsequent skin involvement may be observed [15]. In addition, soft tissue calcifications distinguish dermatomyositis from polymyositis. Calcifications are hypointense on all MRI sequences. In dermatomyositis the symmetrical involvement of the thigh muscles is predominantly observed in the quad-

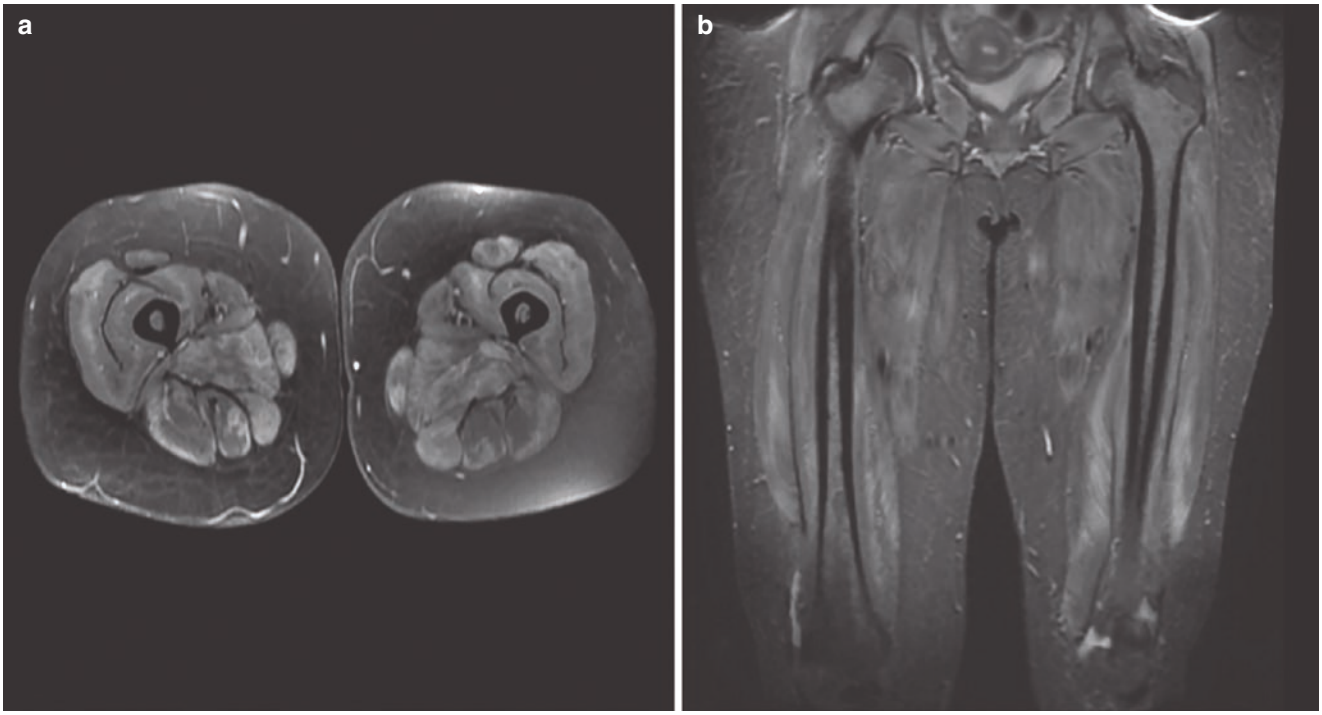

Fig. 8.3 Magnetic resonance imaging of a 41-year-old female patient with scleroderma-myositis overlap syndrome. Oedema in the thigh muscles is observed on axial fat-suppressed T2-weighted (**a**) and coronal plane fat-suppressed T2-weighted (**b**) images. Note that the fibrillary pattern of the muscles is still visible

riceps; however it is more prominent in the adductor muscles in polymyositis [11].

There is generalised muscle oedema, muscle atrophy and fatty infiltration in immune-mediated necrotising myopathy [16]. Compared with other inflammatory myopathies, muscle oedema is more common, less symmetrical and less likely to affect the anterior compartment muscles of the thigh [11].

Sporadic inclusion body myositis frequently affects the medial gastrocnemius, vastus lateralis and flexor digitorum profundus muscles [11, 17, 18]. Less involvement of the rectus femoris in the quadriceps muscle and involvement of the sartorius muscle is important when differentiating it from other myopathies [19]. Unlike other inflammatory myopathies, oedema is not evident on MRI. Fatty infiltration and atrophy are observed in the muscles on T1-weighted images.

The term 'overlap myositis' defines the occurrence of myositis with other connective tissue diseases (Fig. 8.3). Anti-synthetase syndrome is the most common form and is characterised by myositis, interstitial lung disease and joint pathologies [11]. In the thigh muscles, the posterior compartment is most commonly affected.

The radiological appearance of toxic and drug-induced myopathies is nonspecific, and muscle oedema, fascia oedema, muscle atrophy and fatty infiltration are observed.

Radiology in Neuropathies

The radiological findings of neuropathies vary according to the involvement of the central and peripheral nervous systems. Central nervous system pathologies will not be discussed as they are not within the scope of this chapter. Peripheral neuropathies can be evaluated radiologically by ultrasonography and MRI. Although not routinely used in clinical practice, advanced MRI techniques can show nerve tissue involvement more precisely. These include diffusion-weighted imaging, diffusion tensor imaging and MR neurography. MR neurography can be simply defined as a combination of heavy T2 weighting, diffusion weighting and strong fat suppression [20].

A normal peripheral nerve is characterised by a round-shaped, hyperechoic honeycombing-like appearance, formed by fascicles on a hypoechoic background in the axial section ultrasound image. While peripheral nerves are more echogenic than muscles, they are darker than tendons [21]. On MRI, peripheral nerves are isointense with muscle on T1-weighted images and slightly more hyperintense than muscle on T2-weighted images.

Peripheral neuropathy may develop secondary to entrapment, trauma, infection, inflammation or tumours. Regardless of the aetiology, the nerve thickens in the early period in the presence of neuropathy. Its echogenicity decreases in ultrasound, and its signal increases on T2-weighted MRI [22].

The main purpose of imaging in entrapment is to reveal the cause of the compression. Various anatomical structures, variations, repetitive trauma and tumours can cause nerve compression. Entrapment of the ulnar nerve at the level of the cubital tunnel is frequently encountered in clinical practice (Fig. 8.4).

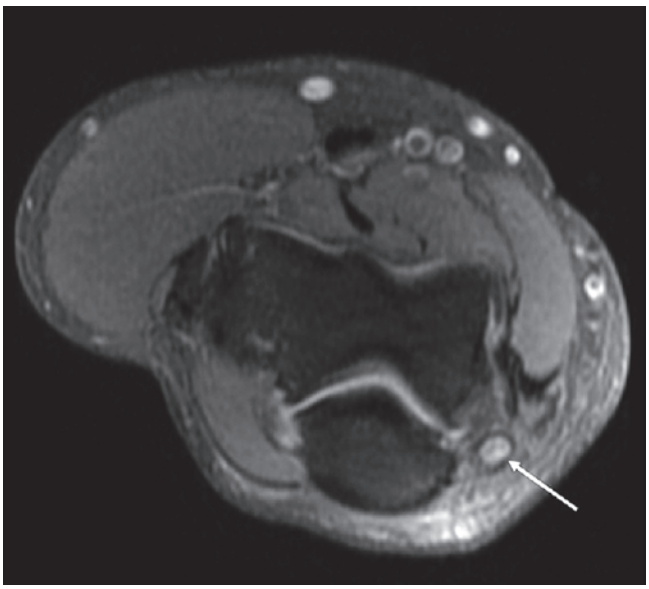

Fig. 8.4 Axial fat-suppressed proton density magnetic resonance imaging of the elbow joint. In the cubital tunnel, the ulnar nerve (arrow) is thickened and has an increased T2 signal. These findings correspond with ulnar nerve entrapment

Inflammatory neuropathies are a rare group of diseases. Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), Parsonage-Turner syndrome, multifocal motor neuropathy and multifocal-acquired demyelinating sensory and motor neuropathy are the most well-known neuropathies of this group [21]. In CIDP, there is symmetrical thickening of the brachial or lumbosacral plexus nerve roots and an increased T2 signal (Fig. 8.5). Parsonage-Turner syndrome is idiopathic brachial plexus neuritis. Multiple nerves may be involved, most notably the long thoracic, suprascapular and axillary nerves. Although the affected nerve is not always visible, there is a thickening of the nerve and an increased T2-weighted signal. Commonly, changes related to denervation are observed in the muscle innervated by the nerve, in accordance with the affected stage [22].

MRI findings in the muscle after denervation vary in the early and late stages. While oedema of the muscle is the prominent finding in the acute phase (usually observed within the first 4 days), atrophy and fatty infiltration develop in the chronic phase (Fig. 8.6) [17]. Diseases such as Parsonage-Turner syndrome, amyotrophic lateral sclerosis and acute motor axonal neuropathy may cause these MRI findings as a result of denervation (Fig. 8.7).

The most well-known of the hereditary neuropathies is Charcot-Marie-Tooth disease. On MRI, bilateral symmetrical thickening and increased T2 signal are observed in the affected plexus and peripheral nerves. In the chronic period, fascicular atrophy develops [21].

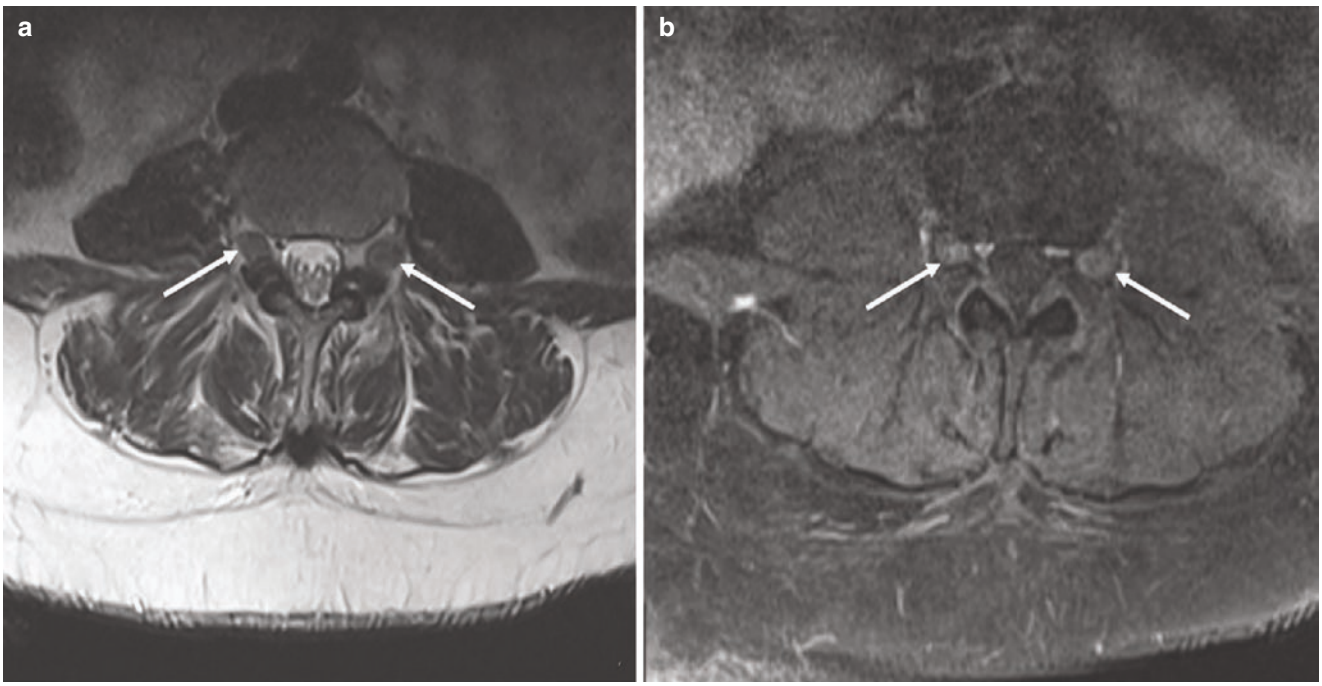

Fig. 8.5 Magnetic resonance imaging of the lumbar spine of a patient with chronic inflammatory demyelinating polyradiculoneuropathy. Axial T2-weighted imaging (**a**) shows bilateral lumbar nerve root thickening (arrows). There is a slight enhancement of the thickened nerve roots (arrows) in the axial post-contrast T1-weighted image (**b**). Post-contrast enhancement of the nerve root is a finding that supports inflammation

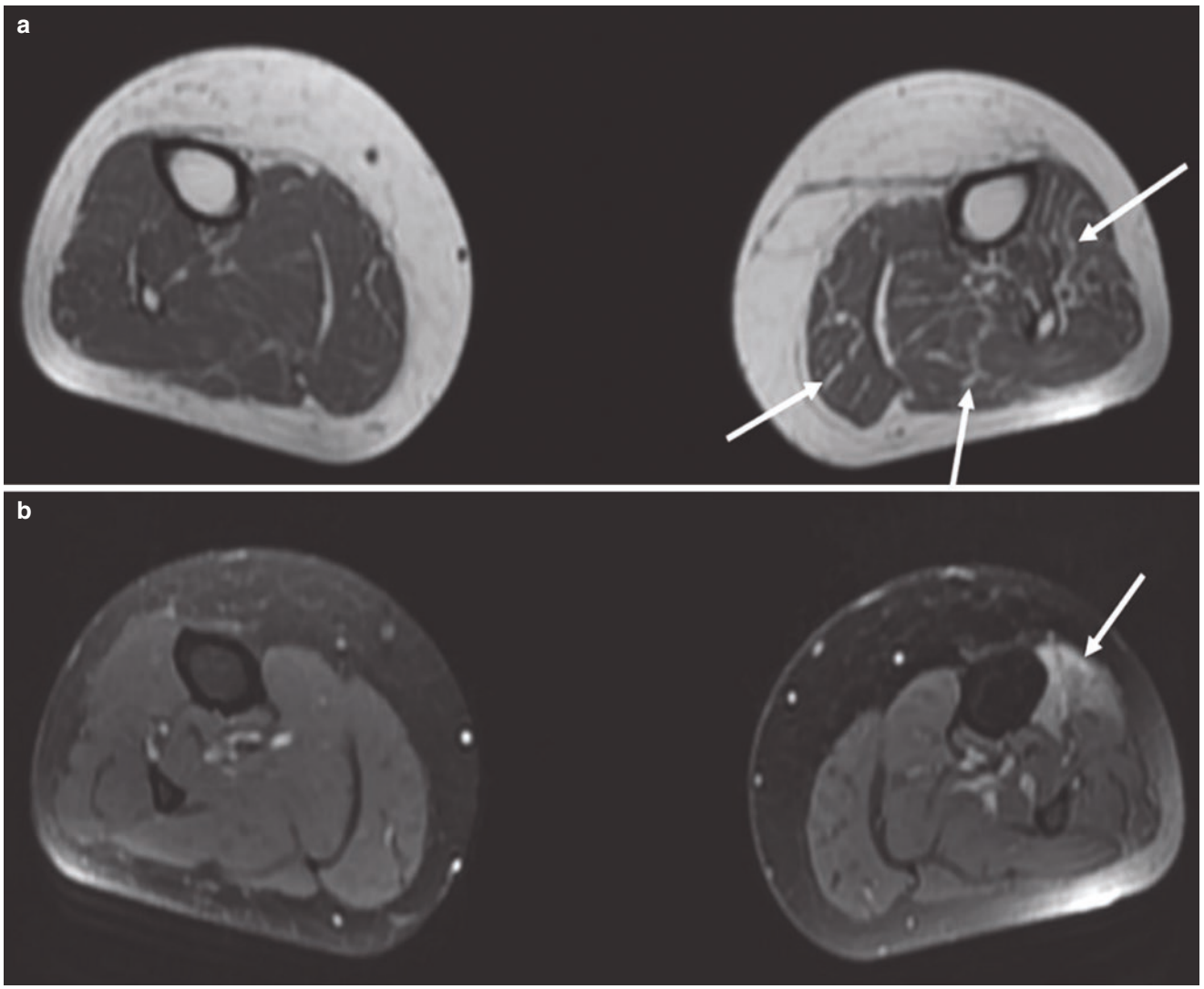

Fig. 8.6 Magnetic resonance imaging of denervation changes in the left lower extremity caused by nerve trauma. On the axial T1-weighted image (**a**), atrophy and fatty infiltration (arrows in **a**) of the muscles of the left lower extremity can be seen more clearly compared to the healthy right extremity. On the axial fat-suppressed T2-weighted image, muscle oedema, which is an earlier sign of denervation, is observed (arrow in **b**)

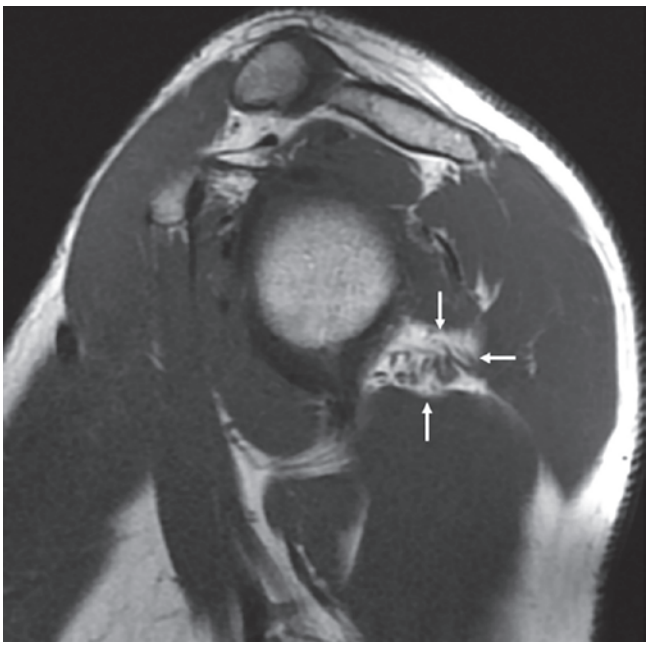

Fig. 8.7 Sagittal T1-weighted magnetic resonance image of a patient diagnosed with Parsonage-Turner syndrome. There is isolated atrophy and fatty infiltration of the teres minor muscle (arrows). Note that the teres minor muscle has a higher signal intensity than the other muscles due to fatty infiltration, and a decrease in the number of muscle fibres is also observed

Conclusion

With the use of the most appropriate modality, radiology plays an important role in the diagnosis and differentiation of neuromuscular diseases, identifying the presence and pattern of involvement. Additionally, radiology provides valuable contribution in biopsy guidance, in the follow-up of disease progression and in the evaluation of treatment response.

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9 Physical Examination, Electrophysiological Procedures and Laboratory Tests Used to Differentially Diagnose Neuromuscular Disorders

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In this section, physical examination, electrophysiological features and abnormalities in laboratory tests, relating to both paediatric and adult neuromuscular diseases, will be explained with patient photographs or illustrations.

Physical Examination in Neuromuscular Disorders

A clinician's expertise in history taking and physical examination is extremely important when trying to reach a diagnosis. Medicine is an art form which requires a thorough knowledge of the underlying anatomy and physiology of the nervous system as well as the neuromuscular system. Clinicians should take the human body as a whole, checking all the organ systems and assembling all information gathered on the pathway to diagnosis. A systematic approach in an orderly manner, careful observation, correlation of symptoms and signs, accurate localisation and interpretation are fundamentals. Patience, adequate time, attention and concentration are necessary. As the experience level increases, a clinician's diagnostic sense will improve. They will be more able to recognise patterns specific to various neuromuscular diseases. Although the introduction of various biochemical/genetic tests and invasive/noninvasive neuroimaging techniques provides valuable additional information, they still cannot replace detailed history taking and localised findings of neurological examination.

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History Taking

Diseases of different organ systems might have similar symptoms. Thorough history taking and detailed physical/neurological examination are essential for reaching a differential diagnosis. The clinical history, duration and temporal development of the present symptoms should be noted in detail. A patient's past medical history, medications taken, eating habits, social and economic standards, occupation, hobbies, toxicant exposure, behaviours, family life and family's medical history should also be questioned. A family tree spanning three or more generations could reveal other family members with similar disease. Complaints could be classified as positive or negative symptoms (Table 9.1). It might be confusing for patients to describe their sensations. For instance, patients could describe limb weakness as numbness or heaviness, but clinicians should guide the patient and help them to express their complaints with different questions. Asking the patient about which routine activities have become challenging, or which behavioural changes have occurred since the beginning of illness would help the patient to describe symptoms more easily. Postural symptoms should not be overlooked. Inability to perform certain tasks due to the primary problems of the skeletal system such as arthrosis and capsulitis should be differentiated from true muscle weakness.

Table 9.1 The most common positive and negative symptoms in neuromuscular disorders

Positive symptoms	Negative symptoms
Twitching	Weakness
Cramps	Numbness
Spasm	Atrophy
Tetany	Slowness
Rigidity	
Tingling	
Pins and needles	
Pain	

G. Diniz (ed.), *Clues for Differential Diagnosis of Neuromuscular Disorders*, https://doi.org/10.1007/978-3-031-33924-0_9

Questioning the temporal development of symptoms yields crucial information. Clinicians should question the onset and development of each symptom, separately. Symptoms could be stable, progressive, episodic or fluctuating which guides the clinician when diagnosing certain disorders. Fluctuating weakness is the prominent feature of neuromuscular junction (NMJ) disorders. Typically, patients with myasthenia gravis have worsened symptoms after exercise or later in the day which partially improve with rest. Triggering or mitigating factors such as hunger, restlessness, physical exercise, certain foods, beverages, medications, supplements, etc. should also be ascertained.

Physical Examination

A systematic physical evaluation is indispensable. Physical examination begins with inspection. Watching the patient walking into the examining room, dressing/undressing, sitting/rising from a chair, their facial expressions, posture, attention, motive, mood, attitude, reaction to questions and interest in objects in the room reveals a lot of information not only about their nervous system but also their general wellbeing.

General inspection should include inspection of the skin and musculoskeletal system. Clinicians should look for coloured lesions such as birthmarks (café au lait spots), hyperpigmentation/hypopigmentation, hypertrichosis, rashes, macular or papular lesions, changes in perspiration and scars.

Posture abnormalities including scoliosis, lordosis, kyphosis, atrophy/hypertrophy in muscles, asymmetry between extremities, pes cavus, pectus carinatum/excavatum, contractures in joints and any deformities should be noted. Then, a general systemic examination including pulmonary, cardiovascular and abdominal systems should be performed.

Neuromuscular Examination

Neurological examination should begin with a mental examination, as cognitive decline or mental retardation can be a major symptom in various neuromuscular diseases including myotonic dystrophy, mitochondrial diseases, amyotrophic lateral sclerosis, frontotemporal dementia and so on. The cognitive capacity of the patient will be important regarding their level of cooperation. Next, the cranial nerves should be tested in a strict order including a fundus examination for cataracts, macula lesions or any retinal deposits as retinitis pigmentosa might be a concomitant sign. Involvement of cranial and neck muscles is quite common and pathognomonic for certain neuromuscular diseases (e.g. ocular myasthenia gravis, mitochondrial dystrophies, oculopharyngeal muscular dystrophy, etc.).

Weakness

Weakness is the most prominent symptom of neuromuscular disease. It is not usually possible to test muscles individually. Instead, muscle groups are tested with manual muscle tests. The British Medical Research Council grading is widely accepted when grading motor dysfunction (Table 9.2) [1]. Quantitative analysis of muscle strength using a dynamometer is usually limited to clinical trials.

Certain diseases have typical distribution patterns. For instance, the extraocular and pharyngeal muscles are involved in oculopharyngeal muscular dystrophy, and the facial and shoulder girdle muscles are involved in facioscapulohumeral muscular dystrophy (Fig. 9.1). A systematic motor system examination of all muscle groups would help to identify subtle weakness in muscles [2].

Patients often have trouble in performing tasks with affected muscles. Individuals with proximal weakness in the upper extremities would have trouble in combing/shampooing hair and reaching for the top shelves. Distal motor involvement in the upper limbs would cause difficulty in fine motor activities such as buttoning/unbuttoning, writing, gripping or using equipment. However, patients with distal weakness in the lower extremities would hit their feet on stones, carpets, sills, small elevations or stairs causing them to fall. Proximal motor involvement in the lower limbs would complicate rising from a chair and stabilising the knee whilst standing/walking. Typically, children with pelvic girdle weakness would rise and stand up from the floor through a series of climbing efforts, known as Gowers's sign (Fig. 9.2) [3]. Positive Gowers sign indicates marked proximal muscle weakness. These patients with proximal lower extremity involvement cannot stand up in a single movement from the sitting position. First, they take to the prone position and rise by pushing off the floor with their hands and feet. Then they put their hands on their legs and stand up using what appears to be a climbing motion [3].

Neuromuscular examination requires patient cooperation. Clinicians should motivate patients to exert full effort and uphold interest throughout the examination.

Table 9.2 Modified Medical Research Council (MRC) scoring

5	Normal muscle strength
5−	Subtle weakness
4+	Movement against resistance, slight weakness
4	Movement against resistance, moderate weakness
4−	Movement against resistance, severe weakness
3	Movement against gravity but not against resistance
2	Movement with gravity eliminated
1	Minor contraction, muscle twitching
0	No contraction

Modified from Paternostro-Sluga et al. Adapted from [1]

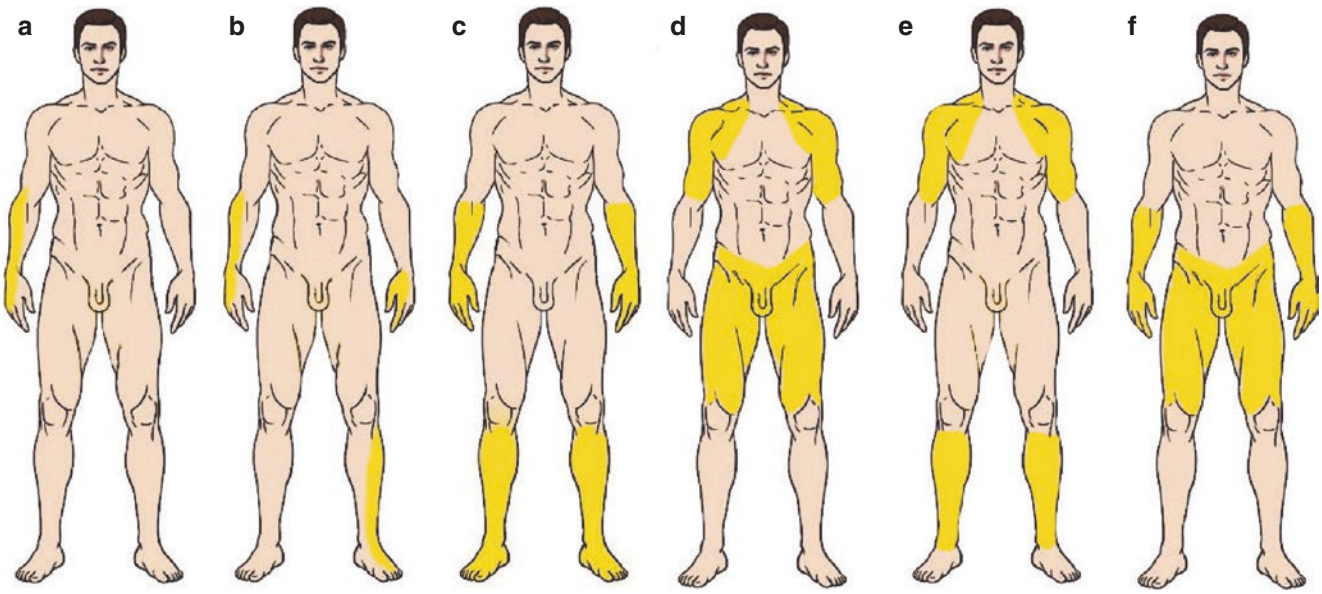

Fig. 9.1 Involvement patterns in neuromuscular diseases. (**a**) Mononeuropathy, (**b**) mononeuritis multiplex pattern, (**c**) distal symmetric pattern, (**d**) limb-girdle pattern: proximal muscles in both upper and lower limbs, (**e**) scapuloperoneal pattern, (**f**) distal upper and proximal lower limb pattern

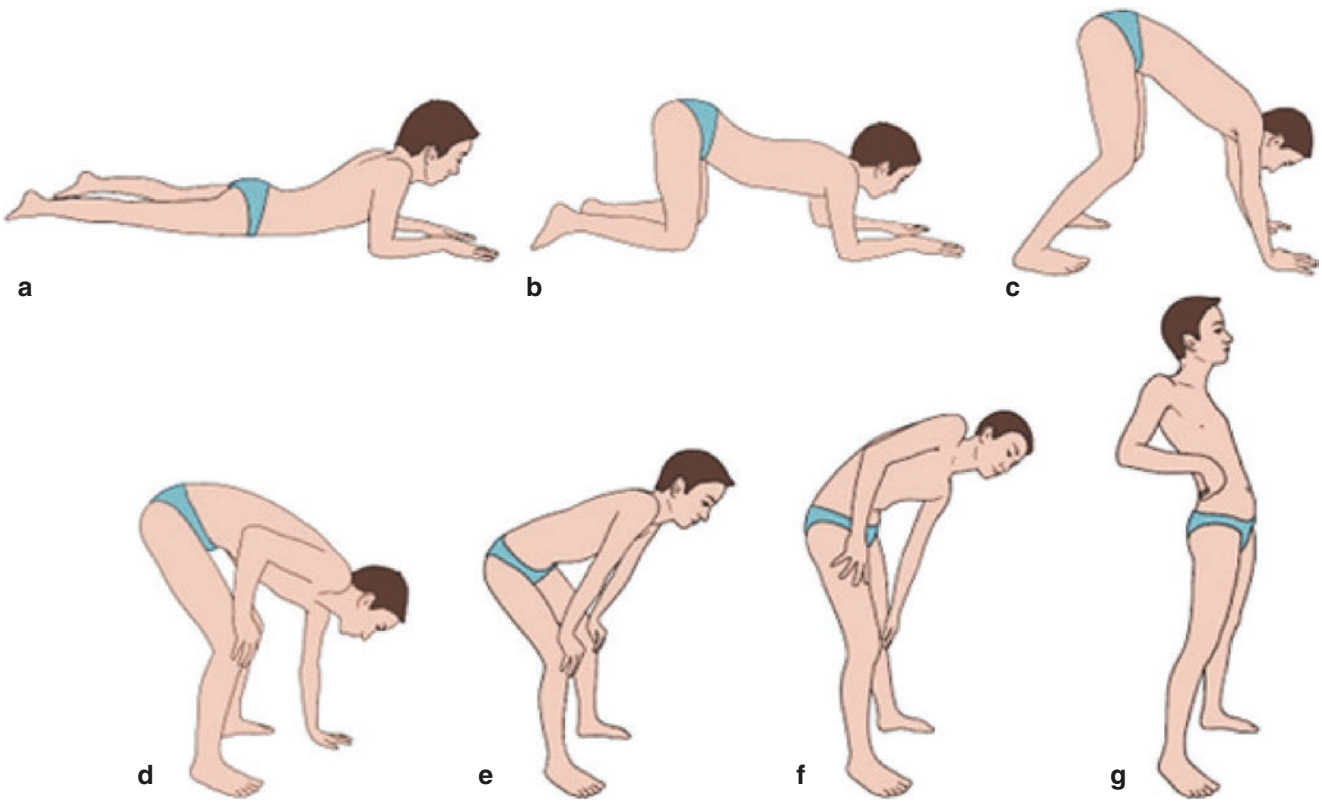

Fig. 9.2 Positive Gowers sign: When a child is asked to stand from a sitting position, they cannot easily stand up. They first roll over into a prone position (**a**), rise by pushing onto their hands and feet (**b**, **c**) and then climb with the help of their hands on their legs to a standing position (**d**–**g**)

Infants or mentally retarded children may be examined whilst reaching/gripping a colourful or noisy object or a toy. Muscle tone during resting state or during voluntary/involuntary movements would give hints to the clinician. Conditions causing muscle weakness or fatigue, such as generalised weakness due to a systemic/infectious/malignant disease, local inflammation, conversion disorder or simulation, should be differentiated from true neuromuscular disease. Clinicians should test the energy and effort of patients during the examination and recognise the early release which usually suggests other conditions mentioned above.

Myalgia and Cramps

Myalgia and painful muscle cramps are frequent in neuromuscular disease. Patients with neurogenic diseases and inflammatory myositis may severely suffer from myalgia. Abdominal girdle style pain might cause discomfort in Guillain-Barré syndrome. Fibromyalgia, polymyalgia rheumatica, spinal canal stenosis and radiculopathies can also cause significant muscle pain. Myalgia with exercise is a typical symptom of metabolic or mitochondrial myopathies. Painful muscle cramp is quite common in neurogenic diseases, metabolic or mitochondrial myopathies. Hyponatremia, hypothyroidism, hypomagnesemia, hypocalcaemia, uraemia, dehydration, low vitamin B12 and folate levels can precipitate these symptoms. Additionally, ischaemia-induced pain and local compartment syndrome should not be overlooked.

Cardiac Involvement

Cardiomyopathy and arrhythmia are common manifestations of neuromuscular disorders in both children and adults [4]. Periodic screening for cardiac involvement should be a routine practice during clinical evaluation. Table 9.3 summarises the most frequent neuromuscular disorders with cardiac involvement.

Respiratory Involvement

Respiratory muscles are frequently involved in neuromuscular disease (Table 9.4) [5]. Respiratory muscle weakness, which could either be neurogenic or myogenic, impairs sleep quality and exercise capacity significantly. Decreased pulmonary ventilation and ineffective coughing predispose patients to lower respiratory tract infections, frequent hospitalisation and premature death.

Table 9.3 Neuromuscular diseases with cardiac involvement

Cardiac involvement	Arrythmia	Cardiomyopathy
Duchenne muscular dystrophy	+/Rare	Dilated
Becker muscular dystrophy	+/Rare	Dilated
Congenital muscular dystrophy	–	Dilated
Limb girdle muscular dystrophy	–/Rare	Dilated
Emery-Dreifuss muscular	+	Rare
dystrophy, X-linked	+	Dilated
Emery-Dreifuss MD, autosomal	+	+/Hypertrophic
dominant	+	+
Myotonic dystrophy type 1	+	Hypertrophic
Mitochondrial myopathies	+	+
Amyloidosis	+	
Friedreich ataxia		
Pompe disease		

Table 9.4 Neuromuscular diseases with respiratory system involvement

Respiratory involvement
Acute onset
Guillain-Barré syndrome
Myasthenia gravis
Chronic failure
Amyotrophic lateral sclerosis
Spinal muscular atrophies
Classic infantile Pompe disease
Duchenne muscular dystrophy
Becker muscular dystrophy
Congenital muscular dystrophy
Limb-girdle muscular dystrophies
Emery-Dreifuss muscular dystrophy
Myotonic dystrophy type 1
Mitochondrial myopathies
Myofibrillar myopathies
Charcot-Marie-Tooth disease
Late-onset Pompe disease
Glycogenosis type V (McArdle)

Extraocular Muscle Involvement

Blepharoptosis (eyelid ptosis) with or without ophthalmoparesis (weakness in extraocular muscles) may be a feature of certain neuromuscular diseases (Table 9.5). It might be a presenting symptom or the only symptom in the patient. Thus, every patient with extraocular muscle weakness or blepharoptosis should be examined systematically for an underlying neuromuscular disease.

Bulbar Weakness

Bulbar weakness causes nasal speech, difficulty swallowing, frequent airway aspiration and increased incidence of both lower respiratory tract infections and hospitalisation. Bulbar function should be checked in all routine clinical visits. Table 9.6 lists neuromuscular diseases that can often cause bulbar weakness during their course.

Cataract

Early-onset cataracts may also develop during neuromuscular disease (Table 9.7). Either congenital or acquired in infancy, childhood, adolescence or young adults, it should be recognised early to preserve vision.

Table 9.5 Neuromuscular diseases with extraocular muscle involvement

Extraocular muscle involvement (Ophthalmoplegia/blepharoptosis)
Acute/subacute onset
Myasthenia gravis
Lambert-Eaton myasthenic syndrome
Guillain-Barré syndrome-Miller Fisher variant
Chronic
Oculopharyngeal muscular dystrophy
Mitochondrial myopathies
Myotonic dystrophy
Congenital muscular dystrophy

Table 9.6 Neuromuscular diseases with bulbar weakness

Bulbar weakness
Myasthenia gravis
Oculopharyngeal muscular dystrophy
Myotonic dystrophy-1
Inclusion body myositis
Focal myopathy
Motor neuron disorders (amyotrophic lateral sclerosis, bulbar-spinal muscular atrophy, etc.)
Cranial neuropathies

Table 9.7 Neuromuscular diseases with cataract involvement

Cataract
Myotonic dystrophy
Walker-Warburg syndrome
Congenital muscular dystrophy
Mitochondrial myopathies
Centronuclear myopathy
Myofibrillar myopathy
Limb-girdle muscular dystrophy
Encephalopathy with necrotising myopathy
Motor neuronopathy with cataracts

Winged Scapula

Weakness in shoulder girdle muscles leads to scapular winging (scapula alata) (Fig. 9.3). The scapular bone is mainly stabilised by the serratus anterior, trapezius and rhomboid muscles, innervated by the long thoracic, spinal accessory, and dorsal scapular nerves, respectively. Isolated nerve damage, plexopathy, myopathy, motor neuron disease or myopathies may result in scapular winging (Table 9.8).

Muscle Wasting or Atrophy

Muscle wasting is an early feature in neurogenic disorders, whereas in myopathies it is associated with the degree of muscle fibre degeneration. Anatomical involvement is specific in some diseases. For instance, diabetic amyotrophy and inclusion body myositis cause significant atrophy of the quadriceps femoris muscle. Atrophy secondary to prolonged immobilisation (disuse atrophy), malnutrition and sarcopenia should be differentiated from muscle wasting due to neuromuscular disease.

On the contrary, in patients with myotonia congenita, a neuromuscular disease with increased spontaneous muscle fibre activity, generalised muscle hypertrophy without weakness may be seen. Characteristically, *pseudohypertrophy* of muscles occurs in dystrophinopathies (Duchenne muscular dystrophy, Becker muscular dystrophy) due to the replacement of fat and connective tissue instead of muscle tissue.

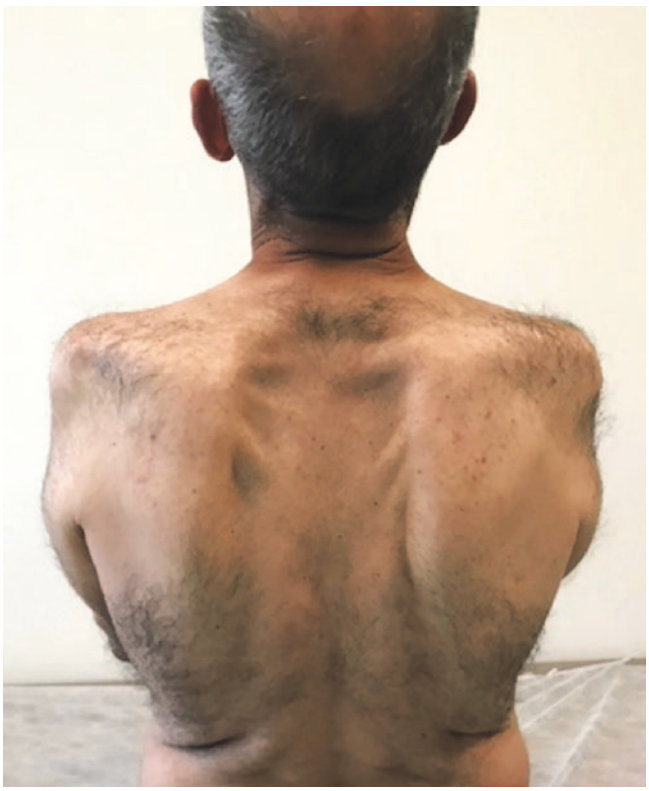

Fig. 9.3 Bilateral scapular winging in a 67-year-old male with amyotrophic lateral sclerosis

Table 9.8 Neuromuscular diseases with scapular winging

Scapular winging
Facioscapulohumeral dystrophy
Limb-girdle muscular dystrophy
Emery-Dreifuss muscular dystrophy
Scapuloperoneal syndromes
Inclusion body myopathy + Paget's disease
Desmin myopathy
Focal muscle weakness
Motor neuron disease

Involuntary Muscle Movements

Involuntary muscle movements might indicate specific neuromuscular diseases.

Fasciculation is random, spontaneous muscle twitching, as an accompanying symptom, which might indicate disease of motor neurons or peripheral nerve fibre degeneration (less likely).

Myokymia is continuous, rhythmic muscle contractions which might indicate post-radiation injury of peripheral nerves.

Myoclonus has a higher intensity and can move joints.

Myotonia is the inability of muscles to relax quickly after a voluntary contraction. It can be painful and may indicate non-dystrophic myopathies such as paramyotonia congenita, myotonia congenita, potassium induced myotonia or myotonic dystrophy (with additional phenomenology). It should be noted that myotonia improves with repeated muscle activity/exercise in myotonia congenita, whereas it worsens in paramyotonia congenita. Patients with paramyotonia congenita also experience worse myotonia when cold and so might describe increased weakness with eating ice cream or swimming in cold water. Electrophysiological evaluation of muscles using needle electromyography reveals these patterns and provides additional information.

Reflex Testing

Deep tendon reflexes measure motor and large fibre sensory nerves. Reflex abnormality in isolated small fibre neuropathy is not common. Neurogenic diseases cause diminished deep tendon reflexes together with muscle weakness and atrophy. In combined lesions of both upper and lower motor neurons (e.g. amyotrophic lateral sclerosis), reflexes are brisk or exaggerated alongside muscle weakness and atrophy. Facilitation in deep tendon reflexes is a characteristic feature of Lambert-Eaton myasthenic syndrome, as reflexes are diminished during rest and become exaggerated with repeated tapping.

Sensory Symptoms

Sensorial symptoms, which are either positive or negative, usually highlight peripheral nerve or dorsal root ganglia involvement in neuromuscular disease [6]. Small fibre neuropathy would primarily affect temperature and pain sensation. However, large fibre involvement would cause loss of pressure, vibration and proprioception sensation (Table 9.9). Dermatomal distribution indicates a radiculopathy. Focal symptoms might suggest plexopathy or isolated disorders of peripheral nerves. Asymmetric proximal painful weakness and atrophy might indicate diabetic amyotrophy. Patchy sensorial involvement can occur in sarcoidosis or lepra, whereas distal symmetric distribution might suggest length-dependent systemic neuropathies.

Table 9.9 Testing methods for sensory modalities and nerve fibre types

Sensory modality	Method	Fibre type
Light touch	Cotton, fine/soft brush	All types
Pain	Pin prick test	Small fibres
Temperature	Hot/cold objects/water	Small fibres
Pressure	Semmes-Weinstein filaments	Small and large fibres
Vibration	Tuning fork	Large fibres
Proprioception	Examiner moves distal joint	Large fibres
Two-point discrimination	Greulich star or caliper	Large fibres

Gait and Posture

Sitting, standing and walking posture should be inspected in detail. Patients should be asked to walk freely in the room. Children should be asked to hop on each foot and run. Proximal muscle weakness in the lower extremities, for example, in limb-girdle muscular dystrophies, causes a waddling gait. Increased lordosis and pelvic tilt will be prominent. Large fibre sensorial involvement will cause a loss of position sense, which eventually yields ataxia and a broad-based gait. Distal weakness characterised by drop foot, due to a loss of dorsiflexion of the feet, will result in a steppage gait. Pes cavus deformity (elevation of the longitudinal plantar arch of the foot) is a typical feature seen in hereditary polyneuropathies.

Contractures may significantly disrupt posture. A contracture is a fixed tightening of muscles, tendons, ligaments or skin. As disease progresses, increased disability, weakness and immobilisation may lead to frozen joints and limb contractures. In most limb-girdle muscular dystrophies, contractures develop late in the course of the disease. However, early-onset contractures are suggestive of other certain neuromuscular diseases (Table 9.10). Early-onset contractures, muscle weakness and hypotonia in infants suggest a neuromuscular disease rather than central nervous system dysfunction. Achilles tendon contractures are an early sign in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). Congenital/intrauterine contractures might be seen in congenital muscular dystrophies. Contractures in the elbow, neck and heels, occurring before weakness, is quite typical of Emery-Dreifuss muscular dystrophy [6].

Table 9.10 Neuromuscular diseases with early contractures

Early-onset contracture
Dystrophinopathies (Achilles tendon contracture in DMD, BMD)
Limb girdle muscular dystrophy 2A
Bethlem myopathy
Emery-Dreifuss muscular dystrophy
Congenital muscular dystrophies
Ullrich congenital muscular dystrophy
Walker-Warburg syndrome

Electrodiagnostic Studies in Neuromuscular Disorders

Electrodiagnostic studies include a wide range of tests and require clinical expertise. In this section, the importance of electrodiagnostic evaluation in differentially diagnosing neuromuscular diseases will be discussed instead of technical details in order to comply with the content of this book.

Nerve conduction studies (NCSs) and detailed needle electromyography (EMG) are valuable tests in the diagnosis of neuromuscular diseases (Fig. 9.4). Clinicians can determine the nature of neuromuscular disease such as motor neuron disease, neuropathy, myopathy or NMJ disorder, based on the pattern, distribution and intensity of abnormalities. EMG should be accepted as an extension of neurological examination, and electromyographers should assess the patient according to their disease history and systemic/neurological examination findings. The level of skill and expertise of the electromyographer is of critical importance. Electrodiagnostic tests are uncomfortable for the patient, so they should be planned wisely. Appropriate and an adequate number of tests with correct interpretation in correlation with symptoms and signs can provide significant information about localisation, distribution, pathophysiology and severity of disease.

Nerve Conduction Studies

NCSs are used to assess electrophysiologic status of motor and sensory nerves. The latency of the action potential propagated in the muscle and calculated nerve conduction velocity reveals the status of myelin sheaths. Amplitude reflects the number of innervated muscle fibres. NCS helps to discriminate between axonal and demyelinating pathologies. Preferably, motor NCSs are completed before sensory NCSs to locate the correct stimulation site and current intensity. This is because beginning with sensory NCSs would oblige the examiner to search the response, unnecessarily, in the case of a peripheral neuropathy which would easily disrupt the sensory NCSs.

Lesions of or distal to dorsal root ganglion cause abnormal sensory NCSs. Normal sensory NCSs in patients with sensory symptoms usually indicate a lesion proximal to the dorsal root ganglia (i.e. nerve roots, spinal cord, brainstem and brain) [7].

In motor fibres, NCSs would mainly reflect the integrity of the axons and myelin sheaths of second order motor neurons, meaning that lesions in lower motor neuron, nerve roots and peripheral neurons would cause abnormal NCSs.

After axonal peripheral nerve lesions, NCSs would be normal until Wallerian degeneration takes place, which occurs within 6–10 days for sensory and 3–5 days for motor fibres, approximately [8]. Subsequently, the action potential amplitudes will decrease depending on the number of diseased fast and myelinated fibres. The conduction velocity will also decrease slightly, although not as much as would be expected from a demyelinating lesion, with an increase in distal latency.

In hyperacute stages of an axonal loss, NCS findings might be similar to that of a pure demyelinating lesion. In that case, NCS should be repeated in 1 week to see if signs of Wallerian degeneration are present.

In pure demyelinating lesions, marked slowing in nerve conduction velocity with increased latencies will be the major finding in NCSs. In addition, if conduction block occurs, action potential amplitudes will decrease. Detection of conduction block in non-entrapment sites (segmentary demyelination) will point out acquired demyelinating peripheral neuropathies such as Guillain-Barre syndrome or chronic inflammatory demyelinating polyneuropathy. However, hereditary demyelinating neuropathies exhibit uniform demyelination.

On the other hand, in proximal demyelination and conduction block, peripheral NCS might be normal since the segments tested are distal to the lesion site. In that case, late responses will be important for diagnosis. Two late responses, the F response and the H-reflex, are used to examine the integrity of more proximal nerve segments, in which the stimulus travels through the entire nerve from distal to proximal and back down again.

Although normal NCSs are expected in disorders other than peripheral nervous diseases, it should be remembered that compound muscle action potential (CMAP) amplitudes might be low in atrophied muscles (whatever the cause is), NMJ disorders and botulism.

Temperature has significant effects on NCSs. In the cold, neuromuscular transmission enhances, which might be a deceptive factor in NMJ disorders. Nerve conduction velocity will decrease.

When an unexpected test result is obtained, electromyographers should reconsider the diagnosis and differential

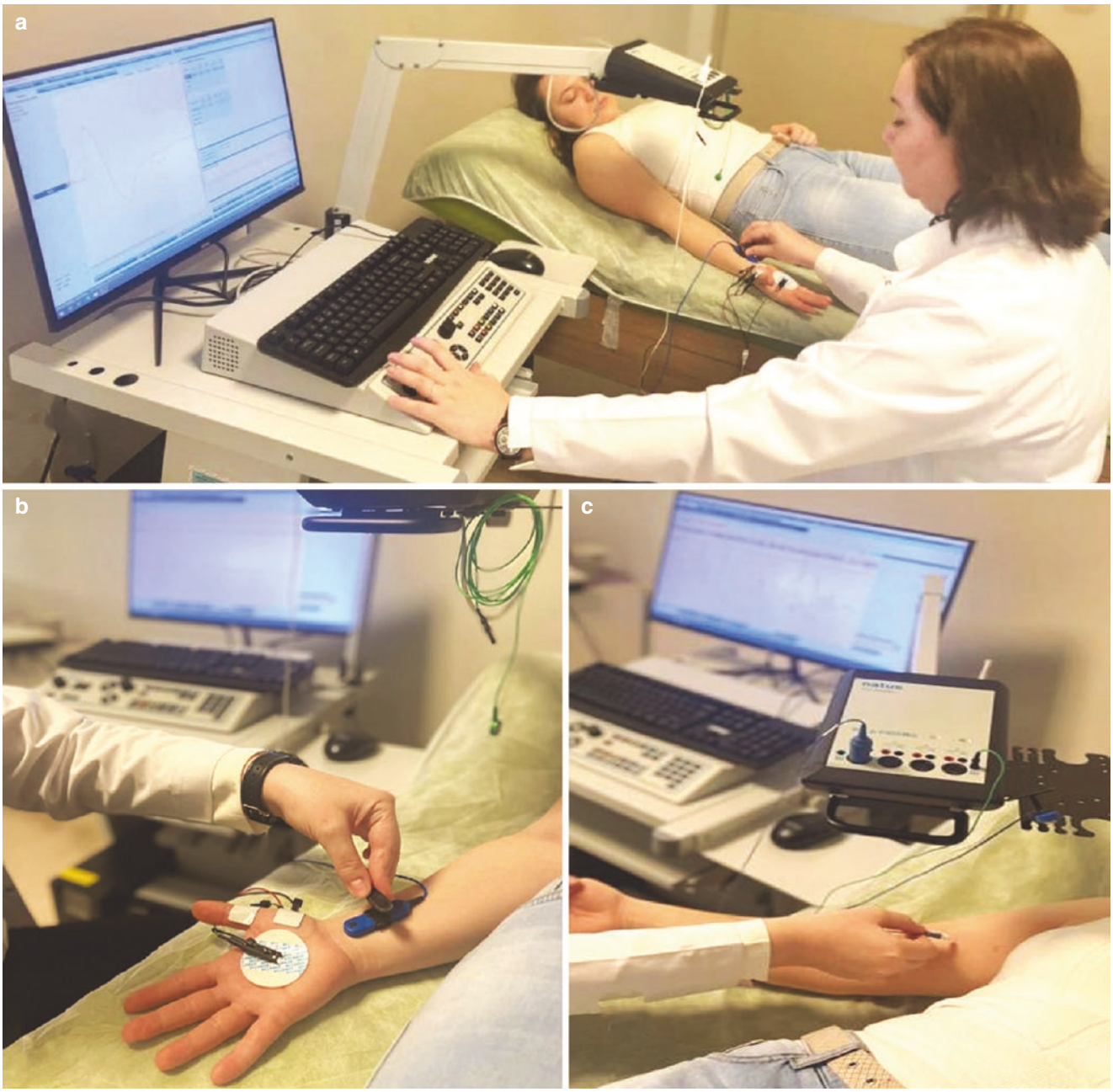

Fig. 9.4 Electrodiagnostic studies are indispensable in diagnosis of the neuromuscular diseases (**a**). Nerve conduction study of the right median motor nerve (**b**). Needle EMG enables to evaluate the electrophysiologic status of the muscles (**c**)

diagnosis as well as re-evaluate the concordance of test results with clinical symptoms and neuromuscular examination findings. Another possibility is that a co-incidental abnormality may be detected in NCSs and/or needle EMG, irrelevant to the patients' symptoms. On the contrary, normal electrophysiological results may be found despite severe neuromuscular symptoms. In that case, the time of testing could be too early for electrophysiological changes to take place, or the sensitivity of the tests is not sufficient for electrodiagnosis. Technical problems should always be kept in mind when clinical and electrodiagnostic correlation fails.

Needle Electromyography

After the NCSs are completed, detailed needle EMG is performed. Electromyographers should decide which muscles to test according to the patient's symptoms and the diseases

in the differential diagnosis. Testing should begin with the weakest muscles, which are most likely to be abnormal, in the case patients may not be able to tolerate extended needle EMG. Insertional and spontaneous activity at rest and motor unit action potentials (MUAPs) during low level voluntary muscle activation (during isometric contraction, if possible) should be carefully assessed in each muscle tested. Normally, muscle should be quiet at rest, except for spontaneous activity in endplate zones. Spontaneous activity is defined as any activity at rest that lasts longer than 3 s. Spontaneous activity includes positive sharp waves, fibrillation potentials, complex repetitive discharges, myotonic discharges, fasciculation potentials and myokymic discharges.

Shape, duration and amplitude of individual MUAPs should be assessed during minimal muscle contraction. The needle can be withdrawn to the subcutaneous tissue and reinserted in four quadrants to assess different regions of the same muscle. After analysis of an adequate number of MUAPs (generally 15–20 different MUAPs), patients should be asked to slowly increase contraction intensity. As the patient increases their force, the firing frequency and the number of MUAPs normally increase (*activation* and *recruitment*, respectively). Then the patient is asked to contract with full power to assess the maximal capacity. During maximal contraction, MUAPs overlap, and an interference pattern occurs where no single MUAP can be distinguished. An incomplete interference pattern may be due to either poor activation or poor recruitment. The range of normal values varies with age and the nerve/muscle being tested. Interpretation of needle EMG requires caution [8]. Table 9.11 summarises MUAP morphology and firing patterns in different pathophysiologies.

Patient cooperation increases the reliability of the data. Inadequate patient cooperation or upper motor neuron disorders (stroke, multiple sclerosis, etc.) may cause decreased activation. Decreased numbers of MUAPs (due to axonal degeneration, conduction block or end-stage myopathy) results in decreased recruitment. On the contrary, in NMJ disorders or myopathies, each motor unit has less muscle fibres, where more motor units fire to generate force even with minimal contraction, called *early-recruitment*.

Abnormal spontaneous activity on needle EMG is a sign of denervation in the muscle, and this will not take place until 2–6 weeks after disease onset, depending on the distance between the muscle tested and the nerve lesion site. In other words, following an axonal peripheral nerve lesion, needle EMG might be normal in the first 2–6 weeks, except for decreased recruitment.

Reinnervation typically takes place after several months or years, resulting in changes in MUAP morphology. These changes include early reinnervation MUAPs with a decreased duration, smaller amplitude and increased phase in the early stages. Then MUAPs with a prolonged duration, increased amplitude and phase appear later in the disease course.

In pure demyelinating neuropathies, axons remain intact and no Wallerian degeneration, denervation or reinnervation occurs. Therefore, needle EMG would be normal except for reduced recruitment in lesions with conduction block.

The presence of abnormal spontaneous activity is a diagnostic sign for certain myopathies. For example, denervation potentials (e.g. positive sharp waves, fibrillation) accompanying myopathic MUAPs would lead to a differential diagnosis of inflammatory or necrotic myopathies (e.g. polymyositis, inclusion body myositis, dystrophies) in the acute stage. In chronic stages of myopathies with denervation potentials, needle EMG might exhibit both myopathic and neuropathic morphologic changes.

Myotonic discharges with myopathic MUAPs are seen in myotonic dystrophies, whereas myotonic discharges with normal MUAP morphology would indicate myotonia congenita or paramyotonia congenita. The effect of cold helps to discriminate these two. In myotonia, myotonic activity increases with cold temperatures, but muscle contraction patterns remain normal. However, in paramyotonia, myotonic discharges disappear with cold temperatures, and muscles enter an electrically silent, rigid contracted phase.

MUAP morphology can be similar and confusing in the chronic stages of both various myopathies and neuropathies. In that case, the recruitment pattern can be diagnostic, where the chronic myopathies would demonstrate early or normal recruitment patterns. However, decreased recruitment is an electrodiagnostic feature of chronic neuropathies.

Table 9.11 Motor unit action potential (MUAP) morphology and firing patterns in different pathophysiologies

	Duration	Amplitude	Phase/turn	Activation	Recruitment
Myopathic-acute	↓	↓	↑↑	N	Early
Myopathic-chronic	↓/↑	↓/↑	↑	N	Early/N
Neuromuscular junction disorders	N	N	N/↑	N	N/↓
Acute axonal neuropathic	N	N	N	N	↓
Reinnervation	↓	↓	↑↑	N	↓↓
Chronic axonal neuropathic	↑	↑	N/↑	N	↓
Demyelinating neuropathy with conduction block	N	N	N	N	↓
Central nervous system disease	N	N	N	↓↓	N

Similarly, reinnervation MUAPs (also called *nascent units*) and myopathic MUAPs may be quite similar in morphology but can be differentiated by looking at recruitment patterns. Decreased recruitment indicates reinnervation MUAPs following an axonal neuropathy. However, normal or early recruitment indicates a primary pathology of the muscle itself (myopathy).

Needle EMG needs to be performed in many different muscles including both proximal and distal muscles, upper and lower limb muscles, paraspinal muscles and cranial nerve innervated muscles, since the distribution of electrophysiologic abnormality is different in each neuromuscular disease. Electromyographers should decide which muscles to test according to the differential diagnosis.

Electrodiagnostic Examination of the Neuromuscular Junction

NMJ disorders might demonstrate slightly similar needle EMG findings but can easily be differentiated with repetitive nerve stimulation (RNS). RNS with supramaximal stimulation should be done in every patient presenting with proximal weakness, ocular symptoms, dysphagia and fluctuating fatigue. RNS should be performed after routine motor NCS to check the nerve is normal. The weakest (symptomatic) muscle should be tested to minimise the risk of false negativity. Extremity muscles are easier to immobilise during testing which is advantageous because fixation lessens the number of motion artefacts. However, facial muscles may also need to be tested if distal muscles fail to demonstrate any decrement. Unfortunately, technical artefacts would be a concern in this case. It must be noted that results should be reproducible.

In myasthenia gravis (typical for post-synaptic NMJ disorders), a decrement >10% in CMAP amplitude is diagnostic in response to a train of stimuli with twitching (slow) frequencies (e.g. 2 Hz, 3 Hz, 5 Hz). In Lambert-Eaton myasthenic syndrome (typical for presynaptic NMJ disorders), CMAP amplitudes might be decreased at rest, but significantly increase (>100% of baseline) in RNS with tetanic (high) frequencies (e.g. 30–50 Hz) or following brief maximal voluntary exercise. Exercise testing can be helpful to identify *postexercise facilitation* (immediately after 10 s of maximal voluntary contraction) and *postexercise exhaustion* (decrement in CMAP after 1, 2, and 3 min after 1 min of maximal voluntary muscle contraction) in patients with impaired NMJ transmission [8, 9].

Needle EMG should be done in every patient with abnormal RNS results. Denervation and/or myotonia might also induce a decremental response in RNS, but this would be different than the typical decrement seen in myasthenia gravis (*U-shaped* decrement), in which the decrease would be maximal in the fourth CMAP and a slight improvement is seen thereafter with an incline in the CMAP amplitudes. Of course, denervation and myotonia would cause many other electrophysiological findings in NCS and/or needle EMG. Electromyographers should take all the normal and abnormal findings in NCS and needle EMG whilst interpreting the results and making an electrodiagnosis.

Botulism also causes severe NMJ dysfunction with a significant decrease in the number of responsive muscle fibres in motor units, which might result in MUAPs with a short duration, smaller amplitude, an increased phase with reduced recruitment and denervation signs such as positive sharp waves and fibrillation potentials.

Single-fibre EMG is yet another specialised test used to examine the innervation characteristics of muscle fibres. It is highly sensitive at identifying abnormalities (e.g. increased jitter, block, etc.). However, its specificity for NMJ disorders is low because any pathology causing innervation defects (e.g. denervation, reinnervation) would yield abnormal results [10]. For instance, motor neuron disorders, neuropathies, reinnervated muscles, neuromuscular blocking agents and botulinum toxin injection would all display abnormal results in single-fibre EMG. The use of botulinum toxin injection for cosmetic reasons should be ascertained before single-fibre EMG examination of facial muscles (e.g. m. frontalis, m. orbicularis oculi) in patients with muscle weakness. A negative test is equally worthy; a diagnosis of myasthenia gravis would be less likely in a case with severe weakness but normal single-fibre EMG.

Cholinesterase inhibitors should be ceased before the examination for at least 4–5 times the half-life to minimise the risk of false negative results, although some do not recommend doing so. Importantly, in critically ill patients or in a life-threatening situation, such as myasthenic crisis, medication should not be stopped.

In conclusion, all electrophysiologic studies should be interpreted according to the patient's symptom history, clinical examination and related differential diagnosis. Limitations of electrodiagnostic tests should be kept in mind. If a definite diagnosis cannot be reached or a clinical-electrophysiologic discordance appears, the electromyographer should re-evaluate the test for any technical artifacts, re-examine the patient and re-consider the differential diagnosis. If there is still doubt, no definite diagnosis should be mentioned in the report, and a repeat test may be suggested later. Overdiagnosis could misdirect the referring clinician and harm the patient.

Laboratory Tests in Neuromuscular Diseases

Molecular and biochemical studies complement each other in establishing the diagnosis of neuromuscular diseases. Biochemical changes detected early in the course of the disease are important for determining the stage of the disease, especially for muscular dystrophy. A diagnosis is usually made in an affected boy at around 3–5 years of age. As the disease progresses, muscle tissue deteriorates and is gradually replaced by fat and connective tissue. As the dystrophic muscle degenerates, its myosin content decreases. As the amount of muscle tissue decreases, the glucose tolerance curve may be slightly abnormal with insufficient glucose excretion in proportion to muscle mass, and additionally, plasma free fatty acids may become elevated. More importantly, noticeable changes occur in creatine and creatinine metabolism [11, 12].

Creatine is largely synthesised in the liver and is delivered to skeletal muscle, where it is converted to creatinine. The amount of creatinine excreted per day does not change, and it is roughly proportional to the total body muscle mass. In muscular dystrophy, plasma and especially urinary creatine levels will increase, and the amount of creatinine in the urine will decrease. However, no abnormalities in creatine and creatinine excretion are seen in female carriers of the disease. Depending on the degeneration of the skeletal muscle, various breakdown products will be released and seen in the urine. For example, 3-methylhistidine, which is a component of both actin and myosin, decreases as muscle breaks down and its urinary excretion increases. Urinary excretion of 3-methylhistidine is an important measure of myofibrillar protein catabolism. Whilst urinary creatinine excretion can be taken as an index of the total muscle mass, 3-methylhistidine excretion is an index of muscle breakdown. As the muscle breaks down, another compound, carnitine, is released and its concentration in dystrophic muscle decreases. Since carnitine is important in the oxidation of fatty acids, reduction in muscle carnitine leads to accumulation of long-chain fatty acid derivatives in muscle tissue [12].

Enzyme Changes in Dystrophic Muscle

It is known that glycolytic enzyme activity is decreased in the muscles of muscular dystrophy patients. The activity of AMP deaminase may be reduced, which occurs in the very early stages of the disease process. Adenylate kinase activity is decreased in the affected muscle, but it does not increase in serum which is also true for AMP deaminase.

Aminotransferases are not significantly reduced in the affected muscle, but their serum levels increase. The isoenzyme patterns of dystrophic muscle more closely resemble those of foetal muscle than adult muscle which was first demonstrated for lactate dehydrogenase (LDH). This enzyme consists of five isoenzymes. The M subunit predominates in adult skeletal muscle and the H subunit in cardiac muscle. LDH-M is clearly dominant, although there are some variations in different skeletal muscles. However, in foetal skeletal muscle, LDH-H predominates, and the isoenzyme pattern is similar to that seen in DMD, even in the preclinical phase. Similar changes in isoenzyme patterns have been reported for creatine kinase, aldolase, isocitrate dehydrogenase, malate dehydrogenase, adenylate kinase and enolase [12–14].

The mechanism of progressive necrosis of muscle fibres in dystrophin-deficient muscular dystrophies is not fully understood.

Serum

Most studies have focused on the serum levels of various muscle enzymes in muscular dystrophy patients. It has been suggested that enzymes can also be released from other organs, especially the liver. The characteristic liver enzymes γ-glutamyl transferase and sorbitol dehydrogenase are within normal levels in muscular dystrophy.

A significant elevation of transaminases is observed in DMD. Before neuromuscular disease is suspected, the possibility of liver disease should be investigated.

Creatine Kinase in Muscular Dystrophies

When diagnosing muscle diseases, especially DMD, measurement of the serum creatine kinase (CK) level is the most valuable screening test. CK is highly specific for muscle diseases. In muscular dystrophies, serum CK levels are 10–100-fold higher than normal reference values.

CK is a dimeric enzyme (82 kDa) that catalyses the reversible phosphorylation of creatine by adenosine triphosphate (ATP). Physiologically, when muscle contracts, ATP is converted to adenosine diphosphate (ADP), and CK catalyses the re-phosphorylation of ADP to ATP using creatine phosphate as the phosphorylation reservoir. CK activity is highest in striated and heart muscle containing 2500 and 550 U/g of protein, respectively [12].

Cytosolic CK enzymes are composed of brain (B) or muscle (M) subunits. These two dimeric forms can exist in three different forms as CK-MM, CK-BB and CK-MB isoenzymes. In skeletal muscle injury or inflammation, particularly the level of total CK-MM increases. Under normal conditions, moderate release of CK into the bloodstream is associated with moderate-intensity physical exercise. In the case of muscle damage, an excess of CK is released into the bloodstream. CK is a common and frequently used blood test parameter in patients with muscular dystrophy. In dystrophinopathies and many muscular dystrophies, CK is a sensitive

biomarker because its high blood levels indicate severe muscle damage [3]. CK activity in the serum of healthy people is almost entirely dependent on CK-MM activity (small amounts of CK-MB may be present) and is the result of physiological cycling of muscle tissue. Exercise, especially if unconventional, and muscle trauma can increase serum CK activity up to tenfold within 24 h of activity [12]. In asymptomatic patients with incidentally detected CK elevation, exercise-induced CK elevation should be excluded. Unnecessary and expensive advanced examinations can be avoided by observing the control CK values after resting.

However, CK is not specific because plasma levels are also elevated in many other forms of muscle damage, and levels are also affected by other factors such as muscle mass, age, ethnicity and muscle activity. When high levels of CK are found in plasma, genetic testing is required to confirm or rule out dystrophinopathies.

Normal blood levels of CK range between 0 and 130 U/L. It is elevated in muscular dystrophy (hyperCKaemia). A diagnosis is made by measuring the blood CK levels, which can be 100 times the normal level. In DMD, serum CK levels are always elevated between 20 and 100 times the upper limit of normal. Elevated creatine phosphokinase levels at birth are a diagnostic indicator of DMD.

HyperCKaemia

HyperCKaemia can be defined as a sustained elevation of plasma CK activity levels. Persistent hyperCKaemia should be confirmed after at least 72 h of inactivity. Persistent elevation is usually determined by confirmation of high CK levels in samples with tests possibly repeated at least 3 weeks apart.

HyperCKaemia can be seen not only in myopathic syndromes but also in amyotrophic lateral sclerosis, hereditary spinal muscular atrophy, post-polio syndrome, bulbospinal muscular atrophy and some neurogenic conditions. Also, some neuromuscular diseases may present with isolated plasma CK elevation in the absence of specific neuromuscular signs and symptoms.

Aldolase

The normal range of serum aldolase is between 0 and 6 U/L. It is elevated in muscular dystrophy but decreases in the later stages of muscular dystrophy. Aldolase is a tetrameric enzyme that catalyses the breakdown of D-fructose-1,6-diphosphate. Aldolase is found in large amounts in all cells, especially in the muscle, liver and brain. Serum aldolase levels may be elevated in primary diseases of skeletal muscle such as progressive muscular dystrophy and polymyositis. Some researchers argue that the increased aldolase activity along with the CK-to-AST ratio is useful in differentiating neuromuscular atrophies from myopathies.

In general, the measurement of aldolase activity in the serum of patients with suspected muscle disease is not easy, and it does not contribute to the diagnosis-making process any more than the measurement of CK. Therefore, aldolase measurement is not recommended in clinical laboratories for muscular dystrophies [12].

Aspartate Transaminase and Alanine Transferase

Muscular dystrophy patients may have higher levels of aspartate transaminase (AST) and/or alanine transferase (ALT), and this may be the first sign of disease. Normal ranges of serum ALT in men and women are 10–40 U/L and 8–35 U/L, respectively. The normal range of serum AST is between 0 and 35 U/L. Both are elevated in muscular dystrophy.

Physiologic levels of serum LDH range between 50 and 150 U/L. It is elevated in muscular dystrophy.

Glucose in the urine, detected in urinalysis, may be associated with muscular dystrophy due to the high incidence of diabetes. Myoglobinuria may also be seen [15].

Elevated serum aldolase, myoglobin, LDH, AST and ALT levels may also be observed in patients with muscular dystrophy. In addition, serum inflammatory biomarkers (erythrocyte sedimentation rate, C-reactive protein and others) may also increase during the active phase of the disease [14].

In addition to CK, which is the most important biomarker in muscular dystrophies, neurofilaments and microRNAs appear to be promising biomarkers.

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Peripheral Neuropathies

10

Figen Tokuçoğlu and Gulden Diniz

Neuropathies are a major branch of neuromuscular disorders (NMDs), and they vary greatly with regard to their aetiology, pathophysiology and clinical presentation. Therefore, different classifications and subclassifications are used to describe different aspects of these diseases. Classifications are useful tools, helping clinicians to reach a diagnosis and decide on treatment. However, diagnosing and identifying the aetiology of neuropathies remains a challenge for clinicians. A multidisciplinary approach is essential at each step from diagnosis to treatment. Motor and sensory disturbances are common symptoms and signs. Of the multiple symptoms, neuropathic pain is the most disturbing, while autonomic involvement may threaten life. As expected, treatment includes aetiological and symptomatic measures. In this chapter we aim to cover the areas of epidemiology, aetiology, signs and symptoms, evaluation, diagnosis and treatment options.

Epidemiology

Peripheral neuropathy (PN) is a frequently seen disorder especially in neurology clinics. The prevalence of peripheral neuropathies is reported to be 2.4% in the population; however, it increases to 8% in the elderly [1]. The worldwide increase of obesity, diabetes and aging has also contributed to the increased occurrence of PN over the years [2, 3]. Diabetic neuropathy (DN) is the most common cause of distal symmetrical sensorimotor polyneuropathy (50%) since it affects almost half of diabetic patients [1, 2]. Idiopathic or cryptogenic PN is the second most common group that is associated with metabolic syndrome and prediabetes [2]. Concerning infections, leprosy is still the leading cause worldwide especially in Southeast Asia [1]. Charcot-Marie-Tooth disease type 1A is the most common genetic polyneuropathy (PNP). In adults carpal tunnel syndrome is the most frequently seen entrapment neuropathy (EN) [1, 5]. The risk factors for developing chronic neuropathies depend on age and socioeconomic status within the studied populations [3]. The terms polyneuropathy, peripheral neuropathy and neuropathy have different meanings. However, they are frequently used in place of each other. Peripheral neuropathy is an inclusive term which encompasses polyneuropathy and any other disorder of the peripheral nervous system such as mononeuropathy, radiculopathy and plexopathy. Plexopathy is a disorder of a network of nerves known as a plexus. It generally develops in the brachial or lumbosacral plexus which sends signals from the spinal cord to the upper or lower extremities. Symptoms of plexopathies include pain, muscle weakness and sensory deficits such as numbness. Radiculopathy can be described as damage to nerve roots in the area where they leave the spine. This condition generally results from disc degeneration, disc herniation or other trauma [1, 4, 5]. Neuropathy is described as the 'damage, disease, or dysfunction of one or more nerves especially of the peripheral nervous system' in the Merriam-Webster medical dictionary [6]. Polyneuropathy is a more specific term meaning generalised involvement of peripheral nerves due to the same pathophysiologic mechanism in a relatively symmetrical distribution, with distal nerves being affected more severely [7]. Deciding a polyneuropathy differential diagnosis should be completed carefully to exclude mononeuropathies, mononeuropathy multiplex and some central nervous system diseases. The term mononeuropathy refers a single nerve injury usually with a local cause such as trauma or a compressive lesion. Mononeuropathy multiplex indicates multiple single-nerve involvement occurring simultaneously or consecutively due to a vasculitic process [7].

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Aetiology and Classification

The aetiology of peripheral neuropathies is diverse and sometimes overlapping. Classification can be based on pathology, aetiology, function, distribution or according to electrophysiological parameters. Discriminating involvement patterns can be very useful in deciding the differential diagnosis of neuropathies (Fig. 10.1). A simple approach that may be used for classifying neuropathies is summarised in Table 10.1. The biopsy findings in some peripheral neuropathies are not familiar to pathologists because nerve biopsy examination is almost never utilised when determining a differential diagnosis of certain disorders such as radiculopathy, plexopathy and mononeuropathies.

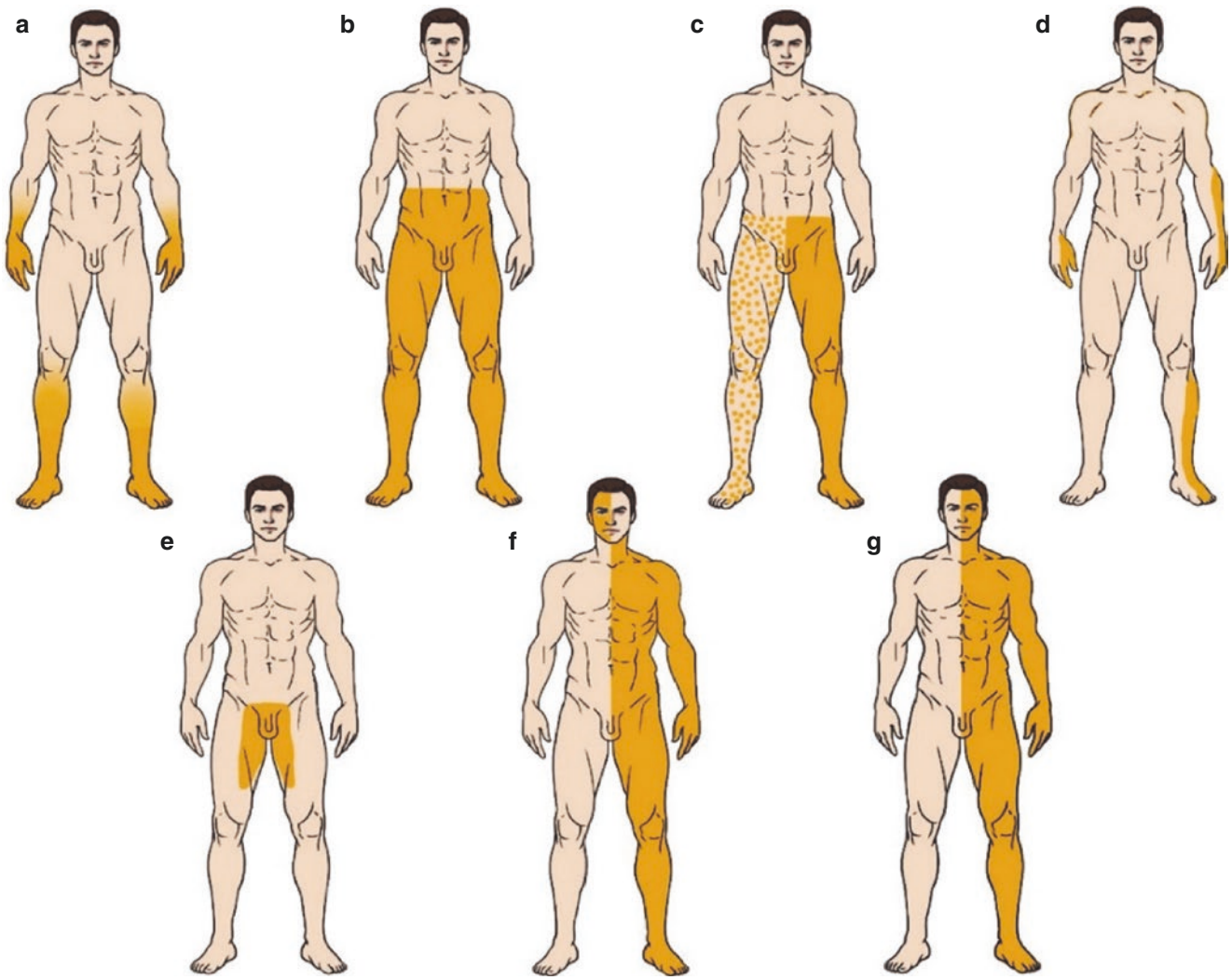

Fig. 10.1 Involvement patterns in different neurological disorders: (**a**) polyneuropathy, (**b**) transverse spinal cord lesion, (**c**) ipsilateral motor defects and contralateral sensory deficits with pain below the level of hemisection of the spinal cord (Brown-Sequard syndrome), (**d**) mononeuropathy multiplex, (**e**) saddle-shaped anaesthesia in compression of the cauda equina nerves, (**f**) cross-sensory defect in brain stem lesion and (**g**) sensory defect in lesions of the thalamus and its surroundings

Table 10.1 A simple classification of peripheral neuropathies

Mononeuropathies of the upper and lower limbs
Plexopathies of the upper and lower limbs
Radiculopathies of the upper and lower limbs
Mononeuritis multiplex (mononeuropathy multiplex)
Polyneuropathies
Hereditary neuropathies
• Charcot-Marie-Tooth
• Hereditary sensory autonomic neuropathies
• Hereditary pressure palsy
• Familial amyloid polyneuropathy
• Giant cell neuropathy
Acquired neuropathies
• Toxic
• Nutritional
• Metabolic
• Infectious
• Paraneoplastic
• Autoimmune

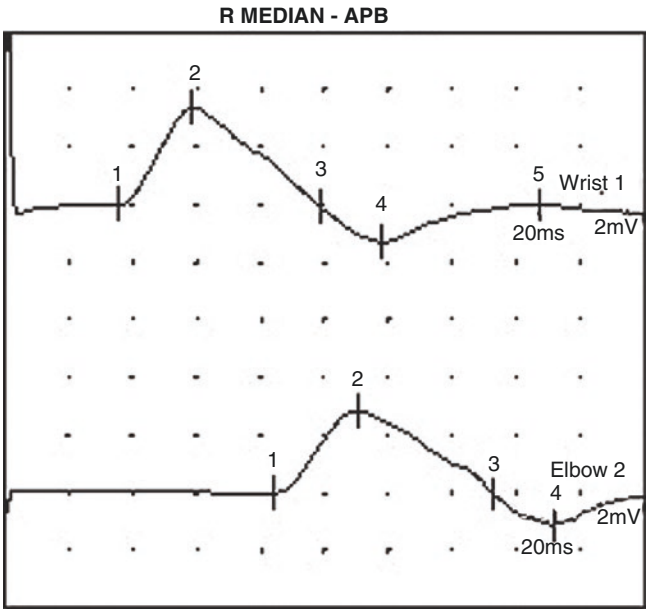

Fig. 10.2 Median nerve compound muscle action potential amplitude is low (3.4 mV), and nerve conduction velocity (51.1 m/s) and distal latency (3.5 ms) are normal, compatible with axonal neuropathy

Mononeuropathies

Mononeuropathies of the Upper Limbs

There a several mononeuropathies of the upper limbs. The most common are briefly discussed in this section.

Median Nerve

Carpal tunnel syndrome (CTS) is the most common neuropathy in adults, and it is more frequent in females than males. The median nerve is compressed while passing through the carpal tunnel, formed by the transverse carpal ligament in the wrist. Initially the symptoms include tingling and hypoaesthesia over the sensory areas of the median nerve with some radiation to the forearm, especially during sleep. In later stages, muscle weakness of the thenar muscles, which are innervated by the median nerve, occurs. CTS is more frequently seen in patients with diabetes and hypothyroidism. A bilateral presence of CTS should be a warning to search for these metabolic conditions [8]. Pronator teres syndrome is entrapment of the median nerve by the pronator muscle or fibrous structures in the forearm. Median neuropathy (Fig. 10.2) in the forearm may be the first sign of mononeuropathy multiplex [9, 10].

Ulnar Nerve

Entrapment of the ulnar nerve at the level of the elbow is the most frequent lesion of the ulnar nerve, and this level is frequently referred to as the cubital tunnel. However not all the lesions at the elbow are in the cubital tunnel. There are three sites: above the elbow, at the ulnar groove and the cubital tunnel. Electrophysiological examination shows the exact localisation where clinical findings are similar for all three sites. *Guyon tunnel syndrome* is entrapment of the ulnar nerve at the level of the wrist. The ulnar dorsal branch of the ulnar nerve leaves the main trunk before entering the tunnel so that it is saved from compression. This feature helps to identify the localization of the lesion [5].

Radial Nerve

Radial nerve entrapments are not as frequent as median and ulnar nerve entrapments. The most frequent lesion occurs due to compression at the spiral groove. Famous ‘Saturday night palsy’ is the result of compression of the radial nerve between the humerus and a solid object. The main clinical finding is wrist drop, meaning inability to extend the wrist and digits. The triceps muscle is saved but brachioradialis muscle involvement usually occurs. Sensory deficit is less prominent. It is usually a benign condition which can rarely progress to axonal degeneration. Fracturing the humerus is another potential cause of radial nerve injury at the proximal level which may require surgical exploration. The posterior interosseous nerve is a pure motor branch of the radial nerve. Lesions distal to the elbow can cause posterior interosseous neuropathy which causes wrist drop, but it spares the brachioradialis muscle [2].

Proximal Neuropathies

There are proximal neuropathies which are not encountered frequently such as suprascapular, axillary and long thoracic nerve neuropathies. The suprascapular nerve is a sensorimotor nerve which innervates the supraspinatus and infraspinatus muscles. Entrapment at the suprascapular notch may cause weakness of innervated muscles and shoulder pain. If

impingement occurs at the spinoglenoid notch, there are only motor symptoms without pain. If it is due to trauma, the axillary nerve may also be involved. Long thoracic neuropathy is important to consider for the differential diagnosis with suspected brachial plexopathy because it arises from the fifth to seventh cervical roots, proximal to the brachial plexus. Damage of the long thoracic nerve results in winging of the scapula (Fig. 9.3). It is noteworthy that scapular wing occurs in other conditions such as accessory neuropathy, dorsal scapular neuropathy, cervical radiculopathy and in some myopathies. Direct trauma, stretching and inflammation may cause long thoracic neuropathy. The axillary nerve is prone to traumatic injuries due to shoulder dislocation or humerus fractures. Clinical features include sensory impairment of a sharply demarcated area over the lateral shoulder with weakness in arm abduction and external rotation. Spinal accessory nerve (a cranial nerve) dysfunction affects the upper extremity because it supplies motor fibres to the trapezius muscle as well as the sternocleidomastoid muscle. Paralysis of the trapezius muscle causes winging of the scapula and a deficit in shoulder elevation. Iatrogenic traumas are common which include radical neck dissection for malignant diseases and biopsy of lymph nodes. External blunt traumas may be a cause of injury too [11].

Mononeuropathies of the Lower Limbs

The lower limbs are innervated via lumbar and sacral roots, spinal nerves, the lumbosacral plexus and peripheral nerves. Major nerves that arise from the lumbar plexus include the femoral nerve, obturator nerve, saphenous nerve and lateral femoral cutaneous nerve. Lower lumbar and upper sacral fibres converge to form the sciatic nerve which has tibial and peroneal components. Inferior and superior gluteal nerves are also distal branches of the lumbosacral plexus which innervate posterolateral hip muscles. Pelvic floor muscles are innervated by the pudendal nerve which only has sacral fibres. Perinea sensation is also carried by the pudendal nerve.

Lower extremity neuropathies occur mostly due to chronic compressive lesions. Acute transection, inflammation, infection, radiation injury and ischaemia are other conditions that affect lower limb nerves less frequently. Detailed history taking and through neurologic examination are a great help when determining a diagnosis and differential diagnosis. Peripheral neurologic symptoms classically involve muscle power, sensation and reflexes. Specifically questioning the time of onset, evolution of symptoms, related events and associated disorders is very important. Pain is the most frequent symptom that brings patients to the doctor. Localisation and radiation of pain should be checked. Identifying sensory abnormalities is important as it helps to localise the lesion. Therefore, the dermatomal and radicular distribution of sensory impairment should be examined [12]. Electrophysiological investigations are usually, but not always, useful for reaching a diagnosis and differential diagnosis. Superimposed disorders, such as dropped foot and suspected radicular involvement in a diabetic patient with lumbar disc herniation, present considerable difficulty for electrophysiologists. Imaging methods are used for locating lesion sites and for defining the nature of the lesions [7, 13].

Peroneal Nerve

A peroneal nerve lesion at the fibular head is the most common entrapment in the lower limbs. The common peroneal nerve passes through a fibro-osseous tunnel at the fibular head. Lesions of adjacent structures may cause compression of the nerve. Prolonged squatting, lying in bed or crossing of the legs may cause both stretching and compression. Dropped foot is the most prominent finding which causes a ‘steppage’ gait [4]. Sensory impairment of the skin over the dorsolateral foot and lateral side of the shin is present, but pain is usually absent. The deep peroneal nerve may be compressed at the level of the ankle where it passes underneath the retinaculum fibres. Motor deficit is restricted to the extensor digitorum brevis muscle which dorsally flexes the toes. Sensory loss occurs over the skin web between the great and second toe [12, 13].

Tibial Nerve

Tibial nerve entrapment at the level of the ankle behind the medial malleolus is called tarsal tunnel syndrome. The tibial nerve is compressed between the bones and flexor retinaculum, while passing underneath tendons of the flexor muscles [13]. Pain over the anterior two thirds of the sole of the foot, which is worse especially during walking, is the most prominent symptom. Sensory disturbances at the same area are reported. Over the retinaculum, there is a positive *Tinel’s sign* which describes a tingling or prickling feeling brought on by the percussion of a damaged nerve. This sign also denotes the regeneration of nerves. Motor signs include weakness of flexion and abduction of the toes. The medial and lateral plantar nerves may be exposed to compression while they pass through the sole of the foot. With this, sensory complaints are more common than motor. Morton’s neuroma is also a painful neuropathy which affects interdigital nerves [12].

Femoral Nerve

A femoral nerve lesion at the level of the inguinal ligament may be caused by lymphadenopathy, haematoma or other space-occupying lesions as well as hip fractures or hip replacement. Pain radiating over the anteromedial side of the thigh, medial shin and arch of foot and weakness of the quadriceps muscle are the reported symptoms. Lateral femo-

ral nerve lesions cause meralgia paresthetica syndrome. Tight belts, tight garments, sitting in the same position for a long time, abdominal obesity and diabetes either alone or in combination may cause sensory symptoms over the lateral aspect of the thigh. Since the lateral femoral cutaneous nerve is purely sensory, there is no motor deficit. If clinical diagnosis is definite with normal neurological examination, other than sensory deficit over the lateral thigh, electrophysiological examination is not necessary. It is difficult to obtain lateral femoral cutaneous nerve sensory nerve action potentials (SNAPs) in overweight people, even on the asymptomatic side.

Sciatic Nerve

The sciatic nerve is a large, deeply localised nerve, and so injury due do external trauma is not frequently seen. However, injuries can occur due to intramuscular injections or compression caused by a deep haematoma, abscess or pelvic mass. Hip dislocation fractures may also damage the sciatic nerve at a proximal site. Piriformis syndrome causes controversies from time to time. Hypertrophic piriformis muscle or a variation of nerve course, due to something like penetrating muscle bulk, may cause symptoms in certain positions. If present, electrophysiological findings are of the sciatic nerve, not the piriformis muscle. At the level of the mid-thigh, femur fractures and vascular lesions may harm the sciatic nerve. Electrophysiological examinations help to determine the site of injury.

Lower extremity nerves can be affected by polyneuropathy or mononeuropathy multiplex and entrapment neuropathies concurrently. Careful neurological and electrophysiological examination is important when deciding a differential diagnosis. When pathological findings are present in one location, homologous nerves should be examined. If necessary, examination should be extended.

Plexopathies

Upper Limb Plexopathies

The brachial plexus is formed by fibres arising from the fifth cervical to first thoracic spinal roots. Fibres are organised as trunks, cords and peripheral nerves which form a mesh. Due to its complex structure, brachial plexus lesions are difficult to localise and differentiate from other disorders. One peripheral nerve may contain fibres from different roots, trunks and cords. Postganglionic sympathetic fibres also join the motor and sensory fibres of the brachial plexus [1]. Different types of pathological processes can affect the brachial plexus such as compression, transaction, ischaemia, inflammation, metabolic abnormalities, neoplastic processes and radiation. Symptoms of brachial plexus lesions depend on the time course. Acute onset symptoms include severe pain over the shoulder that radiates to the upper arm. Chronic cases complain of numbness and increasing weakness of certain muscles of the upper limbs. Trauma, metabolic and inflammatory processes cause acute presentations, while neoplastic involvement or radiation therapy produces a more insidious onset. Electrophysiological examinations are useful for forming a diagnosis and partial differential diagnosis. Imaging studies are also important, especially if neoplastic processes or structural abnormalities are suspected [11, 13, 14].

Traumatic Brachial Plexus Lesions

Traumatic brachial plexus lesions are frequently due to accidents in adults, usually motorcycle accidents and falls, while difficult birth is the main cause in children [11]. Open traumas are associated with lesions of other structures like bone fractures, blood vessel lacerations and haematomas which complicate the diagnosis and management. Root avulsions are caused by forceful stretching of the nerve fibres which results in detachment from the spinal cord. Root avulsion and plexopathy can occur together. A diligent electrophysiological examination is needed in these conditions [13, 14].

Neurogenic Thoracic Outlet

Neurogenic thoracic outlet is less frequent than expected. It usually involves the medial cord and inferior trunk causing thenar atrophy and sensory deficit over the medial side of the hand and forearm. Electrophysiological parameters are compatible with these symptoms showing motor involvement of intrinsic hand muscles innervated by the median nerve with the absence of ulnar and medial antebrachial nerve SNAPs.

Backpack Palsy

Backpack palsy usually presents with unilateral weakness of an arm and/or shoulder. Carrying weight in a backpack or using baby carriers on the back may cause upper trunk injury [14].

Neuralgic Amyotrophy

Neuralgic amyotrophy, also known as *parsonage-Turner syndrome* and *idiopathic brachial plexopathy*, is regarded as an inflammatory illness which can be recurrent. First symptoms include pain over the shoulder and arm which is followed by weakness of the muscles, occurring within a day or 2 weeks. Muscle atrophy appears later in some muscles because brachial plexus involvement is patchy in neuralgic amyotrophy. The long thoracic, suprascapular, musculocutaneous, radial, anterior interosseous and axillary nerves are more frequently affected. In some cases, the homologous limb is also affected simultaneously or on a different occasion. Single-nerve involvement, for example, the anterior interosseous nerve, may be the sole finding which can simu-

late mononeuropathy multiplex. Phrenic nerve involvement occurs in approximately 8% of neuralgic amyotrophy patients [15]. Recovery is slow and can take between 1 and 3 years. However, it may be incomplete in 30% of patients. Biopsy is not performed routinely so verifying histopathological data is not possible.

Hereditary Brachial Plexopathy (Hereditary Neuralgic Amyotrophy)

This is an autosomal dominant disorder caused by a variant of the septin 9 gene on chromosome 17. Hereditary brachial plexopathy patients may have some dysmorphic features like close set eyes, short stature, small face, unusual skin folds and creases on the neck. Four patients with hereditary brachial plexopathy had nerve biopsy during attacks, and in two of them inflammatory changes were seen [14, 16]. Infections, surgical operations, trauma or giving birth may cause exacerbations. Treatment with corticosteroids is reported to improve symptoms, especially pain. During the acute phase, intravenous immunoglobulin (IVIG) treatment may be beneficial [14, 16].

Pancoast Tumours

These may be associated with plexopathy and Horner syndrome. Brachial plexopathy in a patient who had surgery and radiotherapy for malignancy raises questions of malignant invasion and radiation plexopathy. Prominent pain suggests neoplastic invasion, while fasciculations and myokymia in needle EMG favours radiation plexopathy.

Diabetic Brachial Plexus Neuropathy

This is rare and usually occurs with lumbosacral plexopathy which is more severe and draws more attention [14].

Lower Limb Plexopathies

Lumbosacral Plexopathy

The anterior rami of the L1-S4 roots form the lumbar and sacral plexus. As usual for plexus lesions, the symptoms appear asymmetrically. Weakness, pain and sensory disturbances are present in multiple adjacent dermatomes and myotomes. Lumbar plexus lesions affect flexion and adduction of the thigh and/or extension of the knee. Lumbosacral trunk and upper sacral plexus lesions usually involve abduction of the thigh, flexion of the knee and foot movements. Sensory involvement depends on the involved nerves distribution [12, 13].

Diabetic Amyotrophy/Idiopathic Lumbosacral Radiculoplexus Neuropathy

Diabetic amyotrophy, also called *diabetic radiculoplexus neuropathy*, differs from other diabetic neuropathies because the underlying mechanism is highly complex. The nerves and roots are involved in immune, inflammatory and vascular processes. Development of subacute and painful proximal muscle weakness with some degree of autonomic impairment constitute the clinical findings. Electrophysiological examination reveals sensory and motor conduction abnormalities and acute denervation activity in needle EMG. Corticosteroids, IVIG and plasma exchange are effective treatment choices [17, 18]. The only difference between *idiopathic lumbosacral radiculoplexus neuropathy* and diabetic amyotrophy is the absence of diabetes as the aetiological factor. Otherwise, the signs and symptoms are similar and so are the electrophysiological examination results [19].

Neoplastic Invasion

Neoplastic invasion of the lumbosacral plexus occurs due to expansion of primary or metastatic tumours from the organs close by. Colorectal, bone, testis, bladder, uterine and cervical cancers can cause lumbosacral plexus lesions. Pain is prominent. Iatrogenic lesions that occur during surgery are also possible. Compression due to abscess and haematoma masses overlying the psoas muscle can cause damage [19].

Radiculopathies

Upper Limb Radiculopathies

Upper limb nerves are derived from the fibres of the C5 through to T1 roots. The fibres of an individual root can innervate more than one muscle via same nerve or different nerves. Similarly, one individual muscle receives fibres from different roots through the same nerve. When a peripheral nerve is injured, the resulting deficiency in muscle strength is more prominent than that produced from a single-root lesion [20].

Cervical radiculopathy is a common cause of pain and weakness of the neck, shoulder and arm. Weakness of the muscle, dermatomal sensory deficits and reduced deep tendon reflexes are classical findings. Intervertebral disc herniation, spondylosis and degenerative changes of bony structures are common causes of cervical root compression. Traumas, tumours and infections may also cause radicular dysfunction. The presence of *Lhermitte's sign*, increased deep tendon reflexes in the lower limbs, and an extensor plantar response suggest an associated myelopathy.

Electrophysiological findings in cervical radiculopathy include normal or near normal motor nerve conduction velocities (NCVs), normal sensory NCVs and sensory action potentials. Needle EMG findings may also be normal. If axonal degeneration is present, reduced recruitment with denervation potentials may be observed. Large motor unit potentials and reduced recruitment during maximal muscle contraction are compatible with a chronic course. Magnetic

resonance imaging (MRI) of the cervical spine is usually the preferred choice for identifying structural abnormalities.

Lower Limb Radiculopathies

The symptoms of lumbosacral radiculopathy include pain, paraesthesia and muscle weakness. Pain and paraesthesia show a dermatomal distribution. When muscle weakness is not prominent, myotomal distribution is difficult to discern. Lumbosacral radiculopathy usually occurs due to intervertebral disc herniation and neural foraminal stenosis. Tethered cord, diastematomyelia, spina bifida and nonskeletal conditions such as infection, inflammation, neoplasm and vascular disease are defined aetiological factors [12]. MRI, computed tomography and electrophysiological methods are used for evaluating radiculopathy. Prompt radiological evaluation is necessary when neoplasia or an abscess is suspected or if acute progressing neurological deficits, urinary retention, saddle anaesthesia and bilateral neurologic symptoms are present [12, 21].

Mononeuritis Multiplex

Mononeuropathy multiplex is a lesion of two or more peripheral nerves that cannot be explained by other peripheral nerve disorders such as polyneuropathy, root or plexus injury. Mononeuritis multiplex is another term that is used to define same condition [2]. Asymmetric, non-length-dependent involvement of nerves and a subacute pattern constitute the characteristic features. Symptoms occur in different nerves simultaneously or consecutively. Patients may present with paraesthesia, deep, dull pain and weakness in a single peripheral nerve distribution followed by another nerve area. As the process continues, other nerves become involved so that clinical and electrophysiological findings resemble symmetric distal sensorimotor neuropathy [22]. Multifocal nerve infarctions cause mononeuritis multiplex. Systemic vasculitis and nonsystemic vasculitis are the most common aetiological factors. Vasculitis, which accompanies systemic diseases, has some additional symptoms such as weight loss, skin lesions and adult-onset asthma/sinusitis. A prospective study found that patients with systemic vasculitis who had vasculitic neuropathy at the onset of disease had a worse prognosis, even without poor prognostic factors [23].

Shin J. Oh et al. reported nine cases of peripheral neuropathy associated with vasculitis in patients who had malignant disease before or after a diagnosis of neuropathy [9]. They referred to a report of three cases by Torvik et al. dated 1968 as the first report concerning paraneoplastic vasculitis resulting in peripheral neuropathy with malignant diseases. In that article Torvik et al. stated that 'The vasculitis of these cases may remain localised to muscles and peripheral nerves and leave the visceral organs intact' [24]. At present paraneoplastic vasculitis is one of the accepted causes of asymmetric neuropathy or mononeuritis multiplex. Symptoms and signs are responsive to immune suppressant therapy. The most common treatment is pulse intravenous cyclophosphamide with corticosteroids, with transition to azathioprine [2, 25].

Besides vasculitis, diabetes, infection, toxicity and drug adverse effects must be investigated during evaluation [25, 26]. Multiple entrapment neuropathies, a family history of peripheral neuropathy and compression neuropathies are also important to distinguish from hereditary neuropathies accompanying autonomic signs which suggests amyloidosis. Possible aetiologic factors of mononeuritis multiplex are shown in Table 10.2. Electrophysiological tests reveal predominantly motor axonal involvement with denervation activity to some extent (Fig. 10.2). Even asymptomatic nerves should be examined to show involvement of other nerves. An extensive search for the aetiology is very important to identify treatable autoimmune and inflammatory causes. Patients with systemic vasculitis may have vasculitic neuropathy which presented as mononeuritis multiplex treated with corticosteroids and immunosuppressant drugs [26, 27].

Table 10.2 Conditions associated with mononeuritis multiplex aetiology

Infections	**Rheumatologic disorders:**
• Lyme • Leprosy • Acute viral hepatitis A • Hepatitis B • Hepatitis C • Acute parvovirus B-19 • Herpes simplex virus • AIDS and HIV	• Wegener granulomatosis • Henoch-Schönlein syndrome • Sjögren syndrome • Behçet's disease • Temporal (giant cell) arteritis • Systemic lupus erythematosus • Rheumatoid arthritis • Polyarteritis nodosa • Scleroderma
• Chronic conditions: • Diabetes mellitus • Amyloidosis • Neurosarcoidosis • Celiac disease • Tangier disease	• Chronic graft versus host disease • Direct tumour invasion with intraneural spread: lymphoma, B-cell leukaemia, carcinoid tumour • Paraneoplastic: small cell lung cancer
• Haematologic conditions: • Churg-Strauss syndrome • Hypereosinophilia • Cryoglobulinemia • Atopy-related peripheral neuritis • Idiopathic thrombocytopenic purpura	• Miscellaneous conditions: • Amphetamine angiitis • Gasoline sniffing

Polyneuropathies

Hereditary Neuropathies

Hereditary neuropathies (HN) include a wide spectrum of motor, sensory and autonomic nerve involvement. Besides the peripheral nervous system, other organs may be affected. Different HNs share similar clinical and electrophysiological features, which renders the differential diagnosis difficult. It is possible that a patient, who has a HN, may also have a condition that causes acquired neuropathy. In this case, overlapping characteristics cause more complexity [16]. HNs are usually devoid of sensory symptoms and have an early age of onset. The onset is described subacutely by patients. However, symptoms such as pes cavus/pes planus, hammer toes and atrophy of distal limb muscles tell another story. Electrophysiological findings also support a chronic clinical course. The most common type is Charcot-Marie-Tooth (CMT) disease [2, 28].

Foot and toe deformities that can be seen in HNs include pes cavus, pes planus, claw toe, mallet toe, hammer toe and curly toe (Fig. 10.3). Pes cavus or claw foot means a foot with an abnormally high plantar longitudinal arch. In this condition, too much weight and stress are placed on the heel of the foot when walking. Different toe deformities can also be observed with pes cavus. Pes planus or flat foot is the loss of the medial longitudinal arch of the foot. In this condition, the medial arch of the foot comes closer to or in contact with the ground. The claw toe is a bending of the toe at the ball of the foot. At the middle joint, and sometimes the distal joint as well, the toe bends downward in a claw-like or curly appearance. Claw or curly toes can occur in any toe except the big toe. Hammer toe often presents along with hallux valgus which is also known as bunion deformity. The toe is bent in the middle joint causing a curling of the toe in this deformity. This is most common in the second toe but it can occur in any toe. A mallet toe is like a hammer toe except the joint involved is the distal joint instead of the middle joint, giving the toe a mallet-like appearance at the end [6, 18, 28].

Hereditary polyneuropathies (HPNs) include a wide variety of motor, sensory, autonomic and other systems involvement with considerable overlap. The pathophysiological process eventually results in axonal degeneration and neurological dysfunction in all types of HPNs. The morbidity and mortality depend on the neural and systemic involvement. Overlapping genetic and acquired factors increase diagnostic complexity. Neurologic examination and neurophysiologic tests are important for diagnosis. Clinical features, electrophysiological characteristics, the mode of genetic transmission, metabolic deficiency and genetic loci are used for classifying HPNs [29]. The primary HPNs predominantly involve peripheral nerves and symptoms are due to dysfunction of those peripheral nerves. On the other hand, peripheral neuropathies, which are associated with other disorders, also affect the central nervous system and organs. Symptoms of other organ or system involvement may dominate, and peripheral nerve symptoms may go unnoticed (Table 10.3). Genetic diagnosis has improved parallel to next-generation sequencing. Symptoms which were classified as idiopathic or atypical previously may now have a definite diagnosis. Gene-specific therapies have also developed alongside genetic diagnosis. Antisense oligonucleotides, RNA interfer-

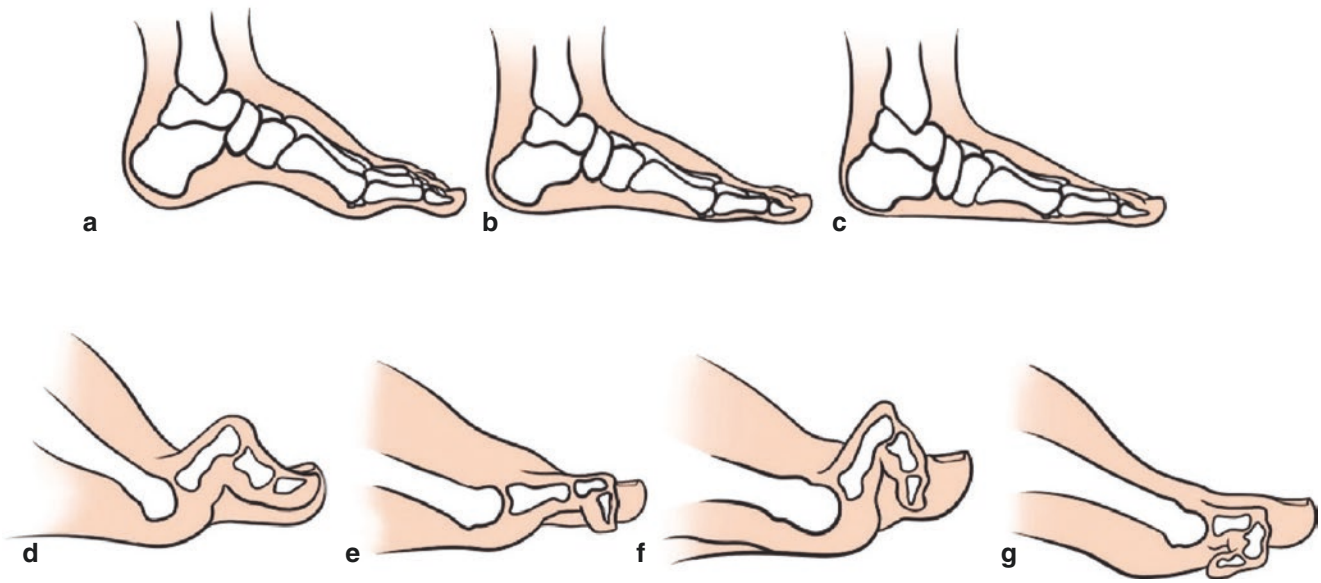

Fig. 10.3 Different feet and toe deformities: (**a**) claw foot, (**b**) normal foot, (**c**) flat foot, (**d**) claw toe, (**e**) mallet toe, (**f**) hammer toe and (**g**) curly toe

Table 10.3 Classification of hereditary peripheral neuropathies

Primary hereditary neuropathies	Complex hereditary neuropathies
Charcot-Marie-Tooth	Familial amyloid polyneuropathy
Hereditary sensory autonomic neuropathy	Hereditary ataxias, including spinocerebellar ataxias with neuropathy
Hereditary neuropathy with liability to pressure palsies	Complicated hereditary spastic paraplegia
Hereditary brachial plexus neuropathy	Lysosomal storage diseases
Distal hereditary motor neuropathy	Mitochondrial disorders
Giant axonal neuropathy	DNA repair disorders (e.g. xeroderma pigmentosum, Cockayne syndrome)

ence, small molecule chaperones and viral gene delivery therapies appear as the new therapeutic options. Treatments for hereditary transthyretin amyloidosis and Fabry disease are already available. However symptomatic treatment and family counselling are the principal therapy options for most inherited neuropathy classes [29, 30].

Charcot-Marie-Tooth (CMT)

Advances in science and technology furthered our knowledge of inherited neuropathies leading to a change in the way they are classified. *Peroneal muscular atrophy* and *hypertrophic interstitial neuritis* became *Charcot-Marie-Tooth* and *Déjérine-Sottas neuropathy*. Dyck and Lambert described demyelinating, axonal and intermediate forms of neuropathies according to motor nerve conduction velocity. These include type 1 demyelinating (NCV below 38 m/s) and type 2 axonal (NCV over 38 m/s). An intermediate form (NCV between 38 and 45 m/s) was added as the need arose. However, due to confusion caused by overlapping phenotypes, the name hereditary motor sensory neuropathy (HMSN) came into use [28]. A new, more comprehensive classification, based mostly on associated genes, has been constructed upon CMT classification. With this evolution, the CMT eponym became more popular again. Subtypes of CMT are described and numbered 1 through 7, and there is also an X-linked category (CMTX). Each category has an assigned letter (CMT1A, CMT2B) which indicates the presence of an associated specific gene. Multiple genes have been found to cause CMT; however most cases have five pathogenic genes which are peripheral myelin protein gene 22 (PMP22), myelin protein zero (MPZ), gap junction protein beta 1 (GJB1/connexin 32) and Mitofusin-2 (MFN2) [29].

CMT1 is autosomal dominant demyelinating neuropathy which is the most common type of CMT. Typical findings are prominent distal muscle weakness and atrophy, reduced sensation, decreased deep tendon reflexes and different foot deformities such as pes cavus and hammer toes [18]. Electrophysiological examinations reveal symmetrical-reduced NCVs, between 15 and 38 m/s, without conduction block and temporal dispersion. Seven subtypes of CMT1 have been identified, with five causal genes [2, 4, 28]. CMT1A accounts for approximately 70% of CMT1 cases and more than 50% of all CMT cases. Electrophysiological findings are compatible with demyelinating neuropathy in the beginning; however by the time of diagnosis, signs and symptoms of secondary axonal degeneration appear. Neurological disability is caused by axonal degeneration rather than demyelination. Histopathological evidence of hypertrophic segmental degeneration and regeneration presents as 'onion bulbs'. The severity of clinical findings is greater in patients with very low NCVs [29]. The causative mutations of the PMP22 gene are most often duplications. However, point mutations may also occur. While duplication causes overexpression of PMP22, point mutations cause a different distribution of PMP22 protein. There is no simple correlation between the expressed PMP22 protein levels and disease severity. Moreover, the severity of neurological findings differs highly within affected families or identical twins. This data suggests that other external factors like epigenetic and environmental changes may have effects on disease severity. An interesting finding is that besides the typical neuropathic symptoms, associated sleep apnoea may be observed in CMT1A patients with duplication of the PMP22 gene. Patients who have 1.5 Mb deletion of the PMP22 gene have hereditary neuropathy with pressure palsy [30].

CMT1B cannot be differentiated from CMT1A on clinical or pathological grounds. Genetic research showed that mutations of the myelin protein zero (MPZ) gene, which is also one of the principal genes of CMT, result in CMT1B. MPZ is a component of compact myelin which is important in maintaining myelin compaction and stability. On rare occasions MPZ mutations can be found in patients with late-onset axonal neuropathy (CMT2J). Adie tonic pupil may also be seen in some of the patients. Adie syndrome affects the pupils in either a unilateral or bilateral fashion. It was described as being almost synchronous with CMT1B by Adie, Morgan, Symonds and Holmes in 1931. This neurological disorder is characterised by a tonically dilated pupil that reacts slowly to light but shows a more definite response to accommodation (light-near dissociation). The affected pupil appears abnormally dilated at rest and shows sluggish pupillary constriction in bright light. Constriction is typically more notable with near reaction. It is caused by damage to the parasympathetic innervation of the eye due to different reasons including the HPNs [6, 28, 31].

CMT1C is an autosomal dominant demyelinating neuropathy which has minor symptoms and doesn't cause significant disability. The causative mutation is in the lipopolysaccharide-induced tumour necrosis factor-alpha factor (*LITAF*) gene. CMT1D is very rare, accounting for

less than 1% of CMT cases. Pathogenic variants of the EGR2 gene, which encodes for early growth response protein 2, cause this CMT form. Most patients have severe symptoms such as delayed motor development and breathing problems. CMT1E is caused by single nucleotide variants in the PMP22 gene. In addition to the classic CMT phenotype, sensorineural hearing loss is also observed in patients with CMT1E. CMT1F is very rare. The neurofilament light (*NEFL*) gene on chromosome 8p21 is defective in this type. Abnormalities in the same gene are also implicated in CMT2E. Roussy-Levy syndrome is a CMT1 phenotype with additional symptoms such as postural tremor, gait ataxia, distal muscle atrophy, pes cavus, areflexia and mild distal sensory loss. Genetic testing of different families found abnormalities that indicated CMT1A and CMT1B type diseases [28, 31].

CMT2 is an autosomal dominant axonal neuropathy. According to epidemiological studies, approximately 8–30% of the CMT cases are genetically confirmed as CMT2 type. The age of onset is usually in the second or third decade which is later compared to CMT1. However, an early onset form also exists with more severe symptoms. It is not possible to differentiate CMT1 and CMT2 on clinical grounds. Distal prominent loss of muscle strength, sensory deficits, reduced deep tendon reflexes, atrophy and deformities are classical findings. However, electrophysiological examination shows reduced CMAP and SNAP amplitudes with NCVs above 38 m/s which are compatible with axonal neuropathy. Needle EMG findings correlate a chronic course with chronic reinnervation potentials. Histopathological examination of the sural nerve reveals loss of large myelinated fibres, regeneration activity without demyelination and hypertrophic properties which are hallmarks of primary axonal injury. CMT2 has more than 30 subtypes and 33 genes have been reported to be involved. Ten more genes are associated with intermediate forms. Despite this, most of the patients with typical findings of axonal CMT do not have a genetic diagnosis. Due to abundance of subtypes, the more common ones are mentioned in this section. Mutations of the mitofusin 2 protein coding gene (MFN2) are responsible for subtype CMT2A. Besides typical characteristic findings of CMT, additional clinical features such as optic atrophy, hearing loss, vocal cord paralysis and diaphragmatic weakness are observed. Specific gene mutations are involved in various specific clinical findings such as CMT2A-MFN2 with optic atrophy, CMT2C-TRPV4 with vocal cord paralysis and CMT2B-RAB7A with prominent sensory loss plus foot ulceration plus mutilation due to the inability to feel pain. Weakness of distal upper limb muscles is more prominent than lower limb weakness in CMT2D-GARS1 cases. As genetic test results are expanding, the clinical spectrum of CMT2 enlarges with many overlaps between subtypes [28–31].

CMT3 consists of two disorders: Dejerine-Sottas syndrome and congenital hypomyelination neuropathy. Dejerine-Sottas syndrome is a severe demyelinating neuropathy which causes floppy infant syndrome. Characteristic findings are motor retardation with severe sensory impairment, distal and proximal weakness of limbs, absent deep tendon reflexes and ataxia. Electrophysiological examinations of peripheral nerves reveal extremely slow conduction velocities below 10 m/s. Scoliosis and contractures can also occur and progress during the disease, but walking ability is preserved through adult age. Clinically, Déjérine-Sottas syndrome is different from both classical CMT and congenital hypomyelination neuropathy. However, as new genetic forms of autosomal dominant or recessive patterns have been described, the genetic overlaps become harder to understand as these described genes are also involved in CMT1 and CMT4. There are pathogenic variants with mutations in the PMP22 gene, which is also involved in CMT1A, and the MPZ gene which is also involved in CMT1B. Congenital hypomyelination neuropathy is one of the causes of floppy infant syndrome. Infants are born with contractures [28, 32].

CMT4 is autosomal recessive. Manifestations of CMT4 include prominent distal muscle weakness and atrophy, sensory impairment and deformities like pes cavus. Electrophysiological examinations show slow NCVs below 40 m/s which is compatible with demyelinating neuropathy. Several subtypes of CMT4 have been reported in consanguineous and nonconsanguineous families [33].

X-linked CMT neuropathies make up around 10–15% of CMT cases. The CMTX1 subtype includes patient with mutations in the GJP1 gene. Almost 90% of CMTX patients have GJP1 mutations which result in connexin 32 (a gap junction protein) dysfunction. Since the mutation is X-linked, dominant females are also affected but less severely than males. Progressive weakness and atrophy of muscles, areflexia, sensory impairment and variable central nervous system symptoms are observed in affected patients. When questioned, patients report a history of frequent falls in their adolescence and early adulthood. Electrophysiological studies reveal intermediate NCVs with mildly prolonged latencies and low amplitudes of CMAPs. Mutations of the recently found PRPS1 gene result in **Arts syndrome**. This syndrome is part of a spectrum of PRPS-1-related disorders with reduced activity of the PRPP synthetase 1 enzyme which includes **CMT5** and X-linked nonsyndromic sensorineural deafness [6, 28, 30]. The **intermediate CMT** category is reserved for cases which do not meet the complete criteria for either demyelinating or axonal neuropathy. This CMT variant is a rare form which causes controversy about its existence and classification. Due to this diversity of views, traditional categories are used as much as possible. Some X-linked types and autosomal recessive forms are in this cat-

egory. Dominant intermediate CMT type A (DI-CMTA) and dominant intermediate CMT type C (DI-CMTC) have been described. Recessive forms of intermediate forms include RI-CMTB and RI-CMTC which are also defined [30].

Hereditary motor sensory neuropathy (HMSN) types 5, 6 and 7 were classically included in CMT and HMSN classifications, but currently these disorders are evaluated with the associated symptoms. Patients with autosomal dominant spastic paraparesis and sensory neuropathy are referred as HMSN 5. HMSN 6 refers to patients with dominant or recessive optic atrophy and motor sensory neuropathy. HMSN 7 refers to patients with retinitis pigmentosa and motor sensory neuropathy [30, 34].

Hereditary Sensory and Autonomic Neuropathies

Other groups of HNs are the hereditary sensory and autonomic neuropathies (HSAN). Sensory and autonomic features are predominantly seen in patients who have the disease [2]. Loss of large myelinated and unmyelinated fibres are prominent features of HSAN. Classification of HSAN is based on clinical characteristics and genetic grounds [35].

HSAN1 is autosomal dominant and is the most frequently seen form of HSAN. Clinical features include distal sensory loss followed by distal muscle weakness and atrophy. Facial sensation is preserved and foot ulcers are frequently seen. Onset is usually in early adulthood. Autonomic abnormalities vary in severity. The underlying pathology is progressive degeneration of motor neurons and dorsal root ganglia. Hearing loss and dementia have also been reported in some affected families. Genetically confirmed, four subtypes are reported. The genes that are implicated in HSAN1 are serine palmitoyltransferase, long-chain base subunit 1 (SPTLC1), SPTLC2, atlastin GTPase 1 (ATL1), ATL3 and DNA-metiltransferaz 1 (DNMT1). Electrophysiological examinations show axonal degeneration of motor and sensory fibres. Sensory neuron action potentials (SNAP) are of low amplitude or normal in the upper limbs, but usually they cannot be detected in the lower limbs. Variability of motor nerve conduction velocities from normal range to the demyelinating range with conduction blocks may cause diagnostic difficulty. This electrophysiological diversity may cause misdiagnosis in patients with prominent motor signs and scarce autonomic findings [36, 37].

HSAN2 is autosomal recessive and is characterised by loss of touch, pressure, pain and temperature sense. Involvement of large myelinated and small unmyelinated fibres cause clinical signs and symptoms. Recurrent infections, fractures of digits, osteomyelitis, spasticity and other autonomic disturbances are frequently encountered. The genes that are implicated in HSAN2 are WNK1/HSN2, FAM134B, KIF1A and SCN9A. Genetically defined, three subtypes are reported in the literature. Recently, a novel RETREG1 (FAM134B) founder allele has been linked to HSAN2B, and the resulting renal disease was identified in a Turkish family. Mutations causing loss of function result in insensitivity to pain and temperature, hearing loss and hyposmia, while gain of function mutations cause excess pain including erythromelalgia [38].

HSAN3 is also called familial dysautonomia and Riley-Day syndrome. HSAN3 is almost exclusively seen in children with Ashkenazi Jewish ancestry. Only a very limited number of cases are reported in other populations. It is an autosomal recessive disorder. The clinical course entails progressive sensorimotor neuropathy with autonomic features. Sympathetic autonomic involvement is responsible for most of the disturbances. Dysautonomic crises are sometimes difficult to manage. Myelin abnormalities are also found in the central nervous system causing dorsal column demyelination. Small stature, vertebral column abnormalities, lack of fungiform papillae causing a smooth tongue surface, dysarthria, mental disability and emotional lability are additional characteristics of the syndrome.

Congenital insensitivity to pain and anhidrosis describes the clinical characteristics of **HSAN4** which is transmitted as an autosomal recessive trait. Severe insensitivity to temperature and pain causes injuries which lead to self-mutilation and osteomyelitis. Seizures and a mild/moderate mental impairment are also seen. **HSAN5** is an autosomal recessive disorder. Loss of pain and temperature sensation is present, while other sensations are normal. **HSAN6** is one of the causes of floppy infant syndrome. Autonomic abnormalities, failure to thrive and absent psychomotor development are characteristic findings. It is an autosomal recessive disorder. **HSAN7** is an autosomal dominant disorder. Signs and symptoms appear at birth or during infancy. Congenital insensitivity to pain, excessive sweating, delayed motor development without cognitive impairment and gastrointestinal dysmotility are characteristic features. Due to the inability to feel pain, self-mutilation, joint dislocation, bone fractures and osteomyelitis are common disturbances. Muscle weakness is not a prominent finding. **HSAN8** is inherited as an autosomal recessive trait. Pain insensitivity causes soft tissue injuries, self-mutilation and tooth loss. There are also some HSAN cases which cannot yet be classified [28, 35–37].

Hereditary Neuropathy with Pressure Palsy

Hereditary neuropathy with pressure palsy (HNPP) is a recurrent, episodic demyelinating neuropathy which is autosomal dominant. Patients typically present with single nerve dysfunction due to compression, usually at the usual sites for entrapment. The most frequently involved nerves are the axillary, median, radial, ulnar, peroneal and brachial plexus nerves. Sensorineural deafness and scoliosis are associated findings. The age of onset is usually in the second decade, but it can develop in early childhood or in the third decade.

Isolated nerve palsies may occur successively with recovery taking days to months. Motor deficits can be persistent, so that in later stages, accumulated deficits may resemble symmetrical neuropathy with entrapment syndromes [28]. HNPP is also called tomaculous neuropathy due to the microscopic appearance of nerves in biopsy materials during tear fibre examination. PMP22 deletion and single nucleotide variants are found in HNPP which render HNPP allelic to CMT1A. The deletion in chromosome 17p11.2 results in decreased expression of the PMP22 gene [38].

Familial Amyloid Polyneuropathy (FAP)

Due to the emerging treatment options, diagnosis of familial amyloid polyneuropathy (FAP) has gained importance. Mutations of the transthyretin (TTR), apolipoprotein A1 (APOA1) and gelsolin (GSN) genes cause this disorder that can be potentially fatal [2]. Accumulation of fibril aggregates of amyloid precursor proteins in the peripheral nerves and other systemic organs results in early autonomic involvement, unexplained cardiomyopathy, bilateral carpal tunnel syndrome and a progressive course in patients with a family history [2, 28]. Length-dependent small fibre neuropathy that causes impaired temperature and pain sensation is the typical manifestation. Autonomic dysfunction may cause life-threatening cardiac arrhythmias with cardiac failure, especially in the elderly [28]. Nephrotic disease is another symptom of FAP. TTR mutations are the most common type. Val30Met was the first identified TTR mutation and is seen most frequently. However, there are more than 100 different amyloidogenic point mutations [2]. Autosomal dominant mutations cause phenotypic heterogeneity [39]. The patients who have the same mutations show different clinical signs and symptoms. They may also have a different age of onset. For example, in Portuguese patients, the age of onset is typically in the third decade, whereas in people from Northern European countries, onset occurs in the sixth decade [28]. Due to the heterogeneous aspects of the disease, TTR-FAP diagnosis is made usually long after the onset of the symptoms. If progressive sensorimotor neuropathy presents with any of the following findings such as a family history, autonomic dysfunction, cardiac failure, gastrointestinal symptoms, weight loss with unidentified cause, bilateral carpal tunnel syndrome, renal dysfunction or ocular involvement, TTR-FAP should be considered in the differential diagnosis [39]. Tafamidis meglumine is a drug approved in Europe and the United States which is administered orally and blocks mutated TTR misfolding and accumulation. The US Food and Drug Administration also approved two gene therapies, patisiran and inotersen. Liver transplantation, which was the only hope for TTR-amyloidosis patients before new molecular therapies were developed, has become a less preferred treatment [28].

Electrophysiological evaluation is very useful for differentiating hereditary and acquired neuropathies. Uniform nerve conduction velocity slowing is the usual finding in inherited neuropathies, whereas patchy slowing, partial conduction blocks and increased temporal dispersion are seen in acquired neuropathies. However, CMTX-GJB1 mutations constitute an exception. In patients with this mutation, nerve conduction abnormalities mimic abnormalities of acquired demyelinating neuropathies. The blink reflex (BR) is an electrical analogue of the clinical corneal reflex. When peripheral nerve conduction studies are ambiguous, blink reflex examination may provide a clue for diagnosing demyelinating HN. Irrespective of severity, a latency of R1 response which is more than 13 milliseconds supports the diagnosis [32]. Autonomic testing can be helpful when distinguishing CMT from other inherited neuropathies that have autonomic involvement such as TTR-FAD and HSAN. R-R interval changes during resting, deep breathing and the Valsalva manoeuvre, as well as a sympathetic skin response and orthostatic blood pressure changes, are accepted as useful tools [28].

Hereditary Brachial Plexus Neuropathy

Hereditary brachial plexopathy is a rare autosomal dominant disorder. Hereditary neuralgic amyotrophy (HNA) is another name for recurrent, painful brachial plexopathy. HNA and hereditary neuropathy with predisposition to pressure palsies (HNPP) are recurrent and episodical disorders [38]. The condition is caused by pathogenic variants in the septin 9 (SEPT9) gene. Childhood onset of hereditary brachial plexopathy is not unusual. Many patients exhibit a relapsing-remitting course characterised by attacks that resolve spontaneously, either completely or incompletely, leaving additive residual weakness. The disorder can also follow a progressive pattern. Physical exertion and pregnancy are reported triggering events. The attacks are heralded by pain and paraesthesia, followed by paresis of the shoulder and arm. While any nerve in the brachial plexus can be involved, injury to the upper part of the brachial plexus is the most frequent feature. The characteristic somatic features of hereditary brachial plexopathy include short stature, hypotelorism, a small face, unusual skin folds and creases on the neck [11, 38].

Giant Axonal Neuropathy

Giant axonal neuropathy (GAN) is a degenerative disorder which affects both the central and peripheral nervous systems. This is a severe autosomal recessive disorder. The genetic locus of the disease maps to 16q.24.1. Symptoms appear in early childhood with frequent falls and gait abnormalities. Weakness of the distal muscles and ataxia are also found. Central nervous system involvement signs include cerebellar dysfunction, spasticity and optic atrophy. A typi-

cal phenotype to develop the disorder consists of red and curly hair with a pale complexion and long eyelashes. Cognitive impairment is present in some cases. Electrophysiological findings are compatible with axonal neuropathy. The progressive course ends with death which is usually due to respiratory insufficiency [28, 38].

Acquired Neuropathies

Toxic Neuropathies

Toxic neuropathies (TNs) are an important category of acquired neuropathies, and they are caused by exogenous neurotoxic substances. Toxic substances enter the body via digestion and inhalation or via parenteral and transcutaneous routes. Acute, cumulative and delayed intoxications may occur [40]. Neurotoxic agents can be classified as environmental, occupational, recreational or iatrogenic [41]. Additionally, the increased survival rates of cancer patients has led to more chemotherapy-related problems. Toxic neuropathy is one of the frequent neurological disorders seen in cancer patients due to the toxic effects of chemotherapeutic agents. More than 30% of patients who are exposed to potentially neurotoxic agents have neuropathi disturbances. The highest prevalence is seen in patients who received platinum-based drugs, vinca alkaloids and taxanes [2, 40]. In developed countries most toxic neuropathy patients are exposed to these group agents. However, in developing countries, TNs are mostly caused by environmental and/or occupational toxic substances including arsenic, lead, mercury and organophosphorus. In addition, some manufacturing processes have moved to less-developed countries which have less control over the occupational hazards related to previously known toxic substances such as hexane, carbon disulphide and 1-bromopropane.

Axonal degeneration, demyelination, neuronopathy, ion channel dysfunction and other molecular pathways are associated with TN (Table 10.4). Most of the toxic substances predominantly cause axonal neuropathy which can be acute, subacute or chronic [42]. But there are no strict rules about this. N-hexane exposure can cause both axonal and demyelinating neuropathies. The effects caused by toxic substances may continue after the offending exposure is removed. This condition is called the 'coasting effect'. Neuropathy due to isoniazid toxicity can occur in patients with tuberculosis.

Table 10.4 Toxic substances and associated neuropathy types

Demyelinating	Axonal	Neuronopathy
Hexachlorophene	Taxanes, vinca alkaloids	Platinum derivatives
Triethyltin	Hexane and 2-hexanone	Methylmercury
Tellurium	Carbon disulphide	Catecholamines
Diphtheria toxin	1-bromopropane	
Disulfiram	Bortezomib	

Isoniazid metabolites inactivate B6 and inhibit the enzymes which convert pyridoxine to active pyridoxal phosphate. Concurrent administration of vitamin B6 prevents the development of neuropathy. However, chronic overdose of B6 also causes axonal large and small fibre neuropathy in susceptible people [43, 44]. Chemotherapeutics, antimicrobials and antiretroviral drugs are also known to cause neuropathy. New treatments such as tumour necrosis factor inhibitors (infliximab) and immune check point inhibitors (ipilimumab) can cause TN.

Alcohol may cause toxic neuropathy by itself. Additionally heavy alcohol use is usually associated with nutritional deficiency which worsens neuropathic disorders. Distal symmetric axonal polyneuropathy is present in 25–66% of chronic alcoholics in the United States [2]. Organophosphate toxicity frequently encountered due to accidental or intentional exposure has severe systemic and neurological effects. One of the neurological effects is axonal neuropathy.

Nutritional Neuropathies

Neuropathies due to the deficiency of essential substances need to be quickly diagnosed and treated before irreversible changes occur. However, associated neurological and systemic disorders may overshadow the signs and symptoms of neuropathies. Nutritional deficiencies occur due to either insufficient intake or reduced absorption. Starvation, consuming foods poor in nutritional content and eating a restricted diet are the reasons for reduced intake. Reduced absorption can be caused by gastrointestinal diseases like inflammatory bowel disease or drugs which impair nutrition uptake. In some conditions, increased metabolic demand can also cause deficiency. Acute and subacute developments of malnutrition and weight loss require a prompt diagnosis and treatment to avoid persistent damage and dysfunction of the nervous system [40–46].

Thiamine (B1) is essential for the metabolism of carbohydrates and amino acids as it is a coenzyme for more than 24 enzymes. A deficiency of thiamine causes 'wet' beriberi with cardiovascular involvement, 'dry' beriberi involving peripheral nerves or Wernicke-Korsakoff syndrome which may result in dementia. Length-dependent, large fibre sensorimotor axonal neuropathy with reduced deep tendon reflexes is a characteristic finding. Autonomic dysfunction may also occur. Tropical ataxia is the result of a diet consisting of cassava which causes thiamine deficiency. Tropical ataxia symptoms consist of sensory ataxia, sensorineural loss of hearing and blindness due to bilateral optic atrophy. Chronic alcohol consumption may also cause neuropathy via thiamine deficiency and/or alcohol toxicity. An increased need for thiamine in acute metabolic stress should be kept in mind, especially for patients in intensive care units [45].

Pyridoxine (B6) is the generic name for pyridoxamine, pyridoxine, pyridoxal and their phosphorylated forms. These

compounds function as co-enzymes in carbohydrate, lipid and amino acid metabolism as well as heme and neurotransmitter synthesis. Malabsorption, increased loss and use of certain medications are causative factors of B6 deficiency. B6 deficiency causes a length dependent, mostly sensory neuropathy with paraesthesia and sensory deficits. Occasional motor involvement is also reported. High levels of B6 also result in sensory neuronopathy which causes sensory ataxia [2, 40, 43, 46].

Folate (B9) deficiency is due to insufficient dietary intake, increased turn over, drug-associated deficient absorption/distribution and folate analogues. Folate acts as a coenzyme in the metabolism of 1-carbon units and DNA synthesis with cobalamin. The neuropathy of folate deficiency has been defined in only a few reports. It appears to be a length-dependent, symmetric, large fibre-predominant sensory neuropathy. Accompanying neurological findings resemble subacute combined degeneration which occurs in B2 deficiency. Megaloblastic changes of erythrocytes are also seen [43, 44].

Cobalamin's (B12) main function is to serve as a cofactor in the methylation process which is very important for DNA synthesis, cell metabolism and erythrocyte function. It also takes part in myelination of the central and peripheral nervous systems. Therefore, deficiency results in demyelination of the posterior and lateral columns of the spinal cord and optic nerves. As expected, the deficiency of B12 causes both haematological and neurological disturbances. The most common deficiency is of vitamin B12 which may be due to insufficient intake or insufficient absorption. Starvation, hunger strikes, low socioeconomic status, inadequate nutrition and vegan diets are some of the causes of insufficient intake. Abnormal absorption is seen in pernicious anaemia, bariatric surgery, short bowel syndrome and gastric bypass surgery. Long-term metformin use can also cause low vitamin B12 levels with a high neuropathy prevalence [47]. Neuropathy is found in one fourth of the B12 deficient patients. Sensorimotor axonal neuropathy, sensory neuronopathy and small fibre neuropathy in a small group of patients are defined in patients with B12 deficiency. Subacute combined degeneration causes impaired proprioception and sensory ataxia with cognitive and psychiatric symptoms. Vitamin E and acquired copper deficiency are difficult to differentiate from B12 deficiency base on only clinical grounds [2].

Vitamin E includes different compounds; however, α-tocopherol is the most common form in human tissue. The functions of vitamin E are very important for survival. It is a cytoprotective antioxidant, it diminishes free radical concentrations in neural tissues, and it modulates glutamate toxicity. Vitamin E also takes part in the construction of cellular membranes, vesicular and cellular transport. Insufficient dietary intake and malabsorption are the main reasons of deficiency. Impairment of large sensory fibre function is reported. However, this is not expected to be a solitary manifestation of vitamin E deficiency because central nervous system dysfunctions such as spinocerebellar syndrome with ganglionopathy create more prominent symptoms. Anaemia and immune system dysfunction are also associated with vitamin E deficiency [44].

Copper is an important essential element which takes part in maintaining the structure and function of the haematopoietic and nervous systems. The most common cause of copper deficiency is surgical operations of the gastrointestinal system. Chronic haemodialysis and zinc toxicity are other frequent issues related with copper deficiency. Large fibre neuropathy occurs in deficient states, but copper deficiency-related vacuolar myelopathy is a more prominent disorder, mainly affecting elderly women [44].

Metabolic and Endocrine Neuropathies

Metabolic neuropathies consist of several peripheral nerve disorders due to the deficiency of organs or glands. The main underlying abnormality is the disorder of metabolic pathways. However, the pathogenetic mechanisms that result in neuropathy are not completely understood yet. There are various associated factors which can cause neuropathy by themselves or worsen present neuropathy. When there is an established metabolic disorder, the symptoms of neuropathy may easily be attributed to the known disease. There may be a causal relationship, but physicians must be cautious and should rule out other possibilities [48–50].

Hypothyroidism can cause multiple central and peripheral dysfunctions. Peripheral complications of hypothyroidism include entrapment neuropathies, polyneuropathy, neuromuscular junction disorders and myopathy. Carpal tunnel syndrome (CTS) incidence is higher in hypothyroid patients compared to the normal population. However, hypothyroidism is not the only factor which causes CTS in patients with hypothyroidism. Other factors such as high body mass index or the presence of other metabolic disorders appear to also contribute to CTS. Deposition of mucin in the perineurium and endoneurium of the median nerve and the deposition of mucopolysaccharides in synovial structures cause increased pressure in the carpal tunnel. CTS symptoms and findings do not differ clinically or electrophysiologically in patients with hypothyroidism. Replacement therapy for hypothyroidism may reverse the changes. However, some patients may be unresponsive. Screening of CTS patients for hypothyroidism is not performed in routine practice. However, it is easy, and thyroid-stimulating hormone and free T4 levels should be obtained, especially in patients with bilateral CTS. Sensorimotor polyneuropathy with stocking and glove distribution is another complication of hypothyroidism. Deep tendon reflexes have a longer relaxation time which is a characteristic finding of hypothyroidism. Painful

neuropathy, which suggests small fibre involvement, is another presenting form of neuropathy. Symptoms and signs are reversible with replacement treatment. Besides this classical presentation of polyneuropathy, polyneuropathy with autoimmune features can be seen in hypothyroidism like CIDP, GBS and multifocal motor neuropathy. Hormone replacement therapy alone is not sufficient, and so that immunomodulatory therapies are applied [44].

Hyperthyroidism-associated sensory polyneuropathy, CTS and myopathy are also reported. Basedow's paraparesis is used to define uniform subacute paraparesis that affects both distal and proximal muscles in patients with hyperthyroidism. Deep tendon reflexes are absent or reduced with severe hypotonia. However, sphincters and sensory examination are normal. Electrophysiological investigations identify reduced CMAPs and acute denervation which suggest axonal degeneration. However, histopathological examinations have not yet been reported [44].

Uremic polyneuropathy is commonly seen in patients with renal failure. In approximately 50% of the patients, neuropathy is asymptomatic. As kidney function decreases, neuropathy frequency increases, reaching up to 80% in end stage patients. The prevalence of uremic neuropathy is higher in female patients than male. Sensory symptoms that affect the distal limbs are the initial complaints. In later stages, motor findings appear. Electrophysiological findings are compatible with axonal and demyelinating features. Abnormalities of beat-to-beat variation (R-R interval) reflecting autonomic dysfunction generally correlate with axonal neuropathy. The cause of neuropathy is not precisely known; however, it may be related to a deficiency of thiamine, zinc and biotin and decreased transketolase activity. An increase of potentially toxic metabolites and hyperparathyroidism are suggested to contribute to neuropathy development because uremic neuropathy shares many features of toxic neuropathy [48].

Acromegaly is generally due to the overproduction of growth hormone from an eosinophilic pituitary adenoma. CTS and length-dependent sensory motor polyneuropathy are the most common peripheral nervous system complications.

Hepatic neuropathy incidence is variable in different reports. In most patients, neuropathy has a subclinical course with minimal symptoms. Length-dependent sensorimotor neuropathy and autonomic neuropathy are reported in patients with nonalcoholic hepatic failure. Autonomic dysfunction may be a life-threatening condition. Porphyrias are inherited metabolic disorders which can cause acute axonal neuropathy. Early diagnosis is important because severe complications such as quadriparesis, autonomic dysfunction and respiratory insufficiency may occur. The proximal upper limb is more frequently affected [49, 50].

Diabetic Neuropathies

Diabetes has become a public health issue in the twenty-first century [51–53]. The prevalence of diabetes in 2019 was estimated to be 9.3% which means 463 million people are living with diabetes worldwide. The prevalence is expected to rise 10.2% by 2030 and 10.9% by 2045 [51]. Around half of the patients with diabetes are affected by diabetic peripheral neuropathy (DPN). Therefore, increased DPN prevalence is also anticipated in the future [52]. DPN prevalence was found to be similar in type I diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) patients in the Rochester Diabetic Neuropathy Study by using clinical and nerve conduction parameters. It is interesting that population-based studies using only clinical findings, without nerve conduction assessment, also showed very close prevalence rates for DPN. The European Diabetes Prospective Complications Study (EURODIAB Study) reported that major risk factors for DPN development were poor glycolic control, age and duration of diabetes. Modifiable cardiovascular risk factors such as hyperlipidaemia (especially triglyceridaemia), obesity and cigarette smoking are also involved in the development of DPN in some studies [52]. However, in a recent meta-analysis, body mass index, hyperlipidaemia and cigarette smoking were found to not be risk factors for DPN. The same study identified diabetic retinopathy as a risk factor for DPN [53]. The inflammatory immune response plays an important role in some types of DPN [17]. DPN is also known to increase mortality, independent of other causes of mortality. The relationship between mortality and diminished vibration sense in DM patients is a very interesting and important finding [51–53]. A widely accepted definition of DPN, described by Boulton, is 'the presence of symptoms and/a sign of peripheral nerve dysfunction in people with diabetes after exclusion of other causes' [54]. However, DPN has many subtypes and is classified based on different aspects. It should be noted that diabetes may cause any type of peripheral neuropathy. In addition, these different types of neuropathies, either with the same or diverse pathophysiology, can be found in the same patients [55]. DPN classification can be made according to the time course, involved nerve types, symmetry and site of involvement or pathophysiology [55, 56]. There is a simple classification list of DPN in Table 10.5 [57].

Table 10.5 Diabetic peripheral neuropathies classified according to neurological involvement patterns

Distal symmetrical polyneuropathy
• Chronic
• Acute
Autonomic neuropathy
Mononeuropathy multiplex
Entrapment neuropathies
Plexopathies
Radiculopathies
Cranial neuropathies

Distal symmetrical polyneuropathy (**DSPN**) with sensorimotor involvement is the most common presentation of DPN. DSNP can be separated into three categories according to the involved nerve fibres. These are predominantly large fibre neuropathy, predominantly small fibre and pure small fibre neuropathy. Treatment-induced neuropathy and hyperglycaemia-induced neuropathy of diabetes mellitus can cause an acute painful DSPN. Typical symptoms of DSPN are numbness, paraesthesia/dysesthesia, pain and a burning sensation which usually affects the distal lower limbs. Positive symptoms like pain appear unprovoked. Patients may feel lancinating pain in otherwise insensitive feet. DSPN is a length-dependent neuropathy which begins in the periphery, affects the feet bilaterally and progresses proximally [56, 58]. Sensory involvement is usually described in a 'stocking glove' fashion. The proximal parts of the limbs are less severely involved than the distal parts, and the upper limbs are rarely involved. If symptoms are present in the upper limbs, they are most probably due to an accompanying mononeuropathy [55]. Asymmetric involvement should be a warning sign to consider other diagnoses besides DSPN. Asymptomatic patients may comprise of 50% of diabetic patients, while 25% experience painful diabetic peripheral neuropathy (pDPN). The use of sensory examinations should not be underestimated, especially in asymptomatic patients. The presence of feet insensitivity creates a predisposition for injury and increased risk for ulceration. Motor involvement signs emerge later. Weakness of the lower limb muscles first affects the toes, then ankles and calves. Difficulty in daily living and self-care activities becomes prominent [56, 58]. Insidiously presenting autonomic neuropathy commonly accompanies DSPN. If there is no adequate intervention, a progressive and chronic course of autonomic neuropathy is inevitable. Quality of life is seriously affected by painful neuropathic pain and foot ulcerations [59]. Patients with pDPN are more likely to have amputations. Psychiatric morbidity such as depression and anxiety as well as sleep disorders appear as symptom progress. Therefore, psychosocial wellbeing is frequently affected [56]. The fast and maintained normalisation of blood glucose can cause treatment-induced diabetic neuropathy (TIND) which mainly affects sensory and autonomic nerves [17]. There is a risk of DSPN occurrence in prediabetic patients, and so some patients already have DSPN by the time of diagnosis [58]. Electrodiagnostic studies usually show mild-moderate slowing of sensory and motor NCVs. Lowered motor and sensory action potential amplitudes with denervation potentials, seen in needle EMG, are characteristic findings which are prominent in the lower limbs. Upper limb examinations may reveal normal results. Electrophysiological findings are especially important for the differential diagnosis if there is a possibility of other neuropathic symptoms.

For instance, diabetic patients are prone to developing entrapment neuropathies. Inadequate diagnosis and treatment are a major problem. Unfortunately, neither patients nor physicians are aware of the importance of the DSPN. Treatment of DSPN is a complex issue and so a holistic approach is preferred. In particular, for diabetic foot care, having a multidisciplinary team consisting of an endocrinologist, neurologist, psychiatrist, physiotherapist, psychologist, podiatrist, orthopaedic surgeon, orthotist, vascular surgeon, microbiologist, pain specialist and a specialist nurse is essential. First, the management of diabetes itself is a priority. Euglycemia may prevent the occurrence or progression of DSPN in type1 DM, but not in type 2 [59]. Reducing other cardiovascular risk factors is also included in the treatment regimen. Targeting the pathogenesis of DSPN is another aspect. Alpha lipoic acid, benfotiamine, epalrestat and actovegin are permitted for use in some countries. Symptomatic treatment constitutes most of the DSPN therapy. Neuropathic pain is one of the worst symptoms that affects quality of life. Analgesics, antidepressants, anticonvulsants and in resistant cases opioid drugs are the best choices for the treatment of neuropathic pain. Local capsaicin treatment is difficult to apply but is reported to be beneficial in some cases. Each treatment has its own limitations. Combination therapy may reduce the individual drug doses and side effects. Neuromodulation therapy like high-frequency (10 kHz) spinal cord stimulation may be beneficial for pharmacotherapy-resistant patients [57].

Diabetic autonomic neuropathy (DAN) involves small myelinated and unmyelinated fibres. Cardiac, gastrointestinal and urogenital system symptoms are frequent. Cardiac effects are evaluated using heart rate changes during deep breathing, the Valsalva manoeuvre and during R-R interval change recordings (Fig. 10.4) [17]. Besides cardiovagal impairment, adrenergic denervation of the heart also occurs.

Fig. 10.4 Normal R-R interval recording

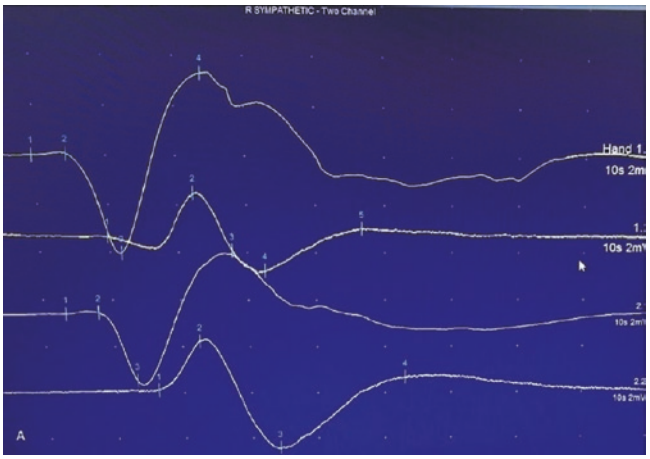

Fig. 10.5 Normal sympathetic skin responses recorded from the hand

Sympathetic involvement is evaluated using single-photon emission tomography or positron emission tomography and sympathetic skin response recordings (Fig. 10.5). Cardiac abnormality prevalence is higher in type 1 diabetic patients. Cardiac autonomic neuropathy is associated with high mortality rates. Orthostatic hypotension is another manifestation of autonomic dysfunction. Baroreflex failure is due to both adrenergic and vagal dysfunction [60]. Postprandial hypotension occurs after a heavy meal with a high carbohydrate content. Due to sympathetic denervation, blood pools in the splanchnic mesenteric bed after such a meal which causes orthostatic symptoms. An exaggerated blood pressure increase in response to direct adrenergic agents is related to denervation hypersensitivity. Gastrointestinal motility disorders are also a major component of autonomic dysfunction. Delayed gastric emptying, constipation or diarrhoea is reported. In diabetic patients with chronic intestinal pseudo-obstruction, a majority were found to have an abdominal vagal neuropathy [61]. Bacterial overgrowth, ischaemia of intestinal mucosa and pancreatic exocrine insufficiency have also been implicated as causative factors [17]. The prevalence of neurogenic bladder in diabetic patients is expected to rise as the duration of diabetes prolongs. Decreased detrusor tone results in an increased bladder capacity. The reduced perception of bladder fullness with the presence of high post-voiding residual urine causes overflow incontinence. The symptoms and signs are due to parasympathetic involvement which is usually part of a generalised autonomic disorder. Insufficient emptying of the bladder can be demonstrated by ultrasonographic examination and urodynamic tests [17, 60]. Penile erection is a function of the parasympathetic system, while ejaculation requires sympathetic activation. Erectile dysfunction may be the earliest symptom of autonomic disorder in diabetic men. After initial parasympathetic dysfunction, sympathetic denervation develops which causes ejaculation failure or retrograde ejaculation. Besides autonomic failure, nitric oxide levels and vascular insufficiency, somatic sensation loss contributes to sexual dysfunction [17]. Sexual dysfunction in females has not been studied as well compared to males. Absent and decreased sympathetic skin response amplitudes were reported in diabetic women [62]. A decrease in libido and lubrication, painful intercourse and anorgasmia are reported sexual problems in diabetic women [17]. Sweating and thermoregulation may be impaired due to sympathetic degeneration. Quantitative sudomotor axon reflex and thermoregulatory sweat tests can be used to detect these abnormalities [17, 60]. Before diagnosing autonomic neuropathy, other causes in differential diagnosis must be excluded such as malignant or vascular disorders.

Diabetic radiculoplexus neuropathy differs from other diabetic neuropathies because the underlying mechanism is highly complex. The nerves and roots are involved in immune, inflammatory and vascular processes. The development of subacute and painful proximal muscle weakness with some degree of autonomic impairment are common clinical findings. Electrophysiological examination reveals sensory and motor conduction abnormalities and acute denervation activity in needle EMG [17].

Infectious Neuropathies

There are many types of primary nerve infections, but infections can cause peripheral nerve dysfunction through different mechanisms.

Leprous neuropathy is a well-known disease, even in ancient times. Leprous neuropathy is one of the earliest symptoms of leprosy which appears as an impairment of sensation which may transform into painful neuropathy later in the course of the disease. In the tuberculoid form of leprosy, this is more likely to be a focal neuropathy. The affected nerves are usually located near the skin lesions. However, with the lepromatous form, a generalised neuropathy occurs. Claw hand, foot drop and lagophthalmos are frequent. The ulnar, median, common peroneal, tibial, facial, radial cutaneous and great auricular nerves are commonly affected nerves. Hypertrophic nerves can be palpated, especially the ulnar nerve at the elbow. Earliest findings include impairment of cold and warmth sensation and sensory nerve conduction abnormalities. Although there is a demyelinating pathology, the mechanism is not well understood [63].

Lyme disease can cause lymphocytic meningitis, facial nerve palsy and radiculoneuritis on top of other systemic involvements. Painful radiculoneuritis, also called Garin-Bujadoux-Bannwarth syndrome or Bannwarth syndrome, is more common in European countries. In some patients, facial nerve palsy and motor weakness with pleocytosis in cerebrospinal fluid can also be found [64].

Human immunodeficiency virus (**HIV**) can cause a distal symmetrical polyneuropathy (DSPN). This common neu-

rologic complication creates considerable difficulty in daily living activities. The underlying pathology includes ganglionopathy, axonal degeneration and decreased intraepidermal fibre density. The effects of HIV infection on peripheral nerves occurs most likely due to the production of inflammatory cytokines and chemokines by infected monocytes and macrophages because HIV does not infect cells devoid of CD4 receptors such as neurons, dorsal root ganglia and Schwann cells. The envelope glycoprotein gp120 also has direct toxic effects on Schwann cells and dorsal root ganglia by initiating a chain reaction. Genetic susceptibility renders some patients prone to developing DSPN. Regeneration and axonal sprouting defects are also found. Symptoms like dizziness, fainting and bladder dysfunction are suggestive of autonomic involvement [65].

Cytomegalovirus (**CMV**) infection causes a progressive radiculopathy and mononeuritis multiplex in patients with HIV infection or in immunocompromised patients.

It was reported in a large meta-analysis investigating COVID-19-related neuropathies that **SARS-CoV-2** does not cause viral neuropathy. However, peripheral neuropathy frequently occurs during COVID-19 infection. Neurotoxicity due to drugs used for treatment and immune mechanisms are suggested factors which can cause neuropathy. Compressive neuropathies are also reported to occur in patients with associated risk factors such as diabetes [66].

Paraneoplastic Neuropathies

Subacute sensory neuronopathy is mostly associated with small cell lung cancer (SCLC). It precedes the diagnosis in most cases [67]. Numbness and tingling sensations might be the first signs that progress to the impairment of proprioception, due to the involvement of large, myelinated axons. Impairment of sensory function begins in one limb and spreads to other limbs, the face and the trunk. Severe, disabling sensory ataxia and pseudoathetosis may prevent performing daily living activities. Patients rarely have mild ataxia but prominent painful neuropathy. Sensory nerve conduction velocities are normal or near normal, but SNAPs are low amplitude or absent. Motor nerves are usually found to be normal in electrophysiological examinations. However, in some cases, minor involvement is found, which presents a difficulty for differential diagnosis. Clinically, one absent or three low-amplitude SNAPs (<30% of normal), with less than two abnormal motor nerve conduction findings in the lower limbs, favour a diagnosis of sensory ganglionopathy. CSF findings are either normal or pleocytosis, oligoclonal bands and a high IgG index can be seen. SCLC-associated cases usually have anti-Hu antibodies. Anti-collapsin-response mediator protein 5 (CRMP5) antibodies are found in some cases [68, 69]. Co-occurrence of CRMP5 and anti-Hu antibodies is also possible. Superimposition of mixed axonal and demyelinating sensory motor neuropathy on sensory ganglionopathy can be found if the two antibodies are present together. Sensory ganglionopathy may also be associated with breast, prostate, colon cancers, lymphoma or uterine sarcomas. Symptoms improve when the primary malignant disease is treated. Immune modulatory therapies like IVIG, plasmapheresis and corticosteroids are of little help. Patients without a diagnosed malignancy should be followed up for 5 years [68].

Acute sensorimotor radiculoneuropathy occurs in patients with malignant diseases, especially in patients with lymphoma and solid tumours. Symptoms and signs of this disorder are identical to Guillain-Barré syndrome (GBS), and it is treated as GBS. The neuropathy can appear at any time during the course of the disease and may be a warning of relapse [67, 68].

Chronic sensorimotor neuropathy occurs in patients with a malignant disorder without any other identifiable cause. Patients are usually at a late stage of disease. Paraneoplastic antibodies are not present, and the onset is slow and not disabling. In the presence of paraneoplastic antibodies like CRMP5, neuropathy is progressive and disabling.

Neuropathies associated with lymphoproliferative disorders are associated with elevated paraprotein levels. These disorders are found in patients with multiple myeloma, POEMS and Waldenström macroglobulinaemia. The POEMS acronym stands for polyneuropathy, organomegaly, endocrinopathy, M protein, polyneuropathy and skin changes. Multiple myeloma-associated neuropathy is also associated with immunoglobulin light chain (AL) amyloidosis or POEMS and is axonal and demyelinating in nature. If neuropathy is the initial symptom, it may cause a misdiagnosis of CIDP. POEMS may be associated with multicentric Castleman disease. Waldenström macroglobulinaemia may be associated with neuropathy during the initial phase of disease, and antibodies against myelin-associated glycoprotein (MAG), GM1 gangliosid or asialo-GM1 ganglioside can be found in the sera of patients [70].

When it appears as a paraneoplastic syndrome, **autonomic neuropathy** is mostly associated with SCLC. Other malignancies associated with autonomic neuropathy are carcinomas of the pancreas, thyroid and rectum; Hodgkin's lymphoma (HL) and carcinoid tumours of the lung. Anti-Hu, anti-CRMP5 and antibodies against ganglionic acetylcholine receptors (AChR) may be found in the sera of the patients.

Vasculitic neuropathy presents as mononeuropathy multiplex or in some cases proximal neuropathy. Nerve biopsy is useful for diagnosis and differential diagnosis. A diagnosis of vasculitis may predate a diagnosis of cancer. The associated malignant disorders include lymphomas and cancers of the lungs, prostate and endometrium [24, 67].

Checkpoint inhibitor-associated neuropathies are possible adverse events of these medications. Combined thera-

pies (ipilimumab and nivolumab) increase the occurrence of adverse events. GBS like syndromes, cranial neuropathy, non-length-dependent neuropathy and autonomic involvement are commonly reported neuropathies. Meningitis may be accompanied with cranial neuropathy. Small fibre neuropathy with antineutrophil cytoplasmic antibody (ANCA)-related mononeuropathy multiplex, neuralgic amyotrophy and some other types may also occur. Mostly axonal neuropathy-compatible changes are revealed by electrophysiological examinations.

Paraneoplastic neuromyotonia, also called **Isaacs syndrome**, is due to peripheral nerve hyperexcitability. Muscle cramps, stiffness and increased sweating are the usual symptoms. EMGs reveal complex repetitive discharges and continuous muscle activity [67]. Findings of sensorimotor polyneuropathy are also observed. In patients with Isaacs syndrome, an underlying thymoma, SCLC and HL should be searched for. If central nervous system symptoms occur, it is called as Morvan syndrome.

Immune-Mediated Neuropathies

Multifocal motor neuropathy (**MMN**) is an immune-mediated and acquired disorder of peripheral nerves, which is defined by asymmetric motor involvement without sensory deficits [71]. Nerve conduction studies show conduction blocks in motor nerves, hence why it is also called multifocal motor neuropathy with conduction blocks. The worldwide prevalence is estimated to be approximately 0.6–2 per 100,000, and males are affected more than females (2.7:1). Age at diagnosis is usually between 30 and 50 years; however, younger and older patients have been reported [72]. Anti-GM1 antibodies, a class of IgM, are frequently found in sera of MMN patients, which provides an explanation for motor nerve involvement. Motor nerve myelin contains a high amount of GM1, especially around the Ranvier nodes and in comparison to sensory nerves [73]. Initially, muscle weakness affects one of the upper limbs and spreads to the contralateral limb, which is followed by lower limb involvement. Brachial involvement is common. Some nerves are severely affected, while others are totally spared or minimally involved. Dropped wrist and weakness of the hand or distal foot muscles are common symptoms. Atrophy can be observed, but it is not well correlated with weakness. Progression is expected in both treated and untreated patients, but the level of progression may vary between individuals [74]. Sensory findings are usually not present but the vibration sense can be minimally impaired. Electrophysiological examinations show motor neuropathy with conduction blocks and normal sensory NCVs. However, in some patients, conduction block cannot be shown, probably due to the proximal location of the blocks. Proximal stimulation of nerves (Erb points), F-wave studies and examinations of the asymptomatic nerves must be performed. Previously, nerve conduction blocks were thought to be caused by severe demyelination, but accumulated data suggest that anti-GM1 antibodies are responsible for the blocks and reduced motor NCVs. The functions of sodium and potassium channels, located around Ranvier nodes, are disrupted by anti-GM1 antibodies, resulting in the failure of action potential propagation. Therefore, MMN can be considered part of the nodoparanodopathy group of neuropathies. However, there is no consensus regarding the function of anti-GM1 antibodies in the pathogenesis of MMN [74].

Radiological examination with MRI and ultrasound can be performed, but there are no certain criteria for the diagnosis of MMN. Diagnosis is made on clinical grounds and electrodiagnostic parameters. A diagnostic criteria for MMN has been proposed by the European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) [75].

The two core criteria for MMN (both must be present) are the following:

1. Slowly progressive or stepwise progressive, focal, asymmetric limb weakness, that is, motor involvement in the motor nerve distribution of at least two nerves for more than 1 month. If symptoms and signs are present only in the distribution of one nerve, only a possible diagnosis can be made.
2. No objective sensory abnormalities except for minor vibration sense abnormalities in the lower limbs.

The supporting criteria and exclusion criteria are also defined. The differential diagnosis is important for treatment planning and predicting prognosis (Table 10.6). The main concern is to distinguish MMN from amyotrophic lateral sclerosis (ALS). The absence of pyramidal signs and bulbar involvement in MMN are important clues. The progression of MMN is relatively slower than ALS. Flail leg syndrome (FLS) is an atypical variant of amyotrophic lateral sclerosis (ALS) characterised by progressive weakness and atrophy of lower limbs alone. Contrarily, flail arm syndrome (FAS), an atypical presentation of ALS, is characterised by upper limbs involvement [75]. The pure motor variant of CIDP is also an important disorder to consider for the differential diagnosis because this variant also has conduction block. CSF findings may help to exclude a variant of CIDP. Treatment of MMN with IVIG is effective. Patients with impairments affecting activities of daily living must be treated as soon as the diag-

Table 10.6 The differential diagnosis of multifocal motor neuropathy

Progressive muscular atrophy
Flail arm syndrome
Flail leg syndrome
Chronic inflammatory demyelinating polyneuropathy
Multifocal acquired demyelinating sensory and motor neuropathy
Hereditary neuropathy with liability to pressure palsy

nosis made. Plasmapheresis and corticosteroid treatment are not recommended because the worsening of symptoms has been reported [73].

Inflammatory Neuropathies

Inflammatory neuropathies are members of autoimmune peripheral nerve disorders. The temporal course of these disorders can be acute or chronic. The exact aetiology or pathophysiological mechanisms are unknown; however, it is widely agreed that an aberrant immune response causes demyelination and axonal degeneration. This abnormal immune response involves both humoral and cellular elements of immune system. Understanding of the underlying etiological and pathophysiological mechanisms is essential to choose appropriate treatments and estimate the prognosis. Acute forms of immune neuropathies are grouped under the Guillain-Barré syndrome (GBS), whereas chronic forms constitute chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

Chronic inflammatory demyelinating polyradiculoneuropathy (**CIDP**) is a rare disease with a reported prevalence of 0.67–10.6 cases per 100,000 people [76]. A meta-analysis reported an estimated incidence of 0.33 per 100,000 and prevalence of 2.81 per 100,000 in 2019 [77]. The discrepancy between reported prevalence rates is probably due to the use of different diagnostic criteria [78]. The risk of CIDP increases with age and males are more likely to be affected than females. No risk factors for CIDP have not been reported yet. CIDP is an autoimmune demyelinating disorder which affects nerve roots and peripheral nerves. Large, myelinated fibres are most severely involved. The clinical course may be monophasic, chronic progressive or relapsing remitting [77]. The symptoms usually show a progressive course for more than 8 weeks. Muscle weakness, sensory loss and areflexia are the characteristic findings. Severe postural tremors may be an accompanying feature that can be resistant to CIDP treatment. Both proximal and distal parts of the limbs are affected symmetrically. Distal muscle weakness is more prominent; however, the presence of proximal weakness is a useful clue for CIDP diagnosis. Patients often have difficulty walking and performing usual activities of daily living. Cranial and respiratory muscle involvement has been reported but is very rare in comparison to GBS. Systemic symptoms such as fever, weight loss, autonomic dysfunction and severe pain are not expected in typical CIDP patients. The presence of these symptoms should suggest other possible diagnoses. A diagnosis of CIDP requires the exclusion of other possible aetiologies.

Diagnosis is based on history, clinical findings, electrophysiological examinations and nerve pathology because there is no available diagnostic marker yet. Different diagnostic criteria have been proposed, but the definition of atypical forms and CIDP-mimics complicates the already complicated diagnosis [77, 79]. Identification of immunoglobulin G4 (IgG4) antibodies in some patients with CIDP-like clinical findings is a notable advancement. Patients with IgG4 antibodies against nodal and paranodal proteins usually do not respond to standard CIDP therapies, but rituximab is an effective treatment [76, 80].

The typical CIDP phenotype has sensory-motor peripheral nerve involvement with a progressive course for longer than 8 weeks. It has a symmetric distribution and is predominantly proximal. At least 50% of patients are in this group. A careful evaluation should be performed to exclude more common types of sensory-motor neuropathies, such as paraproteinemic and hereditary neuropathies. As opposed to the slow progressing group of patients, around 18% of patients are known as acute onset A-CIDP cases. The distinct features of A-CIDP are acute onset of the disease and rapid progression that reaches maximal involvement before 8 weeks. It is also symmetrically distributed but with proximal and distal involvement. The course of A-CIDP is either with relapses or slow progressive. A-CIDP should be differentiated from subacute inflammatory demyelinating polyneuropathy and acute inflammatory demyelinating neuropathy, which have overlapping features. Electrophysiological examination is needed for CIDP diagnosis and differential diagnosis. Electrophysiological findings of demyelination are slowed conduction velocities, long distal sensory and motor latencies, partial conduction block in motor nerves (Fig. 10.6) and increased temporal dispersion. F-response and blink reflex latencies may also be prolonged. Sural saving and more prominent involvement of upper limb sensory nerves are characteristic findings for both acute and chronic acquired demyelinating neuropathies. The only needle EMG finding may be decreased recruitment activity due to slow conduction velocity or conduction block. In the chronic phase, due to secondary axonal degeneration, positive sharp waves and fibrillation potentials appear, as well as reinnervation potentials such as large polyphasic motor unit potentials. Strict criteria are difficult to achieve, and in some patients, when clinical findings strongly indicate CIDP diagnosis, electrophysiological findings are insufficient to fulfil the defined criteria [81]. When standard methods do not reveal the expected abnormalities, magnetic or electrical stimulation of nerve roots might yield rewarding results [82]. Magnetic resonance imaging of the proximal parts of nerves, namely, roots and plexuses, are useful to show thickening and enlargement of the affected structures, especially in the treatment of new patients. Contrast enhancements of the roots are informative. However, the MRI technique is difficult, time-consuming, expensive and sometimes unavailable. Instead of MRI, ultrasound examination can be used to show the t proximal median nerve thickening and brachial plexus enlargement [21, 22]. Nerve biopsy is not required when the presentation is one of typical CIDP. However, nerve biopsy

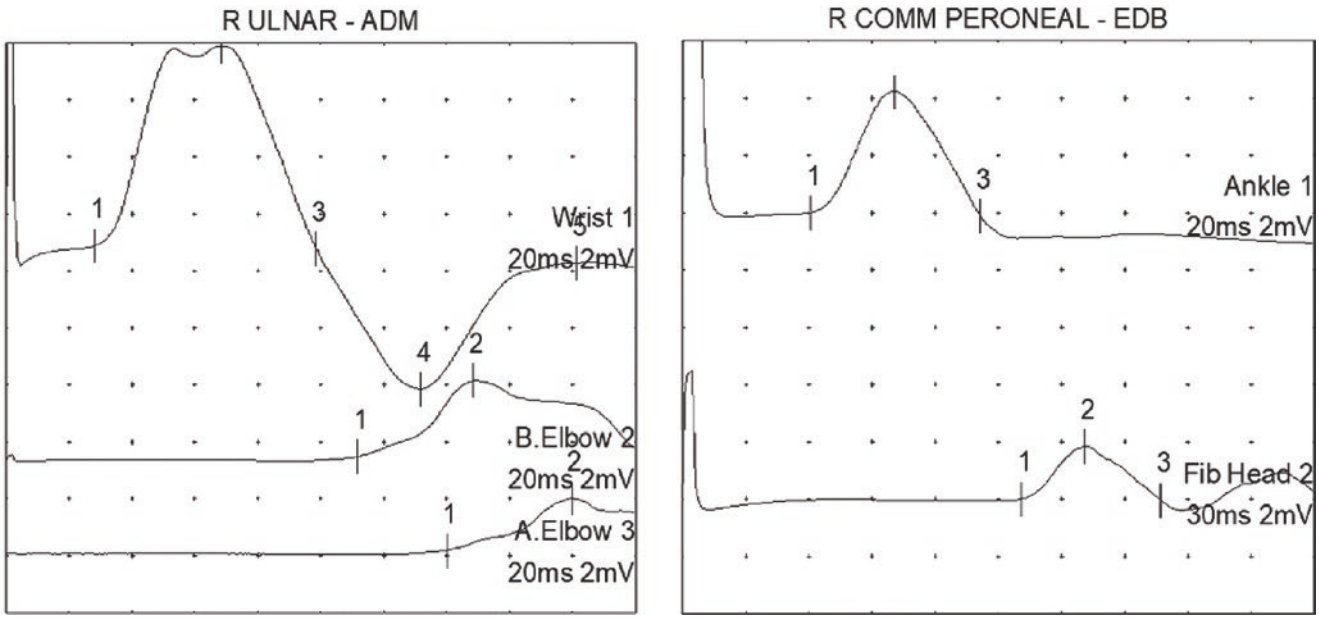

Fig. 10.6 Motor conduction block in an ulnar nerve and common peroneal nerve. Ulnar nerve conduction velocity: 27.6 m/s and 38.4 m/s; peroneal nerve conduction velocity: 27.4 m/s

should be considered in rapidly progressive or treatment-refractory neuropathy, in multifocal cases, or when vasculitis, amyloidosis or a neoplastic process is suspected. Both forms of typical CIDP benefit from IVIG, corticosteroids and plasma exchange. Subcutaneous immunoglobulin treatment is also approved for CIDP treatment by the FDA.

CIDP Variants

The European Academy of Neurology/Peripheral Nerve Society published a revision of the 2010 guidelines for the diagnosis and treatment of CIDP. The use of the term 'CIDP variant' is proposed instead of the term 'atypical CIDP'. CIDP variants consist of distal CIDP, multifocal CIDP, focal CIDP, motor CIDP and sensory CIDP [79].

Distal CIDP, also called **distal acquired demyelinating symmetric neuropathy** (**DADS**), is a CIDP variant which often presents with weakness and sensory deficits which are symmetrical and more pronounced in the distal parts of limbs [79]. Gait instability due to ataxia and action tremor are other symptoms of distal CIDP. Nerve conduction studies show disproportionate prolongation of distal motor latencies compared to nerve conduction slowing. The terminal latency index is equal to or less than 0.25 in motor nerves. A lack of conduction block and absent sural nerve sensory potentials are noteworthy differences [77]. In this group of patients, monoclonal gammopathy of undetermined significance (MGUS) may be present with IgA, IgG or, more commonly, IgM isotypes of immunoglobulins. Around 50% of patients with IgM monoclonal gammopathy have antibodies against myelin-associated glycoprotein (MAG) [76]. It is suggested that DADS with anti-MAG antibodies lies outside of the CIDP spectrum as there is no response to IVIG, corticosteroids or plasma exchange. It is reported that around 50% of anti-MAG positive patients are responsive to rituximab in some series [83]. Monoclonal gammopathy may be associated with a haematological malignancy in some patients with distal symmetric polyneuropathy [76]. Distal CIDP patients without anti-MAG antibodies have a more favourable response to classical CIDP treatments [76, 79, 84].

Multifocal CIDP is also known as many other names such as Lewis-Sumner syndrome, multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) and multifocal demyelinating neuropathy with persistent conduction block [79]. In their original article, Lewis and Sumner reported the findings of five cases with asymmetric sensorimotor neuropathy having a chronic course [84]. Findings were more prominent in the upper limbs with focal nerve involvement. Electrophysiologic manifestations of persistent multifocal conduction block were present. Histopathological examinations of sural nerve biopsies revealed primary demyelinating disorders. Two of five patients had subacute optic neuritis. Two patients were treated with prednisone. Those patients showed improvement with the corticosteroid; however they relapsed when doses were reduced. Untreated patients were reported to have progressed. They suggested that chronic multifocal demyelinating neuropathy with persistent conduction block seems to be a variant of chronic acquired demyelinating polyneuropathy and it may be immunologically mediated [84]. Furthermore, 16 patients with similar characteristics were later reported, and beneficial effects of IVIG treatment were noted. The term MADSAM was proposed to point out

the differences between multifocal motor neuropathies. The definition of multifocal CIDP variant is almost the same except for the name in the European Academy of Neurology/Peripheral Nerve Society guideline on the diagnosis and treatment of chronic inflammatory demyelinating polyradiculoneuropathy [85].

Focal CIDP is rare and usually affects the brachial or lumbosacral plexus but can affect individual peripheral nerves as well [85].

Motor CIDP affects motor nerves in a rather symmetrical fashion with proximal and distal weakness mostly in young males. Clinical and electrophysiological examination show no sensory abnormalities. If two or more nerves have low sensory nerve action potential amplitudes in electrophysiology studies while clinical findings suggest motor CIDP, then it is called motor-predominant CIDP. The most common electrophysiology findings are F-wave abnormalities and conduction blocks. IVIG treatment has beneficial effects, while corticosteroid treatment in motor CIDP is a controversial subject. Patients with motor CIDP may get worse with corticosteroid therapy, but motor predominant cases may benefit. On the other hand, some reports suggest using corticosteroids in IVIG-resistant cases [86].

Sensory CIDP is characterised by the damage of sensory fibres which results in an impairment of the vibration, position and cutaneous senses. An ataxic gait is observed due to the loss of proprioceptive senses. As the name implies, muscle weakness is not an expected finding in sensory CIDP [85]. However, in some cases, motor nerve conduction studies reveal slow velocity and a conduction block. The term sensory-dominant CIDP is used for this type. Treatment with IVIG and corticosteroids yields results similar to typical CIDP [87, 88].

Disorders Not Classified as CIDP

Patients who have clinically suspected CIDP with normal nerve conduction examinations may have chronic immune sensory polyradiculopathy (CISP). When the dorsal spinal roots that are proximal to the dorsal root ganglia are damaged, neurons in the dorsal root ganglia remain intact [85]. Therefore, sensory nerve conduction velocity and SNAPs remain normal. Patients who meet the criteria for CIDP may have IgG4 antibodies against nodal NF140/186 and paranodal proteins such as neurofascin isoforms, neurofascin-155 (NF155), contactin-1 (CNTN1) and contactin-associated protein 1 (CASPR1) [74, 79]. The organisation of myelinated axons allows the transmission of impulses in a saltatory fashion, saving energy. Ranvier nodes with paranodal regions are an important part of this organisation. The site of damage in nodal and paranodal neuropathies is at the paranodal section of myelinated axons [74]. Due to the absence of inflammation and true demyelination, the group with IgG4 antibodies is excluded from CIDP classification [74, 79]. Patients who have antibodies often present with specific clinical features. Corticosteroids have a partial effect, while response to IVIG is poor. Treatment with rituximab and plasma exchange appear to be more effective [74, 89]. Different pathophysiological mechanisms may underly the different responses to treatments [74, 79, 87]. The term autoimmune nodopathies is proposed for these conditions [85]. Nodal and paranodal antibodies are also mentioned in the acute inflammatory neuropathies section.

CIDP Diagnosis

There are numerous proposed diagnostic criteria for CIDP [90]. The European Academy of Neurology/Peripheral Nerve Society published a guideline on the diagnosis and treatment of CIDP in 2021. This guideline is a comprehensive and useful tool for the diagnosis, classification and treatment of CIDP [79]. The complexity of neuropathic disorders forces clinicians to use the guidelines. In this section, diagnostic parameters are briefly discussed. Clinical, electrodiagnostic and supportive criteria are defined as well as criteria for immunological testing in patients with clinically suspected CIDP. A recent proposal suggests clinically classifying CIDP as typical CIDP and CIDP variants. Electrodiagnostic certainty of CIDP is defined as CIDP and possible CIDP. Previously defined definite and probable CIDP criteria are found to be neither sensitive nor specific enough to differentiate the two types [79, 81]. Clinical diagnosis of typical CIDP requires the presence of all the symptoms listed which includes distal and proximal involvement of both the upper and lower limbs in progressive or relapsing forms, in addition to sensory impairment in at least two limbs, symptom evolution for at least 8 weeks and absence or reduction of tendon reflexes in all limbs [79]. Electrophysiological findings of demyelinating lesions include motor conduction velocity slowing, increased distal motor latency, increased F wave latency, partial motor conduction block, increased distal compound muscle activity potential duration and reduced SNAP amplitude/increased latency SNAP/decreased sensory nerve conduction velocity. Different combinations of these abnormalities, out of certain defined limits, are sought during diagnosis. Supportive criteria include CSF analysis, nerve biopsy and imaging of the nerve's radices using MRI and ultrasound techniques [77, 79].

Differential Diagnosis

Clinical and electrophysiological findings of CIDP may be similar to findings of genetic neuropathies such as CMT type 1 or transthyretin familial amyloid polyneuropathy. The haematological malignancies, diabetes mellitus and recently described nodopathies and paranodopathies are also impor-

Table 10.7 Recommended diagnostic tests for the differential diagnosis of CIDP

Complete blood count	Antinuclear antibodies
Fasting glucose, Haemoglobin A1c (HbA1c)	Skeletal surveys
	X-ray or scintigraphy
	Monoclonal gammopathy in serum and urine
Electrolytes	• Electrophoresis
Liver function, renal function	• Immunofixation
Vitamin B12	• Free light chain analysis
Thyroid function	Genetic
HIV	
Neuroborreliosis	

tant diagnoses which should be excluded to achieve an optimum treatment response. Vitamin B toxicity and chemotherapeutic agents may cause CIDP-like features, and so exposure to them should be questioned during history taking. CSF examination results with elevated protein levels and a normal leucocyte count support CIDP diagnosis. Radiological evidence of enlarged nerves and nerve roots are also helpful findings (Table 10.7). In the presence of atypical findings for CIDP such as prominent pain, tremor, autonomic dysfunction ataxia or muscle atrophy at the onset of disease, painless injuries and respiratory muscle involvement CIDP diagnosis should be reevaluated [91].

Treatment

The aim of treatment for CIDP is to stop the immune attack of peripheral nerves which causes demyelination and secondary axonal degeneration. If early and effective treatments are administered, symptoms and long-term prognosis improve. Treatment decisions are made according to symptom severity and disease course. The presence of disabling symptoms which affects quality of life is a predictive factor for treatment. Severe symptoms and findings are difficult to manage. Switches between the treatment types and combination therapies may be required before reaching an optimum treatment point. After symptoms stabilise, the target of treatment is then sustained improvement and further remission. In difficult cases, referral to a neuromuscular specialist may be beneficial for treatment optimisation. As a first step, corticosteroids and IVIG treatment are considered for typical CIDP and CIDP variants. Plasma exchange is also effective; however it is more difficult to administer and requires an intensive care unit. Patients may not tolerate this treatment. Subcutaneous immunoglobulin treatment is approved for maintenance therapy by the FDA. Additionally, some immunosuppressive drugs may be used; however there is no evidence-based information detailing the appropriate drugs or doses that can be used. Symptomatic treatment for pain and motor impairments are also important factors for improving quality of life. Each treatment has its own pros and cons. Both availability and patients' characteristics are important factors that influence the choice of treatment. Treatments can be evaluated as induction therapies and maintenance therapies. Glucocorticoids are more effective at accomplishing long-term remission. However, there is not yet a consensus about the best corticosteroid regimen [79, 92]. Other treatments like azathioprine, mycophenolate mofetil and ciclosporin are suggested as IVIG or glucocorticoid sparing agents for maintenance treatment. Patients who are resistant to the abovementioned proven effective treatments can be treated with rituximab, ciclosporin or cyclophosphamide [79, 92, 93].

The prognosis of CIDP is variable. Most patients respond to any of the standard treatments; however 10–15% of patients are refractory to IVIG, glucocorticoids and plasma exchange. The cure or remission can be accomplished in about 30% of patients [94].

Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) was first described by Guillain, Barré and Strohl in 1916. Previously, Landry published an article regarding ascending paralysis but did not mention the absence of tendon reflexes and albuminocytologic dissociation. GBS is the most frequent reason of acute flaccid paralysis. Most epidemiological information is gathered from developed countries, and therefore, data from other parts of the world are lacking. GBS has a range of incidence of 1.81–1.91/100,000 person years. Incidence increases with age, and males are more susceptible than females (M/F = 1.5). Usually, an antecedent infection is reported before acute limb weakness develops which may progress for up to 4 weeks until reaching a plateau phase. Sensory symptoms may precede motor symptoms; however electrophysiological evidence of sensory involvement is less than that of motor findings. Weakness usually follows an ascending route, but it can start in the proximal muscles. The need for mechanical ventilation arises in approximately 25–30% of patients due to respiratory muscle weakness [95–97]. The most common infection is gastroenteritis due to *C. jejuni.* Investigations disclosed molecular mimicry occurring between nerve structures and antigens of bacteria in *C. jejuni*-related GBS. Zika virus-associated GBS and recently described severe acute respiratory syndrome corona virus-2 (SARS-CoV-2)-related GBS cases are also reported. Determining a clinical diagnosis of GBS is not difficult when classical findings are present, which consist of ascending paralysis and areflexia, and additionally albuminocytologic dissociation is present with an infection history of 2 weeks prior. Although historically, GBS is a single disorder, but now several variant forms have been reported (Table 10.8). The presence of variant forms and overlap between different forms causes a diagnostic challenge. Treatment with IVIG

Table 10.8 Guillain-Barré syndrome variants

Acute inflammatory demyelinating polyneuropathy	*Rare forms*
Acute axonal neuropathies	Acute bulbar palsy
Acute motor axonal neuropathy (AMAN)	Facial diplegia and distal limb paraesthesia
Acute motor and sensory axonal neuropathy (AMSAN)	Pure sensory GBS
GQ1b syndromes	Paraparesis
Miller Fisher syndrome (MFS)	Acute pandysautonomia
Bickerstaff brainstem encephalitis (BBE)	

and plasma exchange improves the prognosis of GBS, and rates of 5% mortality and 20% severe disability are reported. Therefore, it is important to recognise the large spectrum of clinical patterns which constitute GBS for correct and timely diagnosis.

Variant Forms of Guillain-Barré Syndrome

Acute inflammatory demyelinating polyneuropathy (**AIDP**) is the most frequently seen form of GBS which constitutes around 85–90% of cases. During the Zika epidemic, an increased incidence of AIDP was observed in French Polynesia and South America. Even though no causative antibodies have been identified in infection-related AIDP, molecular mimicry between the viruses and human proteins is suggested in recent studies as an underlying mechanism [98]. Classically, AIDP presents with progressive, ascending and symmetrical muscle weakness associated with reduced or absent deep tendon reflexes. Electrophysiological evidence of demyelination such as slow NCV, prolonged distal motor latency and partial conduction block are characteristic findings. However, in the early phase, an absence of F waves or the H-reflex may be the only pathological findings. When peripheral nerve examinations are normal, lumbar root stimulation is a useful method to identify proximal demyelinating lesions which cannot be demonstrated with standard nerve conduction studies [82]. Reduced recruitment activity may be the sole needle EMG finding in the early phase. If secondary axonal degeneration occurs, spontaneous activity with reduced recruitment is observed.

Acute axonal neuropathies consist of **acute motor axonal neuropathy** (**AMAN**) and **acute motor and sensory axonal neuropathy** (**AMSAN**). These primary axonal forms are often encountered in China, Japan and Mexico. In Western countries, axonal forms comprise approximately 5–10% of GBS cases [95, 99]. AMAN was first reported in 1986. AMAN particularly affects young people following *C. jejuni* infection. As the name AMAN implies, in this axonal form of GBS, motor nerves are affected, while sensory nerves remain intact. In some patients, deep tendon reflexes may be spared or even increased. An electrophysiological study demonstrated motor neuron excitability due to dysfunction of the intraneuronal inhibitory circuits in the spinal cord. The proposed mechanism of the dysfunction is immune-mediated damage of the spinal inhibitory intraneuronal network [100]. This finding is associated with *C. jejuni* infection [101]. Although, an absence or decrease of deep tendon reflexes is required for GBS diagnosis, this group of patients may be an exception. Hyperreflexia can be seen in patients with AMAN which is possibly a nodopathy [101]. Electrophysiological findings of AMAN include decreased compound muscle action potentials (CMAPs) with slightly slow or normal motor NCVs. Needle EMG shows reduced recruitment patterns and spontaneous activity approximately 3 weeks after disease onset. Antibodies against the gangliosides GM1, GD1a, GalNac-GD1a and GD1b are associated with the development of AMAN. Molecular mimicry may be the underlying mechanism responsible for the production of antibodies against gangliosides. Antibodies and complement activity cause axonal damage with variable severity or reversible conduction block at the nodal/paranodal parts of the nodes of Ranvier. Both abnormalities can be present on the same nerve. In some patients, conduction block can be resolved rapidly without causing axonal damage. This temporary conduction block is called reversible conduction failure (RCF) which possibly occurs due to loss of voltage-gated sodium channels. RCF is a different condition than classical demyelinating conduction block. It is a part of a nodo-/paranodopathy concept [74].

AMSAN affects both sensory and motor nerves with prominent axonal degeneration. Clinically, AMSAN is like AMAN; however, it is more severe and has a delayed and incomplete recovery with sensory involvement. GM1, GD1a, GalNac-GD1a and GD1b antibodies are involved in the pathophysiological process. Electrophysiological investigations demonstrate axonal damage with low or absent CMAPs and SNAP amplitudes. NCVs are slightly low or near normal. Due to axonal degeneration, active denervation potentials and reduced recruitment activity are demonstrated by needle EMG examination [95].

GQ1b syndromes are forms of GBS which are frequently associated with antibodies against GQ1b. The external ocular muscles, muscle spindles and most likely the reticular formation in the brainstem contains GQ1b antigens. In susceptible patients, infection with microorganisms with GQ1b epitopes may stimulate anti-GQ1b antibodies which result in a continuous spectrum. **Miller Fisher syndrome** (**MFS**), **Bickerstaff brainstem encephalitis** (**BBE**) and pharyngeal-cervical-brachial (PCB) variants are included in this spectrum [102]. *Haemophilus influenzae* and *C. jejuni* infection is frequent before GQ1b syndromes. It is reported that MFS and BBE relapse more frequently than other forms of GBS [103]. Relapsing MFS may be associated with HLADR2 positivity [104]. MFS is characterised by a triad of ophthal-

moplegia, ataxia and areflexia which was first described by Fisher in three male patients in 1956 [105]. MFS is more frequent in Asia and occurs in around 20% of the cases in Asia and in 5–10% of the cases in the United States and Europe. In addition to the characteristic triad, approximately 25% of cases will have some limb weakness during the disease. Autonomic involvement of the pupil is present, with fixed dilated pupils in some patients. Incomplete forms are reported such as ophthalmoplegia without ataxia, ophthalmoplegia and ptosis or ataxia without ophthalmoplegia [106]. Unilateral ophthalmoplegia is also a reported finding. Approximately 80–90% of patients have GQ1b antibodies. Electrophysiological examinations may demonstrate absent SNAPs or reduced SNAP amplitudes without slowing of sensory NCVs which indicates sensory neuronopathy. Motor NCVs are normal in patients without paresis, but abnormalities compatible with axonal involvement can be found in patients with muscle weakness [104]. MFS prognosis is quite favourable in general since it is usually self-limiting and patients recover fully by 6 months [107].

BBE presents with ataxia, ophthalmoplegia and encephalopathy with GQ1b positivity. Altered consciousness in patients with BBE suggests involvement of the ascendant reticular-activating system. The blood-brain barrier (BBB) prevents the entry of large molecules into the brain parenchyma. However, there are some areas like the area postrema where the BBB is relatively more permeable for large molecules. Even though it has not been proven experimentally, there is a possibility that anti-GQ1b antibodies travel through the BBB at this site. In addition, facial weakness, bulbar symptoms and pupillary abnormalities can be found. According to a review of 53 BBE patients, mild limb weakness was found almost in half of the patients, and deep tendon reflexes were normal or brisk in approximately 40% of the patients. The treatment of BBE is like that of classical GBS including IVIG and plasmapheresis. **Rare variants** are also reported such as paraparesis, acute pandysautonomia, pure sensory GBS, facial diplegia and distal limb paraesthesia and acute bulbar palsy [95, 96, 108].

Differential diagnosis is relatively easy when characteristic findings are present. However, a long list of other diagnoses must be considered depending on the subtypes, patient characteristics and geographical localisation. Spinal cord and cranium imaging help to differentiate central nervous system disorders. Electrodiagnostic studies are used for excluding acute myopathies, neuromuscular junction diseases, anterior horn diseases and polyradiculopathic conditions. A change in the state of consciousness is not an expected finding in GBS, except for BBE. A severe neurological deficit that persists without a significant recovery or relapsing pattern suggests another diagnosis other than GBS. On the other hand, sural sparing is in favour of GBS and helps to discriminate GBS from other neuropathies [109, 110]. Treatment with IVIG and plasmapheresis is effective, and studies show no significant difference regarding their effectiveness. Patient characteristics and availability are the factors which affect the choice of treatment [111].

All About the Pathology of Neuropathies

Neuropathy Muscle Biopsy Findings

Muscle biopsy is a method that is used more frequently than nerve biopsy because it is easier to sample and has much less morbidity. Especially in hereditary neuropathies, diagnosing a significant proportion of the cases with genetic examination has greatly reduced the need for nerve biopsy examination. During the course of many diseases that can cause neuropathy, particularly diabetes or systemic vasculitis, nerve biopsy is not often considered necessary. However, for certain diseases, especially for localised vasculitis in the nerves, nerve biopsy examination may be deemed essential. Simultaneous muscle biopsy is recommended during nerve biopsy examination. Muscle biopsy examination is also not uncommon for the differential diagnosis of myopathy in some cases where muscle or nerve involvement cannot be clearly differentiated. Regardless of the indication, it is possible to diagnose any disease that affects nerves from a muscle biopsy specimen because maintaining the nerve supply is directly related to the health of the muscle fibres. The detrimental dysvoluminal alterations in muscle fibres will result from any disruption of these neurotrophic influences. Neuropathies affect the muscles innervated by the involved nerves, and some of these changes are pathognomonic for neuropathies. These myopathies due to neurogenic diseases are generally classified as neurogenic myopathies or neurogenic (denervation) atrophy of the muscle. Neurogenic atrophy may stem from diseases of the anterior horn cell or its myelinated axons [112–114].

The pathological effects of denervation are generally the same in almost all neurogenic atrophies except in SMA type 1. One of the most important diagnostic findings of neurogenic myopathies is the unusual clustering of cell nuclei called nuclear clumping. The presence of nuclear clumping alone in a muscle biopsy suggests a disease of neurogenic origin (Fig. 10.7). Except for small clusters of specialised nuclei at the neuromuscular junction, each skeletal muscle fibre includes hundreds of nuclei just below the plasma membrane scattered along the fibre. Like all other cell types, skeletal muscle fibres have dynamically controlled nuclear positions in both space and time. Nuclear movements are mediated by the cytoskeleton, which transfers pushing or pulling forces onto the nuclear membrane. The cytoskeleton plays a major role in this activity, and various molecular connections between the nuclear membrane and cytoplasmic

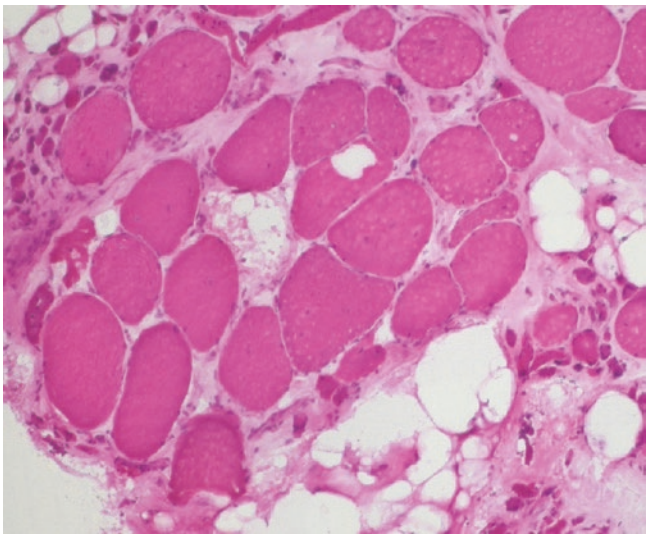

Fig. 10.7 Nuclear clumping in neurogenic myopathy (H&E × 100)

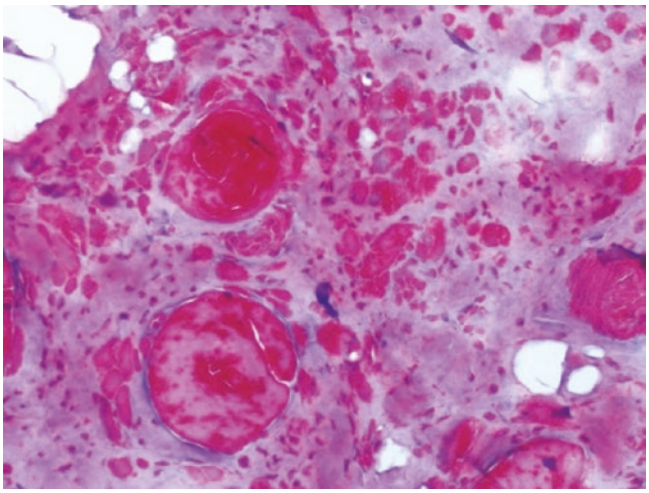

Fig. 10.8 Fibrosis in a later phase of neurogenic myopathy (Masson's trichrome × 200)

components such as actin and other intermediary filaments have been found for various cell types. The mechanisms governing nuclear localisation inside cells have been partly identified in recent studies. However, it is not yet understood exactly how nuclei arrive at positions along fibres of skeletal muscle. Recent studies show that nuclei preferentially localise near blood vessels, especially in slow-twitch-oxidised fibres. In addition, it has been determined that desmin deficiency significantly alters the distribution of nuclei along fibres but does not prevent their close association with vessels. Consistent with the role of desmin in nuclear spacing, denervation has been shown to affect desmin filament organisation and nuclear distribution [115–117].

Denervation is an important cause of nuclear misplacement. During permanent denervation, up to the 90% of severely atrophic muscle fibres present a reorganisation of their nuclear distribution. The permanent injury of lower motor neurons causes skeletal muscle fibre atrophy which mainly occurs during the first few weeks or months following injury. In the later phases of denervation, muscle fibre atrophy consistently progresses. Still, many severely atrophied muscle fibres remain present in the denervated muscle at this late stage, some of which have lost all contractile proteins and the helical distribution of myonuclei, which are aggregated in the core of the muscle fibre (nuclear clumps). Adipocytes and collagen sheets fill the empty areas of muscle tissues, and finally fibrosis replaces muscle fibres (Fig. 10.8). Nuclear clumping, a pathognomonic change due to denervation, becomes increasingly evident, especially in the later stages of denervation. In the first months, or more so in the later months of the first year, there may be a mild myopathic appearance during the histopathological examination of muscle [115].

In cross-sections of muscle biopsy, normal muscle cells are polygonal in shape and similar in size. Each muscle fibre is surrounded by a very thin layer of connective tissue called the endomysium that cannot be discriminated without special connective tissue staining such as trichrome staining. Muscle fibres usually become irregular in size and shape during any neuromuscular disease. After repetitive injuries, the size and shape of the muscle fibres change. However, in the neuropathies, sizes of muscle fibres vary greatly. A few fibres remain as almost normal size, while most fibres are very small, with some fibres disappearing, leaving only the nuclear clumps behind [112–114].

Some atrophic fibres are angular in shape, and these angular fibres are very specific for neurogenic myopathies (Fig. 10.9). During acute denervation, atrophic fibres randomly scatter. At this stage, small fibres are flattened and angular, and most of them can be glycolytic in type (type 2). If the denervation continues, the population of type 1 and type 2 fibres becomes a more irregular mixture. Advanced denervation demonstrates a pattern of atrophy that progresses from a random distribution to a grouping of affected fibres. Rarely, some atrophic fibres appear as regenerated fibres with more basophilic cytoplasm and internally located nuclei (Fig. 10.10). In addition, there is no inflammatory infiltration like that of the muscular dystrophies. In the early phases of neuropathies, muscle biopsy findings are like that of a noninflammatory myopathy. In these phases, histopathological differential diagnosis may be impossible, as the differences muscle fibres' sizes are minimal (Fig. 10.11), and there are no/scarce nuclear clumps or angulated fibres. On the other hand, advanced fibrosis observed in a muscle biopsy in late-stage neuropathies can be interpreted as the last stage of muscular dystrophy. In this situation, the occasional pres-

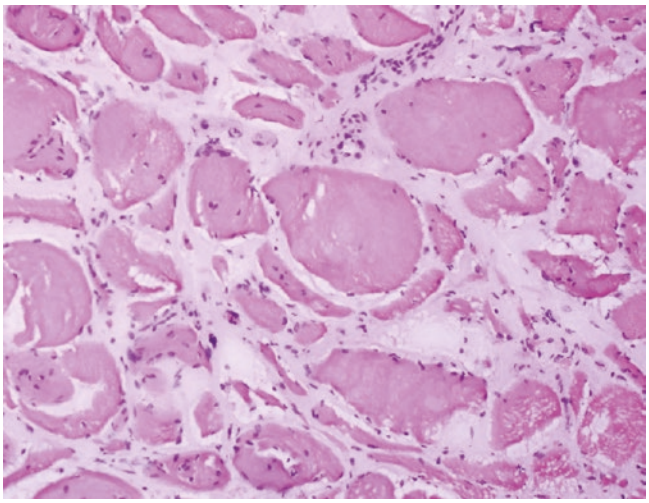

Fig. 10.9 Atrophic fibres with angular shape in neurogenic myopathy (H&E × 100)

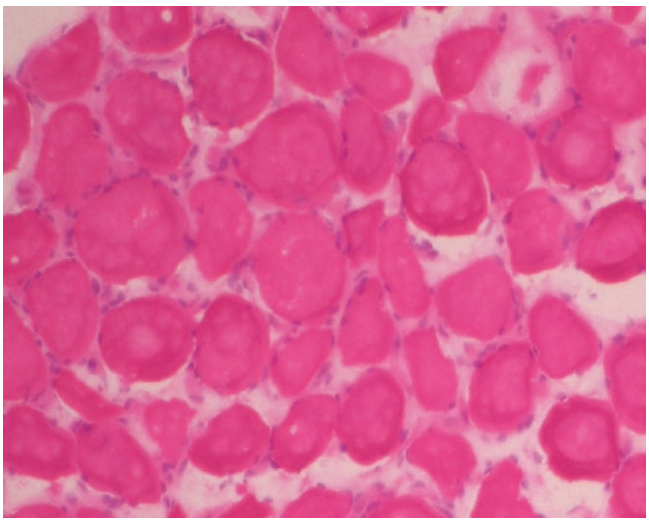

Fig. 10.11 Mild myopathic appearance in a muscle biopsy of a 19-year-old male with vasculitic neuropathy (H&E × 200)

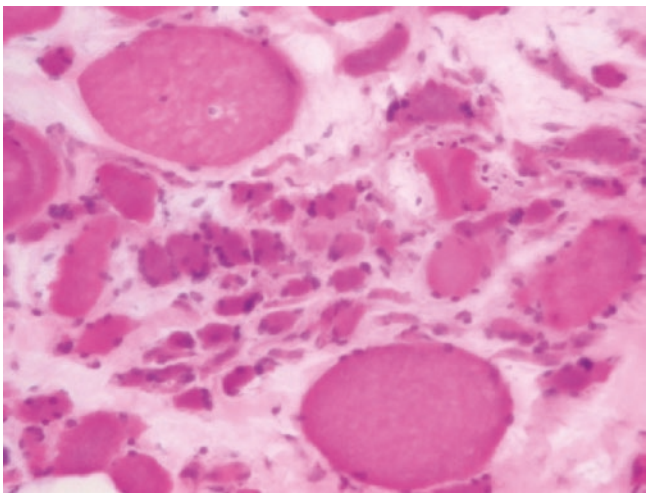

Fig. 10.10 Some atrophic fibres resemble regenerated fibres with more basophilic sarcoplasm (H&E × 200)

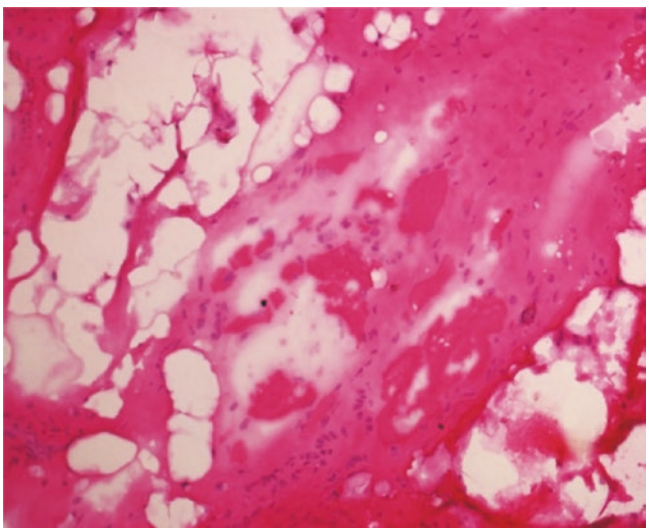

Fig. 10.12 Small atrophic fibres between the adipose and fibrous tissues in a muscle biopsy of a 23-year-old male patient with KIF5A gene variation (H&E × 100)

ence of muscle fibres with almost normal size is in favour of neuropathy. For example, a 23-year-old male patient, who started at 18 years of age and had proximal muscle weakness more prominent in the lower extremities, had creatine kinase levels around 4000 IU/L. Clinically, muscular dystrophy was suspected, and genetic testing was completed. For Becker muscular dystrophy and limb-girdle muscular dystrophies, there were no genetic abnormalities. In the neuromuscular disease panel, a heterozygous c.2263G > A (p.Glu755Lys) variant was detected in the kinesin family member 5A (KIF5A) gene localised on chromosome 12q.13, which was associated with spastic paraplegia type 10 and Charcot-Marie-Tooth type 2 diseases. The same variant was also present in the heterozygous form in his older brother, who had signs of proximal myopathy. The patient had been evaluated as having end-stage muscular dystrophy due to the presence of severe atrophy and interstitial fibrosis in the muscle biopsy (Fig. 10.12). However, when the biopsy was re-examined in the light of genetic tests, it was decided that it would be more accurate to evaluate the pathology as neurogenic muscle atrophy due to the presence of myofibers that remained relatively unchanged and almost normal in size (Fig. 10.13) [112–114].

However, angular-shaped atrophic myofibers are not seen in infantile denervation, and almost all muscle fibres are round. In these cases, the most pathognomonic appearance in the differential diagnosis is the presence of muscle cell groups composed of larger myofibers between small round atrophic muscle fibres. Immunohistochemical analysis of

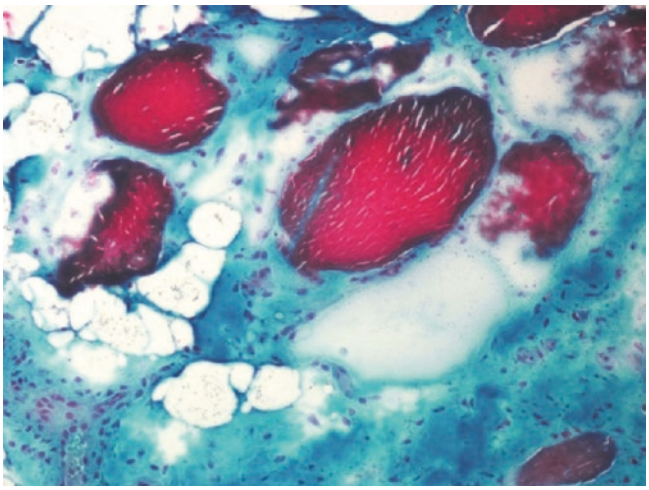

Fig. 10.13 There are normal-sized myofibers with atrophic ones in a patient with KIF5A gene variation (Gomori's trichrome × 200)

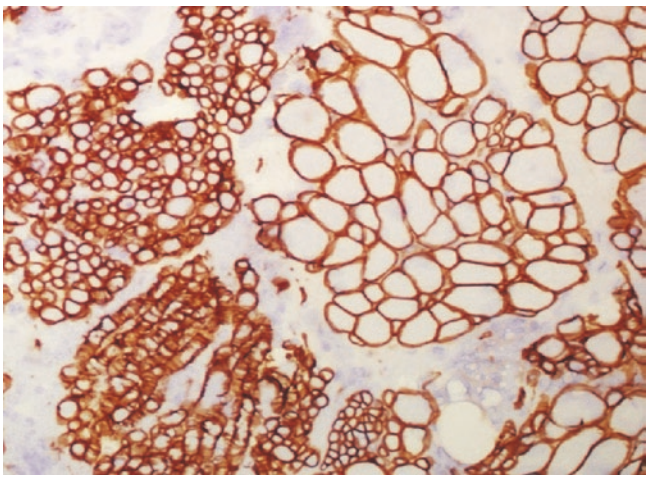

Fig. 10.14 Myofiber groups consist of small or large round fibres which are specific for infantile denervation. Severe size differences of myofibers are highlighted by sarcolemmal merosin expression (DAB × 100)

sarcolemmal proteins enables us to better select cell groups with significant size differences (Fig. 10.14). In summary, denervation atrophy of muscle fibres can be defined by the presence of atrophic fibres with an angular shape, significantly decreased myofiber size and markedly increased myofibrillary disorganisation highlighted with nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) staining (Fig. 10.15). The occurrence of target fibres due to myofibrillary disorganisation is suggestive of reinnervation [118]. The target fibres which have a central pallor area surrounded by an internal very darkly stained rim and external normal-stained sarcoplasm are seen in about 20–30% of cases with neurogenic myopathy. They are more common in peripheral neuropathies than in amyotrophic lateral sclerosis (ALS). Fibres which have a central pallor area without a darkly stained rim are referred to as targetoid fibres [112–114].

Neurogenic myopathies are generally seen in diseases affecting lower motor neurons or peripheral nerves. Before deciding whether to undertake a muscle biopsy, neurogenic disorders can be diagnosed by the presence of sensory deficiency, a distal-dominant distribution of muscle weakness and from the results of electrophysiological tests. In this situation, a muscle biopsy is not performed. However, it can be challenging to distinguish between neurogenic and myopathic weaknesses. During the early denervation phases, there is random atrophy of both fibre types. The atrophic angulated fibres can also be seen as type 1 or type 2 fibres with oxidative enzyme stains except for the NADH-TR because all the atrophied fibres, regardless of the type, are stained darkly like type 1 fibres by NADH-TR stain [113]. During peripheral neuropathies, type 2 fibres can be more atrophied, and atrophic fibres can become angulated in muscle biopsy. The most striking finding of neurogenic changes in muscle is fibre-type grouping, where the same type of muscle fibres group together (Fig. 10.16). Repeated cycles of denervation and reinnervation cause the grouping of atrophied myofibers in neuropathies (Fig. 10.17). Fibre-type grouping is present if more than nine consequent myofibers of the same fibre type are seen together (Fig. 10.18). The formation of groups of atrophied myofibers is one of the most specific changes of denervation. Examples include inherited or chronic polyneuropathies, like Charcot-Marie-Tooth disease, and motor neuron illnesses, like spinal muscular atrophy and amyotrophic lateral sclerosis. Due to reinnervation from the collateral sprouting of surviving axons after denervation, the typical checkerboard pattern of muscle fibre types is lost [114–117].

If neurogenic diseases are chronic and long-standing, myofiber hypertrophy is also seen in the muscle biopsy. It is thought that these hypertrophic fibres represent a compensatory reaction in response to the inefficiency of the atrophied fibres. As this process is not type selective, hypertrophic fibres may be oxidative or glycolytic. The muscle may also exhibit fibre splitting and even necrotic fibres after long-standing denervation. This appearance may create a pseudo-myopathic image. Very little muscle may be seen in a biopsy when there is advanced neurogenic atrophy. The tiny, undetectable bundles of atrophic fibres may be found mingled with adipose tissue (Fig. 10.19). These advanced atrophic myofibers, which cannot be seen in the fibroadipose tissue, can be identified with special dyes (Fig. 10.20) [114–119].

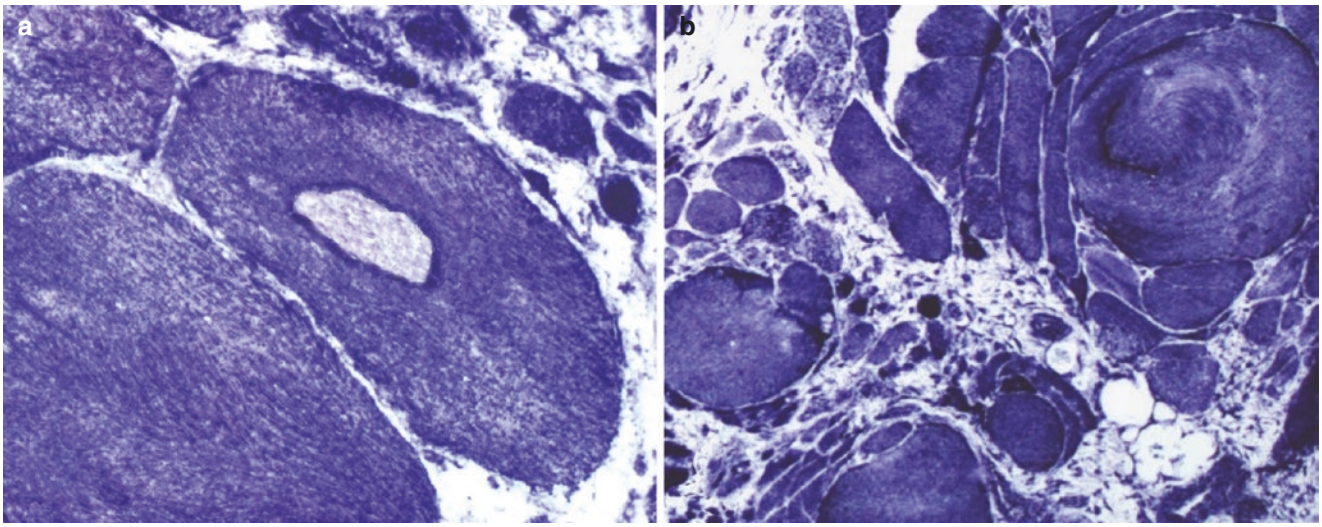

Fig. 10.15 Myofibrillary disorganisation is suggestive of reinnervation. (**a**) Target fibre, (**b**) spiral-like appearance in a fibre due to myofibrillary disorganisation

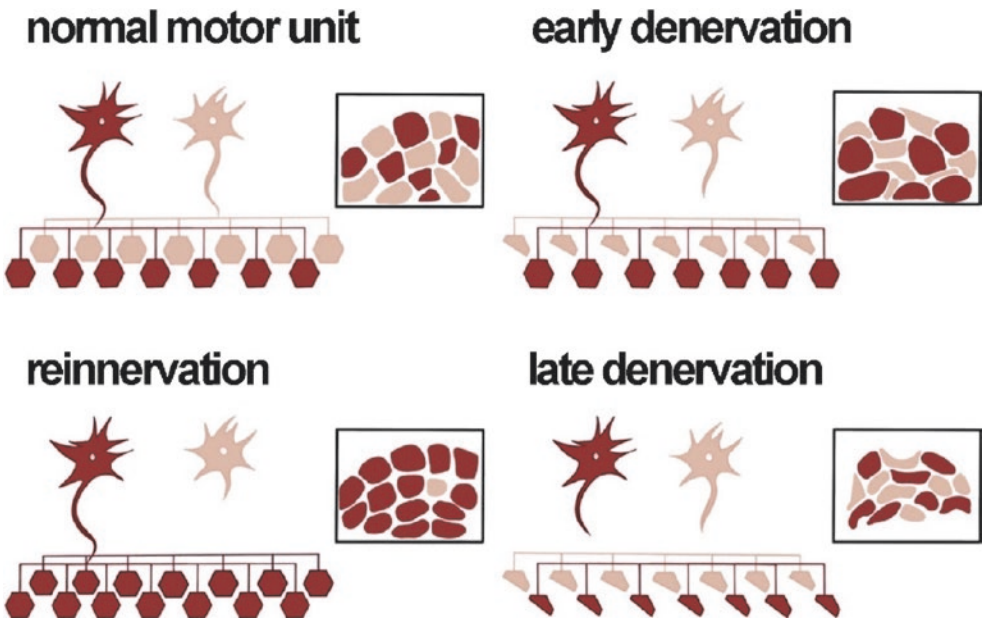

Fig. 10.16 Schematic of the typical motor unit and three steps of denervation are shown

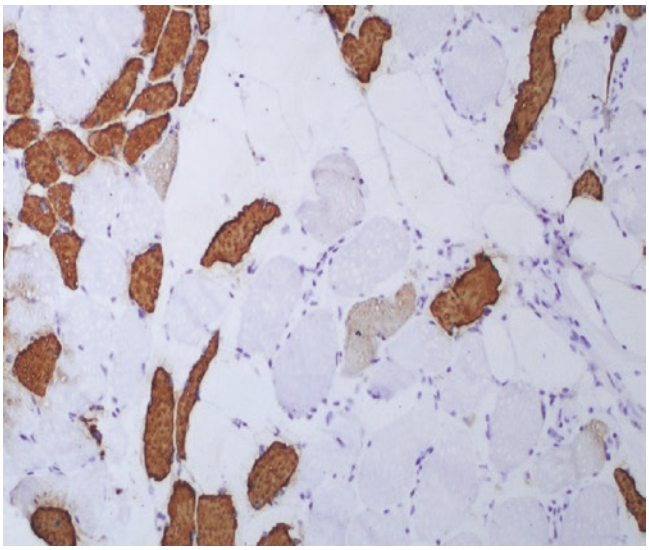

Fig. 10.17 Myofiber grouping with fast myosin staining is suggestive of neurogenic myopathy (DAB × 100)

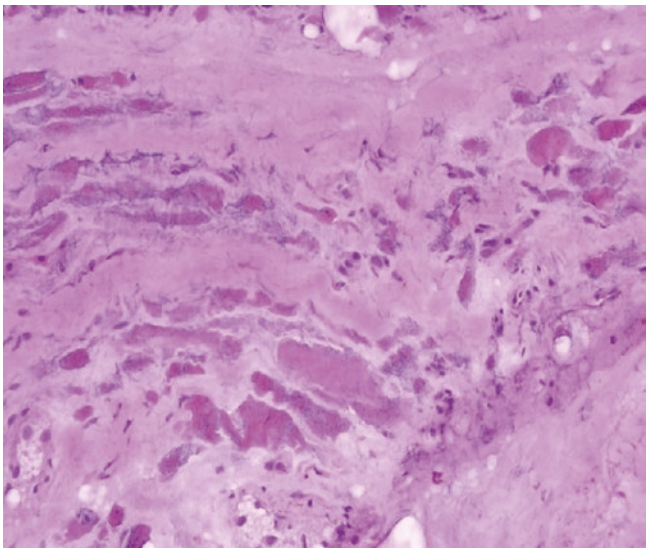

Fig. 10.19 Unnoticeable atrophic fibres among fibroadipose tissue (H&E × 100)

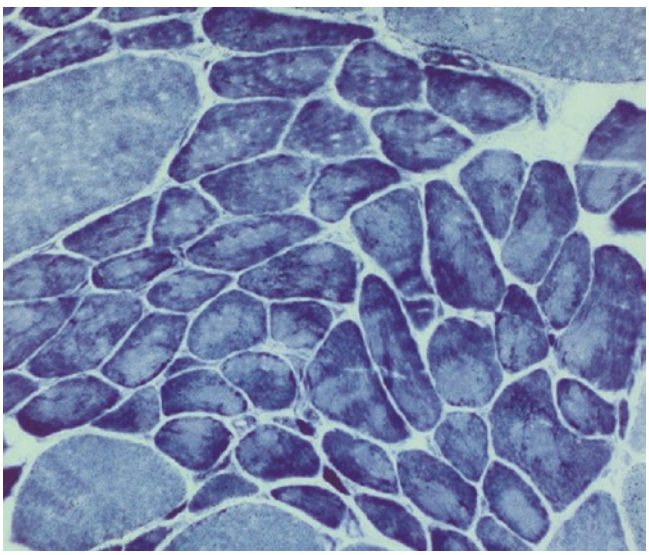

Fig. 10.18 Myofiber grouping can be highlighted with enzyme staining (NADH-TR × 200)

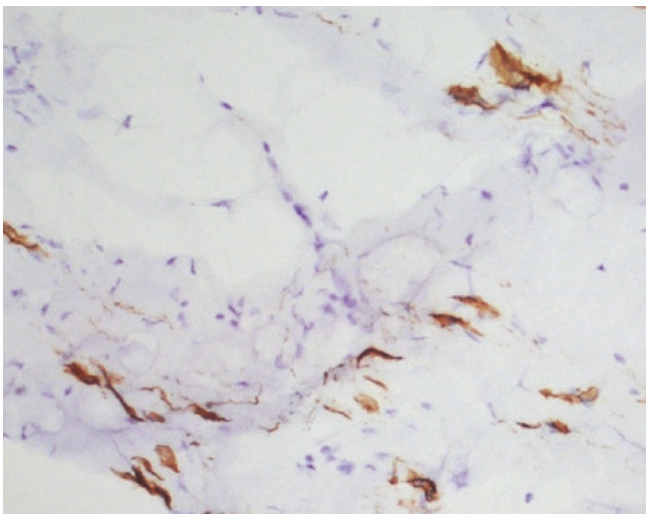

Fig. 10.20 Atrophied fibres are highlighted with immunohistochemical staining with anti-alpha sarcoglycan antibody (DAB × 100)

Neuropathy Nerve Biopsy Findings

It is not uncommon for inflammatory processes which occur in peripheral nerves to cause difficulty in reaching a differential diagnosis regarding noninflammatory or hereditary neuropathies. This is particularly relevant if vasculitides are localised to peripheral nerves or if they are systemic, as this means reaching a differential diagnosis will not be possible without a nerve biopsy examination (Fig. 10.21). It has been reported that concomitant muscle biopsy examination increases the chance of diagnosing vasculitis, even with isolated peripheral nerve vasculitides because there may be damaged or inflamed vessels involved in the neighbouring muscles. On the other hand, the diagnosis of vasculitis cannot be made from time to time because the diagnostic necrotising vascular lesions are not observed homogeneously along the nerve (Fig. 10.22). A 19-year-old male patient, who presented with progressive, ascending, symmetrical muscle weakness associated with reduced or absent deep tendon reflexes, was thought to be at the early stages of Guillain-Barre syndrome. However, electrophysiological evidence of demyelination such as slow nerve conduction velocity, prolonged distal motor latency and partial conduction block was absent. As the course of the disease was atypical, nerve biopsy was also performed. There was no

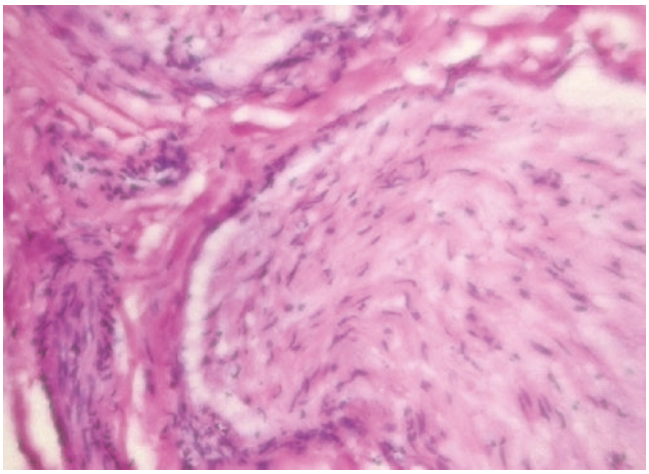

Fig. 10.21 Perivascular inflammatory cells in the perineurium of a sural nerve biopsy taken for the differential diagnosis of peripheral neuropathy (HE × 200)

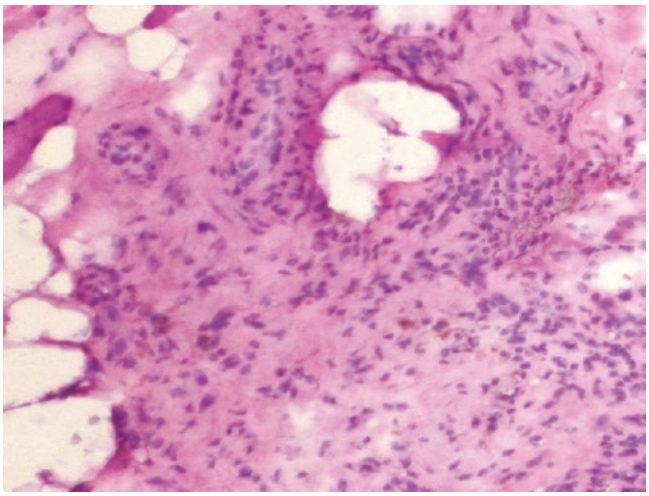

Fig. 10.22 Similar perivascular inflammatory cuff of vessels in the perimysium of the adjacent muscle (H&E × 200)

denervation or axonal degeneration in the biopsy. Severe vascular attack targeting sural nerve vessels, including endoneurial capillaries, were observed (Fig. 10.23). The clinician was informed, and emergency treatment was commenced. It should not be forgotten that vasculitis is a pathology of the peripheral nerves that requires urgent intervention. If vascular destruction is inhibited in the early period, axonal damage and demyelination secondary to ischaemia can be prevented or delayed. As an early diagnosis is very essential for the prevention of ischaemic axonal damage, searching for changes which suggest vasculitis is important. Such changes that supported the presence of neighbour vasculitides include luminal narrowing/thrombosis, intimal hyperplasia, disorganisation of the media, breakup of the elastic lamina, vessel sclerosis, recanalization, proliferation of epineurial capillaries and focal perineurial damage. Multifocal axonal degeneration is also a very important finding suggesting remote vasculitides. Identifying vascular immunoglobulin and complement deposition may also help to diagnosis vasculitis (Fig. 10.24). The presence of hemosiderin, due to old haemorrhages in nerves (Fig. 10.25), or focal calcification of vessel walls is important evidence suggesting neighbour vasculitides [120–122].

Nerve biopsy offers much more limited diagnostic clues compared to muscle biopsy. Furthermore, since the way in which genes cause hereditary neuropathies has been further understood and markers of immune system-mediated processes can be detected via blood tests, the indications for nerve biopsy have diminished. For neuropathies which develop in systemic vasculitis conditions such as polyarteritis nodosa (PAN) or Wegener's granulomatosis (WG), for typical distal sensory neuropathies which develop in diabetic patients or for neuropathies which develop due to an infectious agent, the diagnosis is often confirmed using blood tests and electrophysiological studies; thus, the need for nerve biopsy is eliminated. However, nerve biopsy examination is still essential, especially in atypical and suspicious cases. Clinicians generally have three basic expectations from pathologists who examine a nerve biopsy which include grading the presence of inflammatory cells or vessel damage, grading any deficiency of myelin and grading any axon degeneration. The main question is whether basic pathology is in the production of myelin or in the axon. However, it should not be forgotten that in the advanced stages of demyelination, even in pure myelination defects, pathology is not limited to conduction defects due to the deterioration of the isolation of the axon, and the demyelinated axon also begins to degenerate and eventually disappear. Therefore, in an end-stage nerve disease, differentiating between primary myelinopathy and primary axonopathy may not be impossible.

Electron microscopic examination highlights endothelial cell necrosis and basal lamina disruption and identifies the presence of hypertrophic endothelial cells with prominent intraluminal projections which is a useful clue regarding vasculitis. Similarly, areas of focal axonal degeneration due to ischaemia can be observed in detail with electron microscopic examination. On the other hand, nonspecific axonal degenerative changes and consequent segmental demyelination can be seen with both light and electron microscopic examination. Although focal demyelination can be observed in neuropathy associated with necrotising vasculitis, focal axonal damage is often accompanied by ischaemia, unlike that seen in acute or chronic inflammatory polyneuropathies. With ischaemic injury due to vasculitis, all fibres may be affected, or unmyelinated fibres may be preserved. Detailed discrimination of fibre type in a nerve plexus cannot be ascertained with light microscopic examination. In summary, with electron microscopic examination of ultrathin sections and of semithin sections of resin blocks that can

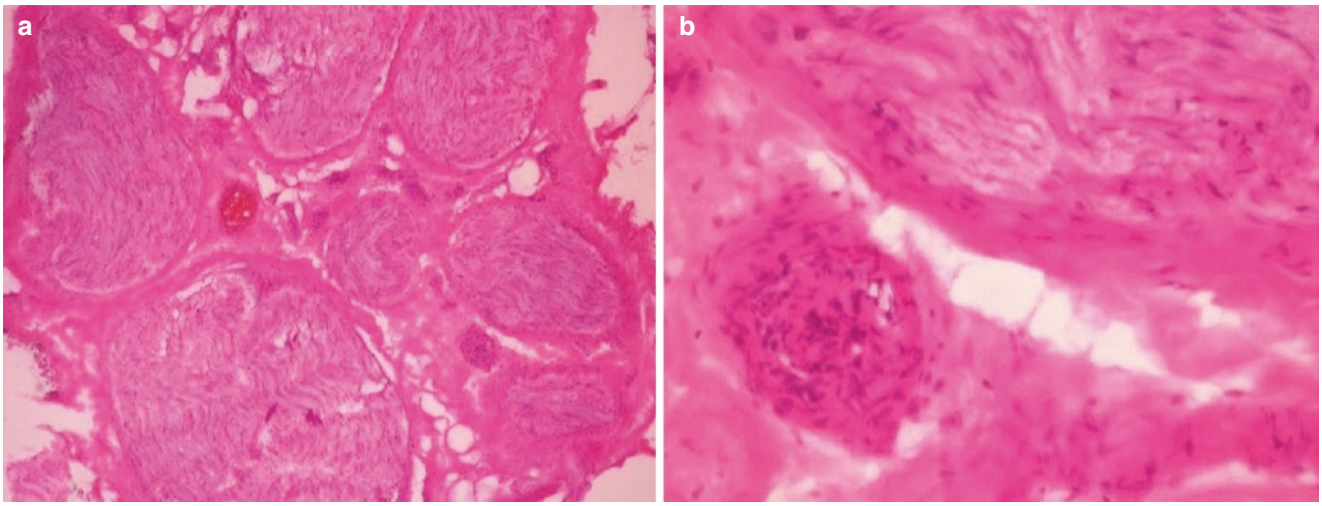

Fig. 10.23 (**a**) Vasculitis in the sural nerve of a 19-year-old adolescent which can be seen in the panoramic picture (H&E × 40). (**b**) A perineurial vessel is entirely infiltrated and damaged with inflammatory cells (H&E × 400)

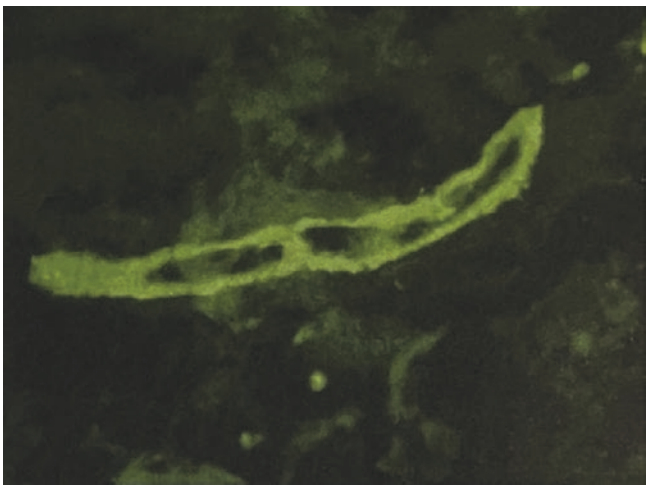

Fig. 10.24 Vascular deposits of immunoglobulin G, observed using direct immunofluorescent (DIF) examination, in a large epineurial vessel of a sural nerve (fluorescein × 200)

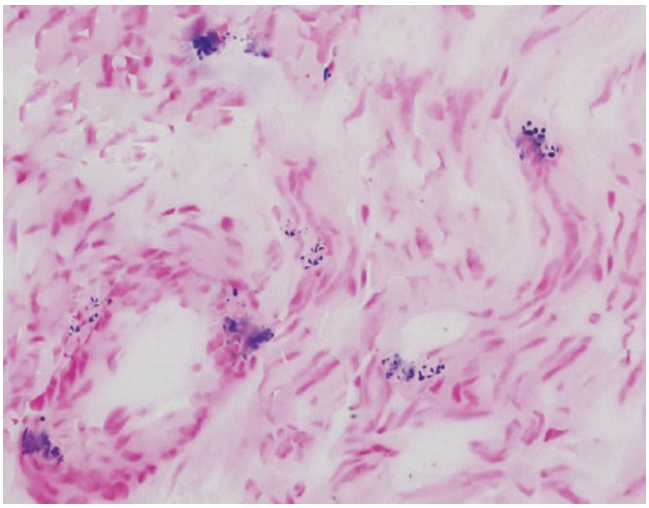

Fig. 10.25 Hemosiderin deposition in the vascular wall is highlighted by Prussian blue (Perl's Prussian blue × 200)

only be stained with toluidine blue, the state of vessels, myelin sheaths and axons can be examined in much more detail. In these examinations, it is even possible to tell which group of nerve fibres the pathology particularly affects. However, the most important part of the nerve biopsy examination relates to the presence of inflammation. For this reason, signs of vascular damage should be investigated first, and then inflammatory cells should be examined. As endoneurial lymphocytes resemble the nuclei of Schwann cells, immunohistochemical examination is required for the differential diagnosis. The majority of inflammatory cells in any vasculitis are T lymphocytes (95%) and macrophages. Depending on the severity and stage of the inflammatory process, varying numbers of neutrophils and eosinophils may also be present [121].

It is suggested that direct immunofluorescence (DIF) examination of combined peroneal nerve/peroneus brevis biopsies reveals immunoglobulin or complement deposits in epineural vessel walls in 70–80% of nerve biopsies in patients with suspected isolated peripheral nervous system (PNS) vasculitis and diabetic neuropathy. In summary, immunohistochemical and immunofluorescent examinations are very important for identifying the presence of inflammatory cells and their immune phenotype in a nerve biopsy, and these examinations can only be performed on frozen or paraffin tissue sections. For this reason, examination of paraffin and frozen sections using different staining methods is indispensable for nerve biopsy examination. Additionally, different histochemical and immunohistochemical examinations can also detail the status of myelin and axons (Fig. 10.26).

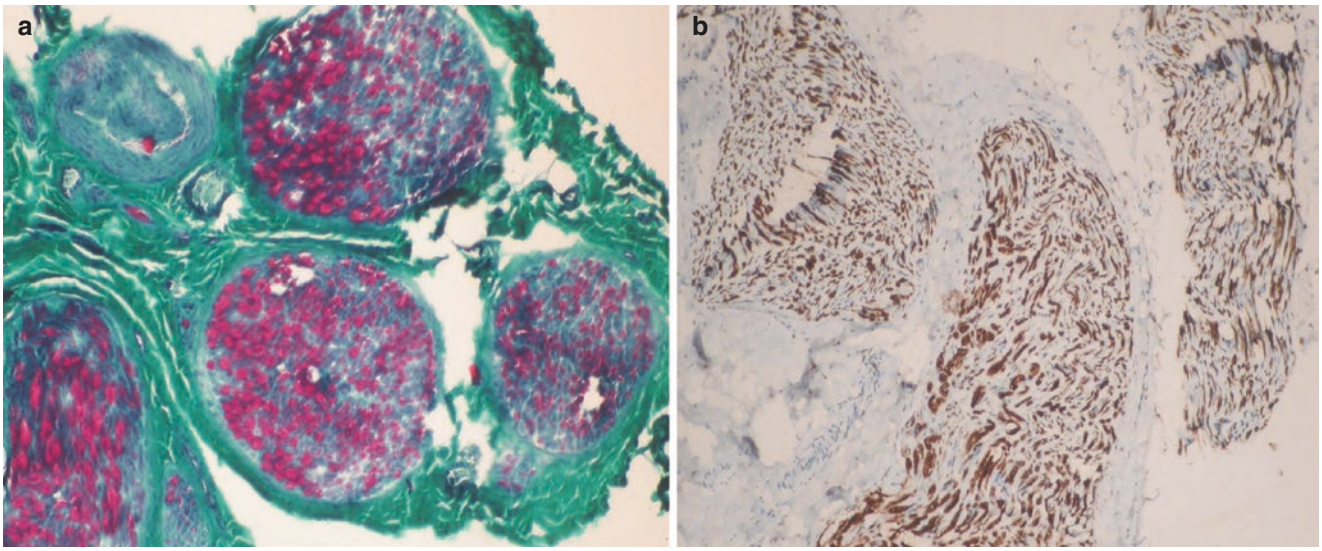

Fig. 10.26 Minimal myelin defect is highlighted using special stains in a patient with systemic lupus erythematosus. (**a**) Myelin is seen as red globes (modified trichrome × 100) and (**b**) immunohistochemical examination by myelin basic protein (DAB × 100)

Nerve dysfunction in vasculitis is assumed to occur based on ischaemia secondary to vessel destruction. Patients who have symmetrical sensorimotor neuropathy, which is an atypical finding for vasculitis, are likely to be affected by several unrelated, mild lesions that together will have a greater overall impact on the longest nerve fibres. Although segmental demyelination caused by ischaemia rarely presents as a conduction block in electrophysiological examinations, axonal destruction is always the dominant defect in vasculitides. The diameter range of epineurial arterioles is 75–350 μm, while that of perineurial and endoneurial arteries is much less than 75 μm. Most vasculitic processes are vessel size-specific, but the much more common injuries are due to disease of epineurial vessels of the peripheral nerves. Epineurial vascular involvement is typical of PAN, Churg-Strauss syndrome (CSS) and WG, while there is a tendency towards the involvement of small vessels of the epineurium in isolated PNS vasculitis. Involvement of veins is more common in WG and CSS than in PAN. Endoneurial vessels are usually <30 micrometres in size, and arterioles, venules and capillaries of this calibre, in both the endoneurium and epineurium, are the typical site of injury in systemic lupus erythematosus (SLE), hypersensitivity vasculitis, Henoch-Schoenlein purpura (HSP) and essential mixed cryoglobulinemia. Both small- and large-sized vessels may be involved in the collagen diseases. It must be noted that overlap syndromes, in which vessels of all sizes are involved, are not uncommon. Activated complement and immunoglobulin deposits are found in almost all inflamed vessel walls, regardless of the aetiology. Even though perivascular immune complexes are seen in vasculitis, they do not cause it; rather, they develop after a cell-mediated attack. Interaction with foreign antigen results in the activation of complement, the adhesion of neutrophils and other inflammatory cells to the blood vessels and the release of toxic substances that can cause vessel wall necrosis. Vasculitis likely involves a variety of disease and tissue-specific underlying mechanisms. Hepatitis B antigen found in PAN is an example of where a causal antigen source has been identified. Regardless of the underlying disease, activated T cells make up a significant portion of the inflammatory infiltrate. The observation that they are primarily of the CD8 subtype points to an important role for cytotoxic T-cell-mediated damage, possibly directed at a vascular antigen or an antigen presented by endothelial cells [120, 121].

Many infectious diseases such as acquired immunodeficiency syndrome (AIDS), leprosy, syphilis and Lyme disease can lead to peripheral neuropathy during their course. Different clinical manifestations such as distal symmetrical sensory polyneuropathy, acute or chronic inflammatory demyelinating polyneuropathy, human immunodeficiency virus (HIV)-associated mononeuropathy multiplex (MNM) syndromes, cytomegalovirus-associated lumbosacral polyradiculomyelopathy, autonomic neuropathy and even paraneoplastic neuropathy secondary to the developing lymphoproliferative disease may be encountered in patients with AIDS. In all infectious diseases, histopathological findings can be very variable, depending on the direct effect of the infectious agent or the reactions that develop due to the immune response. Among these agents, the most specific histological finding is perhaps the granulomas observed in leprosy. Similarly, the detection of granulomas in sarcoidosis nerve biopsies is a pathognomonic and diagnostic finding (Fig. 10.27). However, with infections that have peripheral nerve involvement, pathological findings may not be observed in biopsy specimens because these diseases do not cause diffuse involvement of the nerve, like the vasculitides.

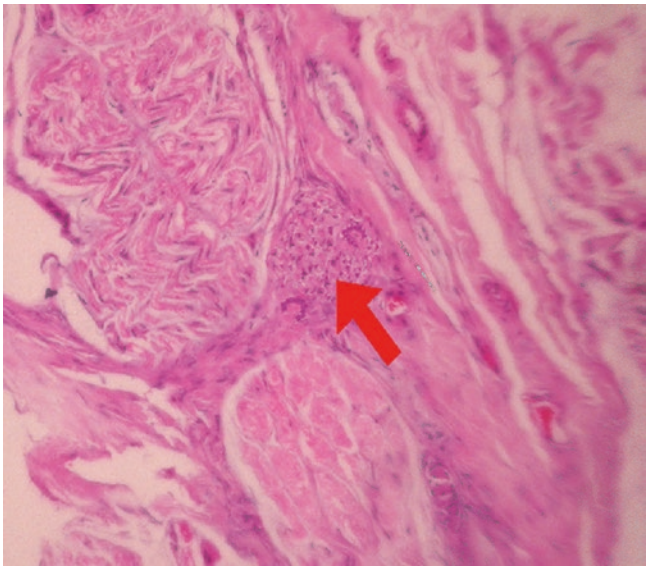

Fig. 10.27 A granuloma with two giant cells between two nerve plexi in a patient with sarcoidosis (H&E × 40)

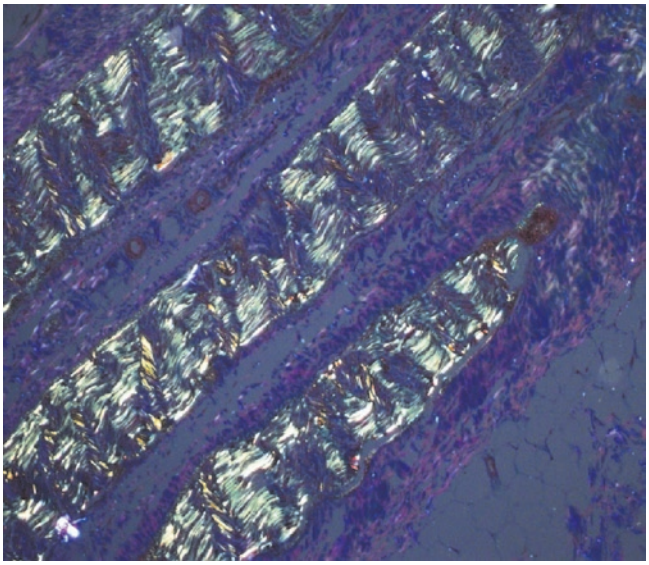

Fig. 10.28 Apple green-yellow birefringence localised to the nerve plexus is observed under polarised light in a patient with amyloid neuropathy (HE × 40)

Therefore, today nerve biopsy examination is not performed in patients with neuropathy if diagnosed with a specific infectious disease [112–114, 119–122].

Amyloidosis is another cause of neuropathy involving primary axons. Amyloid storage is highly diagnostic if observed with nerve biopsy examination. It is one of the most well-known diseases associated with protein misfolding due to gain-of-toxic function. It is characterised by the build-up and aggregation of toxic and dysfunctional proteins that harm tissues and cells. Under optical and electron microscopes, amyloid appears amorphous and fibrillar, respectively, and is recognised as an extracellular proteinaceous material. When stained with Congo red and observed under polarised light, amyloid fibrils exhibit apple green-yellow birefringence, setting them apart from other protein aggregates. Three patterns of amyloid deposits can be seen in the peripheral nerves. Areas of amyloid accumulation, seen according to these patterns, are found in extraneural connective tissue, widely in the endoneurium (Fig. 10.28), or in the walls of vessels in the nerve. Amyloidosis is generally associated with paraproteinaemia, for example, multiple myeloma, or chronic inflammatory disorders such as familial Mediterranean fever (FMF). Stored materials in different tissues in these disorders are a part of special amyloid types such as light chain amyloid (AL) and serum A amyloid (AA), respectively. In addition, there are hereditary familial types of amyloidosis, and up until today several distinct familial amyloid polyneuropathies (FAP), all of which are autosomal dominant, have been described. Mutations of the transthyretin (TTR), apolipoprotein A1 (APOA1) and gelsolin (GSN) genes cause these disorders which can potentially fatal. Transthyretin (TTR), encoded on chromosome 18, is a serum transport protein previously called pre-albumin, which serves as a carrier for several substances, including thyroxine and vitamin A. Apolipoprotein A1, encoded on chromosome 11, is a plasma protein with an extensive α-helical structure synthesised by the liver and the small intestine. The neuropathic pattern of symptoms is associated with the Gly26Arg mutation. Gelsolin is an actin-modulating protein encoded on chromosome 9. An accumulation of fibril aggregates of amyloid precursor proteins in the peripheral nerves and other systemic organs results in early autonomic symptoms, unexplained cardiomyopathy, bilateral carpal tunnel syndrome and progressive course in patients with a family history. The predominant nerve degeneration in amyloidosis is axonal degeneration which involves smaller fibres. The predilection of damage to a special nerve area is very diagnostic for some disorders. Alcohol and diabetic neuropathies also predominantly affect the smaller nerve fibres, like amyloidosis, and they cause neuropathy of smaller nerves [2, 28, 114, 121].

In some biopsies, granular material accumulations consisting of ground substance, extracellular microfilaments and scarce fibroblasts are seen adjacent to the inner perineurium (Fig. 10.29). These cushion-like accumulations are called Renaut bodies. They appear as large, loosely spiralled, elongated connective tissue accumulations in somatic and occasionally autonomic nerves. They generally involve more than one nerve fascicule. They are not seen in foetal life and their numbers increase during age. It is suggested that repetitive trauma or pressure may play a role in their pathogenesis due to their presence at the entrapped site of nerves. The collection of fluid, which may also be slightly granular, all around and under the perineurium is called sub-perineurial oedema.

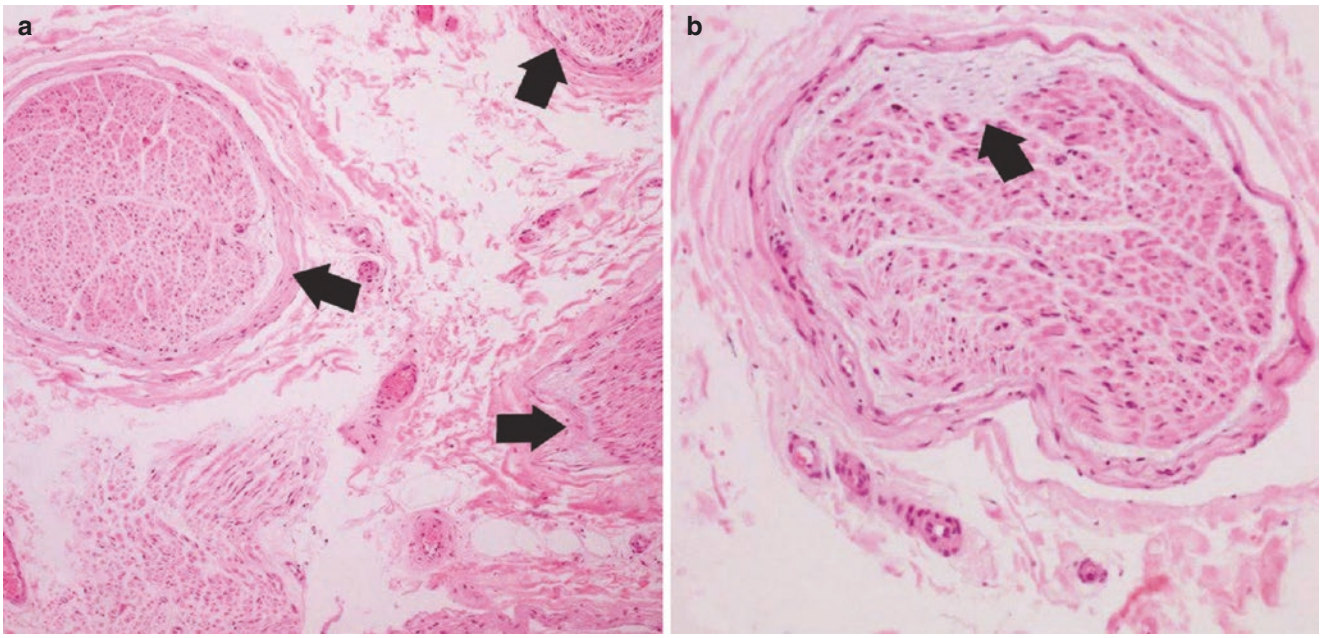

Fig. 10.29 (**a**) Renaut bodies (arrows) in several plexi of a sural nerve biopsy (H&E × 40). (**b**) Close-up view of a Renaut body (H&E × 200)

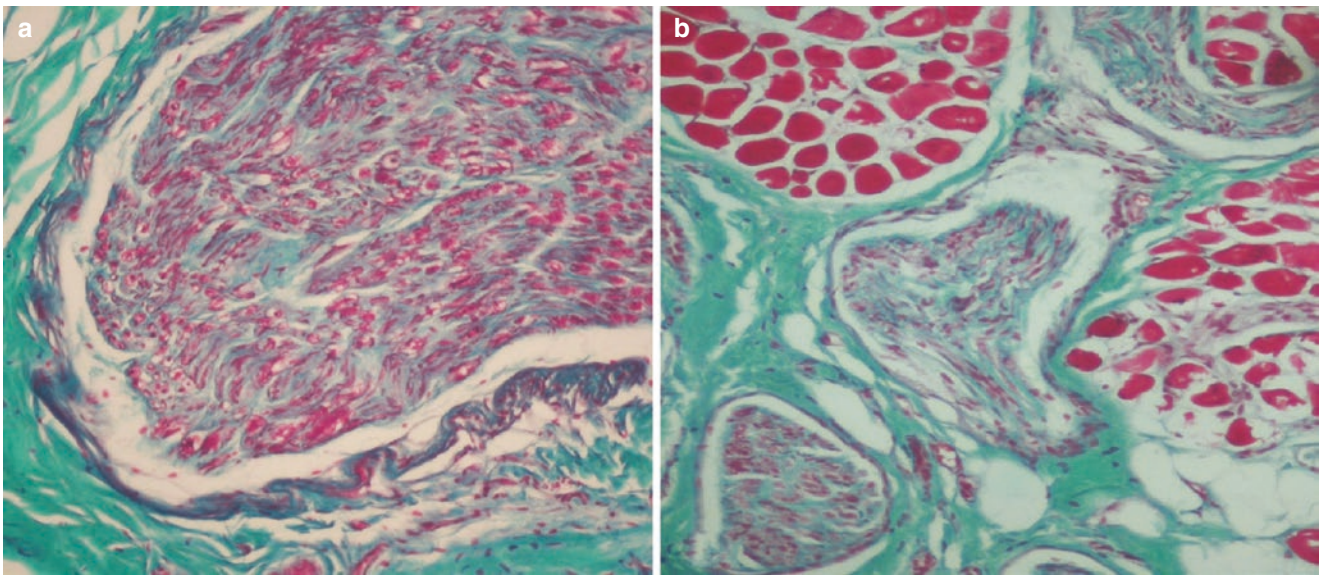

Fig. 10.30 (**a**) Sub-perineurial oedema in a sural nerve biopsy (Gomori's trichrome × 400). (**b**) Sub-perineurial oedema can be seen in several nerve plexi of a muscle biopsy (Gomori's trichrome × 100)

It is better visualised with plastic or frozen sections than fixed sections, and it has scattered fibroblasts, mast cells and macrophages (Fig. 10.30). It may be stained with alcian blue because of the mucopolysaccharides it contains. Sub-perineurial oedema may be seen in different disorders such as thiamine deficiency, leprosy, ischaemic (atherosclerotic) diseases, vasculitides, immune-mediated/hereditary demyelinating diseases, lead toxicity and ipilimumab-associated neuropathy [119–122].

Acquired inflammatory neuropathies are the main peripheral neuropathy group that constitute the most common indication for nerve biopsy examination today. Nerve biopsy examination was widely used to differentiate hereditary neuropathies from acquired inflammatory or immune-mediated neuropathies, especially in times when genetically diagnosing hereditary demyelinating neuropathies was not so widespread. Differentiating primary demyelinating neuropathy from primary axonal neuropathy can significantly narrow the

differential diagnosis (Fig. 10.31). This distinction also has profound implications for prognosis and treatment because many demyelinating neuropathies are inflammatory. Electrophysiological tests are always necessary in this context, as it is often not easy to distinguish clinically. On physical examination, clues of a demyelinating process include early loss of reflexes disproportionate to weakness, greater motor than sensory defects and palpably enlarged nerves. If peripheral neuropathies are classified as diseases that mainly affect axons or myelin production, it is seen that most infectious, toxic, nutritional and metabolic disorders, including vasculitis, mostly cause axonal degeneration (Table 10.9). On the other hand, diseases characterised by myelinisation defects comprise the acquired demyelinating neuropathies (Fig. 10.32), rare toxic neuropathies due to perhexiline, amiodarone, solvents or chloroquine, most subtypes of Charcot-Marie-Tooth disease and a few metabolic diseases such as Krabbe, Tangier or Niemann-Pick diseases [121].

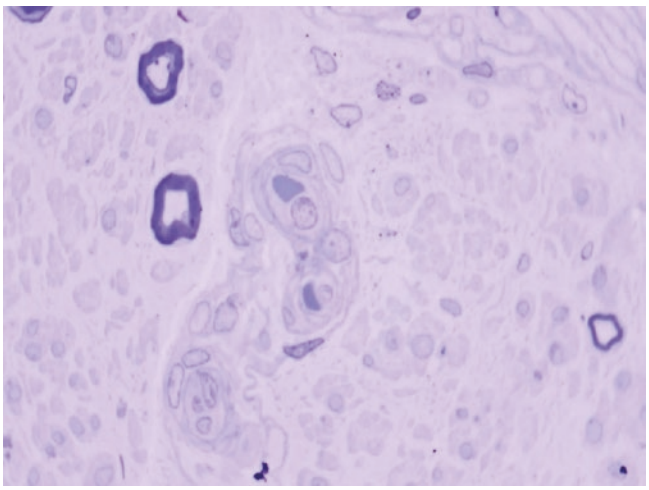

Fig. 10.31 Severe myelination defect in transverse semithin section of a sural nerve biopsy (toluidine blue × 1000)

Due to their chronic and fluctuating course, chronic idiopathic inflammatory polyneuropathies (CIDPs) are one of the most common group of diseases for which nerve biopsy is performed for the differential diagnosis of many disorders, including hereditary diseases. Two important reasons for performing a nerve biopsy are to confirm the demyelinating nature of the disease and to distinguish it from other diseases that may give similar clinical findings (Fig. 10.33). The histopathological hallmark of CIDP is primary demyelination. This feature is the most constant and important finding in the sural nerve biopsy. The presence of thinly myelinated fibres in the biopsy confirms a disturbance of myelination with repetitive demyelination and remyelination processes (Fig. 10.34). Repeated primary demyelination and remyelination eventually lead to the formation of onion bulbs (Fig. 10.35). These structures are layers of supernumerary Schwann cells with intervening collagen arranged in rings around longitudinal nerve fibres (Fig. 10.36). Although hypertrophic neuropathy is a prominent feature of several

Table 10.9 Classification of neuropathies according to pattern of involvement

	Primary axonal	Primary demyelinating
Acute	Vasculitis Porphyria Toxins (misonidazole, nitrofurantoin, arsenic, thallium) Alcohol Nutritional agents	GBS Diphtheria
Subacute/chronic	Diabetes Toxins (alcohol, drugs) Vasculitis Connective tissue disorders B12, B6, B1, vitamin E defects Uremia Hypothyroidism HIV, HTLV-I Lyme disease Paraproteinemia/paraneoplastic Neoplastic infiltration Sarcoidosis	CIDP Paraproteinemia POEMS syndrome HNPP Toxins (perhexiline, amiodarone, solvents, chloroquine) L-tryptophan toxicity Oxalosis
Chronic	CMT-2 CMT-X (in females) HSAN Amyloidosis Spinocerebellar degeneration Fabry disease Mitochondrial neuropathies Adrenoleukodystrophy Polyglucosan body disease	CMT-1 CMT-X (in males) Dejerine-Sottas syndrome Refsum disease Metachromatic leukodystrophy Krabbe disease MNGIE syndrome Niemann-Pick disease Tangier disease

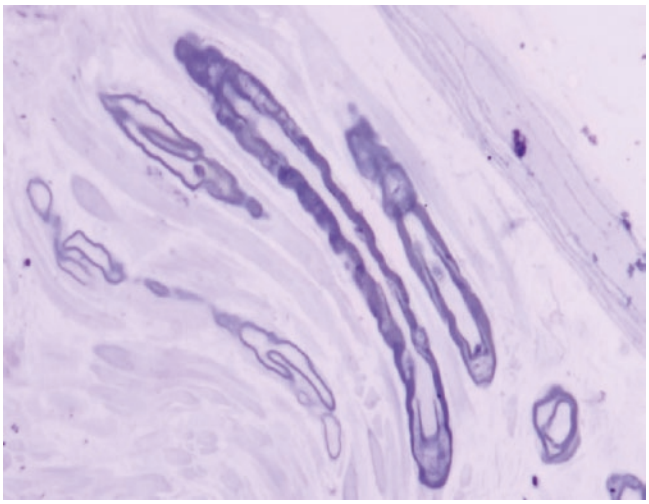

Fig. 10.32 Myelination defect in longitudinal semithin section of a sural nerve biopsy (toluidine blue × 1000)

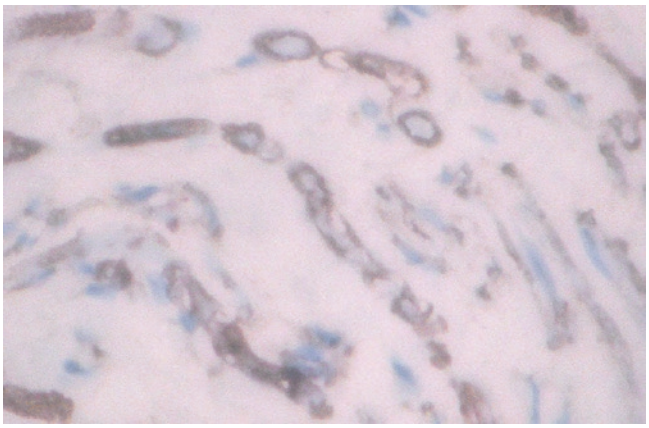

Fig. 10.33 Severe myelination defect with myelin basic protein immune staining (DAB × 400)

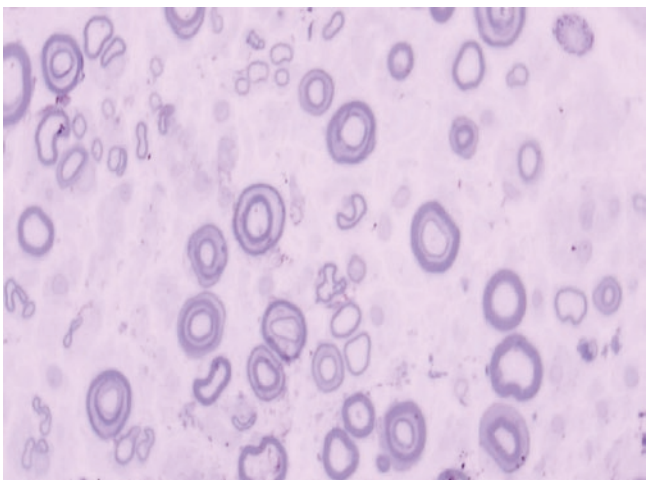

Fig. 10.34 Note the presence of thinly myelinated large nerve fibres in a transverse semithin section of sural nerve biopsy (toluidine blue × 1000)

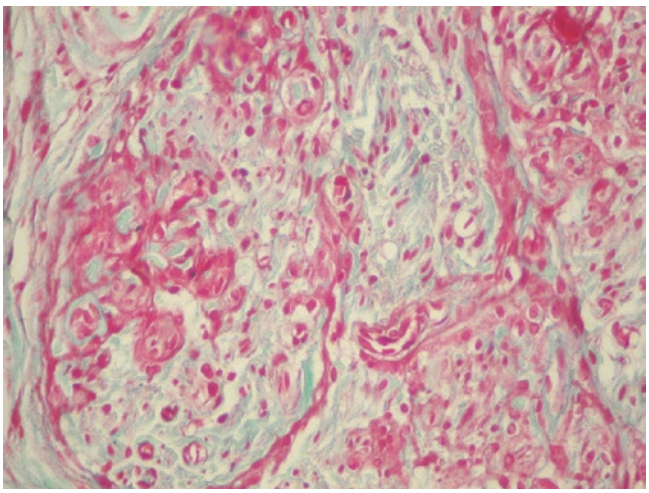

Fig. 10.35 Severe myelination defect with occurrence of onion bulbs (Gomori's trichrome × 200)

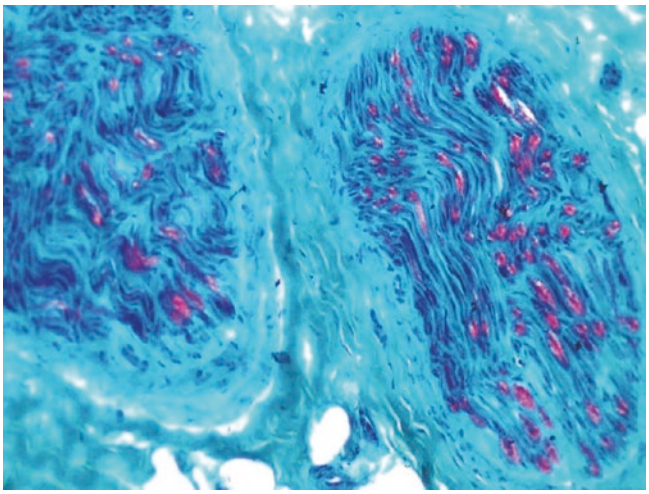

Fig. 10.36 Patchy myelination defect with fibrosis in the sural nerve of a patient with CIDP (modified trichrome × 100)

genetically determined neuropathies, onion bulbs can also occur in CIDP and, to a lesser extent, in diabetic neuropathy and other acquired polyneuropathies. On the other hand, the widespread presence of onion bulbs distributed diffusely among nerve fascicles is the hallmark of most familial hypertrophic neuropathies. Onion bulbs can be visualised in paraffin-embedded material using haematoxylin and eosin (H&E) sections, and their presence may be confirmed with collagen IV staining of Schwann cell basal laminae. Onion bulbs may have a central myelinated or demyelinated axon. While the presence of axons can be highlighted by neurofilament immunoreactivity (Fig. 10.37), axons can even be seen in H&E-stained sections (Fig. 10.38). In some types of inherited demyelinating neuropathies, especially CMT-1, onion bulbs may be seen in 30–100% of the visible myelinated and demyelinated nerve fibres. Axonal degeneration is

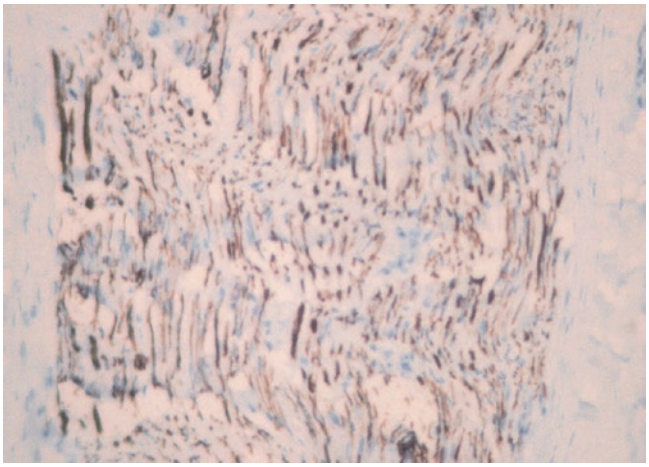

Fig. 10.37 Axons can be highlighted by neurofilament immune reactivity (DAB × 100)

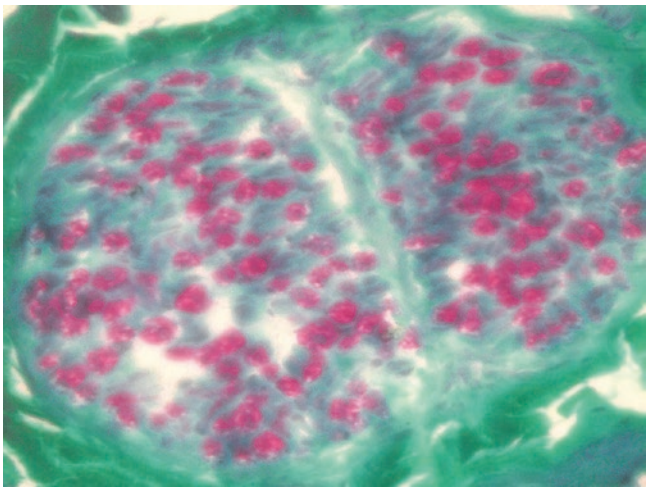

Fig. 10.38 The presence of axons can be discriminated in most fibres (modified trichrome × 200)

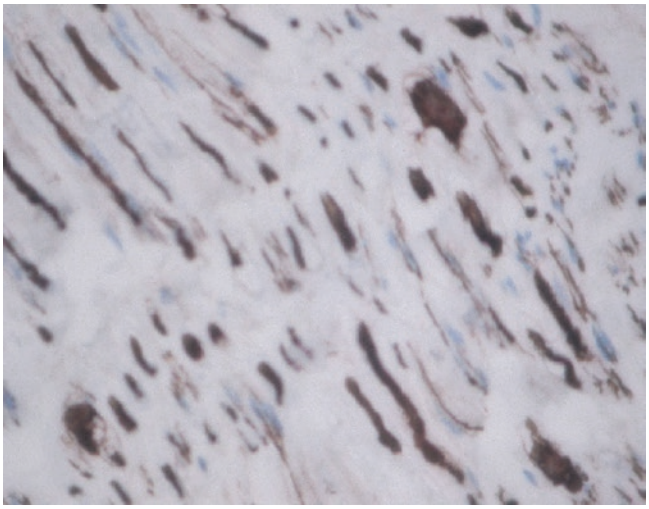

Fig. 10.39 A few giant axons can be highlighted by neurofilament immune reactivity (DAB × 200)

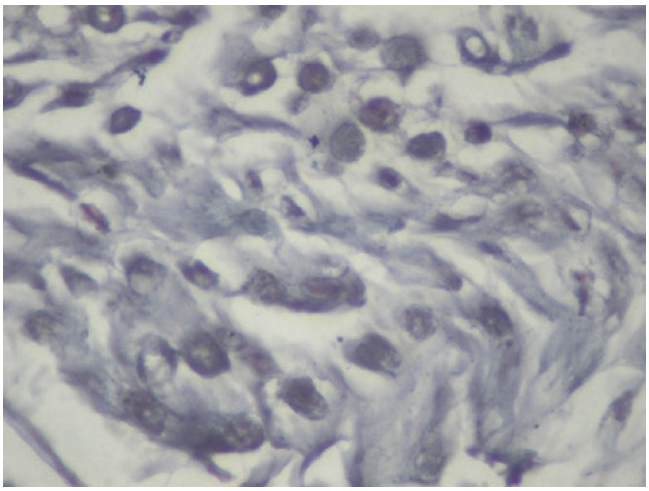

Fig. 10.40 Axonal swelling can be discriminated (Holmes stain × 400)

not an expected finding in hereditary and acquired demyelinating polyneuropathies. However, some reactive changes like axonal swelling and degeneration can be observed in demyelinated axons, especially in the advanced stages of the diseases (Fig. 10.39). Nonuniform involvement within and between fascicles, macrophage-mediated myelin stripping, perivascular lymphocytic infiltrates, specific endoneurial signs of active demyelination such as numerous naked axons, scattered endoneurial macrophages and Schwann cell mitosis are histological features that support CIDP more so than CMT-1. The presence of giant axons is a pathognomonic feature of giant axonal neuropathy (GAN) which is a rare hereditary disease. However, in some atypical CIDPs or toxic neuropathies, including glue and lacquer thinner sniffing toxic neuropathies, there may be giant axonal swellings that can make them difficult to differentiate from GAN. Especially in CIDP, the presence of many myelin-digestion chambers (Fig. 10.40) is indicative of axonal degeneration and the increased demyelination gap between Ranvier nodes [120–122].

Identification of inflammatory cell infiltration is diagnostic, especially in immune-mediated or inflammatory polyneuropathies such as AIDP and CIDP because in hereditary disorders, inflammation is almost never detected. On the other hand, inflammation is not very evident in CIDP, which is an inflammatory process. Therefore, immunohistochemical studies are necessary for the diagnosis of CIDP. The presence of more than three T lymphocytes per plexus (Fig. 10.41), or the presence of T lymphocytes, albeit in small numbers, on the walls of the perineurial and endoneurial vessels, makes the diagnosis of inflammatory neuropathy certain (Fig. 10.42). This is because normally, inflammatory

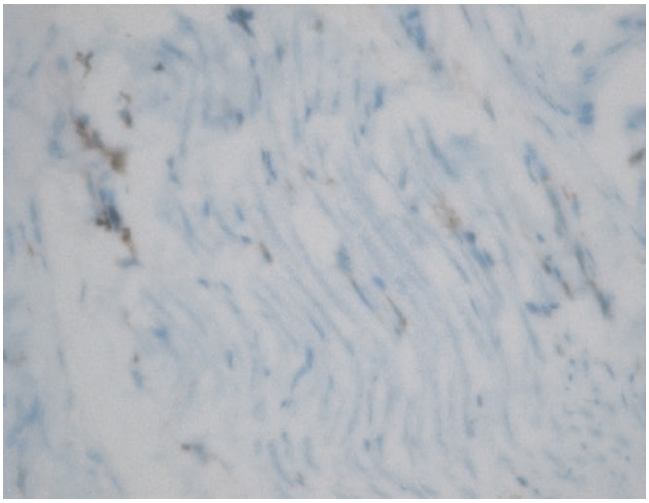

Fig. 10.41 A small collections of T lymphocytes around the endoneurial vessel highlighted by CD3 immune reactivity (DAB × 100)

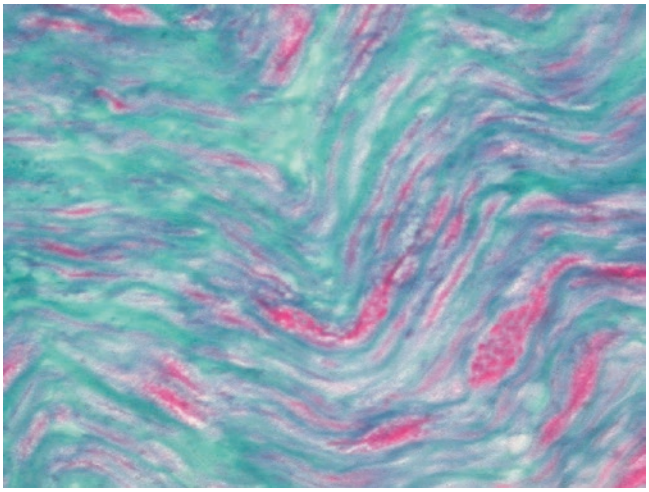

Fig. 10.43 The myelin-digestion chambers occur due to axonal degeneration (modified trichrome × 200)

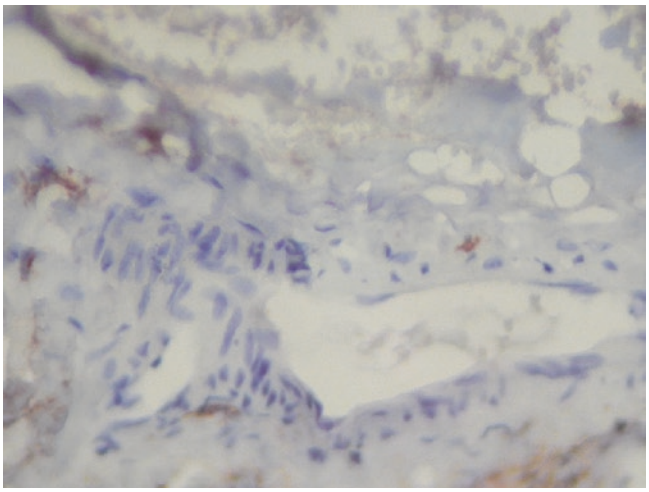

Fig. 10.42 T lymphocytes around the perineurial vessel highlighted by CD3 immune reactivity (DAB × 400)

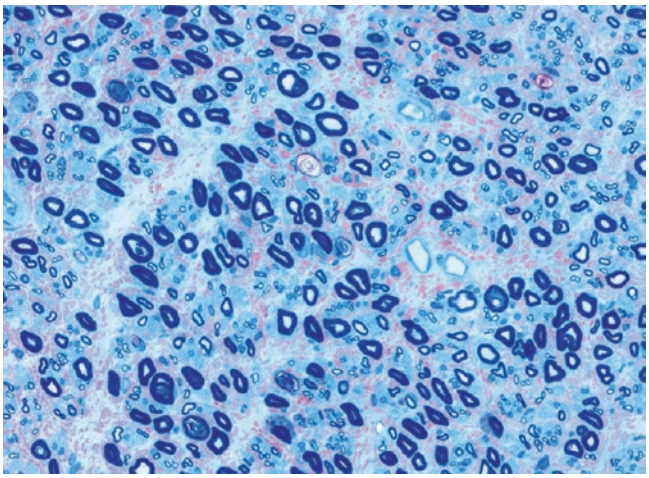

Fig. 10.44 Axonal degeneration and myelin ovoids can be discriminated in a superficial peroneal nerve biopsy (toluidine blue and basic fuchsin stain × 1000). This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London

cells can only be seen in small numbers around epineurial vessels [121].

Axonal degeneration of the nerves is a very important clue for the diagnosis of axonal neuropathy. The features of axonal degeneration include the presence of myelin-digestion chambers (Fig. 10.43) and myelin ovoids (Fig. 10.44). Axonal degeneration can be indirectly diagnosed by the presence of giant axons and the presence of axonal regeneration. Axonal regeneration, like Wallerian degeneration, is diagnosed by the presence of small clusters of small axons surrounding small myelin sheaths (Fig. 10.45). In some patients, axonal degeneration is so silent that axonal atrophy may be the only evidence of axonal degeneration (Fig. 10.46). Except for GAN and vasculitic neuropathy, most axonal neuropathies do not have specific histopathological findings in the nerve biopsy that indicate aetiology [120–122]. On the other hand, some nerve biopsies can be normal. For example, in a sural nerve biopsy of a 33-year-old male with a 1-year history of distal numbness and muscle wasting, no pathological features were determined (Fig. 10.47 and Table 10.10).

Nerve biopsy is rarely used when determining the differential diagnosis of a large spectrum of miscellaneous diseases such as mitochondrial, metabolic and toxic pathologies that may cause peripheral neuropathy. This is because most of these pathologies have no diagnostic findings or it is very difficult to encounter the pathognomonic findings in nerve biopsy. For example, peripheral nerves are frequently affected in mitochondrial diseases. However, it is not possible to diagnose neural mitochondrial pathologies with oxidative enzyme stains. It has been reported that the detection of

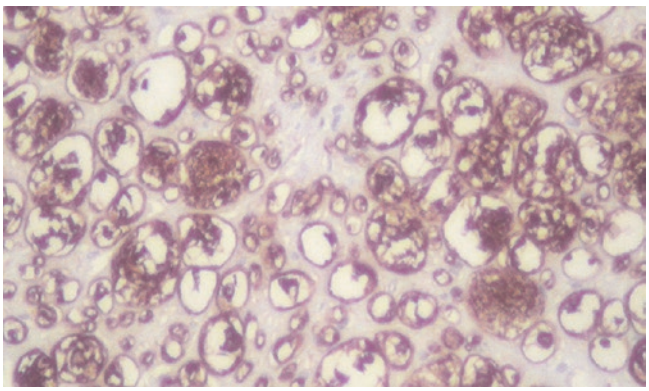

Fig. 10.45 The myelin-digestion chambers can be highlighted by myelin basic protein immune reactivity (DAB × 100)

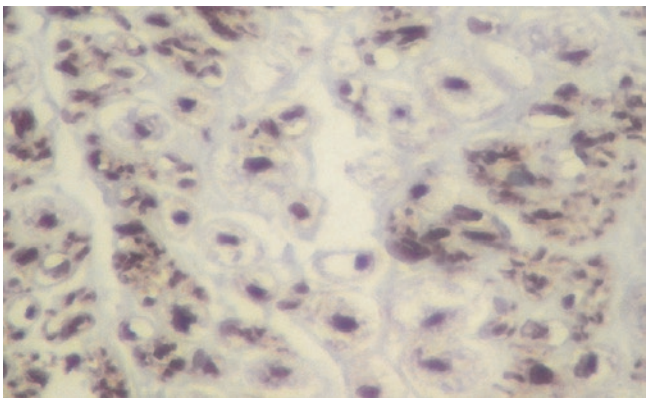

Fig. 10.46 The presence of small clusters of small axons can be discriminated by neurofilament immune reactivity (DAB × 100)

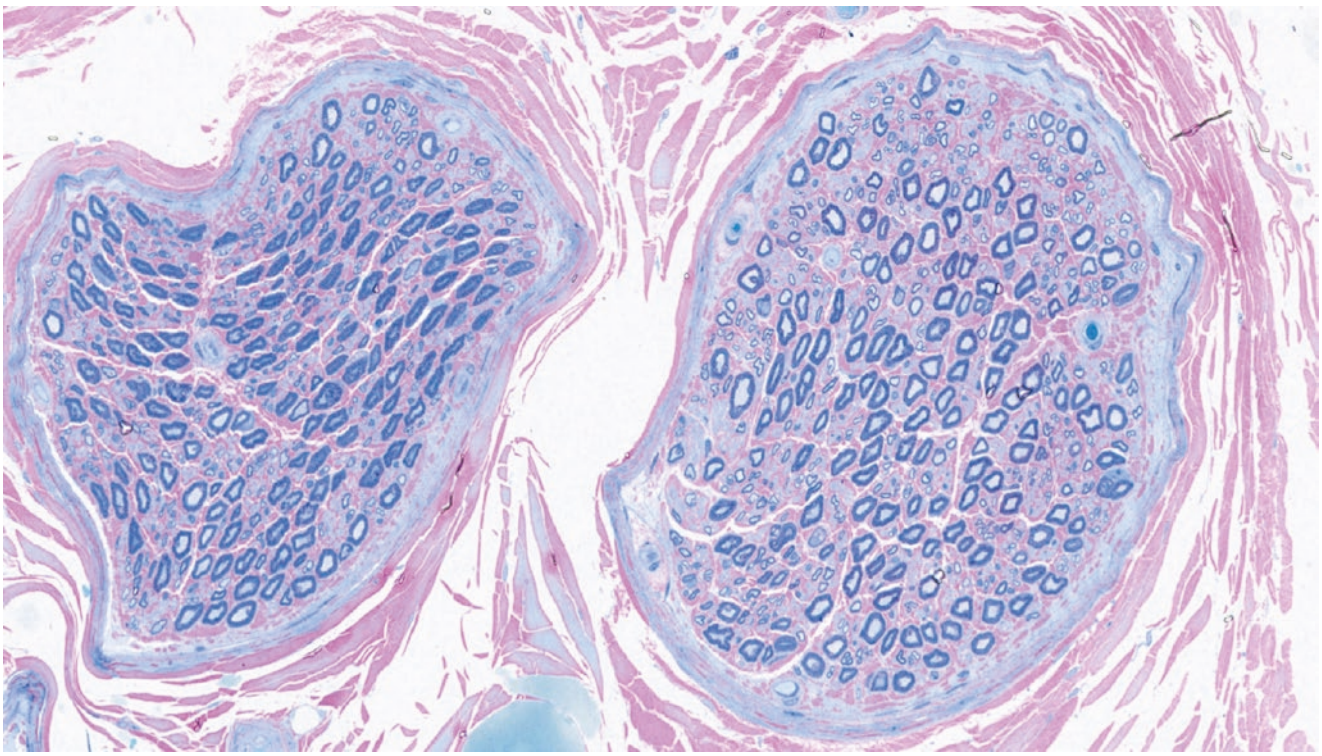

Fig. 10.47 Normal sural nerve with resin semithin section (toluidine blue and basic fuchsin stain × 200). This photograph is from the archive of Rahul Phadke, MD, PhD, Associate Professor of Neuropathology, Dubowitz Neuromuscular Centre, UCL QS Institute of Neurology, London

proliferating mitochondria in bizarre shapes in the cytoplasm of Schwann cells is diagnostic if they can be detected in electron microscopic examinations. Similarly, the presence of intra-axonal periodic acid-Schiff (PAS) positive hyaline concentric lamellar bodies in the nerve plexi in polyglucosan body disease, giant lysosomes in Schwann cells in Chediak-Higashi disease and banana bodies in the cytoplasm of Schwann cells in Farber disease are diagnostic. However, these changes, which can only be detected by electron microscopic examination, are extremely unlikely to be seen in these small tissue sections. For this reason, patients with these rare diseases in which pathognomonic findings can be exhibited in nerve biopsy, have been published as case reports in the literature [121].

Almost all neuropathies mentioned above affect both motor and sensory nerves equally, or in other words, the sural nerve, totally composed of sensory nerves which is sampled for nerve biopsies is almost always involved. However, some disorders predominantly or only involve motor nerves. Motor neuron diseases like amyotrophic lateral sclerosis (ALS), which is the most common form, are neurological disorders characterised by the degeneration of

Table 10.10 Motor and sensory neuropathies

Primarily motor nerve involvement	Primary sensory nerve involvement
Inflammatory/demyelinating	*Inflammatory*
GBS or CIDP with motor predominance (common)	Predominantly sensory GBS (unusual)
MMN with conduction block	Predominantly sensory CIDP (unusual)
Infection associated	Vasculitic neuropathy (unusual)
Diphtheria	*Infection associated*
Metabolic	HIV (DSPN), leprosy
Porphyria	Lyme disease
Hypoglycaemia-associated neuropathy	*Metabolic*
Toxic (lead, mercury, dapsone organophosphate poisoning)	Diabetes, uremia
Neoplasm associated	Hypothyroidism
Lymphoma-associated motor neuropathy	Liver diseases
Paraprotein-associated motor neuropathy	Nutritional B12 deficiency
POEMS syndrome	Pyridoxine excess
Hereditary	Thiamine deficiency (atypical)
CMT-1	Vitamin E deficiency
CMT-2	*Toxic* (cisplatin, chloramphenicol
SMA, ALS	Metronidazole, misonidazole
	Isoniazid, ethionamide
	Nitrous oxide, L-tryptophan
	Thalidomide, ethylene oxide
	Organophosphate insecticide)
	Neoplasm associated
	Lymphoma/leukaemia
	Hereditary
	Friedreich's ataxia, HSAN
	Fabry disease, Tangier disease

motor neurons. ALS generally effects both lower motor neurons (LMN) and upper motor neurons (UMN). Despite the classical clinical presentation of ALS which is usually very diagnostic, diagnosis may be challenging in patients presenting with sporadic progressive disease of the LMNs. Furthermore, motor neuropathy (MN) primarily affects the motor nerves. In most cases, nerve conduction studies differentiate between these situations. While UMN signs are absent, demyelinating features are present in MN. However, demyelination may not always be identifiable, and with electrophysiological examination, purely axonal findings may be found in some cases. As early differentiation between ALS and MN is important for the prognosis and therapeutic approach, a motor nerve biopsy should be acquired. Therefore, in this situation, obturator nerve biopsy with a muscle biopsy from the gracilis muscle should be considered as a potential differential diagnostic tool [120–122].

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Disorders of the Neuromuscular Junction

11

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Neurophysiology of the Neuromuscular Junction

The neuromuscular junction (NMJ) is a type of chemical synapse between motor neurons and skeletal muscles. Each neuromuscular junction consists of a motor nerve terminal, pre-synaptic membrane, synaptic space, and post-synaptic muscle endplate (Fig. 11.1). Synaptic vesicles that contain acetylcholine (ACh) are located in the motor nerve terminal. Synaptobrevin, found in the synaptic vesicle membrane; syntaxin and synaptosomal-associated protein-25 (SNAP-25), found in the cytoplasm of motor nerve terminals, are called soluble N-ethylmaleimide-sensitive (SNARE) proteins. These are essential for the adhesion and fusion of synaptic vesicles and, ultimately, exocytosis of ACh into the synaptic cleft [1]. Action potentials that reach the motor nerve terminal cause voltage-gated calcium channels (VGCCs) to open. A calcium influx into the motor nerve terminal causes ACh to be released into the synaptic cleft [2]. ACh then diffuses through the synaptic cleft and binds to ACh receptors (AChRs) clustered on the post-synaptic membrane. The AChR is an ion channel consisting of two α1, one δ1, one β1, and one ε (adult) or γ (foetal) subunits [3]. The clustering of AChRs is mediated by muscle-specific kinase (MuSK) and its co-receptor, low-density lipoprotein receptor-related protein 4 (LRP-4), which are receptor complexes [4]. Agrin is an extracellular matrix protein that also activates MuSK by binding LRP-4. Rapsyn and Dok-7 are intracellular adaptor proteins. Rapsyn is a membrane-bound cytoplasmic molecule that binds AChRs and is necessary for their clustering. Dok-7 protein is associated with MuSK, and it induces a cascade of phosphorylation that leads to AChR clustering [5].

The binding of ACh to its receptor leads to the opening of voltage-gated sodium channels in muscle fibers which causes depolarisation. All these processes act to trigger excitation–contraction coupling. After that, acetylcholinesterase (AChE) hydrolyses the ACh in the synaptic space to produce choline and acetate. Choline is actively transported into the pre-synaptic cytosol, where the enzyme choline acetyltransferase (ChAT) synthesises acetylcholine from acetyl coenzyme A and choline in cholinergic neurons [6].

All abnormalities related to the neuromuscular junction cause the myasthenic syndromes. Suspicion of myasthenia typically comes from the clinical history. Difficulty in maintaining certain movements, such as an upward glance, talking, or holding out the arms, may also be a sign of abnormal fatigability. Another distinguishing trait of myasthenia is the myasthenic snarl. Pharmacological tests that measure how the body reacts to acetylcholinesterase inhibitors, electrophysiological studies, or detecting serum antibodies against various neuromuscular junction components are frequently used to confirm the diagnosis. The response reduction of the motor action potential to repetitive nerve stimulation or the presence of "jitter" on single fiber electromyography are typical electrophysiological signs of myasthenia. However, myasthenic syndrome is very difficult to diagnose using muscle biopsy investigation. Therefore, pathology laboratories that can perform muscle biopsy examinations only have a small number of muscle biopsies from individuals with myasthenic syndrome [1–6].

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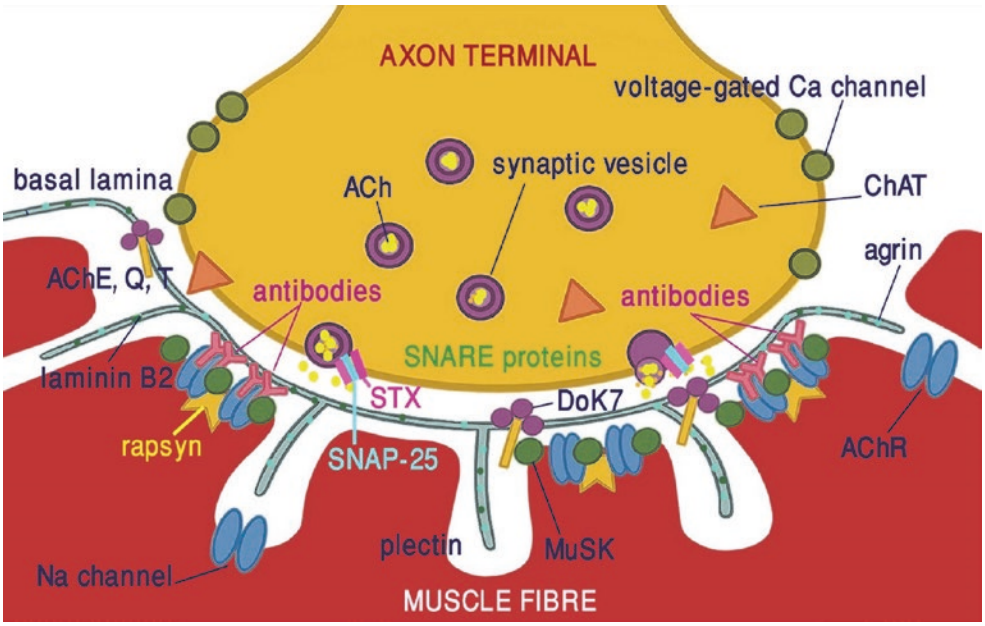

Fig. 11.1 Schematic view of the neuromuscular junction. *ACh* Acetylcholine, *Ca* Calcium, *ChAT* Choline acetyltransferase, *AChE* Acetylcholinesterase, *AchR* Acetylcholine receptor, *STX* syntaxin, *SNAP-25* synaptosome-associated protein-25, *AchE, Q, T* collagen Q tail attached to globular subunits of AChE, *MuSK* Muscle-Specific Kinase, *DoK7* Docking protein 7, *SNARE* soluble N-ethylmaleimide-sensitive proteins

Myasthenia Gravis

Myasthenia gravis (MG) is the most common autoimmune disorder that affects the neuromuscular junction. The annual incidence of MG is nearly 10–29 cases per one million people, and the prevalence ranges between 100 and 350 cases per one million people [7]. There is a bimodal distribution in women, with peaks around ages 30 and 50. In male patients, incidence increases with age, and the highest rates are between ages 60 and 89 [8].

Pathogenesis of Myasthenia Gravis

Eighty percent of patients with MG have detectable antibodies against AChRs (AChR-Ab). These antibodies are of the immunoglobulin (Ig) G1 and IgG3 subclasses binding an extracellular domain of the α1 subunit of AChR [9]. Three main mechanisms are suggested to explain AChR-Ab mediated damage of the NMJ. First, antibodies binding to AChRs cause complement cascade activation, ultimately forming a membrane attack complex (MAC) that leads to a loss of AChR and widening of the synaptic cleft, as well as a reduction in endplate potential amplitudes [10]. Second, AChR-Abs enhance the internalisation and lysosomal proteolysis of AChR [11]. The third mechanism involves the blockage of opening AChR channels due to antibody binding [10]. AChR-Abs are detected and present in 85% of patients with generalised MG and in 50–70% of those with ocular myasthenia [12].

Thymic impairment is suggested to initiate the immunopathogenesis of MG. Nearly 70% of patients with early-onset MG have thymic lymphofollicular hyperplasia, and 10% of patients have a thymoma. Both contribute to tolerance failure responsible for specific Ab production [13]. The thymus in AChR-Ab-positive MG patients has increased B cells. High numbers of germinal centres, which are responsible for B-cell maturation and are almost absent in the normal thymus, are found in thymic lymphoid hyperplasia [14]. Additionally, the thymus is the organ responsible for T-cell maturation and the development of central tolerance to self-reactive T cells. Self-reactive T cells which escape the central tolerance development process are eliminated by regulatory T cells. However, regulatory T cells that differentiate in the thymus are less functional in the thymus and the periphery of MG patients [15]. Sensitisation to AChRs, B-cell proliferation, and ectopic germinal centre formation ultimately lead to the synthesis of pathogenic antibodies [16]. Anti-MuSK antibodies are detected in 1–10% of patients with MG [17]. MuSK antibodies are mainly of the IgG4 subclass, which does not activate complement. MuSK antibodies bind the Ig-like regions of the MuSK ectodomain and cover the binding side of MuSK-associated proteins, interrupting the activation of the agrin–LRP4–MuSK complex [10, 18]. LRP4 antibodies were detected in 7–33% of patients who did not have anti-AChR or anti-MuSK antibodies [19]. LRP4 antibodies disrupt the interaction between LRP4 and agrin, causing inhibition of AchR-mediated neuromuscular transmission [20]. LRP4 antibodies are of the IgG1 subclass, but many patients have additional IgG2 and IgG3 reactivity. Antibodies have also been detected against transmembrane or extracellular proteins such as agrin, ColQ, and the α-subunit of the voltage-gated potassium channel Kv1.4, or intracellular proteins such as titin, ryanodine receptor, and cortactin in patients with MG [17, 18].

Clinical Manifestations

The typical clinical feature is fluctuating muscle weakness which increases with repetitive muscle use as the day progresses. Muscle weakness can be present in the extraocular, bulbar, limb, and axial muscles [21]. Extraocular muscles and the levator palpebrae superioris muscle are frequently affected [22]. Typical symptoms and the first clinical presentation of 60% of patients include intermittent ptosis and diplopia [23]. In 15% of all patients with MG, symptoms and signs are restricted to the ocular muscles. Ocular muscle weakness is often asymmetrical, while weakness in limb muscles is symmetrical. Limb weakness most frequently involves the proximal upper arm, thigh, and hip muscles [18]. The involvement of upper airway muscles and/or respiratory muscles can result in respiratory failure that requires intubation and mechanical ventilation, which is known as myasthenic crisis [24]. MG has been divided into subgroups according to autoantibody pattern, clinical phenotype, age at onset, and association with thymic pathology.

AchR-Ab-Positive MG

MG with AchR-Abs is divided into early onset (if it presents before 50 years) and late onset (if it presents after 50 years) [17]. There is a female predominance in the early-onset MG subgroup [25]. Patients with thymoma are excluded, while thymic hyperplasia is commonly present, and these patients are responsive to thymectomy [13, 26]. There is a slight male predominance in the late-onset MG subgroup [27]. Thymic hyperplasia is rarely detected and, if present, patients are not responsive to thymectomy [17]. Thymoma occurs in 10–15% of all patients with MG, and this subgroup is known as thymoma-associated MG [28]. Nearly all patients with thymoma are AChR-Ab-positive and have generalised MG.

MuSK-Ab-Positive MG

Facial and bulbar muscle weakness are usually the first symptoms of MuSK-positive MG and frequently involve the neck and respiratory muscles [29]. Facial and tongue muscle atrophy may be present. Patients are reported to have symmetrical ophthalmoparesis, mainly of lateral gaze, which is not a usual feature of other MG subgroups [30]. Fluctuation in muscle strength is less profound, and the disease has a more severe course in this subgroup. Most MuSK antibody-positive MG patients do not have thymic pathology [31].

Seronegative MG

MG patients with no positive test results for anti-AChR, anti-MuSK, or anti-LRP4 antibodies are diagnosed as having seronegative MG. AChR-Ab tests are negative in 50% of patients with ocular MG and nearly 10–15% with generalised MG [32]. It has been suggested that having low-affinity antibodies or a low concentration of antibodies to AChR, MuSK, or LRP4 antigen targets are possible underlying features of seronegative MG [33]. Patients with antibodies against antigens that have not yet been determined or patients who are not able to be tested are also in this subgroup. The widely used method to detect AChR-Ab is the radioimmunoprecipitation assay. Recently, the application of cell-based assays has detected antibodies that were not previously detectable. However, commercial use of cell-based assays is not widely available [34].

Diagnosis of MG

The diagnostic approach focuses on confirming the clinical diagnosis by the clinical history and examination findings. The ice-pack test is a bedside test used to demonstrate the healing effect of cold temperatures in neuromuscular junction transmission. An improvement of >2 mm in ptosis after applying an ice pack for 5 min has been shown to have high diagnostic sensitivity [35, 36]. The fast-acting AChE inhibitor, edrophonium, has been used for many years to demonstrate a reduction of ptosis because of the improvement of muscle weakness in MG [37]. However, it has limitations due to cholinergic side effects, so it must be administered with intravenous atropine. Nowadays, slow-acting AChE inhibitors are preferred for pharmacological tests [38]. All patients suspected to have MG are screened for AChR-Ab. If AChR-Ab is not detected, but patients have likely clinical features, then MuSK-Ab and LRP4-Ab are evaluated to identify underlying autoimmunity [39]. Electrodiagnostic tests confirm MG diagnosis and should be performed in weak muscles. Repetitive nerve stimulation (RNS) with a low-frequency stimulus (2–3 Hz) after exercise for 30–60 min shows a 10% decrement in compound muscle action potentials in MG [40]. Single fiber electromyography (SFEMG) is usually used in case of negative RNS. Jitter occurs due to the variability in the time between a depolarising nerve signal arriving at the neuromuscular junction and a muscle fiber action potential being initiated. This time variability is found to be increased in MG [40, 41]. SFEMG is more sensitive than RNS, but increased jitter can also be found in other neuromuscular disorders [18]. CT, MRI, and PET techniques are used to image the thymus in MG, but CT is usually preferred for screening thymic pathology [42].

Lambert-Eaton Myasthenic Syndrome

The Lambert-Eaton myasthenic syndrome (LEMS) is a pre-synaptic paraneoplastic or autoimmune neuromuscular junction disorder. LEMS is a rare disorder and is found to be 46 times less prevalent than MG [43]. Cancer has been detected in nearly 60% of patients with LEMS. Paraneoplastic LEMS is mainly associated with small-cell lung carcinoma (SCLC) [44]. Tumours express the same types of VGCCs as nerve terminals. P/Q and

possibly N-type VGCCs are targets of autoimmunity in LEMS. While P/Q-type VGCCs maintain ACh release from motor nerve terminals, N-type VGCCs control transmitter release from peripheral autonomic nerve terminals [45]. As a result of VGCC impairment, calcium influx into the nerve terminal is reduced, and so less ACh is released into the synaptic cleft leading to failure of NMJ transmission and muscle weakness [46]. The paraneoplastic form has an age onset of around 60 years, and it has male predominance [44]. The non-paraneoplastic form has female predominance, a peak age of onset of around 35 years, and a second peak at 60 years. These features are similar to MG [47].

The classical clinical triad of disease symptoms is lower extremity weakness-gait impairment, hyporeflexia/areflexia, and autonomic dysfunction [46]. The first clinical manifestation is usually weakness of the proximal muscles of the lower extremities. Mild diplopia, ptosis, and dysarthria can be seen during the disease course, and these are not commonly isolated [48, 49]. Weakness extends proximally to distally, and caudally to cranially, finally reaching the oculobulbar region [47]. Dry mouth, constipation, poorly reactive pupils, orthostatic hypotension, and erectile dysfunction are signs of autonomic impairment.

Diagnosis is based on the clinical history, electrophysiological studies, and antibody tests. Antibodies against VGCCs are detected in nearly 90% of patients with LEMS [50]. There are three main findings in EMG. The first is reduced compound muscle action potentials (CMAPs), usually less than 50% in motor nerve conduction studies at rest. The second is an increase in CMAP amplitudes greater than 100% after 10–30 s of maximal voluntary contraction demonstrated by a post-exercise test or by high-frequency (20–50 Hz) stimulation. The last finding is a decrease in the CMAP response to low-frequency (2–5 Hz) RNS [51].

Treatment

The oral AChE inhibitor, pyridostigmine, is an effective drug for the initial and symptomatic treatment of MG. It has also been shown to have fewer cholinergic side effects [52]. Corticosteroids are the first line of immunosuppressive treatment initiated in patients who are still symptomatic despite pyridostigmine treatment or require better symptom control [53]. Oral prednisolone and prednisone are fast-acting drugs with a more rapid onset of action when used in high doses and may be preferred in patients with severe symptoms. If used, patients must be monitored closely, and it should be considered if a bridge therapy with IVIG or plasmapheresis should be used before high-dose corticosteroids [18]. Oral prednisolone treatment usually begins with low doses to avoid corticosteroid-induced exacerbation of symptoms in patients with mild/moderate disorder severity. After low-dose treatment, dose adjustment occurs using weekly dose tapering or alternate-day administration. Oral corticosteroid treatment is also effective for managing ocular MG and has been shown to reduce conversion rates from ocular MG to generalised MG [54]. Long-term corticosteroid treatment has disadvantages, such as increasing the risk of osteoporosis, hyperglycaemia, glaucoma, and weight gain [55]. Azathioprine can be the first choice for non-steroidal long-term immunosuppressive treatment [53]. Azathioprine takes several months to reach peak efficacy; thus, it is usually administered with corticosteroids until that time [56]. Other non-steroidal immunosuppressive treatment options which are shown to have efficacy in MG treatment are mycophenolate mofetil, cyclosporine, rituximab, and tacrolimus [52]. Recent clinical trials have shown that efgartigimod is effective for treating generalised MG, and eculizumab is a treatment option for severe refractory AChR-Ab-positive generalised MG [57, 58]. Thymectomy is beneficial for early-onset generalised MG, which usually features thymic hyperplasia [57]. Myasthenic crisis treatment mainly includes mechanical ventilation of patients and either IVIG or plasmapheresis treatment [53]. Treating MG with MuSK antibodies involves important considerations. First, AChE inhibitors are not as effective or have a worsening effect in this group. Second, patients with MuSK antibodies can remain steroid dependent. Rituximab and plasmapheresis are other treatment options for patients with MuSK-MG, and 3,4-diaminopyridine (3.4-DAP) is also suggested as an effective option for the symptomatic treatment for MuSK-MG [59]. Treatment of LEMS with carcinoma includes the treatment of the underlying cancer. Symptomatic treatment is used in patients with or without cancer. 3,4-DAP binds to VGCCs and increases the time they remain open, leading to an increased pre-synaptic influx of calcium and, thus, increased ACh release. Therefore, it is a symptomatic therapeutic option for LEMS [60]. Patients with LEMS do not usually have a clear response to pyridostigmine, but AChE inhibitors may improve dry mouth [47]. IVIG, plasmapheresis, and rituximab are the therapy options for refractory cases [51].

Other NMJ Disorders

Congenital Myasthenic Syndrome

Congenital myasthenic syndromes (CMSs) are inherited disorders that can manifest with a history of fatigable weakness involving the ocular, bulbar, and limb muscles. CMSs usually occur in the neonatal period or in early childhood, but the disease can manifest in later childhood, adolescence, or even adulthood [61]. CMSs are mainly caused by mutations in genes encoding proteins whose functions are essential for maintaining the integrity of neuromuscular transmission (Table 11.1). The CMSs were previously classified as pre-synaptic, synaptic, basal lamina associated, and post-synaptic. Recently, protein glycosylation defects were also included as

Table 11.1 Classification of myasthenic syndromes according to genetics

Location of defect	Protein	Gene	Locus	Inheritance
Pre-synaptic (5–6%)				
Defects in ACh synthesis	ChAT	CHAT	10q11.2	AR
Paucity of synaptic vesicles				
LE-like congenital M				
Synaptic (13–15%)				
Endplate AChE deficiency	Collagen tail	COLQ	3p24.2	AR
Laminin β2 deficiency	Laminin β2	LAMB2	3p21	AR
Post-synaptic (%65–70)				
Fast channel syndromes	AChR-α	CHRNA1	2q24–32	AR
	AChR-δ	CHRND	2q33–34	AR
	AChR-€	CHRNE	17p13	AR
Slow channel syndromes	AChR-α	CHRNA1	2q24–32	AD
	AChR-β	CHRNB1	17p11–12	AD
	AChR-δ	CHRND	2q33–34	AD
	AChR-€	CHRNE	17p13	AD/AR
ACh deficiency	AChR-β	CHRNB1	17p11–12	AR
	AChR-δ	CHRND	2q33–34	AR
	AChR-€	CHRNE	17p13	AR
Defect in clustering of AChR	Rapsyn	RAPSN	11p11	AR
	MuSK	MUSK	9q31-32	AR
Defect of cytoskeleton	Plectin	PLEC	8q24-qter	AR
Defect in Na channel	Na channelα	SCN4A	17q23	AR
Defects in endplate development and maintenance	proteinDoK7	DOK7	4p16.2	AR
	GFPT1	GFPT1	2p12–15	AR
	DPAGT	DPAGT1	11q23.3	AR

ACh acetylcholine, *AChE* acetylcholinesterase, *LE-like congenital MG* Lambert-Eaton-like congenital myasthenia gravis, *ChAT* choline acetyltransferase, *AChR* acetylcholine receptor subunit (alpha, beta, delta, epsilon), *DOK7* Docking protein7, *GFPT1* glutamine-fructose-6-phosphatetransaminase1, *DPAGT1* dolichyl-phosphate N acetylglucosamine, *AD* autosomal dominant, *AR* autosomal recessive, *Na* sodium

a separate group of CMSs. Most CMSs are autosomal recessive disorders. Nearly 50% of patients with CMSs have mutations in genes encoding AChR subunits (CHRNA1, CHRNB, CHRNAD1, and CHRNE), 10–14% have mutations in Rapsyn, Dok-7, or Col-Q, and 5% have mutations in CHAT [62]. Although CMSs have a wide spectrum, this chapter will discuss the most frequent subtypes and the subtypes which are included in the differential diagnosis of MG.

Pre-synaptic CMS involves CHAT mutations that cause impaired synthesis and catalytic ineffectiveness of the enzyme [63]. The depletion of ACh in vesicles causes reduced CMAPs, especially after prolonged sub-tetanic stimulation. Episodic apnoea and respiratory crises may present in the neonatal period [64]. Pyridostigmine is the treatment option for this subgroup. Asymptomatic patients should also be treated due to the risk of fatal apnoea. Severe phenotypes may not respond to cholinergic drugs and require permanent ventilatory support [65].

Synaptic basal lamina-associated CMS involves Col-Q mutations [66]. Patients present in the neonatal period or infancy with apnoea and generalised weakness. Some patients have spared ocular ductions, and in some, the pupillary light reflex is delayed [62]. AChE inhibitors are not indicated in patients with Col-Q deficiency, but adrenergic agonists such as ephedrine or albuterol can be beneficial [67].

Post-synaptic CMSs, which comprise 2/3 of all CMSs, occur due to AChR deficiency and kinetic changes in AChR function (fast or slow channel syndromes). The nicotinic AChR is a pentameric complex consisting of different transmembrane subunits (α, β, δ, and ε-subunits) encoded by *CHRNA1, CHRNB1, CHRND,* and *CHRNE,* respectively. AChR deficiency is mainly caused by *CHRNE* mutations [3]. Patients with *CHRNE* mutations present with ophthalmoplegia, ptosis, dysphagia, and fatigable proximal muscle weakness with onset in early infancy [68]. Patients respond to 3,4-DAP, pyridostigmine, and add-on β2-adrenergic agonist therapy, but their ophthalmoparesis is frequently treatment resistant [65, 68] Mutations in any AChR subunits can change its ion channel function leading to prolonged (slow channel syndrome, SCS) or shortened periods of opening (fast channel syndrome, FCS). AChE inhibitors and 3,4-DAP are contraindicated in patients with SCS, but quinidine or fluoxetine may be beneficial. Patients with FCS benefit from AChE inhibitor treatment.

Another post-synaptic CMS is Dok-7-associated CMS which frequently manifests in late childhood but can also present in adulthood. Weakness usually occurs in limb-girdle

muscles, and ptosis is often present [69]. Detection of tongue muscle wasting is a diagnostic clue for Dok-7 CMS [68]. Pyridostigmine must be avoided, but most patients have a clear response to the sympathomimetics such as ephedrine or salbutamol [70].

Botulism

Botulism is caused by toxins formed by the anaerobic, gram-positive bacterium *C. botulinum* and rarely by strains of closely related species (*C. baratii* and *C. butyricum)* [71]. Human cases are usually caused by toxin types A, B, E, and rarely by toxin type F [72]. Botulism types include food-borne botulism (from botulinum neurotoxin in food), wound botulism (from botulinum neurotoxin in a wound found due to bacterial colonisation), inhalational botulism (from aerosolised botulinum neurotoxin), and iatrogenic botulism (from botulinum neurotoxin gained from high-concentration botulinum toxin injections for cosmetic or therapeutic purposes) [73]. Neurotoxins produced by *C. botulinum* affect the SNARE protein, blocking the adhesion and fusion of synaptic vesicles and exocytosis of ACh [1]. Typical initial symptoms include acute blurred vision, diplopia/ophthalmoplegia, facial weakness, dysarthria, and dysphagia. Later, descending paralysis occurs, and patients have bulbar and limb muscle weakness. Fixed and dilated pupils, dry mouth, gastrointestinal symptoms, and heart rate abnormalities may be present in patients with botulism [46]. Diagnosis is based on clinical suspicion, a detailed clinical history, and neurological examinations. EMG and laboratory confirmation of botulism can be obtained with serum and stool assays for botulinum toxins which are also used to diagnose botulism. However, these processes take time, and prompt anti-toxin treatment must begin before a laboratory diagnosis is confirmed in the event of clinical suspicion [71].

All About the Pathology of Neuromuscular Junction

Muscle or nerve biopsy examination is almost never required in neuromuscular junction disorders because the patient's history and clinical and electrophysiological findings are often sufficient for diagnosis. However, muscle biopsy examination may occasionally be required in patients with an unusual history and infants with congenital myasthenic syndrome. Histopathological findings that can be seen in neuromuscular junction diseases are minimal and nonspecific. The most common alteration is type 2 myofiber atrophy, but type 1 myofiber atrophy is also reported. In rare cases, nuclear clumping and angular atrophic fibers, similar to those seen in neurogenic myopathies, can be seen [74–77].

Case 11.1
A 45-year-old female patient, who had muscle weakness for 2 years, was diagnosed with myasthenia gravis based on clinical and EMG findings. She was serologically negative for antibodies, and a muscle biopsy examination was performed. No pathology was detected except for slight myofiber size and shape difference and severe myofiber type 2 atrophy (Fig. 11.2).

Case 11.2
A 59-year-old female patient, who had ptosis for several years, was suspected of having myasthenia gravis based on EMG findings. Due to the absence of extraocular involvement, a muscle biopsy examination was performed to investigate a differential diagnosis of mitochondrial disease. No pathological findings were found except for severe myofiber type 2 atrophy. Additionally, ragged red fibers and mitochondrial enzyme deficiency, which are pathognomonic for mitochondrial diseases, were not seen (Fig. 11.3).

Case 11.3
A 19-year-old male patient, who had myasthenia from early childhood, was diagnosed with congenital myasthenia gravis based on clinical and EMG findings. The disease was not genetically confirmed, and a muscle biopsy examination was performed. The muscle biopsy showed slight myofiber size and shape differences and mild group atrophy. In addition, nuclear clumping was also seen, similar to that observed in the neurogenic atrophies (Fig. 11.4).

Case 11.4
A 67-year-old male patient, with muscle weakness for 10 years, was diagnosed with myasthenia gravis based on clinical and EMG findings. He was serologically positive for AChR-Ab, and MG treatment was commenced. A muscle biopsy examination was performed because of the increasing weakness which developed. In the muscle biopsy, an appearance similar to the neurogenic myopathies was observed

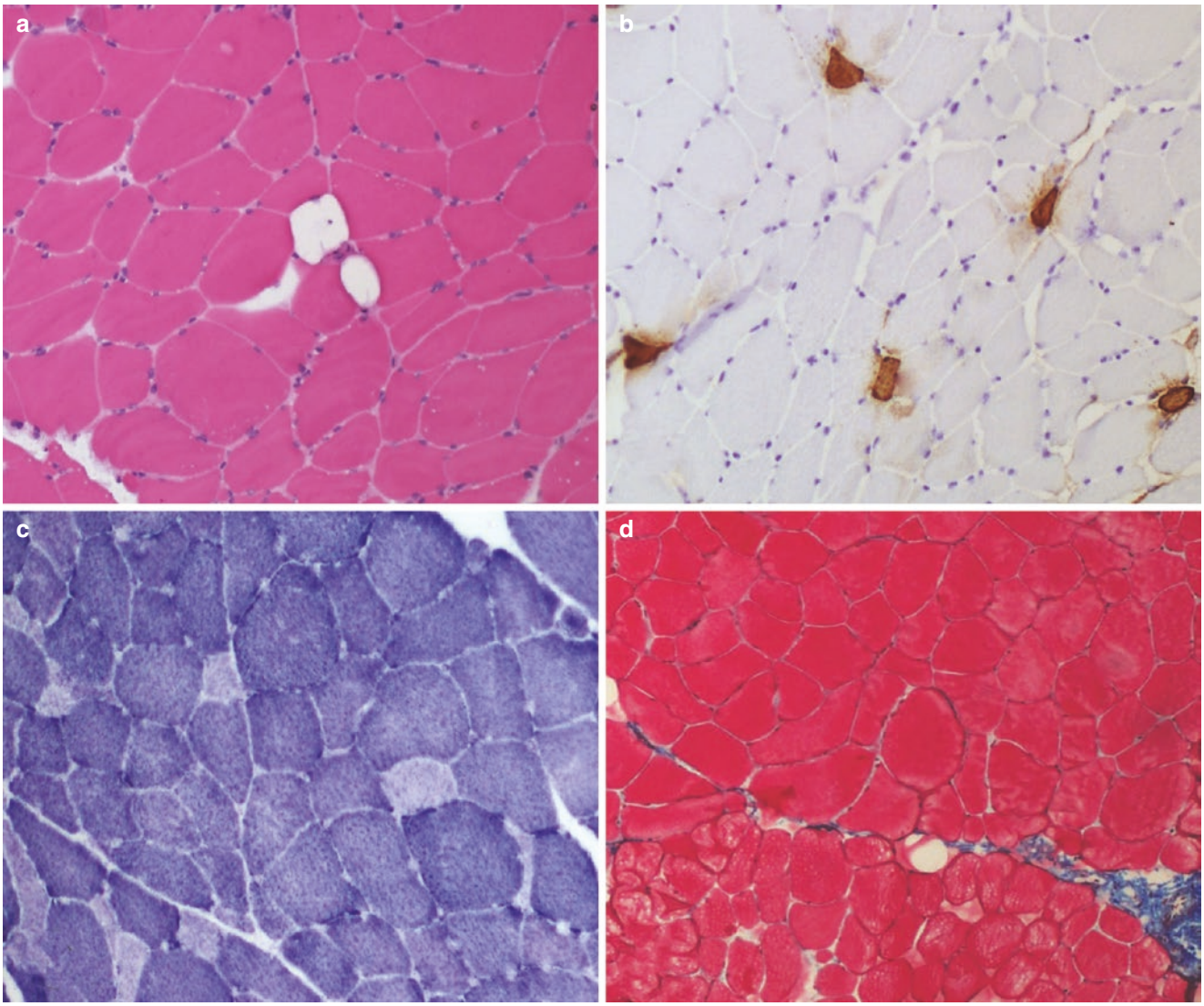

Fig. 11.2 (**a**) Mild variation of fiber size and shape (H&E × 100), (**b**) severe type 2 fiber atrophy with fast myosin immunohistochemistry (DAB × 100), (**c**) type 2 fiber atrophy (NADH-TR × 100), (**d**) normal intercellular tissue (Masson trichrome × 100)

with significant myofiber size and shape differences. Additionally, atrophic angular fibers were present in most type 2 fibers (Fig. 11.5).

Case 11.5

An 18-year-old male patient, who had myasthenia during the last 2 years, was diagnosed with congenital myasthenia gravis based on clinical and EMG findings, but the disease was not genetically confirmed, and a muscle biopsy was examined. There were severe myofiber size and shape differences and group atrophy in the muscle biopsy. In addition, angulation was also determined like in the neurogenic atrophies (Fig. 11.6).

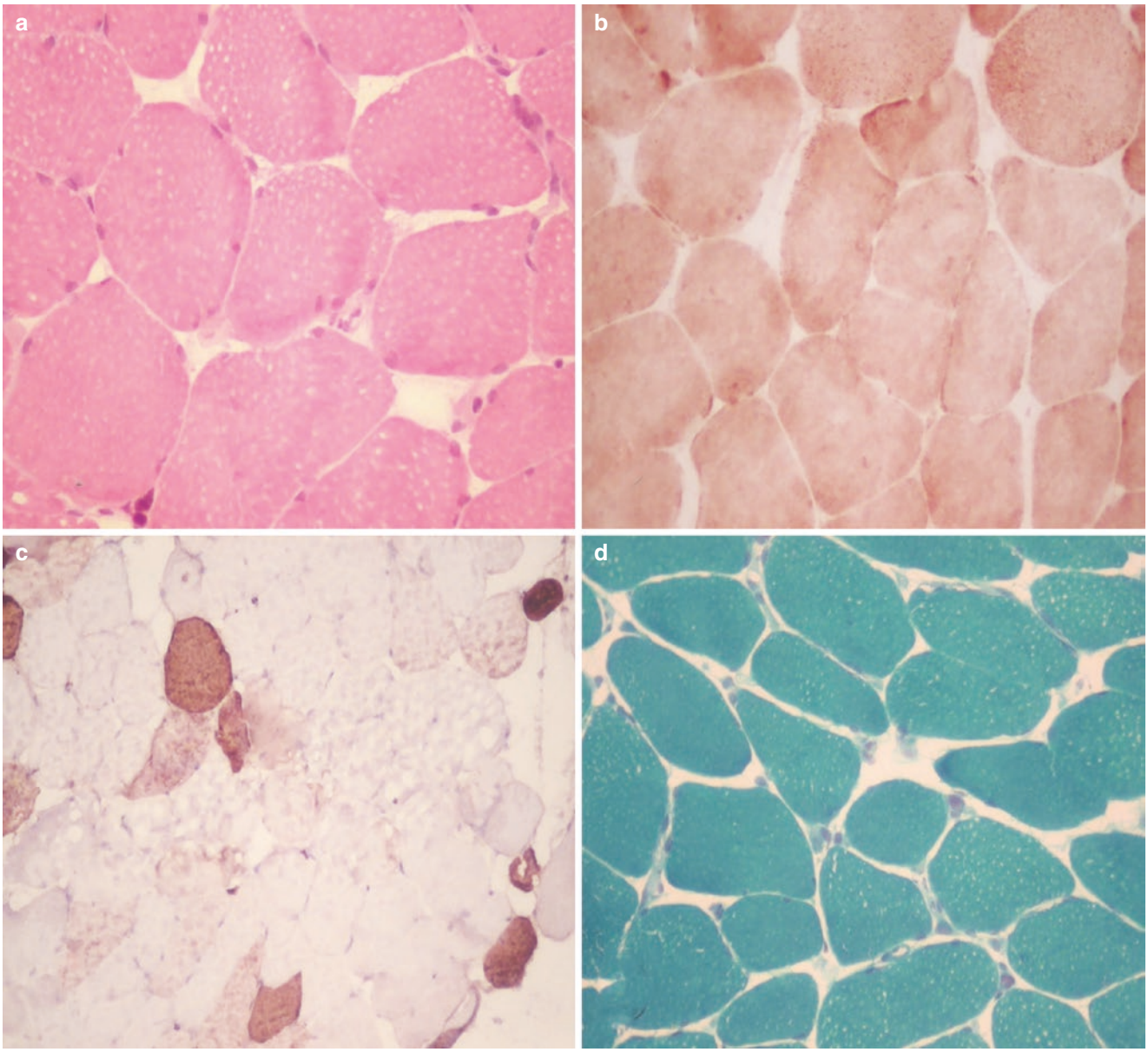

Fig. 11.3 (**a**) Mild variation of fiber size and shape (H&E × 200), (**b**) absence of mitochondrial enzyme dysfunction (COX × 200), (**c**) severe type 2 fiber atrophy with fast myosin immunohistochemistry (DAB × 100), (**d**) absence of ragged red fibers (modified trichrome × 200)

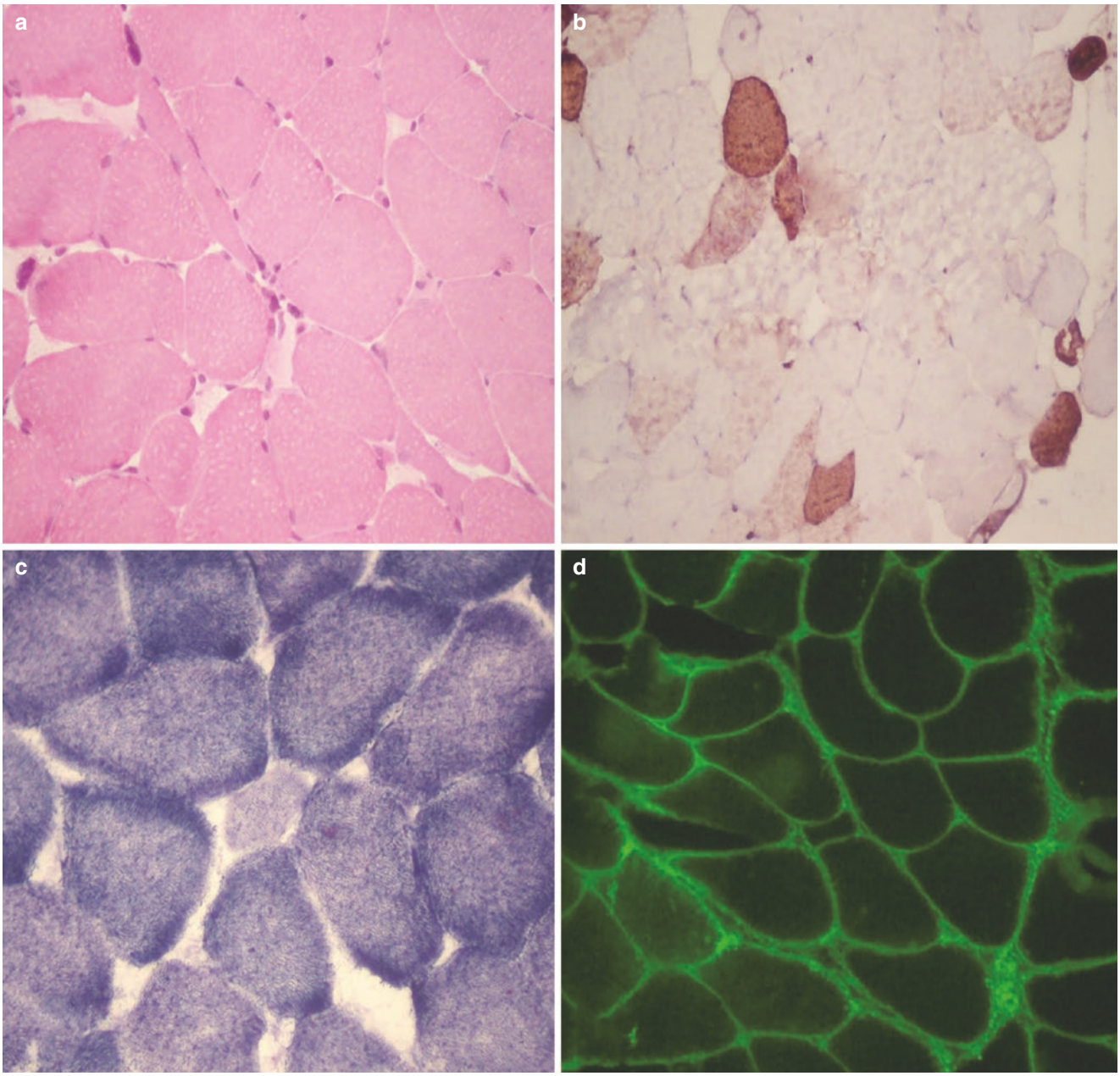

Fig. 11.4 (**a**) Presence of nuclear clumping (HE × 200), (**b**) severe type 2 fiber atrophy with fast myosin immunohistochemistry (DAB × 200), (**c**) an atrophic type 2 fiber (SDH × 100), (**d**) normal fibrinogen expression in the endomysium using direct immunofluorescent (DIF) examination (Fluorescein × 200)

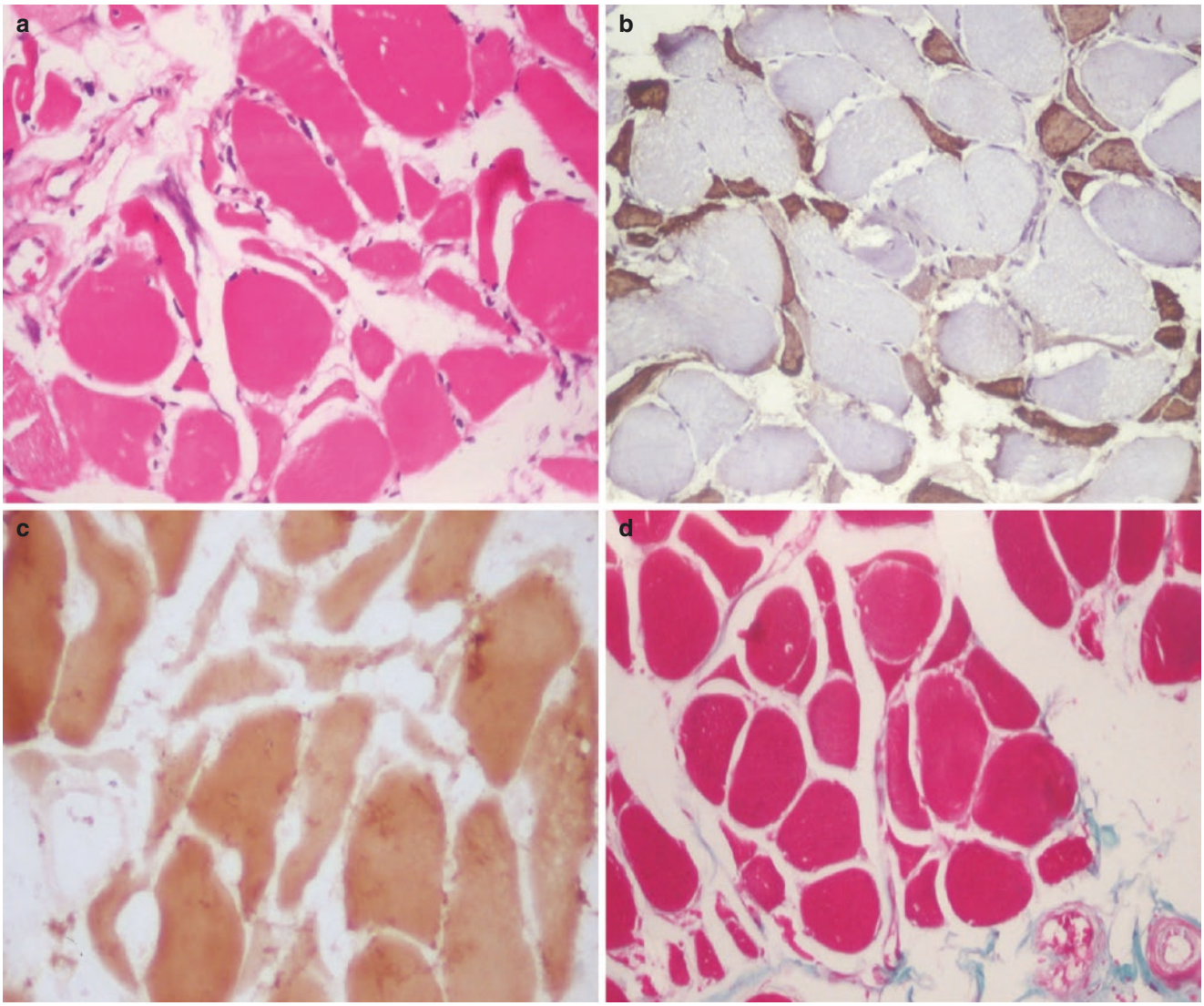

Fig. 11.5 (**a**) Severe variation of fiber size and shape with angulation (HE × 200), (**b**) severe angulation and atrophy of type 2 fibers with fast myosin immunohistochemistry (DAB × 200), (**c**) normal mitochondrial function (COX × 400), (**d**) increasing amount of intercellular tissue (Gomori trichrome × 200)

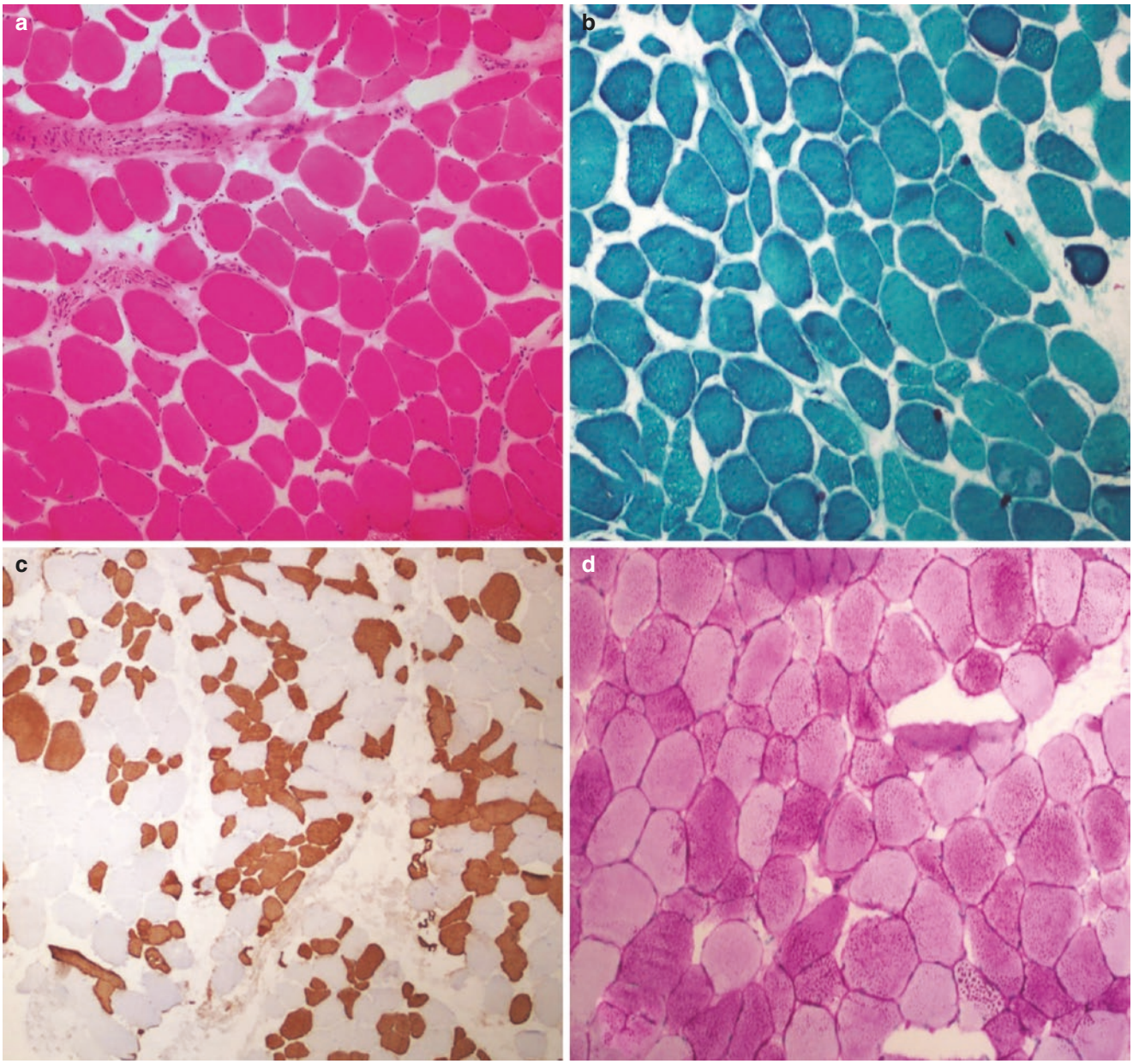

Fig. 11.6 (**a**) Prominent variation of fiber size and shape (H&E × 100), (**b**) absence of the ragged red fibers (modified trichrome × 100), (**c**) type 2 fiber atrophy and grouping with fast myosin immunohistochemistry (DAB × 100), (**d**) normal glycogen presence (PAS × 200)

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Muscular Dystrophies

12

Figen Baydan, Bedile Irem Tiftikcioglu, and Gulden Diniz

Introduction

Muscular dystrophies (MDs) are a group of hereditary and often progressive muscle diseases in which necrosis develops in muscle fibers due to a lack of a structural protein or enzyme, and they are replaced by fat and connective tissue. Structural or functional mutations in one of the proteins in the extracellular matrix surrounding the muscle fiber, sarcolemma, basement membrane, sarcoplasm, or nucleus can result in MD (Fig. 12.1). Limb, axial, or facial muscles are affected in varying degrees. Respiratory muscles, cardiac muscles, bulbar muscles, and smooth muscles might also be affected depending on the pathogenetic basis. Other organ systems or tissues such as brain, eye, and kidney might be involved depending on the diversity of expression of the mutated protein in the body. Accordingly, MDs exhibit a wide range of disease features including age of onset of symptoms, clinical course, pattern and severity of involvement, rate of progression, and concomitant conditions. The pathogenesis of some of *the* MDs has been clarified thanks to developments in molecular genetics (Table 12.1) [1, 2].

Fragility of any component of muscle cells due to genetic defects results in myofibril necrosis accompanied by inflammation and oxidative stress, as the muscle cell cannot withstand the stress during contraction. Over time muscle degeneration progresses and weakness appears due to the loss of muscle cells. In these diseases, attacks of necrosis and regeneration occur in the muscle fibers. First, the sarcoplasmic reticulum dilates due to the membrane defect, and then calcium ions enter the cell and activate endogenous proteases. The number of muscle cells decreases, and variation in muscle fiber size is seen due to the degeneration and apoptosis that occurs. Within 1–3 weeks, the sarcolemmal nucleus swells, its number increases, and regeneration begins. Satellite cells are activated upon injury and differentiate into myoblasts. The amount of connective and adipose tissue increases, which causes the swollen appearance of the gastrocnemius. Fibrosis develops due to repetitive damage; this event also damages the neuromuscular junction, and resulting denervation develops [1–3].

The age of onset of MDs can be seen from birth to the adult period. In a patient with muscle weakness, recognising specific patterns of involvement in MDs helps differentiate the disorder from other diseases that can cause weakness. Muscle weakness can progress with six different clinical findings (Fig. 12.2). For instance, dystrophinopathies are characterised with weakness of the shoulder, pelvic girdle, neck flexor muscles, and gastrocnemius hypertrophy; limb girdle muscular dystrophies (LGMDs) involve the pelvic and shoulder girdle; fascioscapulohumeral muscular dystrophies (FSHDs) involve the face, scapula, and upper muscles; distal myopathies involve distal muscles; and congenital muscular dystrophies (CMDs) are characterised with diffuse muscle involvement. Similarly, in a patient with early contractures and cardiomyopathy, the diagnosis of Emery-Dreifuss muscular dystrophy (EDMD) should be considered, whereas, in a patient with mere extraocular and pharyngeal muscle weakness, the diagnosis of oculopharyngeal muscular dystrophy (OPMD) would be more likely. However, although recognising these patterns might lead to diagnosis, it still does not eliminate the need for a systematic and detailed approach. A systematic approach will facilitate the correct diagnosis in most cases. In other words, a detailed history of symptoms and family medical background, including a fam-

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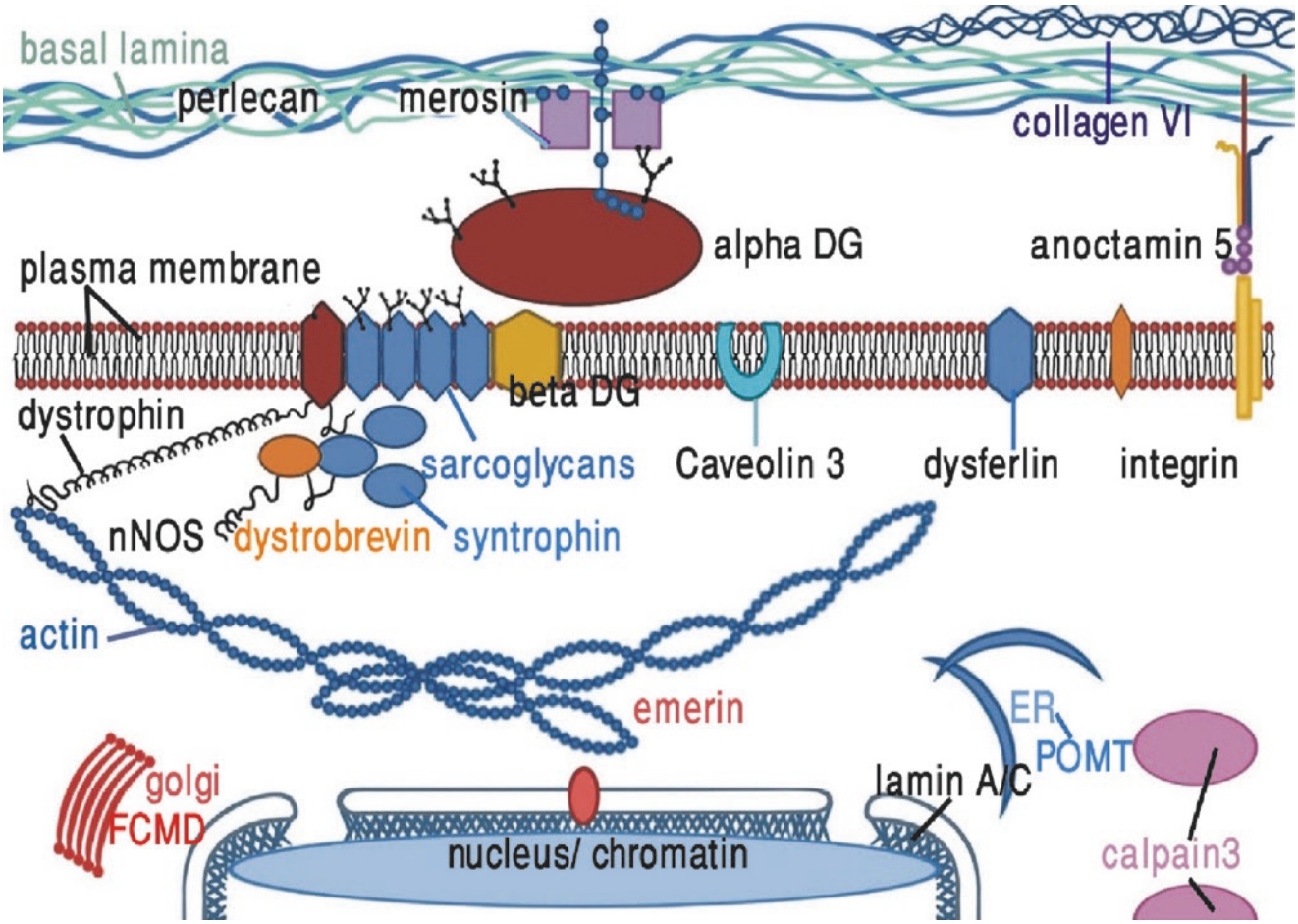

Fig. 12.1 Schematic view of striated muscle cell structural elements that can cause muscular dystrophy

ily tree of at least two/three generations, and comprehensive systemic and neurological examinations remain mandatory. This approach is so important that it may be possible to reach the correct diagnosis later if confirmed by genetic testing, providing a detailed examination and accurate history are taking by an experienced clinician. This will eliminate the need for electrophysiological testing or muscle biopsy, which is often uncomfortable for many patients [1–3].

The clinician should question the patient in detail for a previous diagnosis, the main symptoms, when the symptoms started, course of progression, which movements are difficult to perform, the presence of other accompanying complaints such as pain, and worsening/improving conditions. This helps differentiate primary diseases of muscle from other local, systemic (joint diseases, thyroid disease, anemia, metabolic disorders such as hypoglycemia, hypernatremia, hypomagnesemia, etc.), or neurological (vascular/demyelinating/degenerative diseases of the central nervous system, motor neuron disease, radiculopathy, plexopathy, neuropathy, neuromuscular junction diseases) diseases that can cause muscle weakness. Then, laboratory tests (serum creatine kinase, electrophysiological tests, genetic tests, and histopathological evaluation, where indicated) can be used to confirm the diagnosis. Patients can sometimes use terms such as numbness or dullness to express their symptoms of muscle weakness. In this situation, it is necessary to differentiate the feeling of numbness or paresthesia from a real loss of muscle strength. Pain can restrict functional movement and cause difficulty in walking or grasping. Pain, except for infectious and inflammatory muscle diseases, rarely accompanies MDs. In the presence of pain, it is necessary to further investigate rhabdomyolysis and myoglobinuria in patients with muscle disease. Fatigue is often described by patients as weakness or having a lack of energy, but very rarely, it is secondary to a real muscle disease. In general, fatigue describes the inability to perform more general functions (such as shopping and cleaning) without a special pattern of muscle weakness. Although it is more related to systemic diseases, post-exercise fatigue can typically be seen in metabolic muscle diseases. Fatigue that improves with rest is commonly seen in neuromuscular junction diseases. Fatigue can be a problem in severe MDs if the heart and respiratory muscles are involved [1–4].

Table 12.1 Genetic features of common muscular dystrophies (MDs)

Disease	Protein	Gene	Chromosome
X-linked muscular dystrophies			
Dystrophinopathies (Duchenne muscular dystrophy, Becker muscular dystrophy, manifesting carriers)	Dystrophin	DMD	Xp21
Emery-Dreifuss MD (EDMD)	Emerin	EMD	Xq28
Autosomal dominant muscular dystrophies			
Facioscapulohumeral dystrophy (FSHD)	Double homeobox 4	DUX4	Repeats at 4q35
Myotonic dystrophy, type 1 (DM1)	Myotonic dystrophy protein kinase	DMPK	9q13
Myotonic dystrophy, type 2 (DM2)	CCHC-type zinc finger nucleic acid-binding protein	CNBP	3q21
Oculopharyngeal MD	Poly A binding protein, nuclear 1	PABP2	14q11
Limb-girdle muscular dystrophies type 1 (LGMD 1)			
LGMD 1A	Myotilin	MYOT	5q31
LGMD 1B/EDMD-AD inheritance	laminA/C	LMNA	1q21
LGMD 1C	caveolin3	CAV3	3p25
LGMD 1D	HSP-40 homologue, subfamily B-6	DNAJB6	7q36
LGMD 1E	Desmin	DES	2q35
LGMD 1F	Transportin 3	TNPO3	7q32
Autosomal recessive muscular dystrophies			
Congenital muscular dystrophies (CMDs)			
Merosin-deficient CMD (MDC1A)	Laminin alpha-2 chain	LAMA2	6q22
Dystroglycanopathies, including Walker-Warburg syndrome, muscle-eye-brain disease, and Fukuyama CMD	Defects of O-mannosylation of α-dystroglycan due to several genetic variations	POMT1 POMT2, FKTN FKRP, LARGE, POMGnT1 (O-linked mannose beta1,2-*N*-acetylglucosaminyltransferase)	
Collagen VI-related dystrophies (Ullrich CMD)	alpha1, alpha2, or alpha3 chains of type VI collagen	COL6A1, A2 or A3	21q22
Limb-girdle muscular dystrophies type 2 (LGMD 2)			
LGMD 2A	Calpain-3	CAPN3	15q15
LGMD 2B	Dysferlin	DYSF	2p12
LGMD 2C	Gamma sarcoglycan	SCGC	13q12
LGMD 2D	Alpha sarcoglycan	SCGA	17q21
LGMD 2E	Beta sarcoglycan	SCGB	4q12
LGMD 2F	Delta sarcoglycan	SCGD	5q33
LGMD 2G	Telethonin	TCAP	17q12
LGMD 2H	Tripartite motif-containing 32	TRIM32	9q33
LGMD 2I	Fukutin-related protein	FKRP	19q13.3
LGMD 2J	Titin	TTN	2q31
LGMD 2K	Protein-*O*-mannosyltransferase 1	POMT1	9q34
LGMD 2L	Anoctamin 5	ANO5	11p14
LGMD 2M, LGMD 2N	Fukutin, protein-*O*-mannosyltransferase 2	FKTN, POMT2	14q24
LGMD 2O	POMGnT1	POMGnT1	1p34
LGMD 2Q	Plectin 1	PLEC1	8q24

A multidisciplinary team approach with good communication is paramount for effective, fast, and accurate diagnosis and management of all neuromuscular disorders including MDs. A disease can be diagnosed according to the muscles involved in the first examination, but it may not be given a specific diagnosis since the clinical findings are not fully established in childhood. If the disease progresses slowly over many years, findings such as creatine kinase elevation, the age of onset of the disease, accompanying hearing loss, cardiomyopathy, the muscle group atrophied, form and time of contractures, and family history help to diagnose it specifically. The absence of sick females in a family rules out x-linked heredity. Although a biopsy was performed immediately in previous years, today genetic examination of the presumed disease can be requested, and the diagnosis can be made within a short time span. However, biopsy remains to be the gold standard [3, 4].

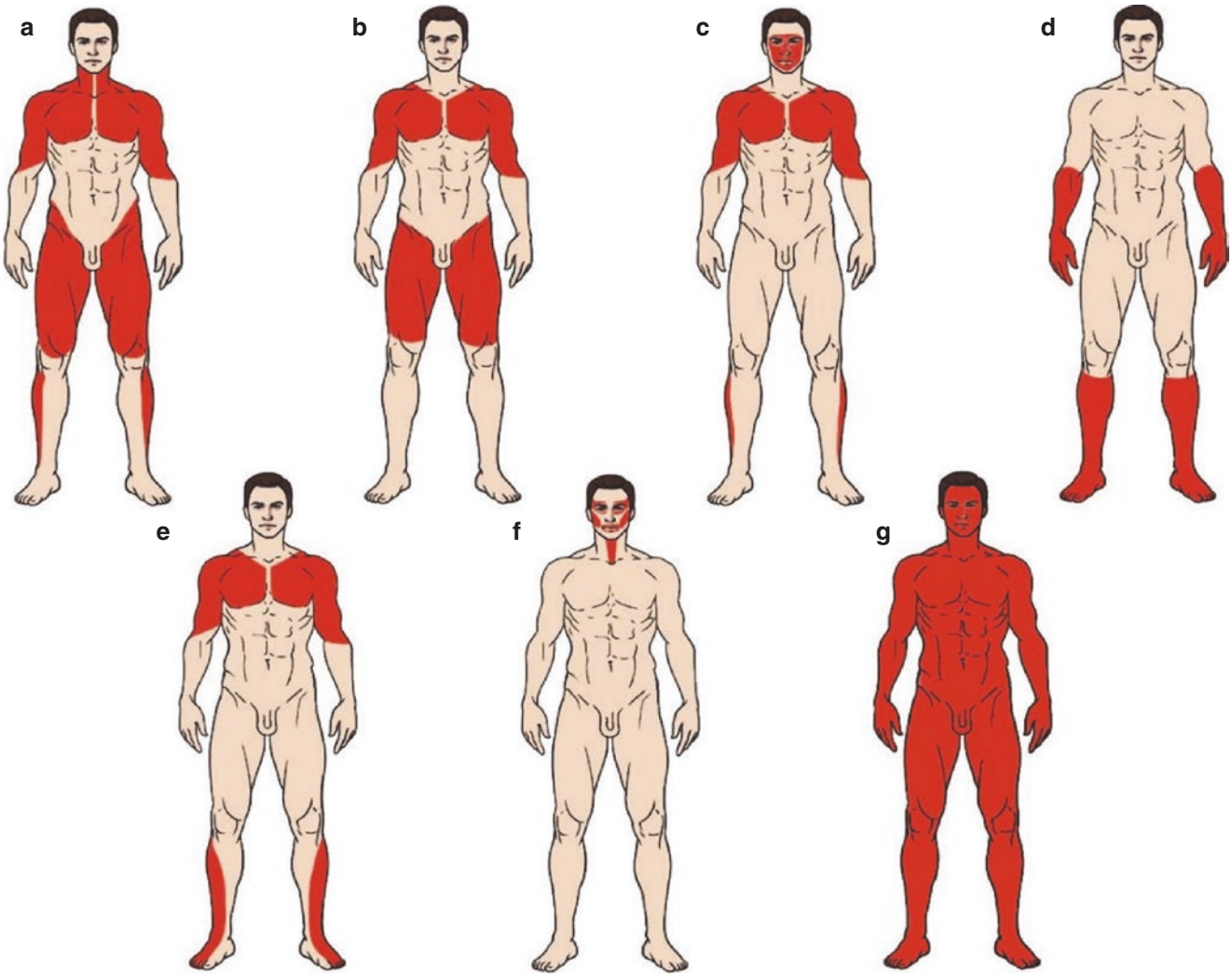

Fig. 12.2 Types of muscular involvement in muscular dystrophies. (**a**) Dystrophinopathies involve the shoulder, pelvic girdle, and neck flexor muscles with gastrocnemius hypertrophy, (**b**) limb-girdle muscular dystrophies involve the pelvic and shoulder girdle, (**c**) facioscapulohumeral muscular dystrophy with fascioscapulohumeral involvement, (**d**) distal myopathies which involve distal muscles, (**e**) Emery-Dreifuss muscular dystrophy with scapulohumeral and peroneal involvement, (**f**) oculopharyngeal muscular dystrophy with oculopharyngeal involvement, (**g**) congenital muscular dystrophies with diffuse muscle involvement

Dystrophinopathies

The dystrophin gene is the largest gene to be isolated in humans to date, consisting of 2.2 million base pairs, 6% of which consists of 79 exons, and the other part consists of introns. In patients, of all the mutations, 65% exon deletion, 6% duplication, and 30–40% point mutation (frameshift, nonsense, splice site, missense mutations) are seen. The nonsense mutation rate of all Duchenne muscular dystrophy (DMD) patients is 10–13%. There are also other mutations that are rarely detected with m-RNA analysis. DMD occurs in 1 in 3500–5000 live male births. In a meta-analysis study conducted in 2022, its prevalence was reported in the male population at 7.1/100,000 (0.9–16.8/100,000) and 19.8/100,000 live male births (1.5–28.2/100,000). However, in this review, it was stated that most of the studies were conducted in Europe, and there were no studies in certain parts of the world. It was also emphasised that more comprehensive studies were needed [1–3]. Dystrophinopathies can present with different clinical courses. DMD, which develops due to the complete absence of the subsarcolemmal protein dystrophin because of a genetic defect in the Xp21.1, Xp.21.2 region on the short arm of the X chromosome, is the most severe form. In Becker muscular dystrophy (BMD), which has a milder course, the dystrophin protein is present, but its quality is poor, and its quantity (Fig. 12.3). The third clinical type is the intermediate type, and its findings may be like that of both DMD and BMD. The fourth clinical type is

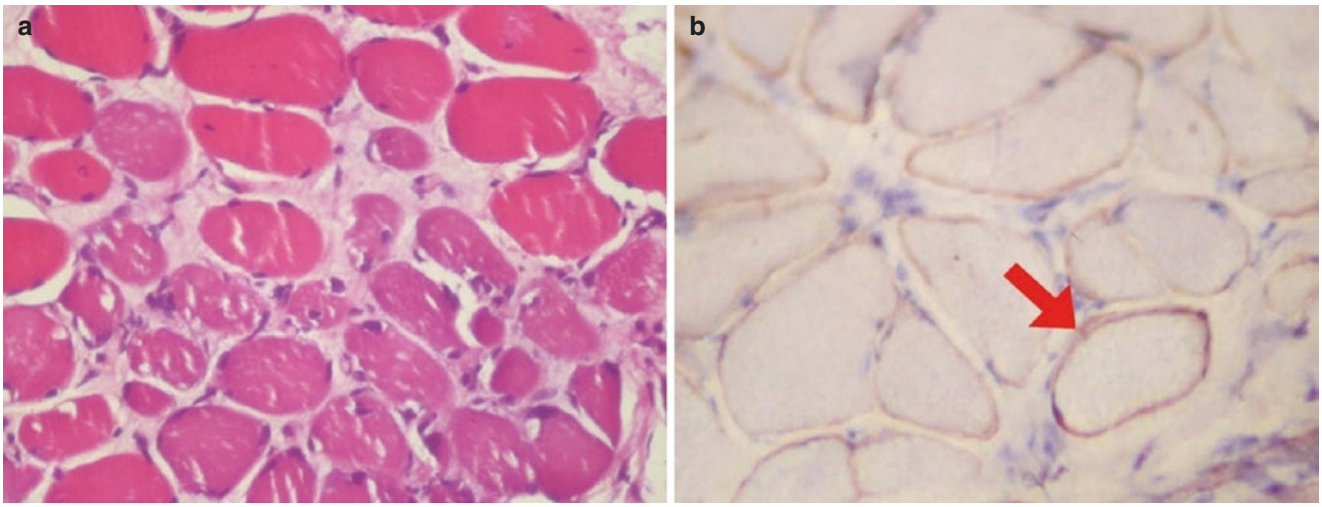

Fig. 12.3 Muscle biopsy from a genetically proven case of dystrophinopathy: (**a**) Typical muscular dystrophy appearance with plenty of regenerated fibers (HE ×100), (**b**) Presence of sarcolemmal dystrophin expression (arrow) excludes the diagnosis of Duchenne muscular dystrophy (DAB ×200)

the clinical type with less severe muscle involvement but with severe cardiomyopathy. The clinical findings in the fifth type include symptoms such as muscle cramps, myoglobinuria, elevated creatine kinase, and creatinuria, while muscle involvement findings are very mild [5–7].

In DMD, the quantity of dystrophin is less than 5% of the normal amount; in intermediate types, it is between 5 and 20%, and it is above 20% in BMD. The difference between DMD and BMD is the age at which symptoms become severe enough to render patients wheelchair bound. While this finding develops at 13 years of age in DMD, it develops later in BMD and in the intermediate type. In patients with DMD, creatine kinase is elevated at birth, and there are histopathological signs of muscle injury, but clinical signs are usually absent. Utrophin is a protein structurally similar to dystrophin. It is found in the cell membrane of many tissues such as , smooth muscle cells of the uterus, myoblasts, myotubules, and intramuscular nerve and blood vessel cells. Utrophin is inherited on chromosome 6q24. In skeletal muscle, dystrophin is found at the plasma membrane, while utrophin is found at the neuromuscular and myotendinous junctions and in the sarcolemma in foetal life. After birth, dystrophin replaces utrophin. In DMD patients, utrophin is still found in the sarcolemma. In animal experiments, it was found that the increase in utrophin can compensate for dystrophin deficiency, but it was already known that utrophin had already increased in DMD. It was therefore understood that the disease progressed more severely in those with utrophin deficiency [8, 9].

In DMD, the mean age at diagnosis is 4 years and 10 months (16 months–8 years) in patients without a family history. Dystrophinopathies involve the shoulder, pelvic girdle, and neck flexor muscles with concomitant gastrocnemius hypertrophy. In childhood, the inability to lift the neck from the ground, short stature, poor language development, delayed sitting and walking without support, flat feet, duck-like walking, toe walking, increase in lumbar lordosis, frequent falls, and gastrocnemius hypertrophy are the first remarkable findings. After the age of 2–3 years, proximal weakness first appears in the proximal lower extremities, and the inability to run as fast as peers, jump and get up from a seat, difficulty in climbing stairs, and frequent falling is noticed. Gowers sign appears (Fig. 12.4). Upon getting up from the sitting position, the patients first turn forward, towards the ground, then cling to their legs and stands up by climbing on their own legs. They cannot get up without support from somewhere. The symptom of touching one leg once when standing up was observed at a young age, and in these children, a full Gowers sign appears within a few years. In DMD patients, the normal development of a child between the ages of 3–6 years shadows disease progression and initiates a false period of well-being, making families doubtful about the diagnosis. Around the age of 6–10, a significant decrease in muscle strength is witnessed. Gastrocnemius hypertrophy appears at the age of 3–4 years. Again, gastrocnemius hypertrophy in asymptomatic young children diagnosed with laboratory findings is the first sign of DMD. BMD patients do not have early hypertrophy. The clinical course of DMD is divided into pre-symptomatic, early ambulatory, late ambulatory, early non-ambulatory, and late non-ambulatory phases [1–3, 5].

The disease primarily involves the proximal muscles and lower extremities. In the later stages, muscle mass decreases in the pectoral, peroneal, and anterior tibial muscles. In the lower extremities, weakness of the muscles in the hip region occurs first, and lordosis increases due to weakness in the

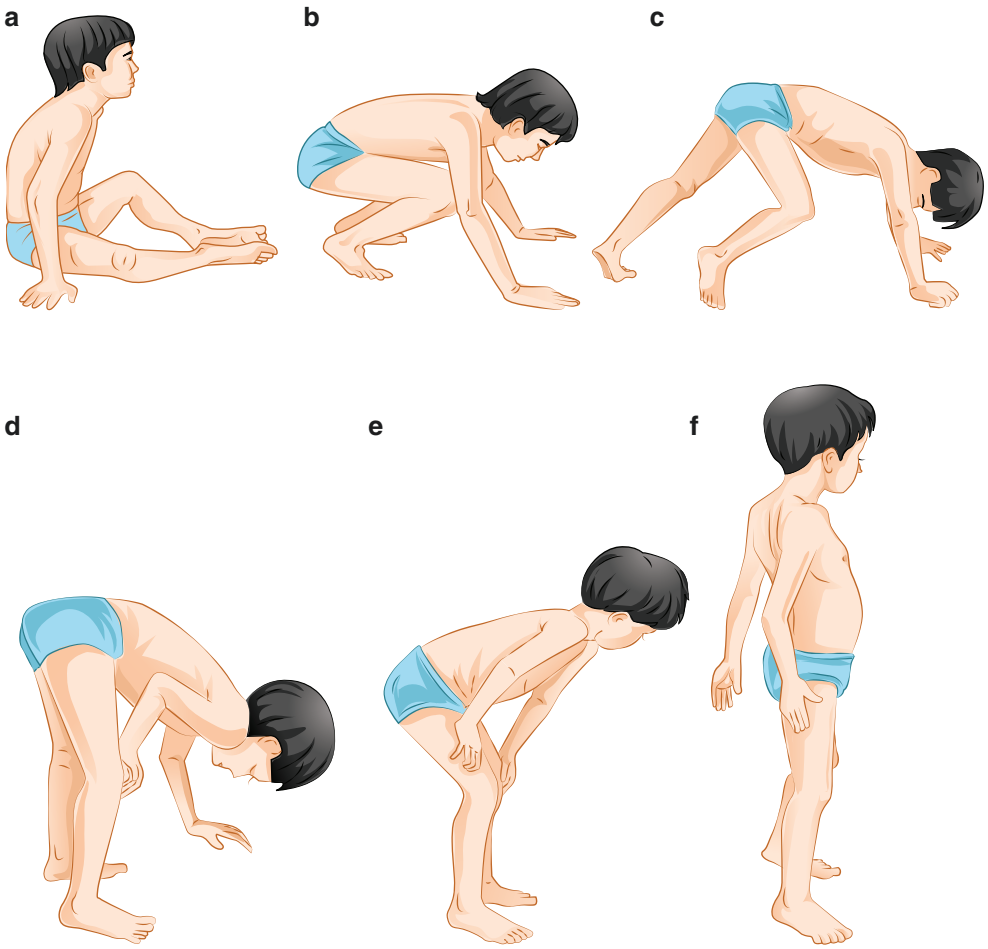

Fig. 12.4 Gower's sign. The sitting child on the floor assumes the hands-and-knees position (**a**, **b**, **c**), and then climbs to stand by "walking" his hands progressively up his shins, knees, and thighs (**d**, **e**, **f**)

extensors resulting in a duck-like gait that draws attention. Hypertrophy may also be seen in the vastus lateralis, infraspinatus, quadriceps, deltoid, and less frequently in the gluteus maximus, triceps, and masseter muscles. When the quadriceps, tibialis anterior, and peroneal muscles become weak, patients begin to walk on their toes to preserve balance and Achilles contracture subsequently develops. Ankle plantar flexors such as the gastrocnemius, soleus, and inverters tibialis posterior muscles are involved in the later stages. Neck flexors are weaker than extensors; biceps and triceps are weaker than deltoids; and quadriceps are weaker than hamstrings. Patients with DMD cannot lift their necks while supine, but patients with BMD and Intermediate types can. In the later stages of the disease, the tongue enlarges, difficulty raising the arms increases, scoliosis occurs, and weakness in the intercostal and diaphragmatic muscles occurs. For these reasons, a loss of pulmonary functions and nocturnal hypoventilation begin. During the development of scoliosis, vertebral fractures occur due to osteoporosis caused by corticosteroids. This makes patient mobilisation more difficult. There are also muscle pains and cramps. The external ocular muscles, levator ani, and external sphincter muscles are unaffected. It was observed that patients play on their phones and tablets more when they are newly immobilised. These children always sit cross-legged and lean to one side, and as a result, talipes equinovarus and scoliosis develop. Due to this contracture, it becomes impossible for patients who are newly bound to a wheelchair to stand up, and they progress to become immobilised more quickly. After being bound to a wheelchair, scoliosis develops with the involvement of the paraspinal muscles. Respiratory function is impaired due to progressive weakness in the respiratory muscles. As a result of insufficient ventilation at night, morning headaches begin first, and then the effects of chronic hypoxia appear. Respiratory muscle involvement and scoliosis make it difficult to overcome an emerging lung infection. Patients die towards the age of 20 or at the end of their 20s. The average life expectancy is 19 years in patients who do

not receive night ventilation, increasing to 27 years in those who receive this support. Spinal surgery is an important operation in the prevention of scoliosis. With spinal surgery and nocturnal ventilatory support, life expectancy can be extended up to 30 years. The development of cardiopathy may cause early death [1–3, 5, 6, 10].

Although females appear only to be carriers, they can be symptomatic if the mutation is carried on the active X chromosome, according to the Lyon hypothesis. Even though the mothers of male patients with DMD are inactive carriers, cardiac problems can be seen. Therefore, they should be under cardiological follow-up. Females carrying the mutation may be completely asymptomatic, or they may have very severe clinical findings. Symptomatic females are explained by the Lyon hypothesis. In early embryonic life, one X chromosome is silenced. According to the Lyon hypothesis, if the X chromosome carrying the mutation is not on the silenced X chromosome, clinical symptoms are seen. In cases where the mutation is carried on the X chromosome in patients with Turner syndrome (X0), the child is also clinically affected. A symptomatic female may also be seen in uniparental disomy. Twenty-two percent of females carrying the dystrophin mutation have signs of disease. Creatine kinase elevation is observed in 70% of carrier females in DMD and 50% in BMD. If no carrier gene is detected in the mother, there may not be a mutation in the mother's peripheral blood, but the mother may still carry the mutation in her germ cells in a mosaic manner. Mosaic carriage is explained by the occurrence of this mutation in the mitotic period of maternal germ cells. Germline mosaicism is suspected if the mother does not carry the mutation and has more than one child with DMD. In cases where the mother is not a carrier, the second child should still be evaluated for DMD, as there may be a possibility of germline mosaicism. Germline mosaicism can also occur in men. The father may not be symptomatic, but the daughters become carriers [1, 11, 12].

Case 12.1

An 8-year-old female patient was examined and referred when the creatine kinase value was found to be 3888 U/L while she was preparing for a tympanic membrane perforation operation. In her personal history, it was stated that she was born with a normal spontaneous delivery at term, walked at the age of 1.5 years, and had no other health problems other than frequent ear infections. There was no consanguineous marriage or any other muscle disease in the family history. She also had a healthy older brother. On physical examination, height = 125 cm (50%), weight = 22 kg (25%), there was gastrocnemius hypertrophy, muscle strength 5/5 in all extremities, and deep tendon reflexes were normal. EMG examination found myopathic findings in the upper and lower extremities and muscles. Duplication was found between exons 45, 46, 47, 48, and 49 in the dystrophin gene with multiplex ligation-dependent probe amplification (MLPA). Her karyogram was normal (46XX). The creatine kinase levels of the patient's older brother and mother were found to be normal. In her mother, 45, 46, 47, 48, and 49 duplications were found in MLPA. The fact that the mother had a normal phenotype despite carrying the mutation is explained by the Lyon hypothesis. The diagnosis was determined by finding a high creatine kinase value when she was admitted to the hospital for a febrile illness or an operation. If the creatine kinase elevation is not noticed, many tests are taken due to suspicion of liver pathologies, and even liver biopsy may be performed. While direct muscle biopsy was previously performed on patients, MLPA testing is now performed for the most common cause of dystrophinopathies due to the advances in genetic testing possibilities in recent years. If a diagnosis cannot be reached with MLPA, further tests can be performed for point mutation and RNA pathologies, or it can be sent to the genetic panel for the second largest group, LGMDs, according to possibilities. If the diagnosis cannot be reached, a biopsy can be performed. Biopsy remains the gold standard in some cases [11, 12].

Case 12.2

A 14-month-old male patient was referred for his incidentally detected high creatine kinase value (15,000 U/L), which was noticed while he was investigated in a health centre where he presented due to an upper respiratory tract infection. In his personal history, there were no abnormalities. He was born with a normal spontaneous delivery at term. There was no consanguineous marriage or any other muscle disease in the family history. He had a healthy older sister. On physical examination, there were no special pathological findings. Duplication or deletion mutations were not identified in the dystrophin gene with MLPA. To investigate the differentials of other MDs, a genetic myopathy panel with next-generation sequencing (NGS) was investigated, and a biopsy from the gastrocnemius muscle was taken. The biopsy had a typical MD appearance with regenerated fibers and fibrosis. Immunohistochemically, sarcolemmal dystrophin expression was completely absent (Fig. 12.5). The clinician was informed, and mutation analysis was recommended for the dystrophin gene. Hemizygous c.1207G > T (p.gly404T) mutation was detected in the dystrophin gene sequence analysis, performed because of the pathological examination, and the patient was diagnosed with DMD.

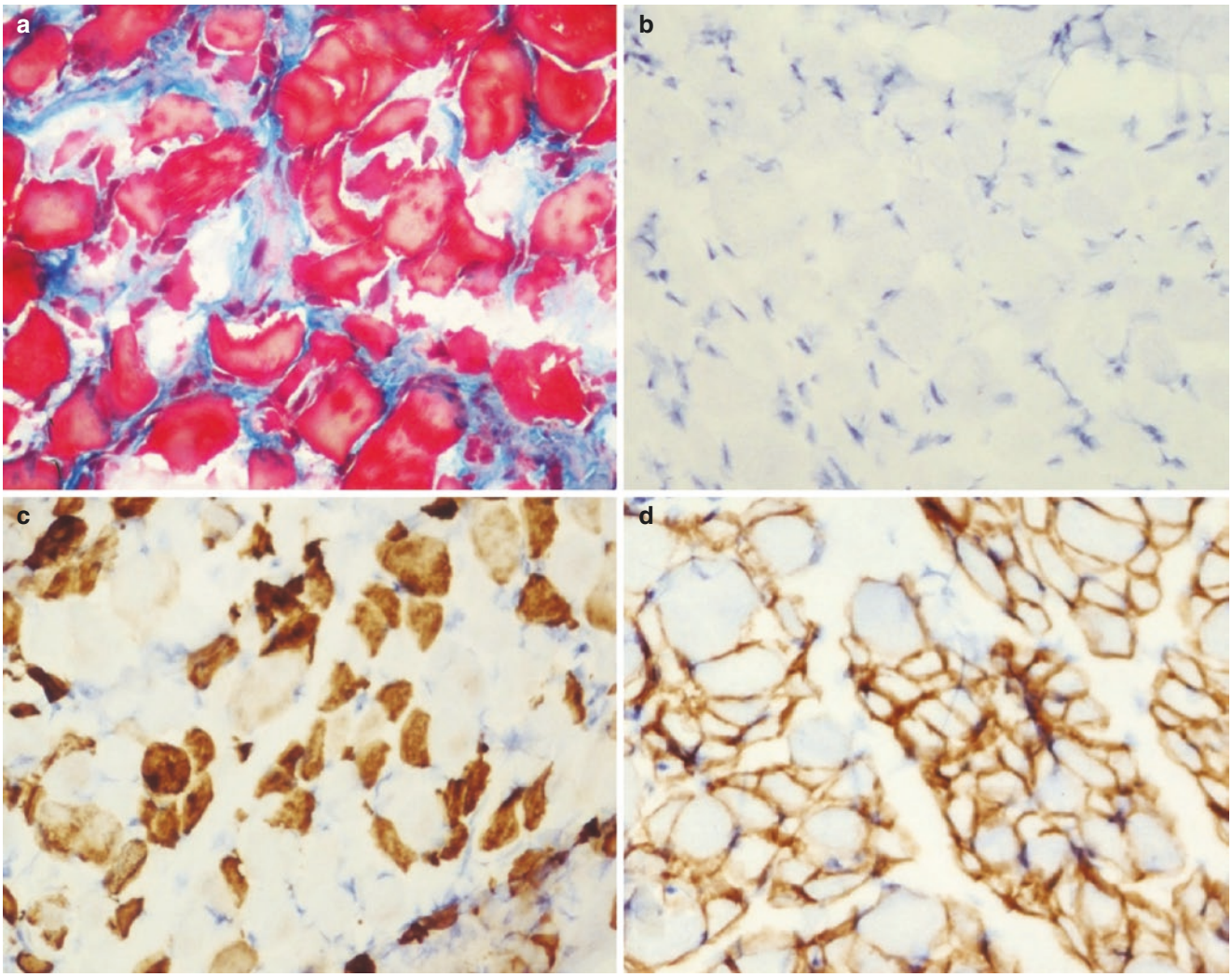

Fig. 12.5 (**a**) Increasing amount of intercellular tissue (Masson trichrome ×200), (**b**) Sarcolemmal dystrophin expression is absent (DAB ×100), (**c**) Many pathological immature myofibers with neonatal myosin immunohistochemistry (DAB ×200), (**d**) Severe myofiber size differences are highlighted with immunohistochemical merosin expressions (DAB ×200)

Clinical Progress and Follow-Up Parameters of Dystrophinopathies

Nutritional status, swallowing function, the presence of gastroparesis, and gastroesophageal reflux must be followed up. Gastrostomy opening in the late period may be required. Dystrophinopathies also affect the smooth muscles in the gastrointestinal tract. Therefore, intestinal hypomotility, megacolon, abdominal cramps, pseudo-obstruction, volvulus, and acute gastroparesis may be seen. Cardiological monitoring should be completed once a year. Cardiomyopathy develops in 90% of patients with DMD. Dilated cardiomyopathy and conduction disorders of intraatrial, interatrial, and atrioventricular type, sinus tachycardia, and other sinus abnormalities have been reported in DMD patients. Although 90% of DMD patients have cardiac involvement, 20% have cardiomyopathy as the cause of death. In the advanced stages of the disease, congestive heart failure and cardiac tamponade can be seen. Fibrosis develops in the anterobasal portion of the left ventricular wall. As the disease progresses, the posterior papillary muscle is involved, and mitral valve prolapse develops. Although muscular involvement is not very advanced in patients with BMD, severe cardiac involvement can be seen. Cardiac involvement is more severe, especially in those with 48–53 deletions. It has been reported that cardiac MRI shows cardiac function more precisely and reliably than echocardiography. In the last stages of disease, cardiac involvement may be masked due to a lack of effort [1, 13].

The pulmonary function of DMD patients should also be carefully monitored. Restrictive breathing difficulties develop over time due to weakness of the respiratory muscles. When secretions cannot be eliminated, aspiration and infections are very difficult to overcome, and the chronic effects of hypoxia, which first occurs at night and then dur-

ing the day, cause a life-threatening situation for patients. A decrease in maximum expiratory pressure indicates decreased muscle strength. It is recommended to monitor the respiratory function of patients once a year in the early ambulatory and ambulatory period and every 6 months in the non-ambulatory period [14].

The patient's mental status, depression, and psychosocial development should be monitored by a paediatric psychiatrist and psychologist. It has been reported that the developmental test scores of DMD patients may be lower than their peers in terms of motor, language, and social skills. It has been reported that reading, language skills, vocabulary, executive functions, attention, and visual memory skills may also be lower. The differences in symptoms and prognosis among patients can be attributed to the isoforms of dystrophin with different functions in different organs. The dystrophin gene can form cell type-specific isoforms of different molecular weights and these isoforms function in different organs of the body. The brain, cerebral cortex, hippocampal cortex, amygdala, purkinje cells, glial cells, Schwann cells, and retina contain dystrophins. Dp71 and Dp140 are shorter isoforms of dystrophin. They are present in the foetal brain and increase in number until adulthood. Dp71 has also been identified extensively in the hippocampus, parts of the cerebral cortex, in the synaptic membrane, microsomes, synaptic vesicles, and mitochondria. More severe intellectual retardation was seen in patients with mutations involving Dp71 and in patients with mutations at the 3′ end of the dystrophin gene. During the illness, increased anxiety and depression can also be seen due to the inability of patients keeping up with their peers in physical abilities. There are publications stating that autism and attention deficits are more common, but the relationship of these conditions with the location of the mutation is unknown [1, 2, 15, 16].

Laboratory Findings of Dystrophinopathies

Creatine kinase (CK) is high in the first year of life, 1000–2000 U/L, but levels then decrease before the age of 5. It can increase 20–200 times in BMD and 50–200 times in DMD, up to a value as high as 10,000–50,000 IU/L. It may increase in the first 10 years with the progression of the disease, and then it decreases by 20% per year with the decrease in muscle mass. In newborns, CK is measured using dry blood samples, and dystrophinopathy is investigated in those with elevated CK levels. Although CK levels are lower in BMD, it can sometimes reach very high values, so the distinction between BMD and DMD cannot be made by looking at the CK value. Excessive increases without an obvious cause should be investigated for an association with another muscle disease or associated metabolic myopathy [1–4].

Due to the leakage of intracellular muscle proteins, other muscle isoenzymes such as lactate dehydrogenase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) are also high. AST is found in the liver, kidney, pancreas, lung, and erythrocytes. ALT is more specific to the liver. CK is found in muscle, liver, and brain. Since other enzymes are found in many other tissues, CK is used more in the monitoring of muscle diseases. The muscle type of CK is MM, or MB and the brain type is BB. Although there is no significant increase after light exercise, myoglobin levels reach the maximum level within 2 h following heavy exercise, and CK levels reach the maximum level after 10–18 h. Myoglobin levels decrease to normal when CK reaches its maximum level. ALT and AST also increased together with CK because they are of muscle origin. In these patients, it is recommended to monitor liver function with gamma glutamyl transpeptidase (GGTP), and kidney function with cystatin C levels, since creatinine and creatinine clearance may be decreased. Malignant hyperthermia can be seen in DMD and other muscle diseases [1, 6, 15–17].

In **BMD**, dystrophin is present, but the quantity is reduced (about 10% of normal). Although dystrophin is quite high in patients with 45–48 distal rod deletions, dystrophin is very low in patients with N terminus deletions. The least amount of dystrophin is seen in those with carboxyterminal deletions. CK levels are around 200–20,000 U/L, but they can be within normal limits in very mildly affected patients. Since CK can increase to high values in BMD, it is not possible to distinguish between DMD and BMD by looking at this value. The age of onset of symptoms in BMD varies between 5 and 20 years (mean age of 12 years). It can be confused with DMD in patients younger than 8 years of age. Ninety-nine percent of patients usually show clinical symptoms by the age of 20, but in very mild types, symptoms may begin in the third and fourth decades. Symptoms first begin in the pelvic girdle, proximal to the lower extremities, and later symptoms develop in the shoulder girdle. Gastrocnemius hypertrophy is observed in most patients, and a myopathic gait is noted. Calf pain and muscle cramps occur after exercise, and myoglobinuria may be seen. The anterior tibial and peroneal muscles are less affected. The forearm, hand, and calf muscles maintain their strength until the last stages of the disease. There is a period of 10 years between pelvic muscle group and shoulder muscle group involvement. There is no involvement of the facial muscles. Contracture and scoliosis develop after being bound to a wheelchair. Gastrointestinal symptoms are usually absent, and cognitive retardation is less compared to DMD patients. Cardiac involvement can be severe, occurring long before the progression of skeletal muscle manifestations with deletions at the amino terminal site. However, much later, deletions at the rod site and hinge 3 occur. The age of access to a wheelchair

varies between the ages of 16–40. Death usually occurs in the fourth decade. Since patients live to adulthood, they can have children, but fertility varies between 10 and 70%. Mental retardation is more common compared to the normal population. Malignant hyperthermia may also develop [1, 2, 16, 17].

Malignant Hyperthermia

Malignant hyperthermia is a condition explained by excessive heat generation due to agents that trigger an increase in cytoplasmic calcium concentrations in individuals with mutations in the L-type calcium channel. The fact that it has not been seen in previously anesthetised events does not rule out the possibility. Its incidence is 1:10,000–15,000. Fever, tachycardia, cardiac dysrhythmia, muscle fasciculations, muscle rigidity, generalised muscle contraction, metabolic acidosis, tachypnoea, skeletal muscle hypermetabolism, and rhabdomyolysis occur with the use of inhalation anaesthetics. If left untreated, it can lead to death. It is caused by drugs such as halothane, isoflurane, and muscle relaxants (succinyl choline, decamethonium, gallamine, d tubocurarine) used in anaesthesia. Myoglobinuria, a rapid increase in creatine kinase and potassium levels, and severe metabolic acidosis are seen. Swelling and necrosis of skeletal muscle, renal failure, disseminated intravascular coagulopathy, and encephalopathy develop. Malignant hyperthermia syndrome is caused by the mutation of genes encoding calcium channel proteins and their receptors in the sarcoplasmic reticulum. It is seen in dystrophinopathies and in some other muscle diseases caused by ryanodine receptor (RYR1), closely associated dihydropyridine (DHP), calcium voltage-gated channel subunit alpha1 S (CACNA1S), and cysteine-rich domains 3 (STAC3) gene mutations. In addition, Native American myopathy, King-Denborough Syndrome, and Noonan Syndrome also predispose patients to the development of malignant hyperthermia. Malignant hyperthermia may also be triggered by exercise or high temperatures and by medications such as statins. The most common genes responsible for malignant hyperthermia are RYR1 and SH3 along with their receptors. Dantrolene is used in the treatment of the disease. Dantrolene sodium is a postsynaptic muscle relaxant that lessens excitation-contraction coupling in muscle cells. This is accomplished by antagonising ryanodine receptors, which prevents the release of calcium ions from sarcoplasmic reticulum reserves. It is the main medication used to treat and prevent malignant hyperthermia. Dantrolene must be administered at the appropriate dose, depending on the weight of the patient before an operation. For a list of drugs that cause malignant hyperthermia, see the recommended resource of the Malignant Hyperthermia Association of the United States [17–19].

Congenital Muscular Dystrophies

Hypotonia and weakness are present from birth. The reason why it is called congenital is that the signs begin to appear during the intrauterine period or in early infancy. The patient may be born with arthrogryposis. Patient with milder forms show symptoms around the age of 2. CK is usually high, muscle biopsies show fibrosis, degeneration, and regeneration, as well as fat and connective tissue proliferation. They progress slowly, in some types the child develops; they can sit, stand and walk. Although the findings are mild in infancy, weakness, developmental delay, and contracture development begin within the first year of life. Findings of congenital muscular dystrophy (CMD) begin to develop during intrauterine life and early infancy, whereas they usually appear at later ages in LGMDs and dystrophinopathies [18].

Primary Merosin Deficiency

Merosin-deficient CMD accounts for 22% of all CMDs. In merosin-negative CMD, a mutation in the laminin alpha 2 (LAMA) gene, inherited on chromosome 6q22–23, is seen. While it can cause severe clinical findings during early infancy, patients may also present at older ages with partial merosin deficiency and milder findings. Patients experience hypotonia, muscle weakness, and growth retardation at birth or in the first few weeks. Contractures may also develop, and some patients are able to sit and walk with little support. Developmental delay and mental retardation may be seen. There is weakness in the neck flexor, extensor, and facial muscles. Upper extremity muscles are mostly affected. Flexion deformities occur in the hip, knee, elbow, and wrist, then rigidity and scoliosis develop, along with muscle hypertrophy. Progressive eye movement limitations with limitation of upward gaze, contracture in the temporomandibular joint, macroglossia, aspiration, and gastroesophageal reflux are observed. These findings increase feeding difficulties. Respiratory functions are reduced in the first decade, and nocturnal hypoventilation may develop. Creatine kinase can rise to 1000 IU/L in the neonatal period and then decrease in later years. There are myopathy findings with EMG, and nerve conduction velocities may be reduced. In the muscle biopsy, there are prominent variations in the size and shape of myofibres, which are generally rounded. Nuclear internalisation is not clearly visible. The increase in fibroadipous tissue can be of varying severity. Large and small rounded myofiber groupings resemble those found in spinal muscular atrophy. However, it is unique to CMDs that all individual myofibers are surrounded by prominent endomysial fibrous tissue. Inflammatory infiltration is not usually observed. Regenerated fibers, the pathognomonic sign of MDs, are few in numbers, unlike in DMD, and can be identified more eas-

ily in the early stages of disease. Merosin is not detected with the immunofluorescent technique or immunohistochemistry (Fig. 12.6). Partial merosin deficiency can also be seen in other CMDs and dystroglycanopathies, although pathological findings are milder in these patients, and disease findings may not be seen until the second decade. Merosin is normally found in Schwann cell basal lamina of cutaneous small nerves, epidermal basal lamina, and basal lamina surround-

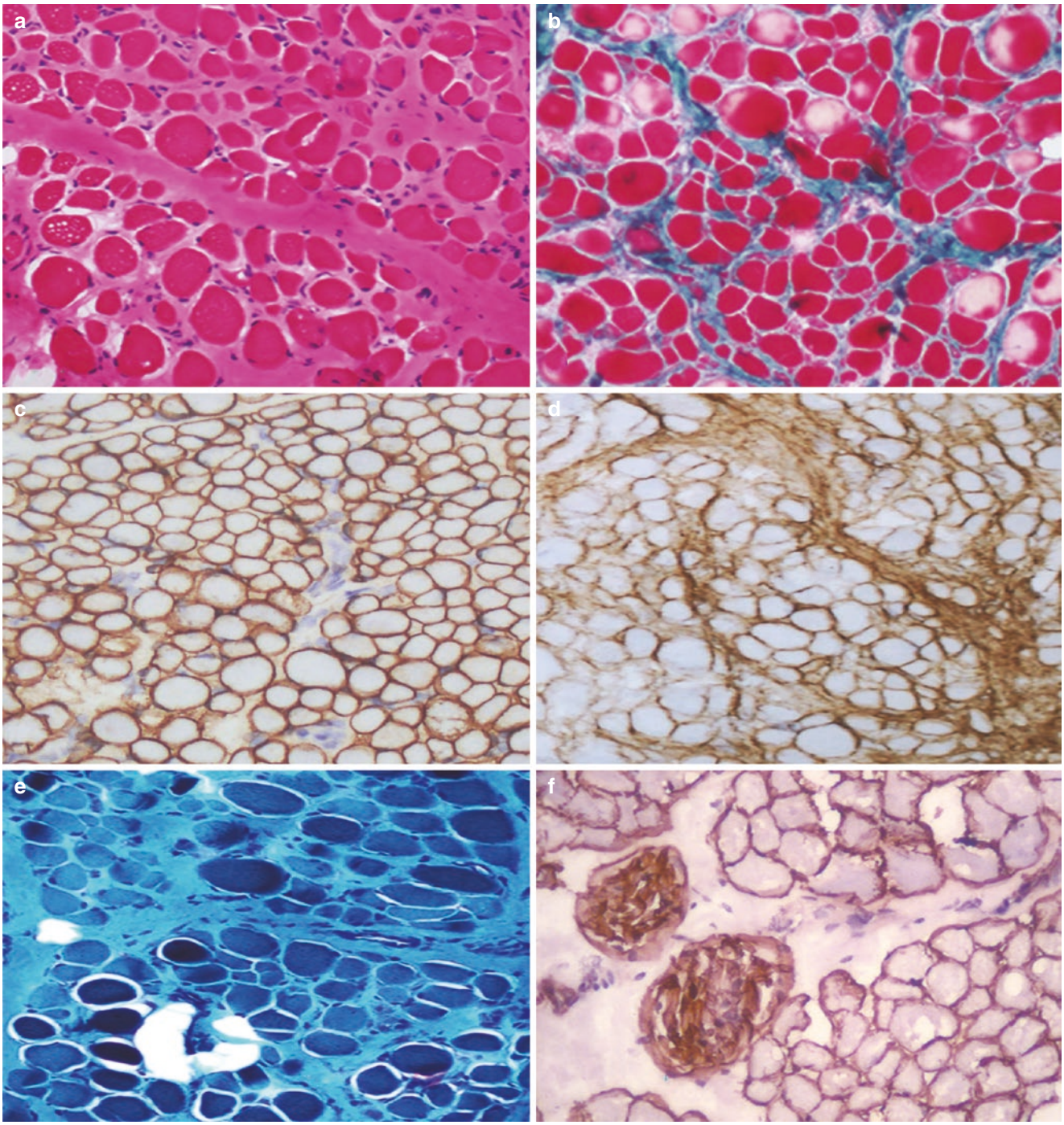

Fig. 12.6 Five different hypotonic infants with merosin deficiency: (**a**) Significant myofiber size difference in a 4-month-old female (CK: 2000 U/L) (HE ×100), (**b**) Significant interstitial fibrosis in a 5-month-old male (CK: 5020 U/L) (Gomori trichrome ×100), (**c**) Sarcolemmal dystrophin expression in a 9-month-old male (2559 U/L) (DAB ×100), (**d**) Normal expression of collagen VI in a muscle biopsy of a 2-month-old female (CK:7000 U/L) who had an older brother diagnosed with merosin-deficient congential muscular dystrophy (DAB ×100), (**e**) Contracted myofibers (Modified trichrome ×100) in a 17-month-old female (CK: 1387 U/L), (**f**) Normal expression of merosin in muscle and two small nerves in the control case (DAB ×200)

ing hair follicles. Therefore, if a muscle biopsy cannot be performed, merosin deficiency of the basal lamina and small nerves can be detected with a skin biopsy [20–23].

Fukuyama Congenital Muscular Dystrophy (FCMD)

The most common autosomal recessive disease in Japan 0.7–1.2/10,000 births. Fukutin is a protein found in the basement membrane of muscle. When fukutin is deficient, the amount of α-dystroglycan and merosin staining is also reduced in muscle biopsies, whilst the dystrophin complex stains normally. Symptoms include hypotonia, generalised weakness, severe growth retardation, febrile convulsions, epilepsy, and microcephaly. Hypotonia and muscle weakness usually begin before 9 months of age, and hypertrophy of the leg muscles is evident at birth or in the first few weeks. There is impaired crying, sucking, and weakness in the facial muscles with a tight upper lip, and developmental delay. Patients can sit without support but cannot walk independently. Contractures are not apparent at birth but form in the hips, knees, elbows, and ankles by the age of 1 year. Severe mental retardation, epileptic seizures, and cardiomyopathy are present in 40% of patients.

Ophthalmological findings include myopia (9%), strabismus (6%), optic disc pallor, and retinal detachment. Creatine kinase (CK) values are high (1000–10,000 IU/L). EMG shows myopathic potential. Cranial imaging shows polymicrogyria, pachygyria, type II lissencephaly, an absence of cortical lamination, and cortical dysgenesis in the temporal and occipital regions. Transient T2 hyperintensities appear in the white matter, the pons is hypoplastic, and there may be cerebellar cysts. Respiratory support requirement develops over the years (16%), as does dysphagia (22%). Fukuyama CMD is caused by fukutin gene mutations which have also been identified as the genetic cause of a type of LGMD with dilated cardiomyopathy and normal intelligence [18, 19, 23–25].

Defects of Extracellular Matrix Proteins: Ullrich CMD and Bethlem Myopathy

Collagen VI consists of three alpha chains governed by three genes. Both COL6A1 and COL6A2 are on chromosome 21q22.3, and COL6A3 is on chromosome 2q37. In Ullrich CMD, CK levels are found to be normal or slightly elevated. Ullrich CMD is autosomal recessive with mutations in the same genes. The milder type is Bethlem myopathy which is autosomal dominant. Congenital generalised weakness, proximal joint contractures, hyperextensibility in the distal joints, spinal rigidity, scoliosis, and early respiratory failure, as well as findings of torticollis, hip dysplasia, and congenital kyphosis, are seen in patients with Ullrich CMD. These findings may become evident at around 12 months. The child's ability to hold up their head develops very late. Some patients can walk independently. Deformities of the feet, calcaneus protrusion, follicular hyperkeratosis of the skin, hypertrophic scars, and keloids are seen. At the end of the first decade, signs of developmental delay and respiratory failure develop, and patients become wheelchair bound. Distal laxity is also present. Bethlem myopathy goes away, and patients have milder clinical findings. Proximal weakness and flexion contractures are seen in the distal joints. Contractures occur in the neck, shoulders, elbows, hips, knees, ankles, and interphalangeal joints of the fingers. Distal laxity is also present. Exaggerated scar tissue develops at operated wounds, and intelligence is normal. Bethlem myopathy can be inherited via the COL6A1, COL6A2, and COL6A3 genes in an autosomal dominant fashion. Additionally, COL6A2 combined heterozygous and autosomal recessive inheritance is possible. Respiratory failure does not develop. There are intermediate types between the two clinical phenotypes. Patients who can walk are wheelchair-bound in the first decade. Reductions in patient-forced vital capacities of nearly 3% annually have also been reported, a marker of pulmonary function. Typically, collagen VI is mostly extracellular in muscle biopsies, and immunohistochemical staining can easily highlight its presence (Fig. 12.7a). As muscle diseases caused by collagen VI deficiency can be of different severities, grading collagen VI defects with muscle biopsy in genetically diagnosed cases, as is done in dystrophinopathies, can help to predict the prognosis of patients [26–28].

Case 12.3

A 7-year-old girl presented with complaints of frequent falls and toe walking. She was born with a normal spontaneous delivery at term, held her head up late, walked at the age of 1.5 years, and complained of fatigue, intermittent contractions, and cramps, which had been increasing during the last 2 years. In her family history, it was stated that her father, in his 20s, was diagnosed with muscular dystrophy by a muscle biopsy performed in a university hospital. However, since dystrophin could not be detected with immunohistochemical staining and genetic tests were not available in those years, it was thought that the patient might have BMD due to his clinical findings. Due to a deformity on the girl's feet, it was suggested that muscle stiffness led to early contracture development. Physical examination revealed: weight 20 kg (25%), height 124 cm (50%), her cranial nerves were normal, gastrocnemius hypertrophy was present, muscle strength was reduced, deep tendon reflexes could not be obtained, and there was distal laxity in the fingers, elbows, and both feet. Hypertrophic scars were seen on the knees of

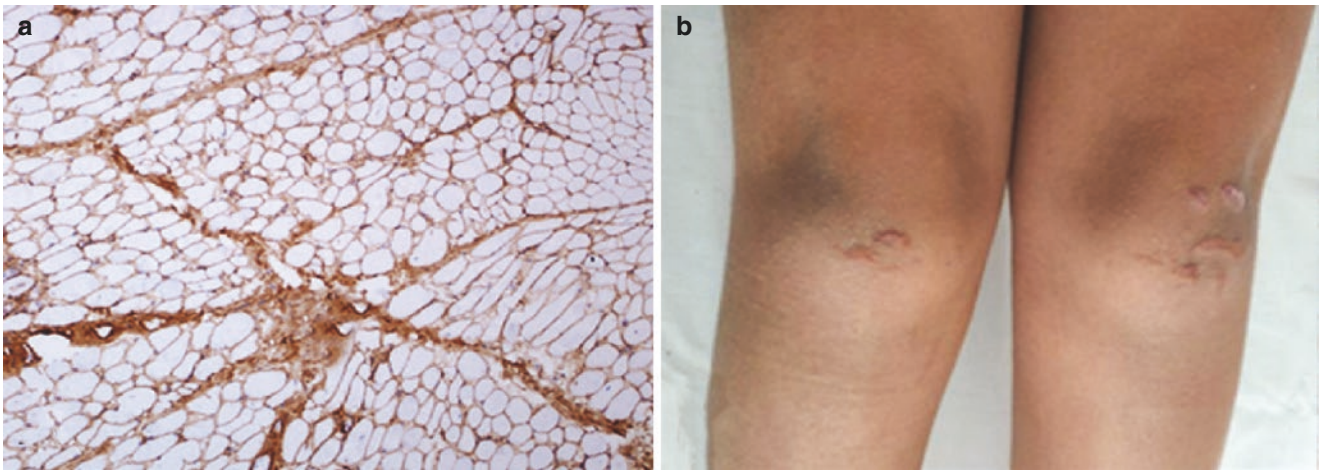

Fig. 12.7 (**a**) Immunohistochemical expression of collagen VI can be seen in both the endomysium and perimysium in the normal muscle (DAB ×100), (**b**) Note the hypertrophic scars on the knees of the patient

the patient, which was stated to have formed after a previous fall (Fig. 12.7b). Her CK level was found to be mildly elevated (312 U/L). Myopathy was found in the upper and lower extremities using EMG which was requested again. MLPA gene analysis was requested from the patient's father and from the patient. No deletions or duplications were found in the dystrophin gene and in the panel for MDs and congenital myopathies, a COL6GA1 (p.gly 284 Arg) variant was detected. She was diagnosed with Ullrich CMD. In the 2-year follow-up of the patient, the calcaneus became prominent, pes equinus deformity developed, antecubital contractures occurred in the arms, and finally, she became wheelchair dependent during the COVID-19 pandemic period when physical therapy could not be performed as intensively.

SEPN1-Associated CMD

Numerous biological pathways depend on selenoproteins, which contain selenocysteine residues. The human genome contains 25 genes that code for selenoproteins. The SEPN1 protein exists within the endoplasmic reticulum (ER) because it has a single selenocysteine residue and ER-addressing and retention signals. Muscles and other foetal human tissues have been shown to express SEPN1 at high levels. SEPN1 involvement is crucial during the early phases of embryogenesis, in early development, and in cell proliferation. However, the level of expression is decreased in adult tissues. A calcium-binding domain on SEPN1 participates in the biochemical procedures that control the release of intracellular calcium. This protein primarily affects calcium pumps in oxidation and reduction processes, changing how calcium is regulated in the ER. For healthy muscle development and differentiation, calcium homeostasis is essential. As a result, the SEPN1 protein plays a crucial role in the development and specification of muscle fibers. SEPN1-associated CMD has been described in the congenital myopathies part of the book [26].

CMD with Integrin α7 Deficiency

Integrins are important transmembrane heterodimeric (α/β) receptors that provide connections between the extracellular matrix and muscle cell structure. Deficiency of integrin α7 is very rare [1].

Facioscapulohumeral Muscular Dystrophy (FSHD)

FSHD is a slowly progressive inherited myopathy. It is one of the most common neuromuscular disorders, and despite this, many affected individuals remain undiagnosed. Although it is a common muscle disease, it is difficult to diagnose in childhood when the child is still young. Weakness of the facial muscles occurs around the age of 5 years or earlier in infantile types, and involvement in the shoulder girdle is noticed around the age of 10 years. The first sign that bothers patients is the inability to fully close the eyelids while they sleep. FSHD occurs with a frequency of 4–7 per 100,000. Inheritance is autosomal dominant, and a de novo mutation is detected in 20% of patients [3, 26].

In FSHD, mutations are found in the 4q35 chromosome in FSHD type 1 and the 18p11.32 chromosome in FSHD type 2. It has been reported that the SMCH1 gene acts as a modifier in the severity of the disease in FSHD type 1. The aberrant expression of Double Homeobox-4 protein (DUX4), which is normally toxic to muscle cells, has been proposed

as being the trigger factor for pathogenesis. DUX4 protein is a transcription factor expressed in the germline, and it is silenced in somatic tissues of healthy individuals. It has been shown that even a brief exposure to DUX4 protein could provoke permanent injury with impaired regeneration. Impaired regulation of DUX4 expression includes genetic or epigenetic mutations in the D4Z4, SMCHD1, or DNMT3B gene loci. FSHD type 1 makes up 95% of FSHD cases in which pathogenic contraction of the D4Z4 repeat array exposes the DUX4 region for gene transcription. FSHD type 2 is responsible for 5% of cases, in which epigenetic mutations occur, such as hypomethylation. The clinical phenotype is similar in both. Typically, larger D4Z4 contractions are associated with early-onset FSHD, and an earlier age of onset of facial weakness is related to greater disease severity [29–34].

FSHD typically presents with weakness of the facial muscles, shoulder girdle, upper arm muscles, and dorsiflexors of the foot, which is referred to as a "scapuloperoneal pattern". It is the most common cause of scapula winging (scapula alata). Usually, bilateral but asymmetric weakness occurs. Although there is weakness in the biceps and triceps muscles, the absence of severe involvement in the deltoid and especially forearm muscles gives the appearance of Popeye the sailorman, which is a famous cartoon hero (Fig. 12.8). Calf, bulbar, respiratory, and pelvic girdle muscles may be involved in severe disease. As the upper abdominal muscles are stronger, the umbilicus slides up during neck flexion, which is known as Beevor's sign. This finding is noticed when patients lie in the supine position and attempt to lift their head off the bed and bring their chin towards their chest (Fig. 12.9). In the later stages, restrictive lung disease occurs. 20% of patients become wheelchair-dependent, and weakness in the lower extremities becomes evident with the involvement of the ankle dorsiflexors. Drop foot is seen due to involvement of the peroneal muscles and ankle dorsiflexors. Hypertrophy of the extensor digitorum brevis muscle helps to differentiate FSHD from neurogenic diseases that cause drop foot. Other pathologies encountered in this syndrome are cardiomyopathy, sensorineural hearing loss, retinal vascular abnormalities (capillary telangiectasia, microaneurysms, protein exudation, retinal detachment, Coat's disease), epileptic seizures, and perception difficulties. D4Z4 deletion in the 4q35 gene leads to the development of more severe disease, and both mental retardation and epilepsy are more common in patients carrying this mutation. Serum CK may be normal or increased fivefold [33–35].

Early-onset FSHD patients with facial muscle weakness occurring before 5 years of age and shoulder girdle weakness occurring before 10 years of age are considered as having the early-onset type. Early-onset patients develop generalised hypotonia, growth retardation, dysarthria, and dysphagia soon after birth. Rarely, patients with Moebius syndrome-like ophthalmopathy findings have been reported. Retinal vasculopathy, cochlear dysfunction, mental retardation, and epilepsy are more common in early-onset FSHD, and most of these patients have genetically large D4Z4 deletions. Symptoms generally begin during adolescence, and a diagnosis is made in early adulthood. However, onset can occur at any age. CK can be normal or mildly elevated. Highly elevated CK levels with proximal lower limb weakness should cause suspicion of limb-girdle muscular dystrophies [34–38].

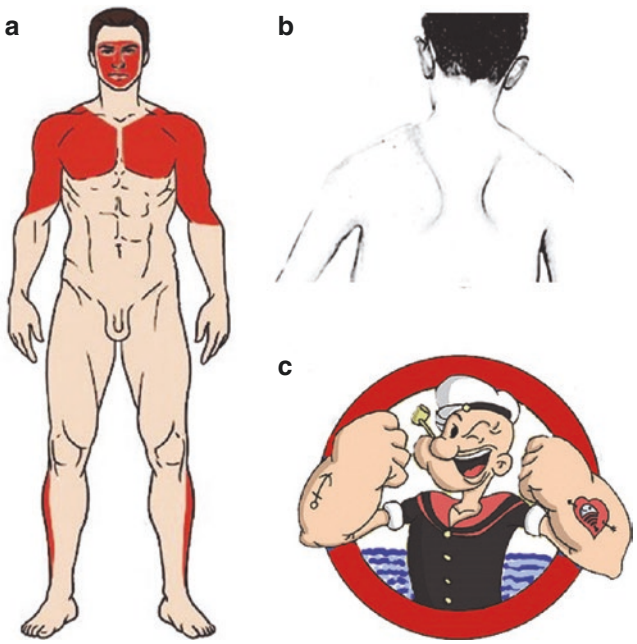

Fig. 12.8 (**a**) Schematic view of the muscles affected in FSHD cases, (**b**) Scapula winging, (**c**) Atrophy of the arm muscles, which is known as Popeye sailor man sign, like the famous cartoon hero

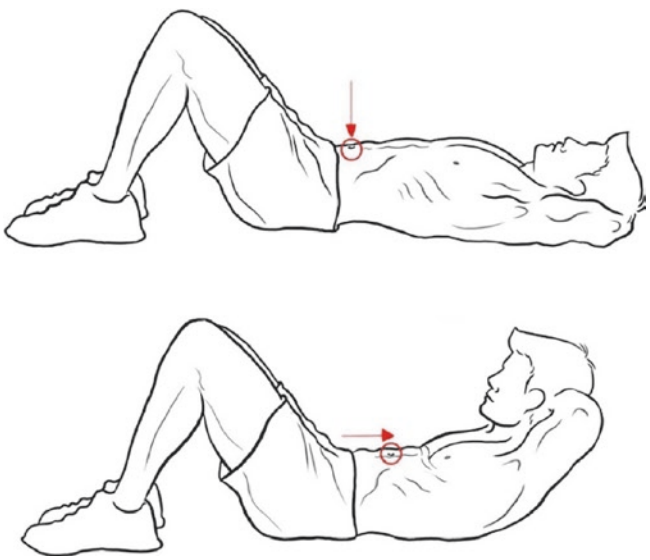

Fig. 12.9 Beevor's sign

Electrophysiological and histopathological tests do not reveal any disease-specific signs except for perivascular complement deposits and the presence of inflammatory cells/proteins. Definitive diagnosis requires genetic confirmation. FSHD should be on the list of differential diagnoses if the following symptoms are present: foot drop, scapula winging, facial muscle weakness with relatively spared bulbar muscles, shoulder girdle weakness, and axial muscle weakness (neck and torso extensors). FSHD should also be considered in patients who were previously diagnosed with inflammatory myopathy but with an inadequate response to anti-inflammatory therapies. Patients with FSHD have asymmetric weakness in the face, shoulders, shoulder girdle, and abdominal muscles. The onset of weakness develops slowly over time and usually appears towards the age of 20. First, involvement of the orbicularis oculi and orbicularis oris muscles occurs, making it difficult to close the eyes, and speech difficulties can occur in older ages. Due to the involvement of the muscles of mastication and the tongue and extraocular muscles, difficulties are observed in movements such as laughing, whistling, inflating balloons, and drinking with a straw. The lips droop and become expressionless, which is known as the "tapir mouth". When trying to close the eyes with force, during examination, the eyelids do not close completely, and weakness of the facial muscles may be asymmetrical [34–37].

Case 12.4

A 7-year-old male patient presented with complaints of frequent falling, the inability to close his eyes, move his upper lip, get up from his seat, and climb stairs and slopes without support. It was stated that he could not move his lips enough, that it took too long to chew, and that he did not have many problems with swallowing. In the family history, it was stated that his mother's paternal grandmother and grandfather were consanguineous, and the mother's two uncles were congenitally disabled and died at an early age. On physical examination: height 128 cm (50–75th percentile) and weight 23 kg (25th percentile). Pectus excavatus, upper lip drooping, and increased lumbar lordosis were present. It was also observed that he kept his legs in a hyperextended position when walking. The right scapula appeared asymmetrical. The right shoulder was noted to be low. Gowers sign was positive. EMG: myopathy in the examined upper and lower extremity muscles. The CK level was 1017 U/L (normal = <171 U/L). No deletion/duplication mutations were detected in the dystrophin gene with MLPA. Genetic testing revealed that exon 7–8 in the SMN1 gene was normal, but exon 7–8 heterozygous deletion in the SMN2 gene was detected. FSHD gene analysis revealed that a contraction-type mutation in the D4Z4 macrosatellite sequence, in the subtelomeric region of 4q35, was causing pathological shortening of the D4Z4 repeat sequence. In a muscle biopsy taken from the gastrocnemius, there was no pathology present except for a slight size difference in the myofibers, and a severe reduction in the amount of type 2 myofibers (Fig. 12.10). Rare immature myofibers were also detected with neonatal myosin. At follow-up, scapula winging became evident, his lower lip developed a tapir mouth appearance, increased weakness of the upper and lower extremities, and weakness in dorsiflexing the feet. It was stated that his eyes remained open while he was sleeping and that his eyes began to open more when closed. Hearing tests revealed sensory-type hearing loss, and cardiological examinations were normal. Scoliosis was detected radiologically when he was 11 years old, and it progressed despite physiotherapy at later ages. The patient became wheelchair-bound when he was 12 years old.

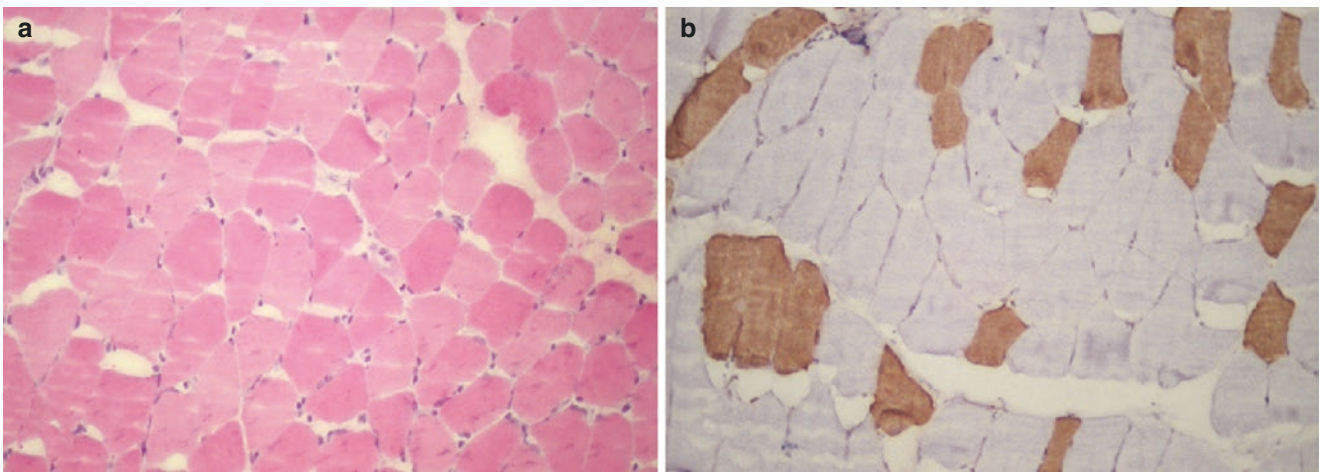

Fig. 12.10 (**a**) Mild size differences of the myofibers (HE ×100), (**b**) Immunohistochemical expression of fast myosin highlighting a reduction in the amount of type 2 myofibres (DAB ×100)

Case 12.5

A 20-year-old male presented with pronounced muscle weakness, especially in the arms. He had a history of recurrent lung infections. On physical examination, scapula winging was observed. There was no consanguinity between his parents. There was no family history of muscle disease. CK levels were within normal limits. Muscle biopsy revealed dystrophic muscle changes as well as inflammatory cells, most of which were T lymphocyte phenotypes (Fig. 12.11). As FSHD is one of the rare MDs that can be accompanied by lymphocyte infiltration, genetic analysis was recommended. In the genetic analysis, a D4Z4 contraction type mutation (1–10 repeat unit) was detected.

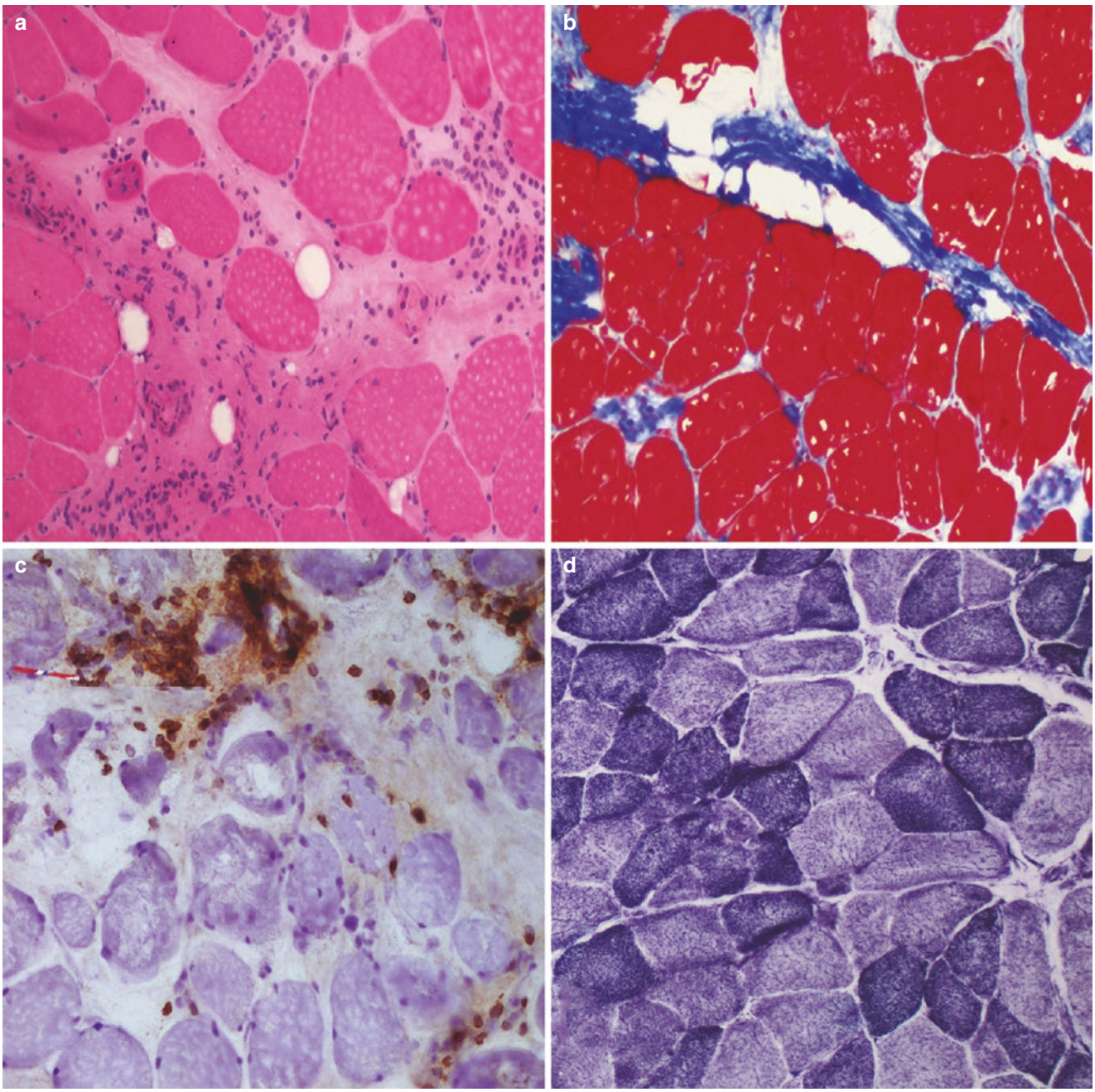

Fig. 12.11 (**a**) Prominent size and shape differences of the myofibers (HE ×200), (**b**) Increased interstitial fibrosis (Masson Trichrome ×200), (**c**) Immunohistochemical expression of CD3 highlights the presence of T-lymphocytes (DAB ×100), (**d**) Normal myofibrillary arrangement observed with an oxidative enzyme stain (NADH-TR ×200)

Limb Girdle Muscular Dystrophy (LGMD)

LGMDs are a group of muscle diseases characterised by slowly progressing weakness, usually involving the proximal muscles of the shoulder and pelvic girdle. LGMDs are mostly autosomal recessive, inherited due to a wide variety of genetic causes. In some types, distal muscles are involved, and these diseases can be confused with neuropathies. Unlike dystrophinopathies, the neck flexor muscles are not involved, and gastrocnemius hypertrophy is not seen. There are many causes that match the symptoms of limb-girdle-type muscle involvement, such as inflammatory, toxic, paraneoplastic, endocrine, metabolic, mitochondrial, structural, and congenital diseases. Various diseases such as spinal muscular atrophies, congenital myopathies, and acid maltase deficiency can mimic LGMD-type muscle involvement. They must be considered in the differential diagnosis. Muscle biopsy findings are very valuable in determining a diagnosis. A new classification of LGMDs is covered in the genetic part of the book. In this chapter, the classification is explained by the inherited proteins, not according to the inheritance type [39–43].

Associated with Sarcolemma

Alpha (α), beta (β), gamma (γ), and delta (δ)-sarcoglycans form a tetrameric structure and provide stability to the muscle cell membrane. Mutation of one of these four proteins causes partial or complete loss of the entire complex. They function to stabilise the dystrophin-associated glycoprotein (DAG) complex in the sarcolemma. 20–25% of all MDs are sarcoglycanopathies. Of the sarcoglycanopathies, 50–60% are severely progressive types, and 10–20% are mildly progressive types. An early onset and severe course are typical findings. Some patients with dystrophinopathies also have sarcoglycan defects in their biopsies. These patients have more severe disease. LGMD 2C, 2D, 2E, and 2F diseases are associated with sarcoglycan defects, and all of them are autosomal recessive. The α-sarcoglycan and γ-sarcoglycan proteins are encoded on chromosomes 17q21 and 13q12, respectively, and they are mostly located in skeletal muscle. The β-sarcoglycan and δ-sarcoglycan proteins are encoded on chromosomes 4q12 and 5q33, respectively, and they are found in both skeletal and smooth muscle. Missense mutations are seen in one-third of α- and β-sarcoglycanopathies [1–4, 39–43].

Sarcoglycanopathies can develop from infancy to young adulthood. The mildest sarcoglycanopathy, due to α-sarcoglycan mutations, only presents with creatinine kinase elevation and lordosis. β and γ-Sarcoglycan mutations result in more severe diseases and may run differently in the same family. The clinical features of α-, β-, γ-, and δ-sarcoglycan diseases are very similar. The average age of onset is around 6–8 years. Early developmental stages usually occur in time. Some children may have delayed independent walking. First symptoms include exercise intolerance with toe walking, a waddling gate, Gower's sign, difficulty in running and climbing stairs, calf hypertrophy, increased lumber lordosis, Achille's contracture, muscle cramps, pain, and a presence or absence of myoglobinuria. Compared to the quadriceps, sartorius, and gracilis muscles, the gluteus and adductor muscles are more involved. Unlike dystrophinopathies, the anterior and posterior thigh compartments are equally affected. Shoulder girdle weakness follows this involvement, with the deltoid, infraspinatus, and biceps muscles demonstrating weakness in the early period. Scapula weakness is more common than in dystrophinopathies. Mild facial involvement, which is called the "transverse smile", macroglossia, and weakness in the neck flexor muscles are seen in later periods, but there is no obvious involvement of the extraocular muscles. In the later stages of the disease, distal muscles are also involved, including the anterior tibial compartment, hip flexors, lateral tract, and knee flexors. Scoliosis also develops. Weakness progresses rapidly and around the age of 12–16, patients are wheelchair-bound. Creatine kinase is elevated up to 10–100-fold. Dilated cardiomyopathy occurs in γ- and δ-sarcoglycanopathies, as well as in patients with heterozygous mutations in the α-sarcoglycan gene, even without skeletal muscle weakness [42, 43].

The cause of cardiomyopathy depends on the involvement of the cardiac muscle cells as well as the pathology in the smooth muscles of the coronary arteries. Cases of cardiac involvement without skeletal muscle involvement have been reported in δ-sarcoglycanopathy. Cardiac examination and follow-up are recommended in carriers. Restrictive lung disease develops in the non-ambulatory period. Lung disease is more severe in patients with α- or γ-sarcoglycanopathy. Intelligence is preserved [39–41].

LGMD 2C occurs due to a defect of the γ-sarcoglycan gene, which functions to stabilise dystrophin. The most common mutation is a single thymidine deletion. In the study produced by Yiş et al., the most common mutations observed in the Aegean region were LGMD 2C (43%), 2A (13%), 2D 13%, and 2E (11%). The most common mutation they detected was c.525delT [43]. γ-Sarcoglycan was also expressed in smooth muscle. It causes a variable type of disease from a mild Becker type to a severe Duchenne type. The age of onset is around 5 years old. It first manifests itself in the gluteus and calf muscles. In the upper extremities, first, the periscapular muscles are involved, then the deltoid and biceps. Unlike Duchenne muscular dystrophy, the quadriceps may be strong. There is hypertrophy of the calf and tongue muscles. Patients develop an excessive lordosis posi-

tion. Cognition is normal, and mild sensory hearing loss is seen. In the later stages of the disease, cardiac involvement becomes evident. Right ventricular hypertrophy occurs. The patients with LGMD 2C lose the ability to walk by an average age of 16. Creatine kinase is elevated more than ten times normal [43, 44].

LGMD 2D occurs due to a defect of the α-sarcoglycan gene, which is on chromosome 17q21. It is expressed in skeletal and cardiac muscle. Generally, symptoms begin between the ages of 2 and 15. Patients with a complete deficiency of α-sarcoglycan have a severe course and die around 3 years of age. The ability to walk in those who start at a late age is preserved until old age. Some patients have high CK levels, even if there is no weakness. It is very clinically similar to γ-sarcoglycanopathy, as scapular weakness is seen in the early period, and cardiac involvement is present in 30% of patients. Cognitive abilities are normal. CK increases up to 5000 IU/L. Cases which benefited from steroid therapy have been reported [45, 46].

In LGMD 2E, there is a β-sarcoglycan defect. It is encoded on chromosome 4q12. This protein enters structures in skeletal muscle, cardiac muscle, brain tissue, and kidney tissue. Twenty-five percent of sarcoglycanopaties occur due to this mutation. There is hypertrophy of the calf muscles. Onset occurs between the ages of 3 and adolescence, and patients are wheelchair-bound between the ages of 10–15. In pathological preparations, besides sarcoglycan, dystrophin staining may be decreased [47, 48].

LGMD 2F is a δ-sarcoglycanopathy. δ-Sarcoglycan is encoded on chromosome 5q33–q34 in the subsarcolemmal region of skeletal and cardiac muscle. It is very rare; missense and nonsense mutations have been reported in Brazil and Turkey [39–43].

Case 12.6

Two Turkish siblings were admitted to the hospital with muscle weakness that resembled the weakness seen in LGMD. Both siblings were found to be negative for deletions and duplications of the dystrophin gene, and they had very high creatinine phosphokinase levels. At age 8, the older son first showed signs which included being unable to climb stairs and walking with an odd gait. He had similar symptoms to his younger brother, who was 5 years old. Muscle biopsy evaluation was performed only on the older brother. α-Sarcoglycan and γ-sarcoglycan, two distinct glycoproteins, were not expressed in the muscle sample, which also revealed dystrophic characteristics (Fig. 12.12). There were sarcolemmal expressions of dystrophin and other sarcoglycans (β and δ). The α-sarcoglycan genes in both siblings were found to have previously unidentified homozygous mutations c.226 C > T (p.L76 F) in exon 3. Both parents shared the same heterozygous point mutations at the same location, but the genes for β-, γ-, and δ-sarcoglycan were normal in the rest of the family. These individuals are an example of how clinical features alone cannot be used to establish a differential diagnosis in medicine. As a result, muscle biopsy and DNA testing are still necessary for diagnosing muscle disorders [46].

Case 12.7

A 12-month-old boy had increased serum CK without symptoms. The persistent elevation of CK prompted muscle biopsy at 18 months of age. He had second-degree consanguineous parents from Turkey without an ancestral history of neuromuscular disorders. Cognitive and motor development was normal. He started walking at 14 months of age and was walking normally. His CK levels were between 9000 and 11,000 U/L (normal <250 U/L), and there was evidence of myopathy on EMG. The muscle biopsy showed dystrophic changes such as contraction, regeneration, degeneration, necrosis, nuclear internalisation, and fibrosis (Fig. 12.13). Immunohistochemically, sarcolemmal dystrophin and spectrin expressions were present at normal levels, whereas sarcolemmal α-, β-, δ-, and γ-sarcoglycans were diffusely absent or there was abnormal sarcoplasmic staining in some myofibers. Sequence analyses of the α-, β-, γ-, and δ-sarcoglycan genes were used to identify mutations and MLPA was used for deletion and duplication analysis for all four sarcoglycans. A large deletion in the β-sarcoglycan gene was identified. In addition, there was a heterozygous c.G860A (p.S287N) mutation in the γ-sarcoglycan gene that was not a disease-causing genetic defect. The results of the molecular analyses checking for mutations in the dystrophin, α-, β-, and δ-sarcoglycan genes were normal [47].

LGMDs classified as type 1 are autosomal dominant (AD). Caveolinopathies, which are common in this group of diseases, will be described here because they are associated with sarcolemmal proteins [48–53].

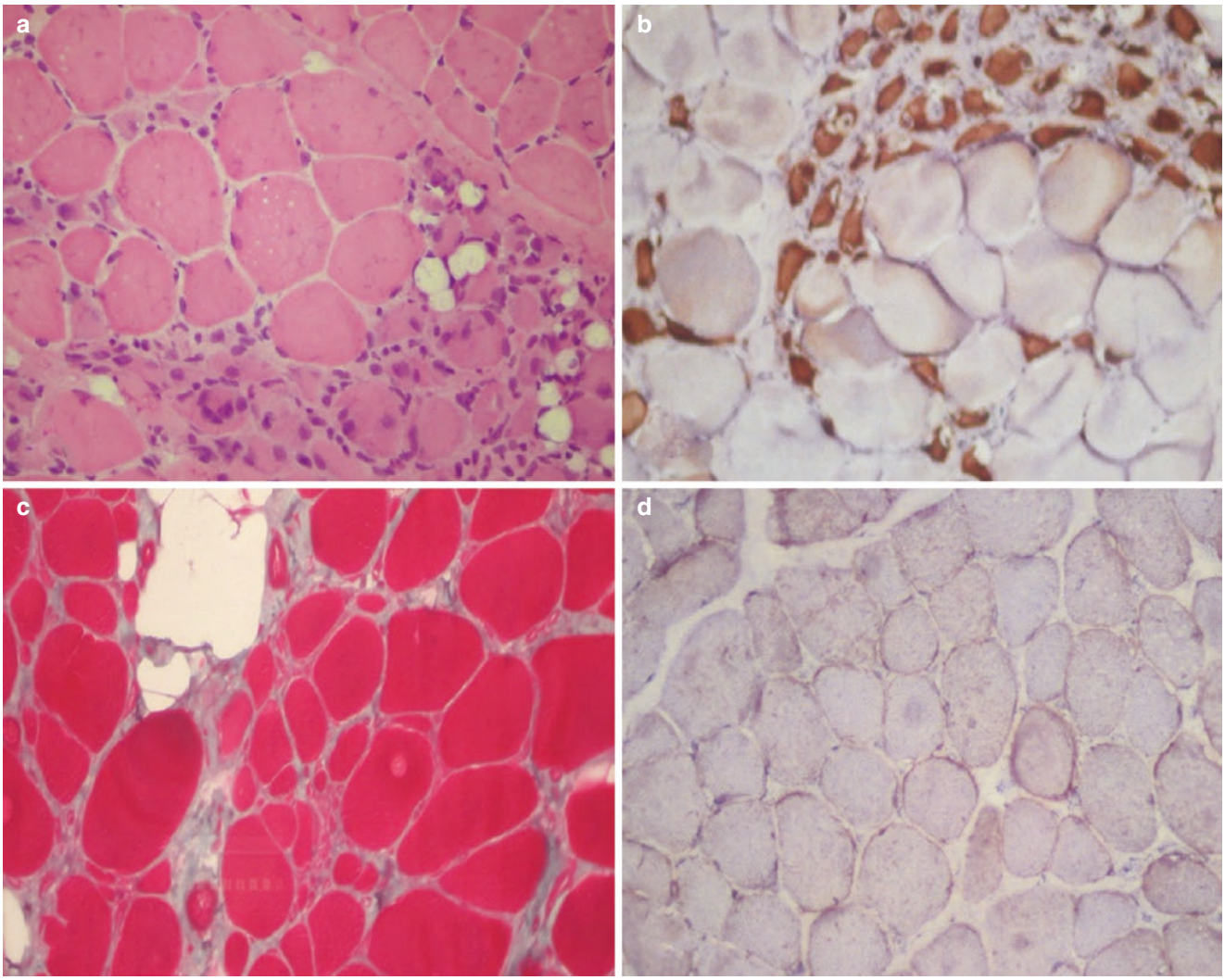

Fig. 12.12 Muscle biopsy from an older sibling with limb-girdle muscular dystrophy 2D: (**a**) Typical muscle dystrophy appearance with plenty of regenerated fibers (HE ×100), (**b**) Immunohistochemically regenerated fibers (arrow) can be highlighted with neonatal myosin (DAB ×100), (**c**) Increased intercellular fibrosis (Gomori trichrome ×100), (**d**) Defect of α-sarcoglycan expression (DAB ×100)

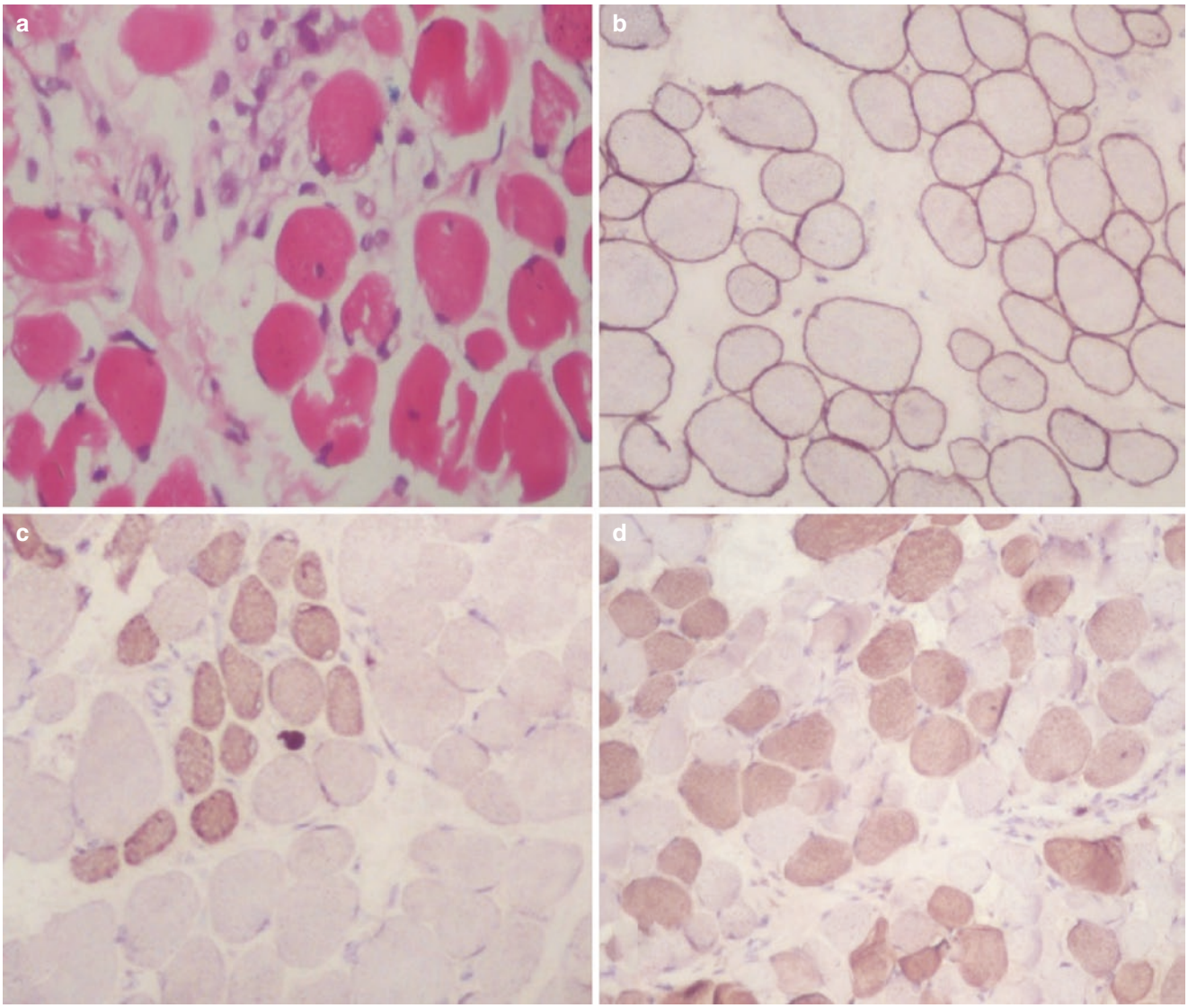

Fig. 12.13 Muscle biopsy from an 18-month-old boy with limb-girdle muscular dystrophy 2E: (**a**) Typical muscle dystrophy appearance with plenty of regenerated fibers (HE ×100), (**b**) Immunohistochemically, sarcolemmal dystrophin expression is normal, (**c**) Regenerated fibers can be highlighted with neonatal myosin (DAB ×100), (**d**) There is aberrant sarcoplasmic α-sarcoglycan expression (DAB ×100)

LGMD 1C, Also Known as Caveolinopathy

The caveolin 3 (CAV3) gene is located on 3p25.3, and defects of the gene are passed on via AD inheritance. Caveola is a small transmembrane protein that oligomerises to form the membrane complex, localises on the cytoplasmic surface, contributes to the robustness of the sarcolemma, directs ion channels, and has a role in signal transduction. It is associated with the formation of T-tubules in muscle differentiation. By binding to the intracellular portion of β-dystroglycan, dystrophin communicates with sarcoglycan complex proteins. The quantity of dysferlin may also be reduced, observed in patient biopsies. CAV3 immunoreactivity is considerably reduced, even in heterozygous inheritances. In patients with LGMD 1C, there is a mutation in the CAV3 gene on chromosome 3p25.3. Many mutations are of the missense or microdeletion type and are AD, but autosomal recessive (AR) cases have also been reported.

This mutation occurs in four different clinical manifestations, which are LGMD, distal myopathy, rippling muscle disease, and asymptomatic CK elevation. Calf hypertrophy is common, and some patients develop muscle weakness in early childhood, while others remain asymptomatic. Patients may also complain of leg pain at night. Muscle pain, cramps, muscle stiffness, fatigue, exhaustion, and hyperirritability occur with exercise [48, 49]. Symptoms begin to show between the ages of 2–10, beginning in childhood and progressing slowly, with patients becoming wheelchair-bound

in the middle stages of adolescence. Patients presenting with evidence of cardiomyopathy, without skeletal muscle myopathy, have been reported. Cardiac conduction problems, such as long QT are described, but pulmonary involvement is rare. Treatment targets the symptoms of the disorder and is not curative. It is also called AD rippling muscle disease. Hereditary rippling muscle disease is a disease in which skeletal muscle is mechanically triggered. When a mechanical stimulus, in the form of percussion, is given to a resting muscle, there are rapid movements in the muscle that spread to neighboring fibers. Although it was revealed in the literature that muscle activity in EMG is silent during the rippling activity that occurs with percussion, a case report remarks bursts of short-duration, low-amplitude spikes, which resemble single muscle fiber discharges [48–51].

LGMD 2B is a dysferlin-deficient myopathy caused by a mutation in the dysferlin gene on chromosome 2p13.2. It is autosomal recessive. Missense/splicing mutations, deletions, and truncations are modes of variation. Variations of the dysferlin gene are not detected in some hotspots; therefore, genetic analysis is not very easy. It plays a role in membrane repair and functions alongside caveolin. Dysferlin is presumed to play a role in maintaining the robustness of the structure and function of T-tubules by participating in the management of calcium homeostasis during muscle injury. It is found in the plasma membrane and cytoplasmic vesicles, without which the integrity of the plasma membrane cannot be maintained. With a deficiency, the repair and regeneration mechanisms of the muscle membrane become insufficient. Due to the very active inflammatory and degenerative changes seen in biopsy, LGMD 2B may be mistaken for an inflammatory pathology, such as polymyositis (Fig. 12.14). It is suspected that there is an abnormal immune response and macrophages are involved. There are large amounts of macrophages, CD4, and CD8-positive T cells in the perivascular area and interstitium. Dysferlin is found in skeletal muscle, heart, kidney, placental, lung, and brain tissue. Although it is found in the heart, cardiac involvement is not seen in its deficiency. Three phenotypes have been described: LGMD type, Miyoshi myopathy, and anterior tibial involvement type, which have even been identified in different family members with the same mutation. Although LGMD 2B can be congenital in onset, the presentation of symptoms may be delayed till the age of 18–20, and even athletes in competitive sports have been reported. Normal development occurs in early childhood [51–54].

Miyoshi myopathy accounts for 1% of LGMDs and 33% of distal LGMDs. Although Miyoshi myopathy shows distal and proximal involvement of LGMD 2B, both are slowly progressive disorders, occurring in late adolescence and causing very high CK levels. In LGMD 2B, progressive muscle weakness and more proximal muscle involvement occur during adolescence. Hyperlordosis develops in patients due to pelvic muscle weakness. Muscles anterior to the distal parts of the lower extremities and distal parts of the arms are relatively normal, even in the later stages of the disease. There is more distal muscular dystrophy in Miyoshi myopathy. The posterior compartments of the lower extremities are involved. One of the first findings is the inability of patients to walk on their tiptoes. In these patients, there is weakness in the gastrocnemius and quadriceps muscles, and the lower extremities are in hyperextension. After the onset of the disease, significant gait disturbance draws attention. After knee and hip flexion, the foot can be lifted from the sole. In this

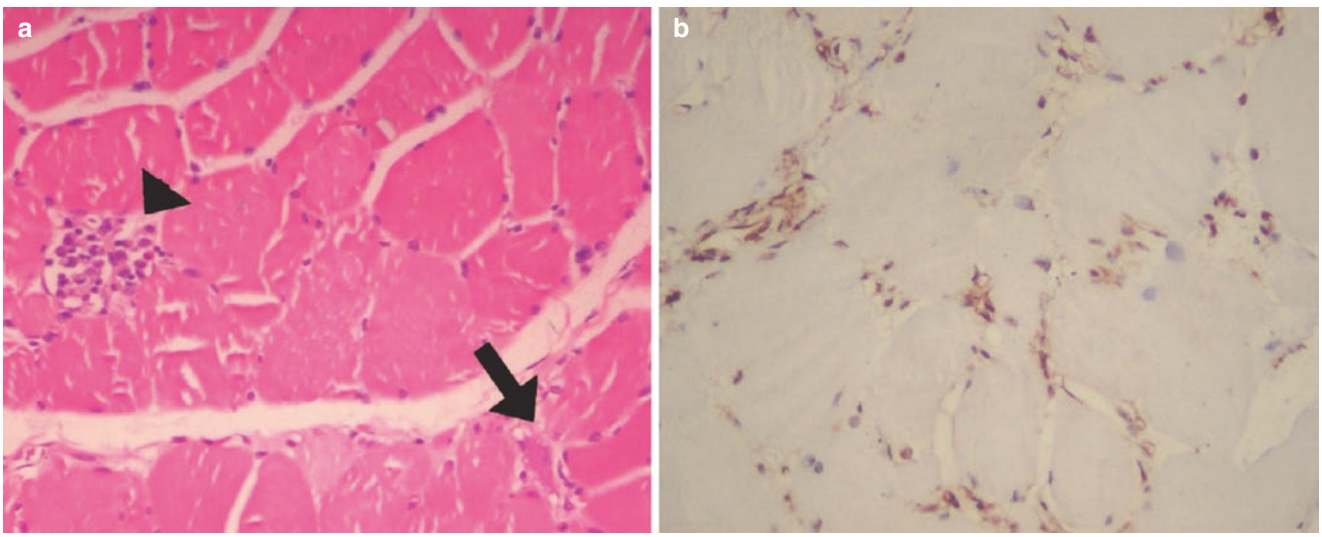

Fig. 12.14 Muscle biopsy from a 27-old-year male with LGMD 2B who was previously diagnosed as polymyositis: (**a**) Atypical presence of cell inflammation (arrowhead) with a single regenerated fiber (HE ×200), (**b**) Presence of interstitial T lymphocytes shown immunohistochemically (DAB ×100)

period, upper extremity weakness also appears. Weakness in the arms is mild at first, involving the distal biceps, deltoid, but the periscapular muscles are spared. Unlike sarcoglycanopathies, and calpainopathy, scapular winging is not observed. The facial muscles and extraocular muscles are also spared. The forearm muscles are weak in the upper extremities, but the intrinsic muscles of the hand are spared. As the disease progresses, the proximal muscles become affected. The disease is common, not only in Japan, but also in Western countries. There is no treatment yet. Despite inflammation, corticosteroids worsen the course of the disease. Physical therapy and orthoses are helpful. Early developmental stages are usually normal, weakness progresses very slowly, and ambulation continues until advanced ages, usually the fourth decade. Involvement of the described muscles in muscle MRI examination has diagnostic value. There is no involvement of the heart or respiratory muscles, and life can be long. Contracture does not develop, except for the type with involvement in the anterior compartment. Amyloid deposition has been observed in the intramuscular vessels. It must be noted that weak staining of dysferlin, secondary to calpainopathy is also seen [48–54].

Extracellular Matrix Proteins

Collagen A1, A2, and A3 proteins help keep the basal lamina in the extracellular matrix anchored. Diseases related to their deficits are autosomal dominant. They can develop from the neonatal period to adulthood. Proximal weakness, atrophy, and distal contractures may be seen. Collagen mutations have been described in congenital muscular dystrophies [34, 35].

Proteins with Enzymatic Activity

LGMD 2A, also known as calpainopathy, is an autosomal recessive disorder. The calpain 3 gene is located on chromosome 15q15. It is the most common type of juvenile-onset LGMD, accounting for 40–50% of LGMD cases. CAPN3 is the gene that encodes calpain 3, a calcium-activated protease found in the cytoplasm and nucleus of the muscle cell. It has a nuclear localisation signal that is suspected of assisting in the processing of transcription factors essential for muscle survival. This signal activates the anti-apoptotic gene and stabilises Nf-kB, thereby preventing its degradation. The occurrence of apoptotic cell death in patients with calpainopathy supports this. It also interacts with titin which is a large sarcomeric protein. It supports membrane repair by interacting with the dysferlin complex. Mutation patterns include large deletions and truncations, with the latter causing a more severe clinical picture. The age of onset is slightly later than sarcoglycanopathies, usually 8–15 years, although occurrences in patients aged 2–40 years have been reported. Early developmental stages are usually normal. Some patients are less skilled in physical activities than their peers. Toe walking and a delay in walking draw attention. After exercise, myalgias and muscle stiffness may precede muscle weakness. As in other LGMDs, symptoms first appear in the pelvic girdle muscles, causing difficulty in climbing stairs, getting up from the ground, and an inability to run fast. Then shoulder girdle and arm weakness develop. The facial, extraocular, and pharyngeal muscles are spared. Very mild weakness in the facial muscles has been reported in very advanced stages of the disease. Weakness in the gluteus maximus and hip adductors of the lower extremities is characteristic. The hip flexors and hamstrings are less involved. Compared to the quadriceps, the hamstrings are weaker, and the hip abductors are stronger. Weakness in the shoulder muscles is very pronounced. Involvement of the latissimus dorsi, rhomboid, serratus anterior, and pectoralis major muscles causes scapular winging and high-set scapula, mimicking facioscapulohumeral muscular dystrophy. The deltoid, biceps, and brachioradialis muscles are less involved, and the triceps and neck flexors are preserved. The abdominal muscles are relaxed. Hypertrophy of the calf muscles is very rare; they appear to have more of an asthenic nature. Contractures of the Achilles tendon, elbows, and spine can be seen in some patients. The disorder mimics Emery-Dreifuss muscular dystrophy. CK levels are very high (520 times normal) and decrease later in the disease. Around the age of 14, patients lose the ability to walk without support. Cardiac involvement generally is not seen. But right bundle branch block and ventricular failure have been reported. Respiratory system disease can develop in very advanced stages. In muscle MRI, selective involvement of the posterior part of the thigh and hip is seen. Eosinophilic myositis is detected in biopsy, and calpain 3 deficiency, secondary to primary dysferlin mutations can occur [48, 55, 56].

Case 12.8

An 18-year-old male with no obvious complaints in childhood or relevant family history was admitted to the hospital with complaints of muscle weakness, gait difficulty, and muscle pain that started about 5 months prior and gradually progressed. His CK values were found to be very high (>10,000) with repeated measurements. Inflammatory myopathy was suspected and treated, and when the findings became more severe, muscle biopsy was requested. Active degeneration and regeneration was observed in the muscle biopsy material. It was also observed that some muscle fibers were surrounded by macrophages, and a substantial number of T lymphocytes were found in between (Fig. 12.15). Since lymphocyte infiltration is not expected in muscular dystrophies, it has been reported that inflammatory myopathies

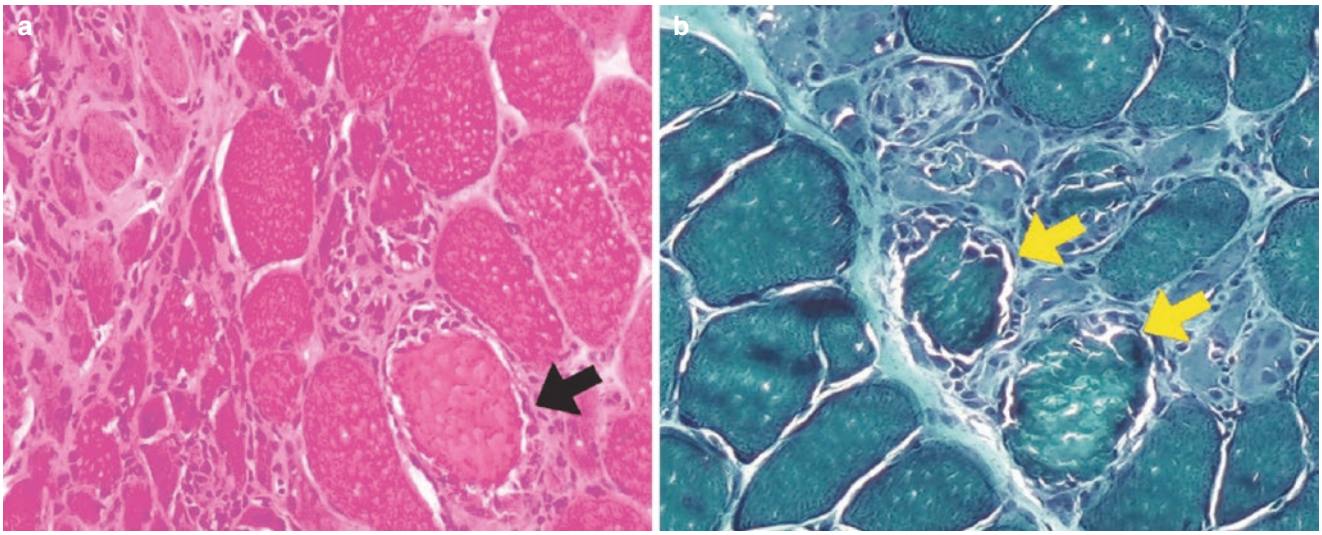

Fig. 12.15 Muscle biopsy from an 18-year-old male with LGMD 1C: (**a**) Presence of several regenerated fibers and a vacuolar, larger fiber (black arrow) covered with macrophages (HE ×200), (**b**) There are two autophagic fibers (yellow arrows) with many regenerated fibers and inflammatory cells (modified trichrome ×100)

cannot be definitively excluded, but myopathy with excessive autophagy may be present with these features. Using genetic analysis, a pathogenic homozygous c.2092C > T mutation was found in the CAPN3 gene of the patient [56].

LGMD 2I, Also Known as Fukutin-Related Myopathy

A major element in the connection between the cytoskeleton and the extracellular matrix (ECM) is dystroglycan (DG), which is a glycosyltransferase, functionally glycosylated by several proteins such as the fukutin (FKTN) and fukutin-related protein (FKRP). CMD and LGMD are two dystroglycanopathies with a range of severity caused by mutations in the FKRP gene. Eleven percent of LGMDs are due to mutations in the FKRP gene. Dystroglycanopathies are defined by aberrant O-glycosylation of DG. The ECM and intracellular cytoskeleton are connected by DG in a basic way, but DG must be appropriately glycosylated for this to happen. More than 20 proteins are involved in this intricate, sequential process, including FKTN, FKRP, protein *O*-mannosyl transferase (POMT1), POMT2, protein *O*-mannose beta-1,2-*N*-acetyl glucosaminyl transferase (POMGNT), and like-acetylglucosaminyltransferase (LARGE). Each of these proteins can be mutated to cause a different type of dystroglycanopathy. FKRP is mainly found in skeletal muscle, heart, and placenta. Mutations of FKRP can cause severe disorders such as Walker–Warburg syndrome with central nervous system involvement or LGMD 2I with a milder course. FKRP is responsible for the glycosyltransferase activity of the rigid endoplasmic reticulum. It takes part in synthesizing α-dystroglycan (α-DG) and mannosyl-glycan. Milder limb girdle involvement may be present, as well as a severe DMD-like course and early loss of ambulation before the age of 10. The age of onset is 0–27 years. It begins before the age of 5 in 61% of patients. There is the involvement of the shoulder girdle and proximal muscles of the extremities. There is difficulty in shoulder adduction, internal rotation, elbow flexion, hip flexion, hip adduction, and ankle dorsiflexion. The axial muscles, neck flexors, and proximal muscles of extremities are also affected. Lordosis is prominent, and scoliosis develops. In some patients, the shoulder girdle muscles are weaker than the pelvic girdle, and atrophy occurs in the deltoid and pectoralis muscles. Hypertrophy of the tongue, calf, and brachioradialis muscle can be seen. The hamstrings are more affected than the quadriceps. Cases with mild facial involvement have been reported. Scapular winging is not common. CK levels may be 10–30 times higher than normal. Nocturnal ventilatory support may be required in the later stages of the disease. No specific treatment has yet been found. Symptomatic treatment and timely support with night ventilation for cardiomyopathy and respiratory failure are essential. Muscle MRI findings are consistent with the way the muscles are involved [43, 57–59].

Autosomal recessive CMDs associated with α-DG glycosylation, which include three subgroups known as MDDGA1 (type A), MDDGB1 (type B), and MDDGC1 (type C, also known as **LGMD 2K**), are linked to mutations in POMT1, which maps to chromosome 9q34.13. There are two similar diseases which are LGMD 2N, caused by POMT2 gene defects located on chromosome 14q24, and LGMD 2O, caused by protein POMGNT1 gene defects located on chromosome 1p32. The most severe manifestation of the disease is Walker-Warburg syndrome (WWS), but there is considerable genetic heterogeneity associated with the WWS pheno-

type as it can result from defects in the genes for POMT1, POMT2, POMGNT1, FKTN, FKRP, LARGE, or the isoprenoid synthase domain containing (ISPD) gene, which maps to chromosome 7p21. WWS has been associated with a loss of α-DG glycosylation. POMT1 mutations which are less severe may exhibit milder forms of LGMD. Despite the lack of a clear genotype–phenotype association, the type and location of the mutations may have an impact on the phenotypic severity. The POMT1 protein has loops in both the cytoplasm and the lumen. Patients who have mutations only in the cytoplasmic loops are more likely to exhibit the LGMD phenotype's milder form. Severe CMD with brain and eye abnormalities develops with POMT1 mutation. Experimental studies have shown that the disruption of muscle cell attachment to the basal lamina plays a role in pathogenesis. Type A patients develop severe brain and eye anomalies. Type B patients develop less severe brain lesions, and no eye anomalies with or without mental retardation. Type C (LGMD 2K type) patients only develop myopia and suffer no mental impairment. POMT1 and POMT2 are gene mutations that cause hypoglycosylation of α-DG. LGMD 2O is a mild disease with normal intellect. These patients have minimal muscle weakness and can live to adulthood [24, 43, 57–61].

Case 12.9

A 7-year-old girl presented with complaints of muscle weakness and difficulty in walking. Her CK value was found to be 2600 U/L. There was no family history or consanguinity between parents. Muscle biopsy was performed for the differential diagnosis. The biopsy findings were reported as non-inflammatory myopathy. During genetic analysis, a homozygous c.2005 G > A variation was detected in the POMT1 gene (Fig. 12.16), and she was diagnosed with LGMD 2K.

Myotilinopathy is an autosomal dominant disorder caused by abnormalities in the myotilin gene (MYOT), which is found on chromosome 5q31–33. A diagnosis of **myofibrillar myopathy-3 (MFM3)** is currently used instead of the formerly used term, LGMD 1A, in appropriate patients with myotilinopathy. Like titin, myotilin is a myofibrillar sarcomeric protein. Its N-terminus is distinct, but its C-terminus has two Ig-like domains which are like those found in titin. Both skeletal and cardiac muscles express myotilin. There is an interaction between myotilin and α-actinin in sarcomeric I bands. It has been shown that myotilin efficiently binds F-actin, crosslinks actin filaments by itself or in combination with α-actinin, and inhibits latrunculin A-induced filament disassembly. It functions to establish a structural and functional connection between the cytoskeleton and the sarcolemma. Additionally, it was shown that myotilin is essential for the stabilisation and anchoring of thin filaments, which may be a requirement for the proper organisation of the Z disc. Myotilinopathy is an adult onset, distal myopathy. There are two missense mutations at the N-terminus: Ser55Phe and Thr57Il. The functional significance of these mutations is unknown. Symptoms begin to appear at the end of the second decade. The disease progresses slowly and involves the proximal leg and arm muscles. Patients do not lose their ability to walk. Many patients

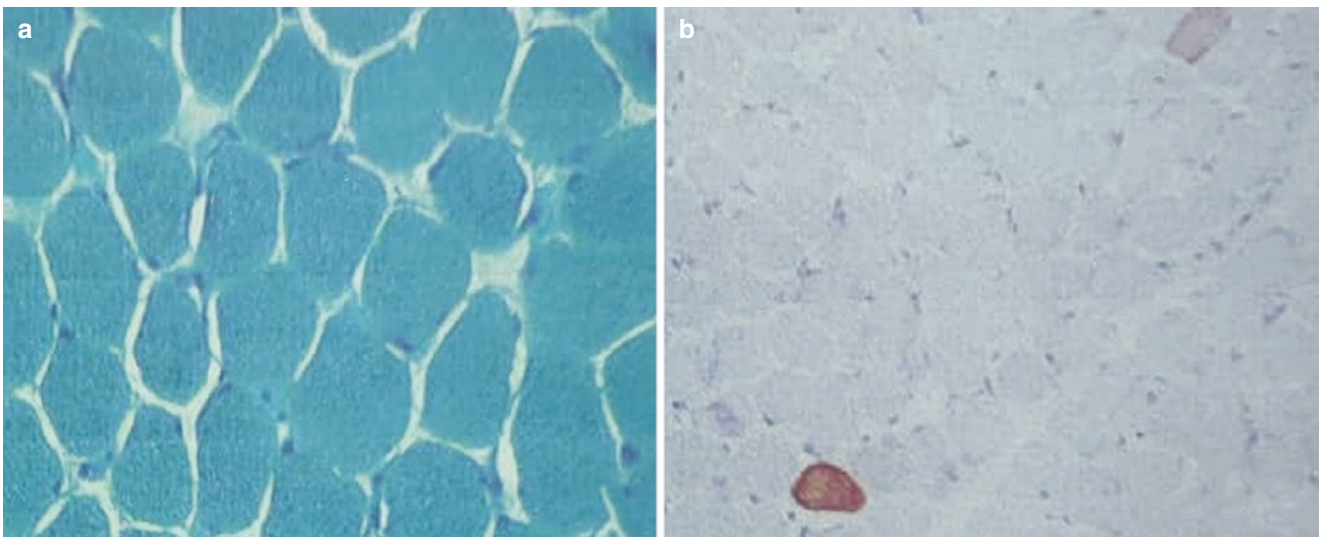

Fig. 12.16 (**a**) Mild size differences of the myofibers (Modified trichrome ×200), (**b**) Immunohistochemical expression of neonatal myosin highlights the presence of immature type 2 myofibres (DAB ×100)

develop nasal speech, which is mistakenly considered dysarthria. Achilles contracture may also develop. CK levels can rise to 1.6–10 times normal. There is no involvement of the heart or respiratory muscles. Dystrophic findings, autophagic vacuoles, structures resembling nemaline rods, and Z-band pathologies can be seen in muscle biopsies [62].

Case 12.10

An 8-year-old girl presented with complaints of getting tired quickly, frequent falling, and muscle weakness. Her CK value was found to be 890 U/L. There was no family history or consanguinity between parents. Muscle biopsy was performed for the differential diagnosis. Muscle biopsy showed a mild myopathic pattern with increased fiber size variability, increased amounts of central nuclei, and mild fibrosis. Sarcoplasmic myotilin expression was diffusely absent in repeated controlled stainings (Fig. 12.17). The biopsy findings were reported as LGMD 1A.

LGMD 2G is caused by a genetic defect of the sarcomeric telethonin protein encoded on chromosome 17q11. This protein is present in the Z disk of both skeletal and cardiac muscle. By binding to the N terminus of titin, it increases its mechanical resistance. Titin is phosphorylated by the serine kinase domain, also called T-cap. Mutations of the C-terminus region (the titin phosphorylation region) have highlighted the important role of telethonin-titin interactions in skeletal muscle. The age of onset of LGMD 2G is 12–13 years. As with other LGMDs, patients first present with complaints of difficulty in walking, running, and climbing stairs. Most patients go on to develop weakness in the distal lower extremities and foot drop. Calf hypertrophy is prominent. CK levels can be increased up to 10–30 times. Cardiac involvement has not been reported. Ambulation is lost in the third or fourth decade. There is also decreased telethonin immunoreactivity in immunohistochemical staining and Western blot analysis [63].

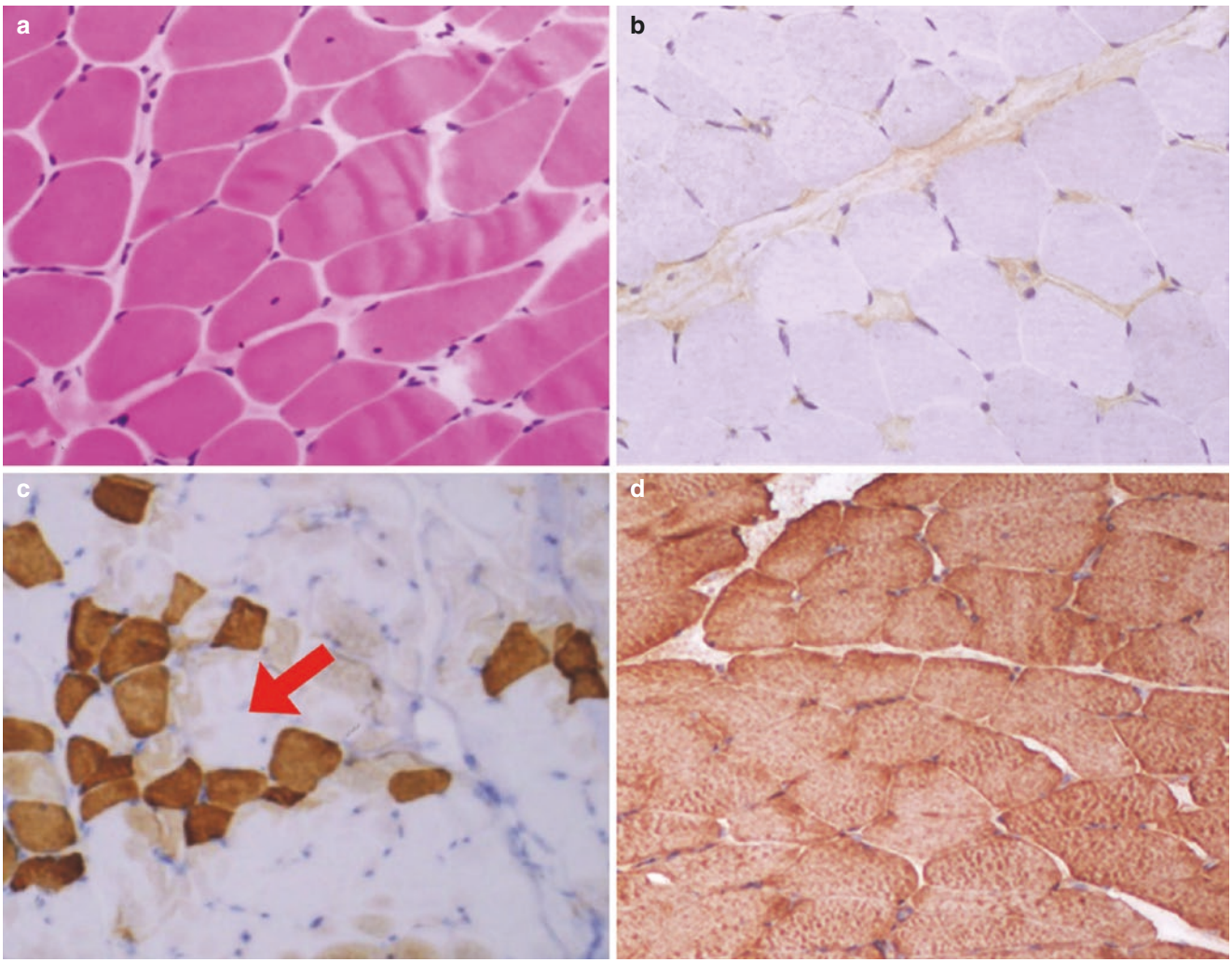

Fig. 12.17 (**a**) Increased numbers of internal nuclei (HE ×200), (**b**) Sarcoplasmic myotilin expression is absent (DAB ×200), (**c**) Increased numbers of pathological immature fibers (arrow) are highlighted by neonatal myosin (DAB ×200), (**d**) Presence of myotilin in a control case (DAB ×200)

LGMD 2J (titinopathy) is caused by a genetic defect of titin protein, which is encoded on chromosome 2q31.2. Titin is one of the largest sarcomeric proteins in the body. Titin provides muscle elasticity by keeping the contractile elements of the sarcomere in their position by providing multiple ligand binding sites for muscle proteins such as muscle-specific protease calpain-3, α-actinin, myosin, myomesin, and telethonin. Titinopathies are autosomal recessive. There is limb girdle-type muscle involvement. Adult-onset hereditary myopathy with tibial muscular atrophy, dilated cardiomyopathy, and early respiratory failure was also found to be inherited by an autosomal dominant titin mutation. A compound heterozygous mutation of titin has been identified in some patients with a diagnosis of centronuclear congenital myopathy. Different clinical presentations are described. Non-progressing arthrogryposis and Emery-Dreifuss-type clinical symptoms are also seen. There is diffuse weakness, but no ophthalmological findings in these patients [64–66].

LGMD 2L (anoctaminopathy) is caused by a genetic defect of anoctamin-5 (ANO5) protein, encoded on chromosome 11p14.3. It is an autosomal recessive disorder and has been associated with adult-onset LGMD. Some patients have distal myopathy, much like dysferlinopathy, while others have quadriceps atrophy. ANO5 is a 107-kDa protein. Although it is found mainly in skeletal muscle, it may also be found in cardiac myocytes and bone. Its function is not fully understood. Although ANO5 is similar to the family of calcium-activated chloride channels, its possible role in maintaining membrane integrity and function is controversial. The most common mutation is a truncation mutation in exon 5, but heterozygous missense mutations have also been reported. The average age of onset is around 40 years. Patients can be completely normal in their youth, and some have gone on to be marathon runners and athletes in competitive sports. Initial symptoms include difficulty in walking long distances, climbing stairs, and walking on tiptoe. Some patients initially have distal involvement like in Miyoshi myopathy. As the disease progresses, the proximal muscles are also involved. Remarkably, there is an asymmetrical involvement between the extremities. CK levels are moderately increased (mean of 4000–5000 U/L). The disease progresses very slowly, especially in women who may remain asymptomatic. Muscle MRI images show asymmetrical fat accumulation in the semitendinosus, semimembranosus, and adductor muscles. The gracilis and sartorius muscles are preserved. The lateral compartment of the lower leg is not usually involved. Changes in the quadriceps muscle occur later in the disease. Muscle biopsy shows nonspecific dystrophic changes, and proteins such as dysferlin and calpain-3 stain normally. Similar to dysferlinopathy, amyloid deposits are seen in intramuscular vessels. Although there are case reports of cardiac involvement, such as premature ventricular contractions and subclinical dilated cardiomyopathy, it is not generally described. Pulmonary involvement is not seen [67].

The onset of **LGMD 2H** occurs between 8 and 27 years old. Initial findings include fatigue, myalgia, and muscle weakness which develop after exercise. Mild weakness of the facial muscles may occur. Affected patients usually have proximal involvement of the lower extremities. In the later stages of disease, weakness in the trapezius, deltoid, anterior tibial group, and brachioradialis muscles develops. Serum CK levels are slightly elevated. Cardiac involvement has not been reported, but pulmonary involvement has. The progression of the disease is very slow. Mutations in the TRIM32 gene, located on chromosome 9q33.1, have been identified to be responsible. TRIM32 protein is involved in proteasomal degradation, and it plays an active role in skeletal muscle regeneration, satellite cell maintenance, and myoblast differentiation [68, 69].

LGMD 1B (laminopathy) is an autosomal dominant disorder caused by a genetic defect of lamin A (LMNA) protein, encoded on chromosome 1q22. Lamin A/C congenital myopathy, LGMD 1B, and autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) are different diseases, but they are all related to lamin A and lamin C. Lamin A/C is an intermediate filament which takes part in the nuclear membrane and envelope. These molecules bind to the intranuclear protein emerin. Laminopathy (LGMD 1B) and autosomal dominant EDMD are diseases related to chromosome 1q. CMD (Laminopathy) and EDMD show clinical symptoms in early ages, whereas LGMD 1B develops between the first and fourth decade. In LGMD 1B, patients have weakness in the pelvic girdle, increased lordosis, Gowers' sign, and difficulty in running from early childhood. There is mild weakness in the facial muscles and anterior tibial compartment. CK values may increase up to 1.5–3 times normal. Cardiac problems may first manifest in the third and fourth decade with a first-degree atrioventricular (AV) block that progresses to complete AV block. Sudden cardiac death may be seen. Biceps and Achilles contractures may occur. A diagnosis can be made by evaluating clinical findings, genetic mutation analysis, and biopsy findings together. In LMNA CMD, patients are divided into two groups. In the severe form, motor weakness occurs early, and spontaneous movements are rare. There is respiratory failure, ventilatory requirements, and severe ventricular tachycardia. In the second type, the symptoms are milder, and patients can maintain the ability to walk. Since the neck extensor muscles are involved, patients cannot fully raise their head and so the disease is known as a dropped neck disease. The peroneal muscles are weak, and the proximal muscles of the lower extremities are involved. CK levels are increased by 2–10 times. There may be contractures in the elbows, an excessive

increase in lordosis, and rigid spine deformity can be seen. Patients with LMNA CMD may have been previously misdiagnosed with Emery-Dreifuss syndrome type 2 [70, 71].

Emery-Dreifuss Muscular Dystrophy (EDMD) is characterised by muscle wasting, early-onset contractures, cardiomyopathy, and humeroperoneal weakness. Autosomal dominant, autosomal recessive, and X-linked inheritance patterns may be seen. Movements are restricted due to contractures rather than muscle weakness. EDMD is an inherited muscular dystrophy, which typically results from a structural or functional defect in proteins of the nuclear membrane. More than seven subtypes have been reported in EDMD, with a huge phenotypic overlap among them. The two most common genetic mutations are in the EMD (EDMD type 1) and LNMA (EDMD type 2) genes coding emerin and lamin A/C proteins, respectively. These account for around 40% of EDMD cases. Although some other genes have also been reported, most are unknown. EDMD is characterised by progressive muscle weakness and atrophy, early contractures, and cardiac manifestations, which frequently precede significant skeletal muscle weakness, including cardiac conduction abnormalities and cardiomyopathy. The weakness pattern involves the proximal arm and distal lower limb muscles, which is known as a humeroperoneal pattern. Contractures frequently begin in childhood, before severe weakness occurs. Elbow flexion and neck extension also occur, causing cervical spine rigidity. Achilles tendon contracture is also common in EDMD and should be differentiated from DMD and BMD patients with early contracture in the Achilles tendons. Other diseases in the differential diagnosis of early contractures include Bethlem myopathy, congenital muscular dystrophies (Ullrich congenital muscular dystrophy, Walker-Warburg syndrome, SEPN1-associated myopathy), and arthrogryposis multiplex congenita. Neck weakness and scapular winging are common. CK levels can be normal or elevated. In electrophysiological testing, nerve conduction studies are usually normal, but with low compound muscle action potentials (CMAPs) if the muscle tested is atrophied. Needle EMG reveals frequent abnormal spontaneous activity and myopathic motor unit action potential (MUAP) findings, i.e., MUAPs with a low amplitude, short duration, and early recruitment. The gold standard diagnostic test is genetic testing. Muscle biopsy reveals a histopathological MD pattern and an absence of perinuclear emerin expression [72–74].

Case 12.11

A 4-year-old boy had a high serum CK without symptoms. Persistent elevation of CK values prompted muscle biopsy. He did not have consanguineous parents or an ancestral history of neuromuscular disorders. Cognitive and motor development were normal. He started walking at 14 months of age and was walking normally. Pulmonary function tests were normal. His CK levels were between 700 and 900 U//L (normal < 250 U/L), and there was evidence of myopathy on EMG. Muscle biopsy showed myopathic changes such as contraction, degeneration, nuclear internalisation, and mild fibrosis. Immunohistochemically, perinuclear emerin expression was totally absent (Fig. 12.18). During sequence analyses of the emerin gene on chromosome Xq28, a hemizygous G724T p.E146X mutation was found. A similar mutation was also present on one of the two X chromosomes of his mother. This patient is an excellent example of how to manage an MD. His educated parents with a high socioeconomic status have successfully managed the disease without reducing his quality of life in the 10 years that have passed, with cardiological follow-up, Achilles tendon correction operations, regular exercise, and physical therapy practices.

Oculopharyngeal muscular dystrophy (OPMD) is an uncommon trinucleotide (GCG) repeat disorder, usually gained via autosomal dominant inheritance. Symptoms typically begin in adulthood with an age at onset in the fourth–sixth decade. Dysphagia and bulbar muscle weakness are predominant; such that the diagnosis might be confused with bulbar amyotrophic lateral sclerosis, progressive bulbar palsy, or even with myasthenia gravis. However, stationary weakness in the eyelids (ptosis) and ocular muscles is a characteristic feature in patients with OPMD. Extraocular muscle involvement is also seen in progressive external ophthalmoplegia, mitochondrial myopathy, and thyroid ophthalmopathy. Weakness may involve the shoulder girdle in advanced stages. Serum CK levels are normal or mildly elevated. In terms of electrodiagnostic studies, needle EMG and repetitive nerve stimulation (RNS) could be of use in differentiating the disorder from myasthenia gravis. Myopathic electrophysiological changes would be detected in needle EMG and RNS would be normal. Single-fiber EMG shows increased jitter in affected muscles. Therefore, it cannot be used for the differential diagnosis of myasthenia gravis. Genetic testing and muscle biopsy would confirm the diagnosis. Muscle biopsies demonstrate dystrophic changes, rimmed vacuoles, and cytoplasmic or nuclear filaments, or both [73, 74].

Myotonic Dystrophy is an inherited, slowly progressive, multisystem disorder, characterised by myotonia and dystrophic muscle fibers resulting in weakness. It is one of the most common muscular dystrophies in adulthood. However, congenital and childhood onset forms also exist. Myotonic dystrophy type 1 (DM1), also known as Steinert disease, is a trinucleotide (CTG) repeat disorder of the myotonic dystrophy protein kinase gene (DMPK) on chromosome 19, being the most common myotonic muscle disease. Distal muscles are primarily affected in DM1. Patients have distal muscle

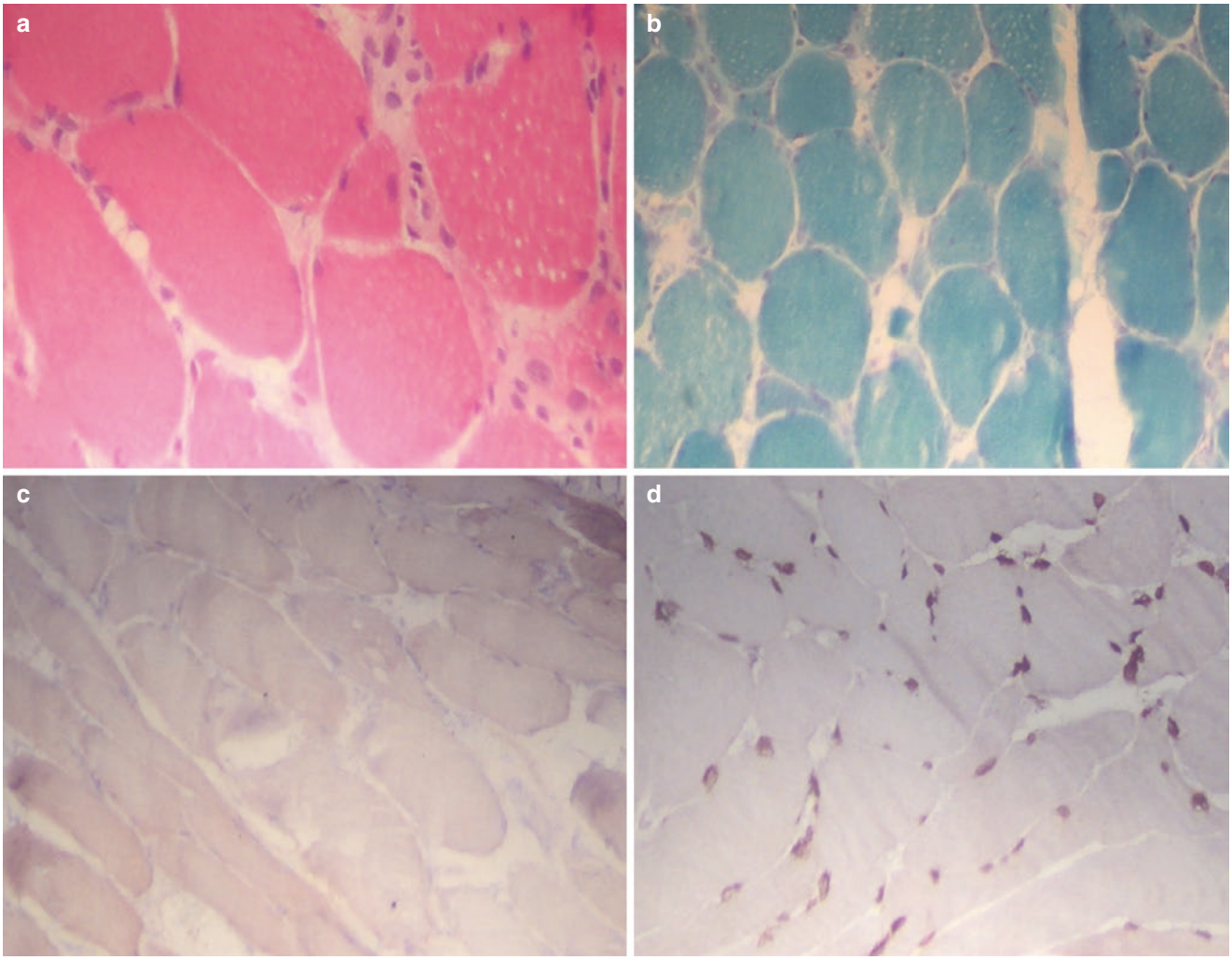

Fig. 12.18 (**a**) Prominent size differences of the myofibers (HE ×200), (**b**) Normal myofibrillary arrangement (Modified trichrome ×200), (**c**) Absence of perinuclear emerin expression (DAB ×200), (**d**) Normal immunohistochemical expression of emerin in a control case (DAB ×100)

weakness with prolonged relaxation of contracted muscles (often painful), improving with repeated contractions. Patients with DM1 have a phenotypic appearance characterised by bifacial weakness, blepharoptosis, frontal balding, and temporal muscle atrophy with an elongated face. Involvement of other systems is common including cardiac conduction deficits, cataracts, hypersomnolence, pulmonary/endocrine dysfunction, hyperglycaemia with decreased insulin sensitivity, and cognitive impairment. The clinical severity of symptoms reflects the number of CTG repeats. Earlier onset usually indicates a severe disease course. Individuals who are asymptomatic, except for early-onset cataracts, may be recognised during examination (e.g., myotonia) and electrophysiological testing for other indications. Serum CK is often normal or mildly elevated. In electrodiagnostic studies, NCS would be normal except for small CMAP amplitudes in affected distal muscles. Myotonic dystrophy type 2 (DM2), also known as proximal myotonic myopathy1 or PROMM, is a disorder where weakness is milder than DM1, and it involves the proximal muscles, predominantly. Patients also have bifacial weakness, blepharoptosis, and prolonged relaxation in contracted muscles (difficulty in opening hand after griping). Multisystem involvement is like that of DM1, except cognition is rarely affected. The most important histopathological finding in myotonic dystrophy is increased nuclear internalisation up to 30 (Fig. 12.19). Needle EMG usually demonstrates myotonic discharges with waxing and waning frequencies, together with dystrophic/myopathic MUAP findings (low amplitude, short duration, polyphasic) and early recruitment [73, 74].

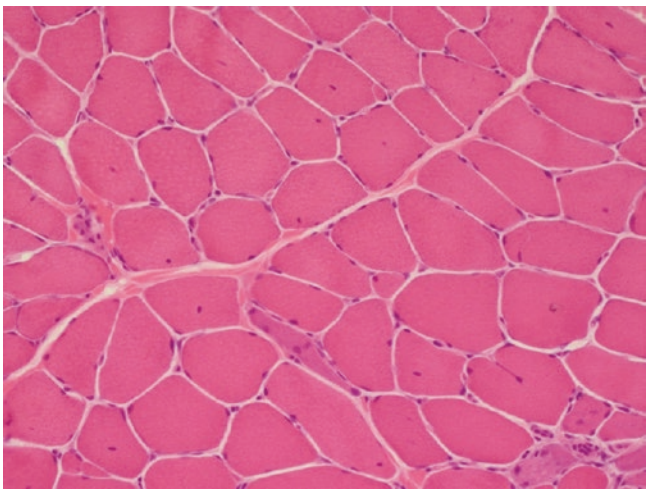

Fig. 12.19 Note the prominent increase in the number of internal nuclei (HE ×200)

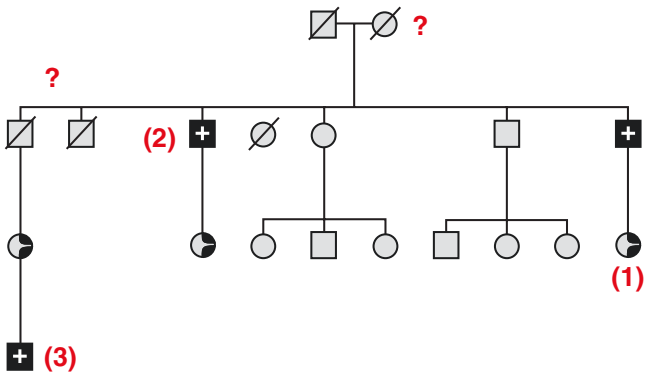

Fig. 12.20 A pedigree diagram of a girl with dystrophinopathy

What Have We Learned from Our Patients?

- **Signs and symptoms may not be fully revealed at the age at which a child is examined. Therefore, diagnosing neuromuscular disorders in childhood may be difficult. Patient must be followed up and changes in physical findings should be observed over time.** For example, in a 3-year-old boy who was diagnosed with FSHMD, scapula winging was not fully visible at the age when he was first examined. As he got older, involvement of the facial muscles, a tapir mouth appearance, and scapula winging became evident. The family reported 2 years later that his eyes were open while he slept. Facial involvement occurred at the age of 5 years, and scapula winging became a full symptom around the age of 10.
- **The patient's history should be listened to patiently, and then the signs and symptoms that will lead to the diagnosis should be questioned.** The same findings should be questioned again at each visit as the patients or family members can forget some things when first asked.
- **Other members of the family should also be evaluated, and the patient's pedigree should be established to determine the family history**. Dystrophinopathy was investigated with MLPA in a 15-year-old girl (Fig. 12.20) who applied to our clinic with elevated CK levels, and 45–55 exon deletions were found. (Patient 1). No mutation was found in her mother. When her father and middle uncle (Patient 2) applied to our centre, the same deletions were found in MLPA. Finally, her father and uncle were diagnosed with BMD, but her father had never had any muscle complaints in his life. The grandson of her oldest uncle also applied to our centre when he complained of fatigue, and a 45–55 exon hemizygous deletion was found in MLPA (Patient 3). When this child's mother and middle uncle's daughter were also examined, it was found that they carried the same deletions.
- **It must be noted that patients can carry more than one disease at the same time. This is especially true in societies where consanguineous marriages are common.** For example, a 15-year-old male patient applied to our clinic with complaints of difficulty in getting up from his seat, going up/down stairs, limitations in raising his arms, and tiring quickly. Both myopathy and neuropathy findings were found on EMG, and this was confirmed in the genetic examination of the patient. His parents were first-degree cousins. EMG findings were accompanied by chronic axonal polyneuropathy affecting the lower extremity proximal muscles, and electrophysiological findings indicating primary muscle fiber involvement in cranial nerves and innervated muscles. In the genetic analysis of the patient, two different pathogenic variants, both autosomal dominant and autosomal recessive, on two different genes were identified. These variants are a heterozygous form of a c.1756 A > T variant in the RYR1 gene, and a heterozygous c.604C > T(p.Arg202Ter) variant, related to distal hereditary neuropathy type VA and Silver Spastic paraplegia, in the BSCL gene.
- **The genetic diagnosis of a disease is also very important.** When the genetic diagnosis of a patient is made, clinicians can predict what findings the patient may encounter, and a more precise view can be gained about the prognosis. Additionally, an idea can be obtained as to whether there is a drug under development for this disease. In the future, information on how to screen and follow-up when a close relative becomes pregnant can be obtained.

All About the Pathology of Muscular Dystrophies

It can be claimed that the development of molecular genetic diagnostic methods should, in theory, render the requirement for muscle biopsy unnecessary in the diagnosis of muscular disorders. Nonetheless, muscle biopsies are still performed, and with rare exceptions, evaluating a patient's muscle biopsy is crucial to examining a patient with a suspected myopathy, especially for MDs. This is because there can be some pathognomonic features or at least there may be some clues that help to differentiate the main group of muscle disease such as neuropathic, inflammatory, mitochondrial, and so on. A pathologist experienced in neuromuscular diseases can tell which diseases are not present, even if they cannot say exactly what disease it is. In this way, the molecular examination can narrow the spectrum of diseases to be investigated [36].

Severe changes in fiber size and shape (Fig. 12.21), muscle fiber necrosis with regeneration (Fig. 12.22), and the presence of interstitial fibrosis are the most prevalent histological characteristics of MD (Fig. 12.23). Even though this trio, collectively known as the dystrophic feature, is the hallmark of dystrophies, migration of nuclei to the centre of the muscle cell is also an important sign of muscle cell injury, with an incidence of up to 30%, particularly in myotonic dystrophy [26, 36, 74–76].

In most cases, fiber necrosis serves as a catalyst for future regeneration. Therefore, even in the absence of necrotic fibers, the presence of regenerated fibers, in a muscle highlighted with neonatal myosin staining, is a likely sign of prior necrosis. The proliferation of satellite cells, as well as the production and fusion of myoblasts, are thought to be the primary causes of fiber regeneration. Injured muscle is returned to a state that is morphologically and functionally equivalent to that of uninjured muscle through a complex cellular process. Degeneration and regeneration are two separate processes of skeletal muscle regeneration. Sarcolemmal damage or myofiber necrosis are the primary features of the degenerative phase, which is then characterised by an influx

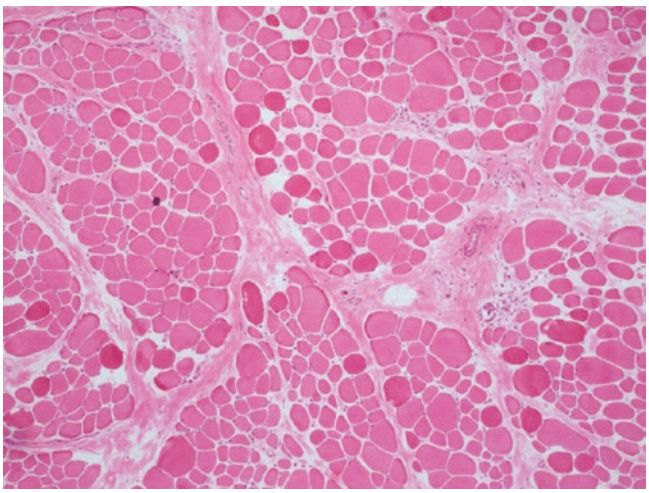

Fig. 12.21 Note the prominent size and shape differences of myofibers in a patient with muscular dystrophy (H&E ×40)

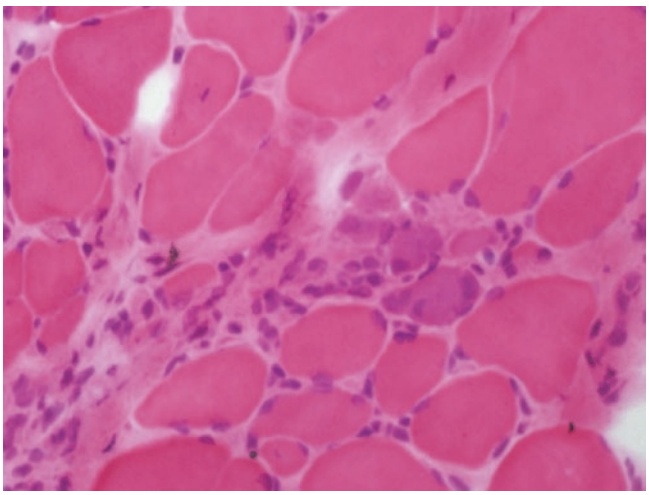

Fig. 12.22 Note the basophilic staining in the regenerated fibers (HE ×400)

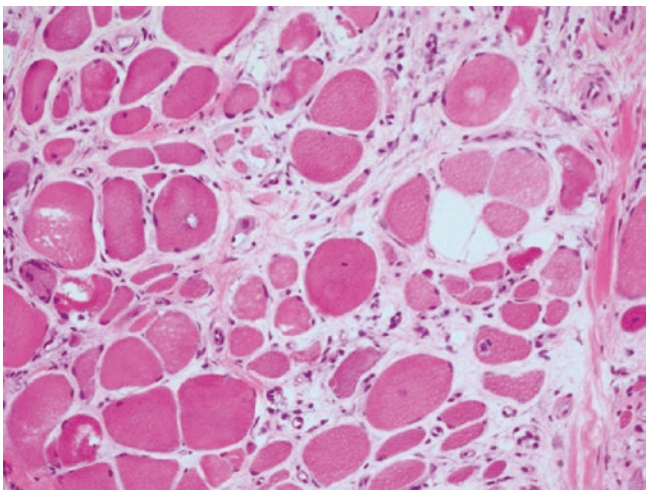

Fig. 12.23 Note the prominent intercellular fibrosis (H&E ×200)

of mononucleated inflammatory cells, an increase in fibroblasts, and myofiber necrosis. Chemical mediators released from damaged myofibers initiate an inflammatory response that recruits neutrophils and macrophages and activates fibro/adipogenic progenitors to facilitate the removal of cellular debris and regulate muscle repair. The basal lamina remains intact, acting as a scaffold for the next phase, muscle regeneration. Several molecular signals, such as growth factors, chemokines, and cytokines, are released, which activate satellite cells both locally and systemically within the first 24–48 h following injury. Myoblasts then terminally differentiate, becoming post-mitotic myocytes, which then fuse with other myocytes or myofibers to regenerate or repair damaged myofibers. Regenerating fibers are most readily visualised in hematoxylin and eosin (H&E) sections by the basophilia of their sarcoplasm. The nuclei are typically increased in number, are larger than normal, have vesicular chromatin and prominent nucleoli, and are often internally placed. Ultra-structurally, the regenerating fibers are replete with ribosomes, which explains the sarcoplasmic basophilia at the light microscopic level. An inflammatory reaction is generally lacking, except in the scattered necrotic fibers in MDs. These cells consist mostly of macrophages, scarce T lymphocytes, myoblasts, and mast cells [75].

Regenerated fibers are present in three conditions which are MDs, inflammatory myopathies, and diseases caused the rhabdomyolysis. The presence of lymphocyte infiltrations is not found in MDs except in rare cases of FSHD, or dysferlinopathy. Therefore, it must be kept in mind that the presence of regenerating fibers and prominent lymphocytic infiltration is a hallmark of inflammatory myopathies (Fig. 12.24), while there are no conspicuous lymphocytes around the regenerated fibers in MDs. This is because like apoptosis, the necrotic-regenerated fibers in MDs are cleaned up mostly by macrophages without creating inflammatory responses. In the case of rhabdomyolysis, there are also regenerated fibers with almost no lymphocyte infiltration. However, in rhabdomyolysis, the number of regenerated fibers is almost higher than the normal number of myofibers, and this appearance is helpful in the differential diagnosis (Fig. 12.25) [75, 76].

In muscle biopsies of some patients with MDs, exclusive myofibers known as trabecular fibers can be seen. These pathognomonic fibers, also known as lobulated or lacey

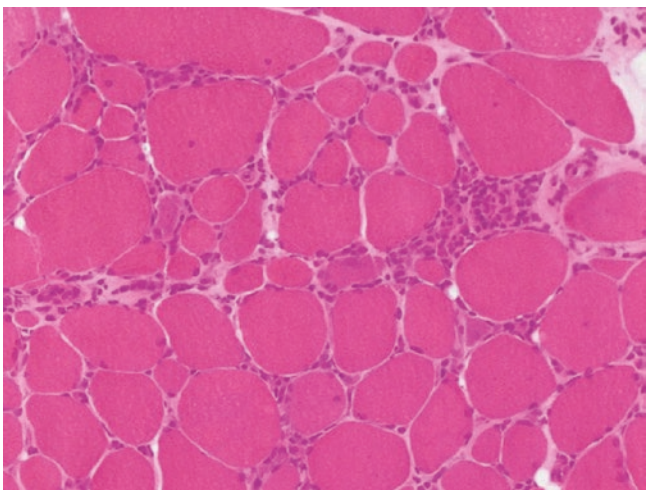

Fig. 12.24 Note the presence of lymphocyte infiltration associated with the regenerated fibers (H&E ×200)

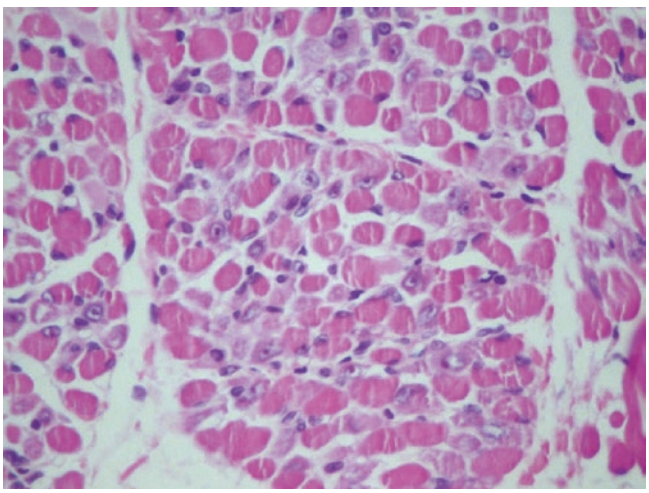

Fig. 12.25 Extensive rhabdomyolysis after chemotherapy in a child with acute lymphocytic leukaemia (H&E ×100)

fibers, have been reported in various neuromuscular disorders. Trabecular fibers are accepted as a maldistribution of intermyofibrillar mitochondria, and they can be well detected by an oxidative enzyme reaction in histochemical preparations of muscle biopsies (Fig. 12.26). They are often seen in LGMDs and may help determine the differential diagnosis [54].

An important point that should not be forgotten during the evaluation of muscle biopsy is the fact that there can be secondary losses in any structural proteins of the striated muscle associated with another muscle disease. For example, the expression of sarcolemmal dystrophin was found diffusely negative with immunohistochemical evaluation (Fig. 12.27) in a 4-year-old boy who presented with difficulty in walking and a high CK level (2400 U/L). Although no defect was detected in the dystrophin gene in the MLPA and sequence analysis, a compound heterozygous pathogen mutation was detected in the plectin (PLEC) gene [45, 46].

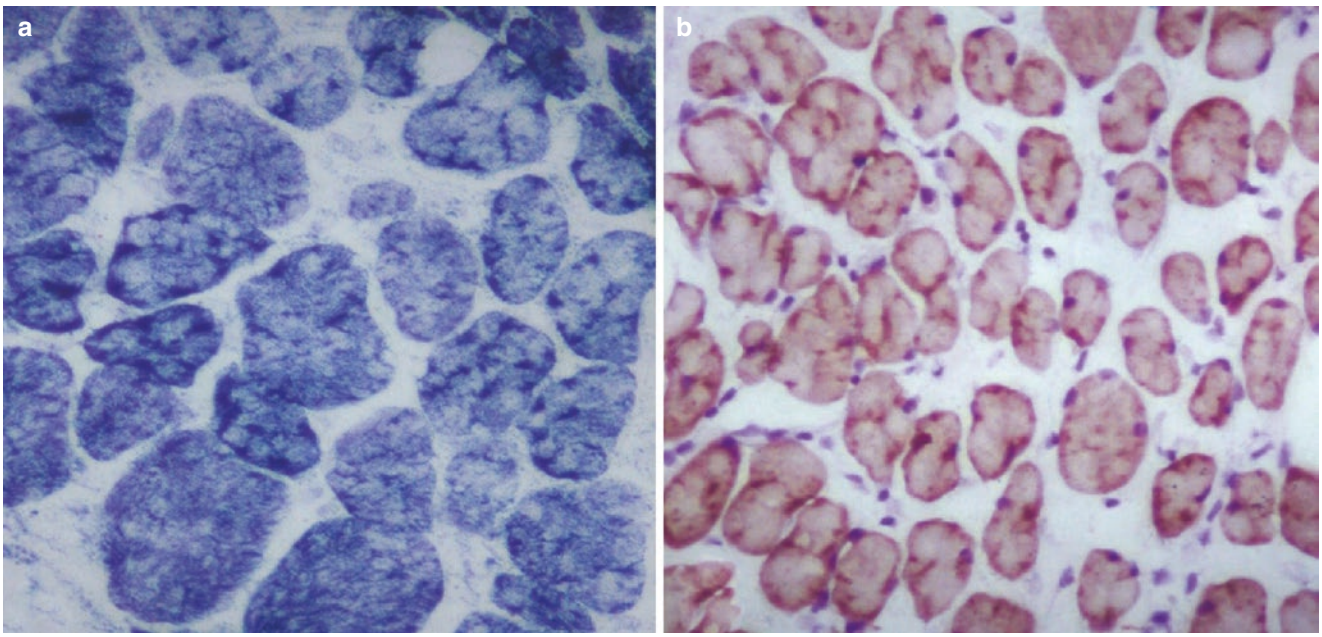

Fig. 12.26 Trabecular fibers in a patient with limb girdle muscular dystrophy 2B: (**a**) NADH-tr ×200, (**b**) COX ×100

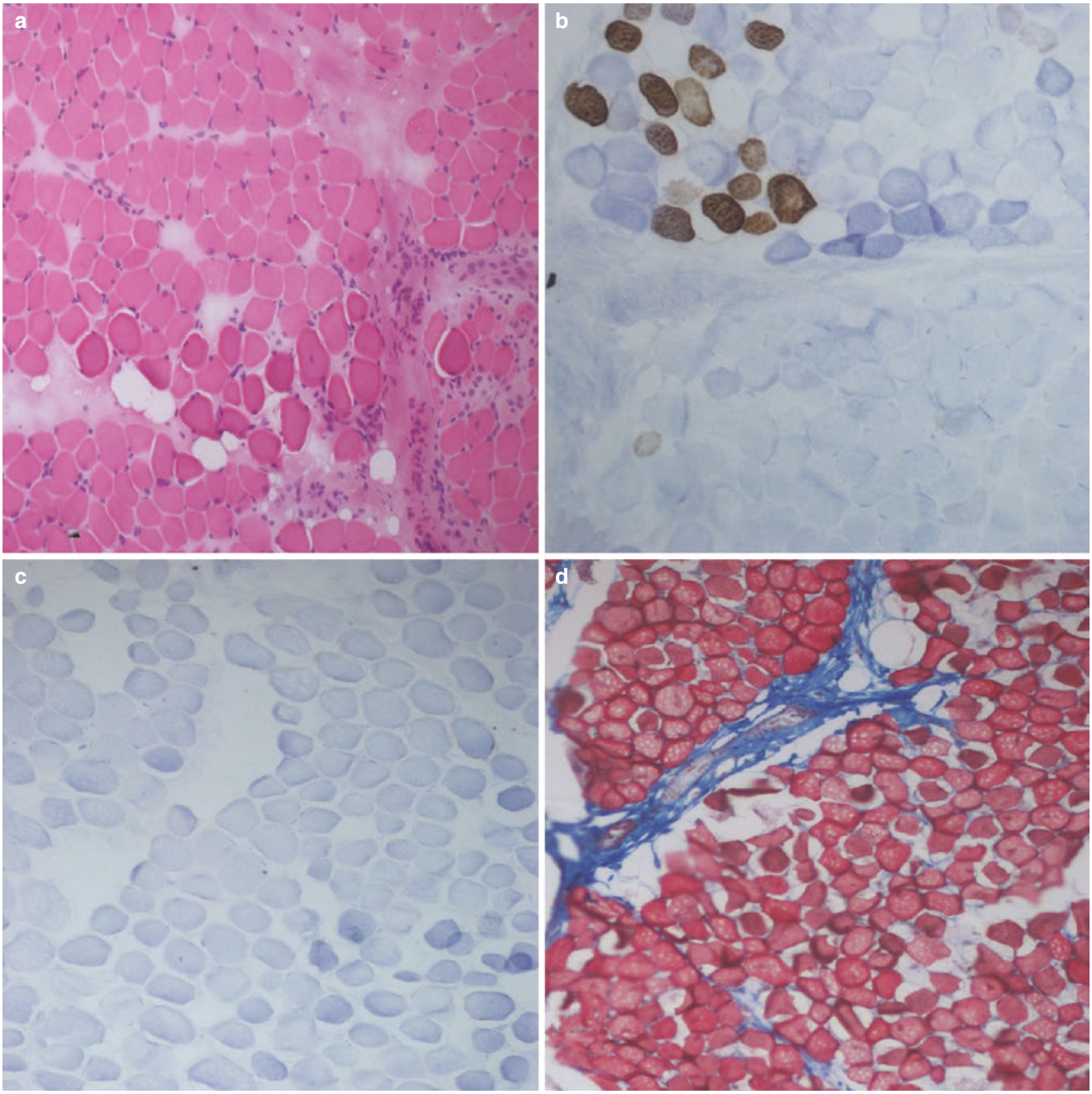

Fig. 12.27 (**a**) Typical muscle dystrophy appearance with plenty of regenerated and contracted fibers (HE ×100), (**b**) Immunohistochemically regenerated fibers can be highlighted with neonatal myosin (DAB ×100), (**c**) Defect of dystrophin expression (DAB ×100), (**d**) Increased intercellular fibrosis (Masson trichrome ×100)

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Congenital Myopathies

13

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Congenital myopathies (CM) form a clinically, genetically, and morphologically heterogeneous group of neuromuscular disorders (NMDs) [1, 2]. Years ago, with the introduction of histochemical examinations and electron microscopy (EM), some abnormal features in muscle structures were associated with certain clinical phenotypes and were named congenital non-progressive myopathies [3, 4]. Although, they generally present with hypotonia (±muscle weakness) in the neonatal/infantile period, in some cases, the first symptoms occur in the juvenile or adult period [1, 2, 4, 5]. Studies report the prevalence of all CMs as 0.96–3.62/100,000, but in the <15–19 age group as 0.52–5.01/100,000 [6]. Core myopathies (*RYR1* gene variants that are autosomal recessive/dominant) are the most common type of CMs, followed by nemaline myopathies [7]. The most common congenital myopathies and the responsible gene loci are summarized in Table 13.1.

CMs occur due to structural defects within muscle fibers, affecting the contractile matrix, excitation-contraction coupling, T-tubules, and sarcoplasmic reticulum, while muscle membrane stability is spared. They are also a type of NMD, pathologically characterized by myopathic changes, such as differences in myofiber sizes, internalized nuclei, and the presence of degenerating fibers [1, 2, 8]. Muscle fibers might also be undifferentiated, and small/ hypotrophic type-1 fiber predominance is an important finding [1, 5]. When there is a disproportion between the sizes of type-1 and type-2 muscle fibers in a biopsy, this is considered a fiber-type disproportion (FTD) [1]. Fibrosis and replacement with fat tissue are observed in all severely affected muscles [8]. Necrosis, inflammation, and dystrophic changes due to sarcoplasmic membrane protein defects in congenital muscular dystrophies (CMD) do not occur in CMs [1, 2, 8]. Structural abnor-

Table 13.1 Most common CMs and loci of responsible genes

Disease	Protein	Gene	Chromosome
Core diseases (AD or AR)	Ryanodine receptor 1	*RYR1*	19q13
	Selenoprotein N1	*SEPN1*	1p36
Nemaline myopathies (AD or AR)	Sarcomeric thin filaments	*ACTA1, CFL2, KBTBD13, NEB, TNNT1,TPM2, TPM3*	1q42.13,14q13.1,15q22.31,2q23.3,19q13.42,9p13.3,1q21.3
Congenital fiber-type disproportion (AD or AR)		*ACTA1, MYH7, RYR1, SEPN1, TPM3*	1q42.13,14q11.2,19q13,1q36,1q21.3
Centronuclear myopathies (X-linked, AD or AR)	Myotubularin	*MTM1*	Xq28
Centronuclear myopathies (AR)	Amphiphysin	*BIN1*	2q14
Centronuclear myopathies (AD)	Dynamin 2	*DNM2*	19p13

AD autosomal dominant, *AR* autosomal recessive

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malities in a muscle biopsy are classified according to abnormally located organelles and intracellular bodies. These classifications include nemaline myopathy (NM), central core disease (CCD), multi-mini core myopathy (MmCM), centronuclear myopathy (CNM), congenital fiber-type disproportion (CFTD), and others [1, 4]. NM is known as the accumulation of Z-line proteins. Cap disease, zebra body myopathy, intranuclear rod myopathy, and myosin storage myopathy are other CMs with protein storage pathology. Cores seen in CCD and MmCMs are areas devoid of oxidative activity [9]. In some cases, different structural pathologies such as cores and rods, and rods and caps could be seen together [5]. A centrally located nucleus is the typical pathological finding in CNM and X-linked myotubular myopathy. Selective atrophy of type-1 fibers without any other structural abnormality indicates CFTD, which clinically and genetically overlaps with CM [9].

With developments in the field of molecular genetics, these diseases have been associated with more and more genes. In terms of phenotypes, clinical signs, histopathological features, and genetic features, there is overlap between CM, CMD, congenital myasthenic syndromes (CMS), and even mitochondrial myopathies [8]. Variant-specific clinical features are reported for some genes. Every gene that causes CM generally acts as a member of a clinicopathologic spectrum instead of acting individually [4]. Until now, almost 60 genes have been defined in CM cases [10]. Pathological classification and a classification based on molecular genetics are used together [1, 2, 6].

Clinical recognition of CM is rather easy, but difficult to diagnose genetically (genomic era). There is histopathological overlap between CMs themselves, and boundaries between subgroups are not always clear. Inheritance can be autosomal recessive (AR), autosomal dominant (AD), or X-linked [4, 5]. There may also be different mutations (deletion, duplication, frameshift, nonsense, missense, splice site, etc.) on the same gene, or, the variant in the same gene, which can cause both AD and AR inheritance [5]. De novo mutations are also frequently seen [4]. The variants in different genes can lead to similar histopathological changes [4]. A variant in a gene may also lead to different clinics by interacting with various gene products [4]. Additionally, the same gene variant within the same family can cause varying disease severity due to somatic mosaicism or epigenetic and environmental modifiers [1, 5]. CMs are rather rare among all NMDs but they are a heterogeneous group. Therefore, gene panels, whole-exome sequencing (WES), and whole-genome sequencing (WGS) have advantages and disadvantages compared to each other for determining a definitive diagnosis. Large genes like *RYR1, TTN,* and *NEB* are frequently found in CMs. Variants of uncertain significance (VUS) have been detected and even new mutations and genes have been discovered [2]. Thus, muscle biopsy materials are needed for functional studies, and molecular diagnostic methods may not be sufficient for determining a definitive diagnosis in some patients [2].Histopathological features, data from molecular genetic analysis, and muscle imaging should be evaluated as part of diagnostic process, and supported with omics studies, if necessary [2].

Common Clinical Features

CMs are one of the causes that lead to various degrees of clinical hypotonia and 'floppy infant' inthe neonatal/infantile period [4]. They are responsible for 14% of all newborn hypotonia cases [11]. (Table 13.2) Muscle weakness and motor retardation are other common findings [11]. Respiratory insufficiency disproportionate to muscle weakness is another typical finding [5]. Intelligence is usually normal [11]. They are a type of primary myopathy because they do not involve the central nervous system or peripheral nerves [4]. Generalized weakness that is predominantly proximal is usually evident in the neonatal/infantile period [4, 8]. Proximal involvement is more prominent in the lower extremities. Rarely, some cases display findings in the adult

Table 13.2 Classical clinical features of some common CMs

NM	CCD	MmCM	X-MTM	CNM
Early-onset	Usually, AD	Usually, AR	Severe IU onset	Diffuse weakness
Generalized hypotonia	Hypotonia	Early onset	Polyhydramnios	Facial weakness
Proximal-axial weakness	Motor delay	Scoliosis	Neonatal hypotonia	Ptosis
Respiratory insufficiency	Proximal-axial-hip-girdle weakness (LL > UL)	Spinal rigidity	Ophthalmoplegia	Ophthalmoplegia
Facial-bulbar involvement	Orthopedic complications	Respiratory involvement	Respiratory insufficiency	
		Preserved ambulation	Feeding problems	

NM nemalin myopathy, *CCD* central core disease, *MmCM* multiminicore myopathy, *X-MTM* X-linked myotubular myopathy, *CNM* centronuclear myopathy, *AD* autosomal dominant, *AR* autosomal recessive, *IU* intrauterine, *LL* lower limb, *UL* upper limb, *IU* Intrauterine

period [1, 8]. There is usually a static or slow progressive course of the disorder [4]. In some cases, axial and facial muscle weakness, ophthalmoplegia, and ptosis are seen. Some patients also present with the signs of distal arthrogryposis [4, 8]. Facial weakness is a discriminative characteristic of CM from CMD and spinal muscular atrophy (SMA). The lower half of the face is particularly involved, an open mouth, a tented upper lip, and drooling may be present [9]. Axial weakness is more prominent in AR *RYR*-related CM and *SELENON*-related CM, and distal weakness in *NEB* and *MYH7, TPM3,* and *DNM2*-related CM [7, 9, 11]. There might be decreased fetal intrauterine movements and polyhydramnios due to prenatal onset [8]. Polyhydramnios and contracture in major joints are more frequently seen in X-linked myotubular myopathies [1]. There is also a risk of dysmorphic features associated with arthrogryposis, micrognathia, high arched palate, and dolichocephaly due to decreased fetal movements [8]. In severe NM and CCD cases (*MTM1*, severe *DMN2*, severe *RYR1*), these dysmorphic findings appear more often [4]. Long typical myopathic facies are more specific for NM [4].

Ptosis and ophthalmoplegia are commonly seen in NM and CNM cases (*RYR1, DNM2, MTM1*), especially in the neonatal period, and are distinctive from CMSs [8, 9]. Eye involvement is relatively less in AD inherited core myopathy [12]. Diaphragmatic involvement is very severe together with generalized weakness in NM and MmCMs[4]. A weak, hoarse cry, and difficulty in sucking and swallowing are findings of bulbar involvement. Respiratory insufficiency and feeding problems are life-threatening in the neonatal period [1]. In studies, the need for respiratory support at birth is reported as 14–30% [7, 13]. For all CM cases at every age, 64% of patients have respiratory insufficiency, and almost half require respiratory support [7]. In the first 2 months of life, mortality is reported as 8% [14]. In AR inherited core myopathy cases, respiratory insufficiency is more prominent [12]. Particularly in NM, CNM (*MTM1)*, and severe *RYR1* cases, severe respiratory insufficiency, the need for nasogastric tube (NG tube) feeding in the neonatal period and prominent bulbar involvement are common [7, 11]. The spine is often hyperlordotic, and spinal rigidity occurs in some cases [1]. Although scoliosis is seen in the early period, 13.6% of patients need surgical intervention [7]. Scoliosis becomes prominent in the period of transition during puberty and the phase of rapid growth [1, 8]. Hip dislocation is commonly seen in CCD [4]. While cardiac involvement is the most important reason for morbidity and mortality in NMD, respiratory complications are the leading cause of morbidity and mortality in CMs and determine the prognosis [1, 5, 8, 13]. However, cardiac involvement should be considered especially in *TTN* and *MYH7* gene relatedCMs [8, 11]. In *ACTA1, RYR1, TPM2,* and *SPEG* gene-relatedCM cases, cardiac involvement is rarely reported [11, 13, 15–17] (Table 13.3).

Table 13.3 Clinical clues in congenital myopathies and related genes

Some common clinical features	Related genes
Premature birth	*MTM1, ACTA1, RYR1* (AR)
Fetal akinesia, arthrogryposis, polyhydramnios	NM (*ACTA1, NEB*), *MTM1, RYR1, KLHL40*
Respiratory insufficiency at birth	*MTM1, ACTA1, RYR1* (AR)
Neonatal respiratory/bulbar insufficiency/NG tube feeding	NM (*ACTA1, KLHL40, NEB*), *MTM1, RYR1* (AR)
Increased mortality in the first year of life	*MTM1, ACTA1, KLHL40*
Facial weakness	NM, CNM (*MTM1, RYR1, DNM2*)
Ptosis/ophthalmoplegia	CNM (*MTM1, RYR1, DNM2*), MmD-CCD, NM (*KLHL40, LMOD3, CFL2, NEB*)
Predominant axial hypotonia	*RYR1, SEPN1*
Acquisition of ambulation	*NEB, SEPN1, RYR1*
Club feet	NM, *RYR1*
Scoliosis	NM, *RYR1, SEPN1*
Rigid spine	*RYR1, SEPN1*
Cardiomyopathy	*TTN, MYH7, ACTA1, RYR1, TPM2 FLCN, SPEG, MYPN, TNNT1*
Foot drop, distal involvement	*DNM2, MYH7, NEB, TPM2, TPM3*

Investigations

Differential diagnoses of CM are CMDs, CMSs, congenital myotonic dystrophy, Pompe disease, metabolic myopathies, and SMA. Investigations are planned according to these differential diagnoses [11]. On the suspicion of NMD, the first test to be used is the level of serum creatine kinase (CK). In CM cases, serum CK levels and electrophysiological studies are not helpful for diagnosis [4]. In CM cases, serum CK is usually normal or minimally increased however, if it increases more than five times or if CK > 1000 IU/L, this finding is suggestive of muscular dystrophy [7, 8]. In electrophysiological examinations, there may be polyphasic, small amplitude motor unit potentials, myogenic features, and early recruitment or the test may be normal [1]. In patients that present with severe neonatal involvement, neurogenic changes can occur [1]. During muscle ultrasonography, increased echogenicity could be detected in the affected muscle, and it is also useful to select, which muscle to use for a biopsy sample [4]. Muscle pseudohypertrophy suggests CMD or Pompe disease [11]. In the thigh and lower leg, muscle involvements can be associated with certain gene mutations [18]. Muscle biopsy supported with immunohistochemical studies and electron microscopy is very effective for CM diagnosis and for referral to molecular genetic tests [4]. On the other hand, molecular genetic analyses are preferred over invasive tests in some centers [8]. However, pathological studies and molecular genetic analyses are generally used together to reach a definitive diagnosis.

CMs comprise genetically, clinically, and pathologically heterogeneous groups. They show overlapping features with each other and with other NMDs. Despite next-generation sequencing techniques, such as gene-targeted exome sequencing or WES, only 50% of cases could be definitively diagnosed.Clinical studies (muscle MRI and muscle biopsy) and preclinical research data may be helpful to understand pathogenicity of variants [11]. As the genetic causes of CM cases are determined, and the pathophysiology and mechanisms of the disease are clarified, we will have the opportunity to develop new treatment options.

Congenital Myopathies and Malignant Hyperthermia

Malignant hyperthermia is an emergency clinical picture in which a life-threatening, hypermetabolic catabolic state develops due to triggering agents such as depolarising muscle relaxants including succinylcholine, and inhaled volatile anesthetics like isoflurane causing hyperthermia and muscle rigidity. Factors increasing the risk of malignant hyperthermia include a family history of malignant hyperthermia susceptibility (MHS), serious problems during anesthesia, previous history of cardiac arrest, and a diagnosis of *RYR1, STAC3, and CACN1S* mutations are at risk. Dantrolene is the mainstay treatment, which suppresses the release of intracellular calcium sustained by *RYR1*[11].

Subgroups of Congenital Myopathies

Nemaline Myopathy (NM)

NM ranges from very severe forms to mild forms with a likelihood of survival until late adulthood [9]. The prevalence of NM is 0.08–0.56/100,000 in children [6]. Clinical subtypes of NM include fetal akinesia syndrome with congenital contractures, severe congenital neonatal onset, and severe respiratory insufficiency at birth and intermediate congenital with severe hypotonia. Furthermore, there is the typical congenital subtype, which presents with spontaneous respiration at birth, and predominantly facial and axial muscle weakness, motor delay and hypotonia, which develop in early childhood. Additional subtypes include, mild childhood onset with a milder clinical course, gammopathy related adult onset and distal arthrogryposis [4, 19, 20]. In subtypes presenting with neonatal and infantile hypotonia, common findings include, symmetrical proximal, neck flexor, axial, and facial muscle weaknesses along with muscle atrophy, respiratory insufficiency, and feeding difficulties [4, 19, 21]. The extraocular muscles are often spared [4]. In some cases, distal muscle involvement is experienced and foot drop may develop as a result of peroneal muscle weakness [19, 20]. Signs of respiratory insufficiency and reduced vital capacity should be closely monitored, because hypoventilation develops slowly or suddenly in cases without any previous findings, and if left untreated it may cause cor pulmonale [19, 20]. Due to weak genotype-phenotype correlation, and overlap between subgroups despite pathological and genetic classification, Sewry C.A. et al. proposed a new NM subgroup classification as follows [21];

1. Severe nemaline myopathy (ACTA1, *NEB, KLHL40, KLHL41, LMOD3, TPM2, TPM3,TNNT*),
2. Congenital nemaline myopathy (*NEB, ACTA1, CFL2, TPM2*),
3. Childhood-juvenile onset nemaline myopathy (*ACTA1, NEB, TPM2, TPM3, KBTBD13,MYPN,*AD-*TNNT1*),
4. AR-*TNNT1* (Amish) NM,
5. Childhood onset NM with slowness of movements and core-rod histology (*KBTBD13*)

In NMs, the typical feature is rod-like structures, stained red with Gomori trichrome dye in skeletal muscle fibers [19]. If only a few fibers are affected or if they are small fibers, the rod-like structures can only be detected with electron microscopy or semithin sections stained with toluidine blue[19]. Rods derived from the Z-line, and include similar proteins, such as α-actinin, actin, and Z-band filaments [4, 11]. Rods cluster at the periphery of the fibers or in areas close to nuclei [4]. There is no correlation between the number of rods and the clinical severity [4]. Rarely, based on histopathologic findings the associated defective genes can be predicted. Nuclear rods, accumulation of thin actin filaments, and expression of the cardiac actin isoform are all seen in *ACTA1*-relatedNM. Secondary nebulin deficiency might also accompany the clinical picture [4, 21]. The accumulation of thin filaments in *CFL2*-relatedNM and nuclear rods in *MYPN*-related myofibrillar myopathy, have also been reported [21]. Rods are found in both type-1 and type-2 fibers. If rods are restricted to type-1 fibers this suggests *TPM3*-relatedNM, and if restricted to type-2 fibers it suggests *TNNT3*-related NM [21]. Another typical characteristic of NM is the predominance of type-1 fibers. A variation of fiber size and small type-1 fibers may be found [4]. Although not frequently found, fibrosis may accompany *TNNT1*-gene-related NM cases [21]. Immunohistochemical investigations rarely aid in the diagnosis of CMs. A total absence of nebulin protein is not expected in *NEB*-related NM, in only severe neonatal cases with antibodies against the SH3 domain, nebulin protein might be absent [5]. Additionally, very small fibers can be found with fetal myosin antibodies, but this is not a typical finding for NM [5].

There are four basic structures in a sarcomere including Z-discs, thick filaments, thin filaments, and titin (Fig. 13.1).

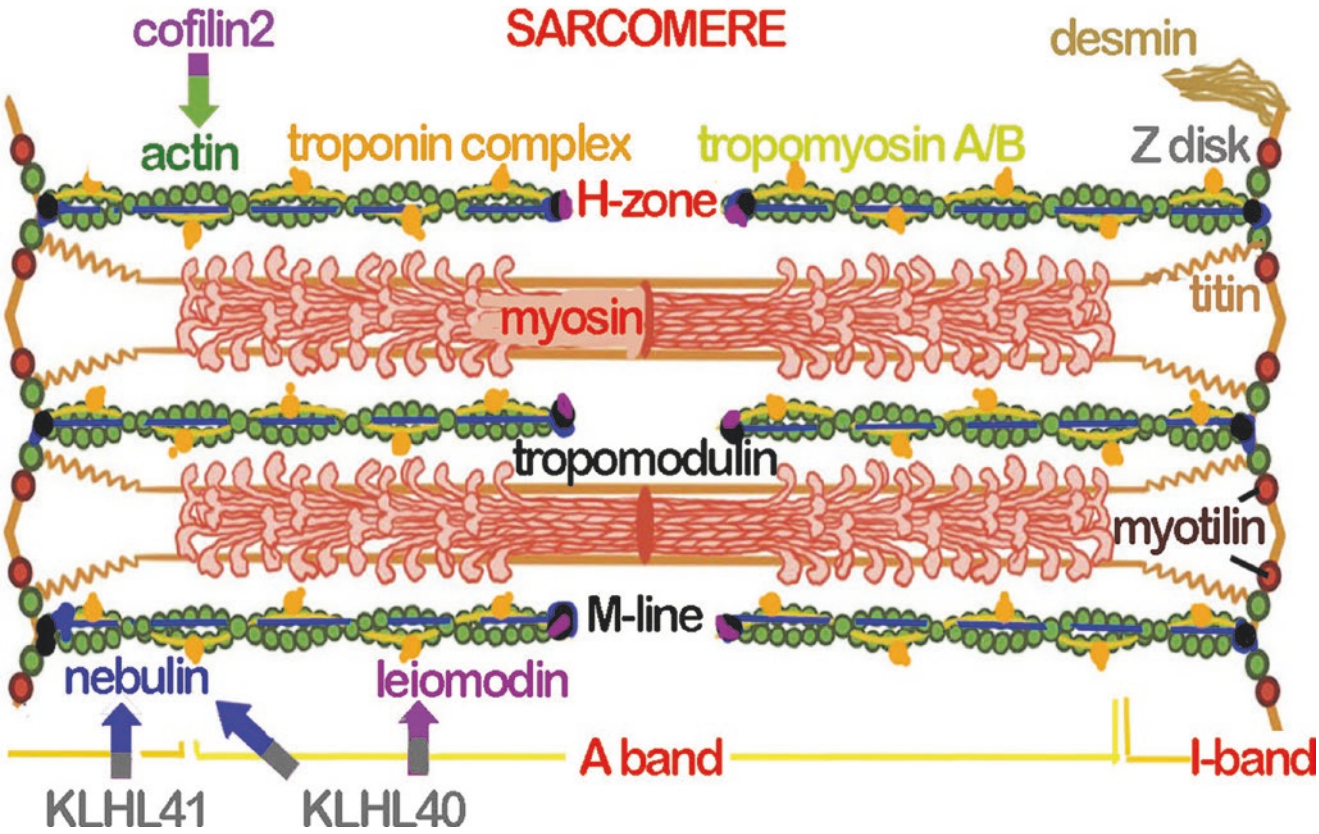

Fig. 13.1 Sarcomeric structures those are associated with the congenital myopathies

The dysfunction of these sarcomeric structures causes structural abnormalities like nemaline rods, Z-disc integrity loss, abnormal mitochondrial organization, and muscle atrophy [22]. Thin filament is formed by proteins that play a role in the actin-based backbone, actin length regulation and the actin-myosin interaction. So far, more than ten genes have been associated with NM [10, 22]. Nebulin (*NEB*), skeletal muscle actin alpha-1 (*ACTA1*), tropomyosin-beta (*TPM2*), tropomyosin-alpha (*TPM3*), and myopallidin (*MYPN*) are integral structural proteins that form the thin filament [10, 20, 22]. Nebulin anchors to Z-disc with its C-terminus, and interacts with thin filament, tropomodulin, and leiomoidin with its N-terminus. Skeletal muscle actin alpha 1 protein forms the backbone of the thin filament [22]. The AR inherited *NEB*mutation is found in 50% of NM cases and the AD inherited *ACTA1* mutation in about 25% [23]. *TPM2* and *TPM3* defects are seen in less than 10% [20]. Tropomyosin beta and alpha modulate the interaction of myosin-binding sites with actin [22]. During muscle contraction, troponin complex proteins (troponin T1, a slow skeletal protein and troponin T3, a fast skeletal protein) are necessary for thin filament function [20]. Troponin T1 modulates tropomyosins[22]. Other proteins play a dynamic role in the regulation and function of structural proteins. Kelch repeat and BTB domain containing 3 (*KBTBD13*), Kelch like family member 40 (*KLHL40*), and Kelch like family member 41 (*KLHL41*) stabilise thin filament proteins [20, 22]. Cofilin (*CFL2*) regulates actin polymerizations[22]. Leiomodin3 (*LMOD3*) is an actin-nucleation protein and organizes the length of thin filaments [20, 22]. Gene variants of these proteins cause muscle weakness through length dysregulation, cross-bridge cycling kinetic disruption, and alteration of calcium-sensitivity to force generation [20, 22–24].

Nebulin (*NEB*)is an actin-binding protein that is localized to the thin filament of sarcomeres in skeletal muscle. It is a very large protein coded by the *NEB* gene and binds nearly 200 actin monomers. Nebulin is thought to function as a regulator of thin filament during sarcomere construction, as it serves as a template for the actin filament by extending along the course of the thin filament determining its length. AR inherited *NEB*-related mutations are the most common cause of NMs, and they are identified in about 50% of cases. They usually cause the typical subtype in which proximal axial involvement is prominent and distal involvementmay develop later. In addition, they may also cause severe, intermediate, mild, or rode-core forms. Distal only involvement—distal nemaline myopathy is discriminated by the absence of nemaline rods in the biopsy. Patients with the typical form can't raise their head while lying supine, and knee flexors are more affected than knee extensors. Additional

signs include facial/bulbar weakness, a nasal voice, and palatal reflexes may be absent [19]. Muscle magnetic resonance imaging (MRI) may show sparing of the thigh muscles with selective involvement of the tibialis anterior and soleus muscles [24]. Missense variants are the most frequent type of mutation, but if this genetic variant lies outside the coding regions for actin or tropomyosin binding sites, it could be welltolerated [20]. Therefore, the most frequent pathogenic variants are splice-site, frameshift, or nonsense mutations. Additionally, although not causing frameshift, exon 55 deletion is also frequently seen in patients because it impairs the modular super-repeated structure of nebulin [20]. Findings from muscle MRI also suggest that the rectus femoris is selectively involved, and at the level of the calf, the tibialis anterior is involved in the early period [18]. These findings help discriminate NM from CCD. Unfortunately, there is no curative treatment and supportive care is needed [24].

Case 13.1

A 7-year-old boy was admitted to hospital with muscle weakness and mild intellectual disability. His weakness developed during infancy, and he was able to walk after the age of 5. He also had delayed speech development. On examination, axial and predominantly proximal muscle weakness, with generalized muscle atrophy and areflexia were noted. The CK levels were normal (104 IU/L). Needle EMG showed myopathic motor unit potentials. WES analysis revealed a pathogenic homozygous variant in the *NEB* gene, c.736dup, p.Leu246ProfsTer6 and a heterozygous VUS variant in the *NEB* gene, c.19993C > T, p.Pro6665Ser. He was diagnosed with Nemaline Myopathy 2.

ACTA1 (Skeletal muscle alpha-actin) defects are found in about 25% of cases, and most are dominant mutations [8, 19, 25]. De novo missense mutations are present in about 90% of pathogenic variants and with its dominant-negative effect it causes severe disease [20]. In 50% of severe onset neonatal cases, *ACTA1* gene variants were found [8, 19]. Rarely, it also causes typical, intermediate, and mild forms. Knee extensors become weaker than knee flexors and ankle dorsiflexion is preserved. Recessive mutations often cause null mutations and disease severity depends on the expression of cardiac actin isoform in skeletal muscles in the postnatal period [19]. NM-related *ACTA1* mutations occur on six coding exons. The hydrophobic core structure of subdomain 1 is the only area where NM related mutations are not seen [19, 25]. Nemaline rods are not seen in every patient with *ACTA1* mutations. In some cases, cores or FTD may not be detected in biopsy [19]. Types with nuclear-located rods have a poor prognosis. There is no curative therapy [24].

Defects of the *TPM2* and *TPM3* (β-tropomyosin and α-tropomyosin) genes because inherited NMs. Dominant missense mutations in both genes, and in-frame mutations that cause an amino acid deletion, are frequently seen [20]. *TPM2* dominant mutations are common causes of typical and mild NM and distal arthrogryposis. Recessive mutations cause a NM-related Escobar sequence with a severe course and fetal akinesis [8, 19]. In *TPM3* mutations, the disease is more severe. A homozygous mutation, which causes deletions in the *TPM3* gene promoter and first 2 exons, leads to severe disease [20]. FTD is seen in biopsies in *TPM3* related NM, and nemaline rods are commonly seen in small type 1 fibers [8, 19].

Defects of the slow skeletal muscle troponin T-1 (*TNNT-1*) gene cause NMs.Recessive mutations in *TNNT-1*are characterized by tremor, progressive contractures, muscle stiffness, progressive restrictive lung disease, and the findings of respiratory insufficiency [21]. In the Amish community, a homozygous nonsense mutation causes a stop codon in exon 11 and mutant protein breaks down [20]. AD inherited *TNNT1* presents with a mild course. In the heterozygous missense mutation, the mutant protein is not degraded, which causes adominant-negative effect [20]. Defects of the fast skeletal muscle troponin T-3 (*TNNT3*) gene cause respiratory insufficiency, continuous ventilator dependency, contractures, and hip dislocation different from other NMs, which are the typical features [21]. Nemaline rods are limited to type 2 fibers [21]. Recessive loss of function mutations in the Myopalladin (*MYPN*) gene are seen in cases with a mild NM disease and slowly progressive cap myopathy [20, 26]. Cofilin-2 (*CFL2*) gene-related NM is rare and cases with *CFL2* mutations have a typical presentation [20]. Facial weakness and foot drop may not develop in homozygous mutations [19]. Recessive missense mutations and deletions are reported and in the absence of cofilin-2, growth control of actin filaments is impaired, and the structure of the sarcomere is affected [20].

The recessive nonsense and frameshift variants of the Leiomodin-3 (*LMOD3*) gene are often seen in some cases with NM [20]. Protein deficiency may lead to short and disorganized actin filaments. Kelch proteins play a role in the stability, turnover, and regulation of thin filament proteins [20]. Defects of the Kelch repeat and BTB/POZ domain containing protein 13 (*KBTBD13*) cause NMs. AD inheritance is detected in *KBTBD13*-related NM [19]. Both nemaline rods and core-like structures are seen [19]. With its defect, regulation in thin filament relaxation is impaired [20]. Apart from that, patients can't adjust their body position to avoid falling, and their muscle movements are slow [19]. The Kelch-like 40 (*KLHL40*) protein also regulates nebulin and leiomodin-3, and prevents degradation. *KLHL40* dysfunction causes several clinical phenotypes. The recessive mutations of *KLHL41* cause mild, typical, and severe NM phenotypes [20].

Case 13.2
An 18-month-old boy who was born to consanguineous parents was admitted to our clinic with motor delay. Reduced in-utero movements, birth with a femur fracture, and hip dislocation were noted in the prenatal and perinatal history. His examination revealed generalized weakness with contractures of the knees, interphalangeal joints, elbows, and mild laxity of the ankles. He had mild facial weakness, kyphoscoliosis, and absent deep tendon reflexes. His CK levels were normal. A targeted gene panel revealed a likely pathogenic variant in the KLHL40 gene, c.1607 + 3A > T. He was diagnosed with nemaline myopathy 8.

Myosin XVIIIB (*MYO18B*) gene defects may cause an unusual phenotype with a Klippel-Feil abnormality and dysmorphic features, as well as muscle weakness and nemaline rods in biopsy [20]. A compound heterozygous mutation of the ryanodine receptor (*RYR3*) gene was reported in a patient with childhood-onset proximal weakness with an elongated face. Muscle biopsy showed fiber size variation, and type 1 fiber predominancy with nemaline bodies that were in perinuclear regions, the subsarcolemmal area and within the cytoplasm [27]. Severe forms, commonly caused by *ACTA1* and *KLHL40* defects, are first seen in the first 2 years [7]. Typical forms have a static/slow progressive course, and even with milestones development, some clinical improvement may be seen [21]. In the childhood or juvenile period, patients with a new onset may have mild findings. However, AR *TNNT1* mutations have a rather progressive course with progressive thorax stiffness, tremor, progressive contractures, muscle stiffness, restrictive pulmonary disease, and findings of progressive respiratory insufficiency in the early period [21].

Nemaline rods in biopsy are not pathognomonic for NM. Adenylosuccinate synthase-like (*ADSSL1*) gene defects may cause distal myopathy with nemaline rods and lipid bodies in biopsy. Similarly, filamin C (*FLNC*) may cause a distal myopathy with cardiomyopathy, as well as nemaline rods and ring fibers in biopsy [20]. Although rods, cores, and cap-like structures are reported in *RYR1* defects, *TTN* gene defect-related CM cases, and the findings are not compatible with NM [11, 20].

Additionally, nemaline rods are not universally seen in all cases with known NM gene mutations [20]. In some cases, rods and cores coexist. This is reported with *RYR1, ACTA1, NEB, CFL2, TRIP4, TNNT,* and *KBTBD13* gene defects [4, 28]. Zebra body myopathy is also a NM variant. It is related to the *ACTA1* gene [1]. Cap myopathy is a NM variant with well-demarcated cap-like structures, disorganized thin filaments, and Z-disc structures in the periphery of muscle fibers [1]. There may be decreased ATPase activity in cap structures, and pale eosin staining with H&E stain [5]. In *TPM2* related cap myopathy, immunolabeling with weak slow myosin can be detected [5]. Cap structures may be present in 4–100% of the fibers. The proportion of affected fibers is related with clinical severity. It is related to the *TPM2, TPM3, ACTA1, NEB,* and *MYPN* genes [11].

Case 13.3
A newborn baby, who was born from the first pregnancy of a 29-year-old mother by vaginal delivery on the 36th gestational week, was admitted to hospital due to severe respiratory failure soon after birth. His family history did not reveal any significance in terms of NMDs and there was no consanguinity between his parents. His antenatal screening tests were normal. However, fetal movements in the third trimester of pregnancy, felt by his mother and observed by the obstetrician on fetal ultrasonography, were decreased and marked polyhydramnios had emerged after the 32nd week of pregnancy. At 36 weeks, vaginal delivery had to be induced because of the onset of membrane rupture and fetal distress. Due to his ongoing severe hypotonia, weak cry, and lack of effort to breathe, muscle enzymes, and screening tests for congenital metabolic disorders were performed and they were found to be completely normal. EMG and cerebrospinal magnetic resonance imaging (MRI) could not be performed because of his dependency on mechanical ventilation. Eventually, to exclude congenital neuromuscular disorders, a muscle biopsy was taken from the gastrocnemius at age 8 weeks, and the diagnosis of NM was established. The rods were not visible with H&E staining but appeared as red or purple structures against the blue-green myofibrillar background with the modified Gomori trichrome stain (Fig. 13.2). The distribution of rods within myofibers showed a tendency to cluster around nuclei. Furthermore, increased oxidative enzyme activity with cytochrome oxidase enzyme stain was also demonstrated. Immunohistochemical stains were per-

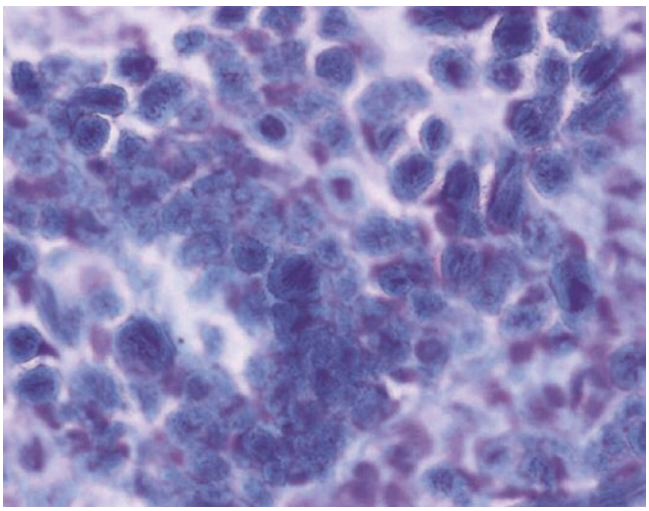

Fig. 13.2 Note the characteristic, purple-colored rods in the perinuclear region of most of the myofibers (Modified trichrome ×400)

formed using antibody against sarcomeric actin, smooth muscle actin, desmin, and vimentin. Only focal desmin positivity could be demonstrated on these rods. Genetic analysis for specific gene mutations could not be performed. The diagnosis was based on clinical findings and the presence of the characteristic, thread-like or rod-shaped structures (nemaline bodies) in muscle biopsy [29].

Case 13.4

A male infant who was 34-gestational-weeks old, and had a birth weight of 2100 grams, was born to a 22-year-old woman by normal spontaneous vaginal delivery. He was referred to the neonatal intensive care unit after delivery due to hypotonicity. His prenatal history was unremarkable. The parents were first cousins, and his 5-year old brother was healthy. There was no family history of neuromuscular disease. On admission, he was hypotonic and had little spontaneous activity. There was bilateral chorioretinal atrophy. Serum CK levels and the cerebrospinal fluid analyses were normal. Cranial magnetic resonance imaging revealed the presence of corpus callosum agenesis. The results of metabolic screening tests and electroneuromyography (EMG) were within normal limits. Muscle biopsy was performed at the age of 142 days. Microscopic examination revealed the presence of red-black colored, short, rod-like structures which were condensed predominantly on small-sized myofibrils and compatible with nemaline myopathy (Fig. 13.3). The patient was fed via an orogastric tube because of swallowing problems. He died at the age of 10 months due to a severe respiratory tract infection [30].

There is no current curative treatment. Monitoring and management of findings, preservation of muscle power/mobility/joint range of motion, maintenance of daily activities, and monitoring of respiratory and orthopedic complications are the basis of the multidisciplinary approach [21].

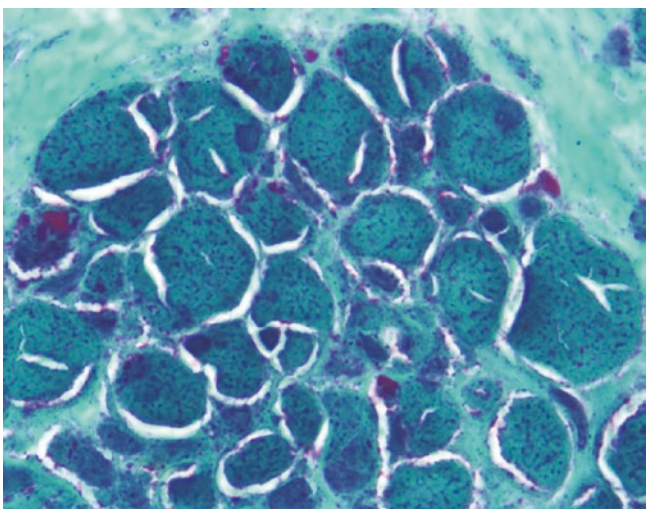

Fig. 13.3 Note the characteristic, purple-colored rods in most of the myofibres (Modified trichrome ×400)

Core Myopathies

The central core, multiminicore, dusty-core, and core-rod myopathies are clinically, pathologically, and genetically heterogeneous groups that are characterized by cores/minicores in muscle fibers [6, 24, 26]. In the pediatric period, its prevalence is 0.08–0.23/100,000 [6]. Cores are well-demarcated areas devoid of mitochondria and oxidative enzyme activity [11]. The absence of oxidative enzyme activity can be illustrated with nicotinamide adenine dinucleotide (NADH) and cytochrome c oxidase or succinate dehydrogenase dyes [4, 8]. Electron microscopic investigations prove the absence of mitochondria within cores [12]. Cores may be located centrally or peripherally, and the number of cores may be one or many [11]. There may be a large central core in CCD, and multiple cores in MmCM[11]. In some cases, cross sections in light microscopy may not be enough to distinguish central and minicores[12]. In CCD, cores extend along the whole longitudinal section within type 1 fibers.However, minicores reveal shorter areas and are seen in both type 1 and type 2 fibers [11, 12]. Other myopathic biopsy findings including variation in fiber size, increased internal nuclei, and rarely endomysial fibrosis, and fatty infiltration may be seen in biopsy specimens [4, 12]. Immunocytochemical investigations may show abnormal desmin reactivity in cores, but it is not helpful in distinguishing the myopathy subgroup [12]. Myotilin, filamin C, triadin, and αβ-crystallin can accumulate within the cores [4, 26].

Central core myopathy (CCM) or central core disease (CCD) is the most frequent CM and more than 90% of patients have dominant RYR1 gene mutations. Recessive SELENON causes multiminicore myopathy (MmCM) and is the second most common form of core myopathies[6, 12]. In contrast to CCDs, MmCMs are known to be mostly autosomal recessive [4]. *SELENON, RYR1, MYH2, MYH7, TTN, CCDC78, ACTN2, MEGF10, and UNC45B* are the genes that are reported to be causative for MmCMs[26, 28]. Core-like structures are seen in not only CCD, but also in neurogenic atrophies, myasthenias, and other CMs [4, 5]. Bi-allelic mutations of *RYR1* can cause dusty-core myopathies [28]. The coexistence of core and central nuclei is reported in CCDC78 gene-related CMs [4]. Furthermore, *RYR1, NEB, ACTA1, CFL2, TRIP4, TNNT1, and KBTBD13* gene-related myopathies may demonstrate core-rod structures in muscle biopsy [28].

CCM is the most common form of CMs and more than 90% of cases have a *RYR1* mutation [1, 8]. Basic clinical features include hypotonia and a delay in motor milestones.

It has a static, non-progressive course with prominent proximal/axial muscle involvement, and involvement of the lower extremities more so than the upper extremities, which are typical findings[4]. In pathology, core structures are more often found in type 1 fibers, but there may also be type 1 fiber predominance[4]. Fiber size variation is partially seen[4]. An absence of mitochondria and sarcoplasmic reticulum in core areas, and an irregularity in Z-lines can be visualized with electron microscopy [4]. There may also be small fibers stained with fetal myosin [4]. There may be peripheral ring-shaped PAS staining in core boundaries, but cores are stained with glycogen and phosphorylase [4]. Core structures are structures that develop secondarily and do not cause direct weakness. Its underlying mechanism is impairment in calcium homeostasis that alters excitability in muscle cells [1, 4]. In recessive cases, like *SELENON*-related MmCM, there may be multiple cores in a few sarcomeres instead of only one central core [4, 5]. In immunohistochemical examinations, cores are stained with desmin[4]. Additionally, αβ-crystallin, filamin C, and myotilin accumulate in the cores [4]. Core structures are not found in every genetically proven case, and type 1 fiber predominance alone may be present [4]. Although internal nuclei, increased connective tissue, fat between fascicles, and increased fibrous tissue are not classical findings, they are rarely seen and can be confused with muscular dystrophies [4, 5]. Central nuclei can be seen and may be confused with CNM [5]. Core is age-related, and in young cases only type 1 fibers are predominant and core is absent [4, 5]. In conclusion, central nuclei, core, and type 1 fiber predominant seen in pathological examination suggests *RYR1*-related CCD. There may be type 1 fiber predominance without core and additionally, core and rod may coexist [4, 5].

Case 13.5

A 9-year-old girl who was born to consanguineous parents was admitted to our clinic with muscle weakness. Her CK levels were normal. Muscle biopsy revealed that type 1 fiber predominance was present, and there were central cores in some type 1 fibers (Fig. 13.4). Nuclei internalization was

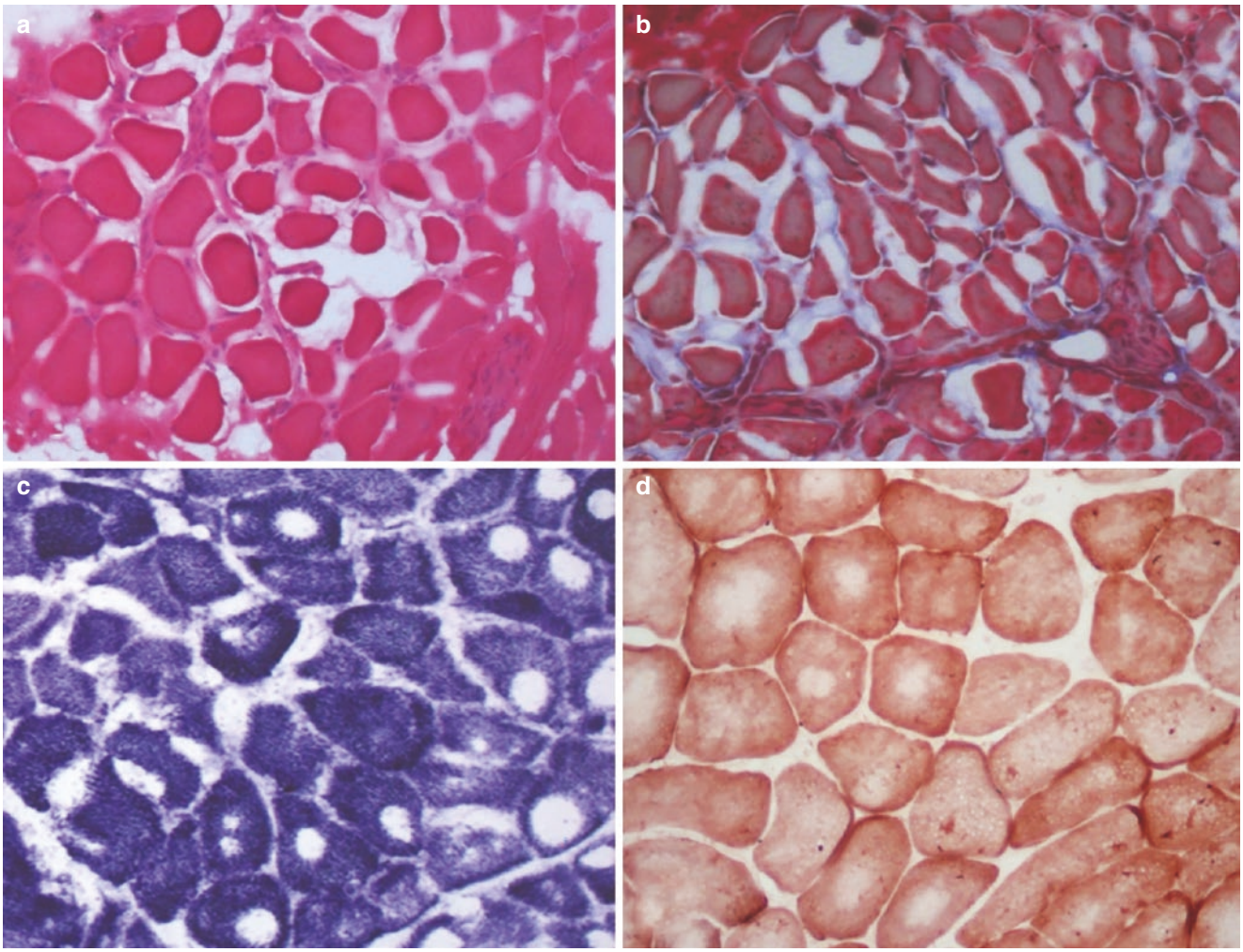

Fig. 13.4 Muscle biopsy of Case 13.5 diagnosed with CCD: (**a**) Muscle biopsy shows variation in fiber size and multiple internalized nuclei (HE ×100), (**b**) Mildly increased fibrous connective tissue (Masson trichrome ×100). Central cores are present in most type 1 fibers, (**c**) NADH-TR ×200, (**d**) COX ×200

mildly increased in both fiber types. A diagnosis of CCD was based on clinical and histopathological findings, and genetic analyses could not be performed.

The *RYR1* gene encodes the skeletal muscle ryanodine receptor protein, and it is a large gene with 106 exons [4]. The protein is a transmembrane calcium-channel protein within the sarcoplasmic reticulum that maintains cytosolic calcium homeostasis and plays a role in excitation-contraction coupling [4, 8]. Dihydropyridine receptors (DHPR) sense depolarizations induced by action potentials and then undergoconformational changes, which lead to interactions with RYR1 receptors. The C-terminal of RYR1 protein forms the calcium channel. The direct interaction between DHPR and RYR1 proteins precipitates the opening of calcium channels, and the release of calcium from the sarcoplasmic reticulum is the hallmark of excitation-contraction coupling [31–33]. *RYR1* mutations are mostly located in the transmembrane domain [11]. Dominant *RYR1* variants are clustered in three regions [32]. Mutations in cytoplasmic N-terminal and central domain often cause susceptibility to malignant hyperthermia [4]. Mutations in C-terminal exons are related to CCD [4, 5]. Mutations, especially in C-terminal 95–102 exons cause classic type CCD and are found in 66% of CCD cases. Non-C terminal *RYR1*mutations cause milder cases with atypical cores [4, 28, 31–33]. Recessive mutations can be seen in any region of the gene and result in more severe clinical presentations like fetal akinesia deformation sequence [4, 28, 31–33]. Heterozygous missense mutations are often identified [1]. Atypical cores can be found in patients with non-C terminal *RYR1* mutations. Some young patients with a family history of confirmed CCD may present with a predominance of type 1 fiber without detectable cores. This condition is defined as a congenital neuromuscular disease with uniform type 1 fibers (CNMDU1). Here, fiber type conversion occurs before core formation [12, 28]. Cores are initially found at the peripheral boundaries of fibers, and with aging, they begin to displace longitudinally toward the central area of the fiber. Therefore, the placement of the cores correlates with the stage of the disease in CNMDU1 cases [28].

Dusty core myopathies overlap with CCD, MmCM, and CNM. Core structures in dusty core myopathies have characteristic features, including unclear borders, irregular size, and non-ovoidal shape [28]. Recessive *RYR1* mutations are linked to dusty core myopathies and have an earlier onset and more severe phenotype than other CCDs [28]. As *RYR1* is a large gene, variants of uncertain significance are often found [4]. The demonstration of decreased protein content by immunoblot analyses of RYR1 proteins can support diagnoses in suspicious cases [4]. *RYR1* variants are common and digenic pathologies should be kept in mind [4]. CCD presents with a wide spectrum of clinical manifestations, ranging from mild to severe fetal akinesis [8]. Typical signs include hypotonia, motor delay, skeletal malformations (e.g., congenital hip dislocation), ankle contractures, hyperlaxity in other joints, pes cavus, pes planus, club foot, scoliosis, pain on exertion, and weakness typically of the proximal and hip-girdle muscles [1, 4, 23]. Contractures are rare except for Achilles tendon stiffness. Due to the presence of hyperlaxity, CCD may be confused with Ullrich muscular dystrophies [1, 4]. In dominantly inherited cases, the disease is mild beginning in childhood, and motor delay and weakness benefit from physical activity [7, 8]. Furthermore, facial and bulbar weakness is mild but patients may not be able to fully close their eyelids. In rare *RYR1* recessively inherited cases, the disease is more severe with signs including neonatal hypotonia, axial weakness, and ophthalmoplegia. These cases can beconfused with NM [4, 8, 25]. About 80% of both AD *RYR1* and *AR RYR1* patients can become ambulatory over time [7]. In cases presenting with severe signs such as fetal akinesis sequence, ventilation dependence and death may occur at an early age [4]. In AR *RYR1* cases, signs including decreased intrauterine movements, premature birth, respiratory insufficiency and bulbar insufficiency are seen more often than in AD *RYR1*cases [7]. In AR *RYR1* cases, the need for respiratory support and NG tube feeding develops in the neonatal-infantile period[7]. In contrast to *ACTA1, MTM1, NEB*-related CMs that cause severe neonatal symptoms, the need for support decreases as the age increases. Except in severe neonatal cases, respiratory insufficiency is milder than in other CMs, and primary cardiac involvement is rare [4, 7].

Typical muscle involvement is demonstrated with MRI, and differs from other CMs. Quadricep muscle involvement and sparing of the rectus femoris are typical characteristics [4]. The vastus, sartorius and adductor magnus muscles are involved in the upper leg, and the rectus femoris, adductor longus, and hamstring muscles are spared. At the calf level, the soleus, head of the gastrocnemius and peroneal muscles are involved, and the medial head of the gastrocnemius and anterior muscles are spared [18].

RYR1 mutations may be related to a susceptibility to developing malignant hyperthermia, exertion-induced rhabdomyolysis, King-Denborough syndrome, Escobar syndrome, fetal akinesia, distal myopathies, and late-onset axial myopathy [4, 5, 28, 31–33]. Histopathological studies may show central cores, multiminicores, central nuclei, core-rod structures, fiber type 1 predominant, dusty cores, and uniformity of type 1 fibers [31–33]. Presentations show similar findings, and the mild finding may be present in biopsy, but core-like structures may not be found in all cases [5]. Diagnosing *RYR1* gene defects is important because they cause susceptibility to malignant hypertension [1]. In cases with malignant hyperthermia, biopsies may be normal, and in less than 30%, there are central cores present [12]. In human and mouse experimental studies, an antioxidant

N-acetylcysteine proved to be efficient [24]. However, more research studies are required.

Multiminicore myopathies (MmCMs) are characterized by multiple minicores which are regions of disrupted sarcomeres that lack mitochondria [1]. These minicores differ from the cores that are seen in CCM. They are not restricted to type 1 fiber and they don't extend along the whole longitudinal section of the fiber. They are also smaller in size, and multiple cores are seen within the muscle fiber [34]. In contrast to *RYR1* related CMs, triadin, SERCA, DHPR, and calsequestrin are not found in cores in *SELENON* related MmCMs with immunocytochemical investigations [28]. MmCMs are clinically and genetically heterogeneous disorders. Multiple cores can be seen in different CM subgroups linked to several genes. Therefore, clinical correlation is required [4]. It has been classified into four categories including a classical form, moderate form, ophthalmoplegic form, and in-utero-onset severe form [34]. The most common phenotype, which constitutes almost 75% of MmCM cases, is the classical form. This form has a distinctive clinical feature and is associated with recessive *SELENON* mutations [12, 31]. Recessive *RYR1* variants are commonly seen in the other forms and these remaining three subgroups have overlapping clinical features [31].

Classical MmCM is characterized by predominant axial/neck weakness, an early onset rigid spine, scoliosis, bracket-like thighs with severe atrophy of the inner thigh muscles, and respiratory insufficiency which is disproportionate to muscle weakness [1, 4]. Poor head control and generalized hypotonia with motor delay are the common features seen in the first 2 years of age [12]. An asthenic phenotype with muscle atrophy, short stature, and joint hyperlaxity are the other common findings [12]. Recessive *SELENON* mutations compose almost 50% of classical MmCM patients [12]. *SELENON* related MmCM and *LMNA* related muscular dystrophy are the main causes of severe neck muscle weakness with poor head control [9]. Rigid spine and scoliosis cause the development of rapid-onset respiratory dysfunction, while the patients can still walk [12]. Respiratory muscle weakness, disproportionate to muscle weakness, can also present in patients with *NEB, ACTA1, or TPM3* related NMs [9]. Muscle MRI of patients with *SELENON* related MmCM shows selective sartorius involvement [18]. The S*ELENON* gene encodes an endoplasmic reticulum glycoprotein; selenoprotein N, which is predominantly found in fetal muscle [4, 12]. It is also associated with RYR1 protein [26]. It plays a role in myogenesis and sarcomere organisation. Mutations of *SELENON* can be found throughout the entire gene [1]. Recessive loss of function mutations in *SELENON* cause a group of NMDs known as *SELENON*-related myopathies, including core myopathies, rigid spine muscular dystrophies, CFTD, and desmin related myopathy with Mallory body-like inclusions [26].

Fewer than 10% of MmCMs are of the moderate form [12]. Distal upper limb weakness with hand muscle atrophy, hip girdle weakness, and joint hyperlaxity are the common features [1, 12]. The respiratory, bulbar, and paraspinal muscles are spared. It is mostly caused by recessive *RYR1* gene mutations [1]. Additionally, fewer than 10% of MmCMs are of the ophthalmoplegic form [12]. These group of patients have a similar phenotype to the classical form, but they exhibit ophthalmoplegia over time [1]. Extraocular muscle involvement is abundant with abduction and upward gaze impairment. Respiratory involvement is milder compared to the classical form [31]. The severe form of MmCM is characterized by an antenatal onset, arthrogyriposis, dysmorphism, and respiratory involvement [1].

Currently MmCMs have a wide phenotypic variability with newly found genes. For example, the multiple EGF-like domain 10 (*MEGF10*) gene, which encodes transmembrane protein of the multiple epidermal growth factor family. Bi-allelic truncating mutations of the *MEGF10* gene are characterized by early onset myopathy, areflexia, respiratory distress, and dysphagia (EAMRDD) [12, 28]. Infantile-onset weakness, adult-onset respiratory insufficiency, and distal involvement are the reported clinical presentations in patients with compound heterozygous mutations [12, 28].

Titin (*TTN*) gene mutations may cause different clinical phenotypes including familial hypertrophic cardiomyopathy (AD), dilated cardiomyopathy (AD), early-onset myopathy with fatal cardiomyopathy (AR-EOMFC), Salih myopathy, tibial muscular dystrophy (AD), CNM related to *TTN* (AR), and MmCM-related to *TTN* (AR) [10, 28]. Homozygous mutations of *TTN* cause MmCM with central nuclei and a secondary calpain deficiency on muscle biopsy [1]. Congenital-onset forms reveal early onset hypotonia with contractures, respiratory insufficiency, and cardiac involvement [8].

Heterozygous mutations of the beta-myosin heavy chain protein gene cause Laing distal myopathy with weakness of the ankle dorsiflexors and great toe [12]. In addition to multiminicores, non-specific myopathic changes including fiber size variation, central nuclei, moth-eaten like fibers, ring fibers, and fiber splitting are detected pathologically [28]. Except for cor pulmonale, cardiac involvement has not been reported in MmCMs[8]. Myosin heavy chain-7 (*MYH7*) and *TTN* mutations should be considered first in patients with cardiac involvement and multiminicores on muscle biopsy [8]. *MYH7* mutations are more commonly found to be related to hypertrophic cardiomyopathies [15]. Mutations regarding the globular head of *MYH7* protein have been found to be associated with dilated cardiomyopathy [15]. Dominantly inherited myosin heavy chain-2 (*MYH2*) gene mutations cause congenital contractures and hip dislocation. However, recessive *MYH2* mutation-related myopathies have a wide range of disease onset, ranging from childhood to adulthood.

Proximal/neck muscle weakness, facial muscle weakness, scoliosis, and ophthalmoplegia, but not ptosis, are the common clinical features. Recessive mutations usually cause truncated proteins, and in muscle biopsies, type 1 fiber uniformity with an absence of type 2A fibers is found. In rare cases with recessive missense mutations, type 2 fibers without multiminicores can be seen. In contrast to biopsy findings in cases with recessive mutations, multiminicores are present predominantly in type 2A fibers [28].

Coiled-coil domain containing 78 (*CCDC78*) gene mutations cause myopathy with the predominancy of type 1 fibers, and both central nuclei and core-like structures are common biopsy findings. Cases present with neonatal hypotonia, distal weakness, fatigue, myalgia, and mild cognitive decline [28]. Bi-allelic mutations in the Unc-45 myosin chaperone B (*UNC45B*) gene were reported in patients with childhood-onset proximal muscle weakness, calf hypertrophy, and eccentric cores on muscle biopsy [28]. Muscle biopsies showed multiple subsarcolemmal cores in cases with dominant mutations in the alpha-actinin 2 (*ACTN2*) gene, which encodes alpha-actinin-2. Patients, either neonates or adults, may exhibit asymmetrical distal weakness, ptosis, ophthalmoplegia, and cardiac/respiratory involvement [28].

The calcium voltage gated channel subunit alpha 1S (*CACNA1S*) gene encodes the DHPR receptor alpha-1 subunit. Homozygous mutations are implicated in congenital myopathy with ophthalmoplegia [12, 28]. The thyroid hormone receptor interactor 4 (*TRIP4*) gene encodes a transcriptional coactivator ASC-1 protein [35].*TRIP4* mutations lead to the depletion of ASC-1 protein which directly binds to transcription factors[12, 35]. Cases with *TRIP4* variants may present with neonatal-onset axial/proximal weakness, a rigid spine with scoliosis, and respiratory involvement, or dilated cardiomyopathy during adult life [35]. Multiminicores and other structural features such as rods, caps, and central nuclei can be seen on muscle biopsies [35].

Myotubular Myopathy (MTM)/Centronuclear Myopathy (CNM)

CNM is characterized by vesicular, large nuclei localized in the center of muscle fibers and a high proportion of small myofibers [11, 36]. This is easily shown with H&E, NADH, and electron microscopy. With NADH, the area around the nucleus is seen as a peripheral halo devoid of myofibrils [1, 5]. While *MTM1* gene mutations may cause X-linked inherited myotubular myopathy (XLMTM), the other CNMs are AR or AD [11]. XLMTM's clinical picture is typical and severe with global hypotonia and respiratory insufficiency, while autosomal CNM's clinical picture is more variable. While AR CNMs often develop in the infantile and early childhood period, AD CNMs are more frequently seen in adulthood with a milder and slowly progressive course [36]. Its incidence is reported with a rate of 0.25–0.66/100,000 in pediatric cases [6]. In XLMTM/CNM suspect cases, *RYR1*and myotonic dystrophy should be excluded because clinical overlap could occur and both central nuclei and core may be coexistent in the biopsy [4].

There are underlying mutations which may affect complex membrane systems such as membrane traffic, endocytosis, triads (junctions between two sarcoplasmic reticula and one T-tubule), T tubule formation, and excitation-contraction coupling [4, 8]. XLMTM, which is caused by *MTM1* gene defects, is one of the most severe types of CM [8]. Severe generalized hypotonia at birth, ophthalmoplegia with marked facial and bulbar weakness, and respiratory insufficiency are the most typical findings[1, 8, 23]. CNMs are found to be associated with AR or AD *DNM2, BIN1, RYR1, TTN*, *MYF6, CCDC78, MTMR14, SPEG,* and *ZAK* genes [1, 4, 8, 10, 11]. *DNM2* mutation are less frequent, followed by *BIN1, CCDC78, SPEG,* and *TTN* with increasing frequency[5]. *MTM1, DNM2*, and *BIN1* play a role in intracellular membrane pathways, endosome traffic, and T tubule formation. The dysfunction of excitation-contraction coupling is the cause of weakness [1, 8, 37]. Although single and central nuclei are typical findings in *MTM1* and *DNM2*, multiple internal nuclei are seen in *BIN1* and *RYR1*-associated CNM [38].

MTM is the most severe CM with utero-onset. In utero fetal movements are reduced. Polyhydramnios, miscarriages, and preterm deliveries are more common with MTM compared to other CMs [7]. It is an X-linked disorder. The most common clinical features include: great height and large head circumference by the week of gestation in affected male cases, hip/knee contractures, severe neonatal hypotonia, ophthalmoplegia, respiratory insufficiency, feeding problems, andundescended testicles[1, 4, 5]. If patients do not receive enough respiratory and nutritional support, they may die in the first year, and this mortality is reported to be between 25 and 50% [7, 8, 39]. Approximately, 80% of survivors live with ventilatory support and require NG tube feeding [40]. Some patients with mild forms due to missense mutations may reach adulthood [1, 8]. About 20% of cases are mild, with patients maintaining the ability to walk, but the need for bilevel positive airway pressure (BiPAP) against nocturnal hypoventilation and feeding support may need continuing [40]. The severity of the disease is defined as severe, intermediate, or mild according to the need for ventilatory support[36]. Familial intrahepatic cholestasis-like liver disease and some hepatic vascular abnormalities were reported [40]. Female carriers may either be asymptomatic or display various clinical severities [4, 40]. In carrier females, mild facial weakness can be seen similar to mild adult forms, as well as early hypotonia, and a loss of ambula-

tion. This clinical diversity can be explained by skewed X-inactivation. In addition, asymmetrical weakness may be prominent in these cases, and this may be a finding of asymmetrical X-inactivation [1, 4, 8].

XLMTM gets its name from recognisable pathological features [40]. Severe fiber size variation with very small fibers and fibers with large central nuclei are common. Disruption of T-tubules, sarcoplasmic reticulum, triad structures, and excitation-contraction coupling are the reasons for severe weakness [40]. Like other CMs, type 1 fiber predominance is present, but most of the fibers are small due to insufficient myofibrillary growth [5, 40]. Necrosis and fibrosis are not expected to be present [1, 4, 5]. During pathological examinations, the number of large central nuclei is variable. Central nuclei can be seen in both fiber types [4, 5]. Although both small and large fibers are seen in the early stages of the disease, large fibers atrophy with age and clinical progress, and small-fiber uniformity is observed [40]. Contrary to the chain-like arrangement of central nuclei in regenerating fibrils, XLMTM appears to be located at regular intervals on longitudinal sections [4, 5]. The atypical location of mitochondria, sarcotubular structures, and other organelles are other typical features [40]. Darkly stained areas, with oxidative enzyme and PAS, are the areas where mitochondria aggregate, and glycogen accumulates. Areas around nuclei, appearing as holes with H&E and ATPase staining, are areas where organelles are absent. In addition, the area around the darkly stained area, with oxidative enzyme, also shows a pale subsarcolemmal halo, which is also present in some autosomal CNMs. Unlike female carriers, basophilic loops, known as "necklace fibers", are associated with internal nuclei and strongly stain with oxidative enzyme and PAS, drawing attention to just below the sarcolemma [4, 5]. Congenital muscular dystrophy should be considered in the differential diagnosis of a hypotonic newborn with central nuclei on muscle biopsy. The pathological distinction is difficult, and so it must be supported with molecular diagnosis [4].

The *MTM1* gene encodes myotubularin which acts as an endosomal phosphatase and plays a role in signaling pathways, membrane traffic, and endocytosis [4, 8]. Various mutations are distributed throughout the entire gene without any hot spots [5, 40]. These mutations include nonsense mutations (more frequent), frameshift mutations causing premature stop codons, and splice site mutations causing loss of protein expression [5, 40]. Some missense mutations do not completely inhibit protein function and have been associated with more mild clinical symptoms [40]. Some missense variants have also been reported in the phosphatase domain, reducing protein function [40]. Increased dynamin 2 (*DNM2*) expressions in muscle tissue has been demonstrated in an XLMTM case [26]. Therefore, *MTM1* is thought to be a negative regulator of *DNM2* expression, and some secondary therapeutic options are being studied [26]. Tamoxifen, as well as Dynamin 2 modulation, are other potential therapeutic options, in addition to gene therapy, enzyme replacement, and PIK3C2B inhibition [24, 36].

Case 13.6

An 18-month-old girl who was born to consanguineous parents was admitted to our clinic with motor delays and muscle weakness. Her CK levels were increased (up to 500 U/L). Muscle biopsy revealed that type 1 fiber predominance is present, but most of the fibers are small. Nuclear internalization was mildly increased in both fiber types. There were darkly stained areas, with oxidative enzyme and PAS, and in the middle of these areas were unstained areas. Areas around nuclei appeared as holes with enzyme stains. In addition, there were necklace fibers, seen as areas around the darkly stained area, with oxidative enzyme, also seen as a pale subsarcolemmal halo (Fig. 13.5). A MTM diagnosis was based on clinical and histopathological findings. Genetic analyses could not be performed.

Case 13.7

A 10-month-old boy was admitted to the hospital with motor delays and muscle weakness. He was a hypotonic infant and had respiratory distress. There was no consanguinity between his parents. In the histopathological examination of the first biopsy material, the presence of an increased number of central nuclei and myofibers of various sizes were detected. The patient was evaluated as a suspected case of CM, such as CNM, CFTD, and so on. Two years later, a repeated muscle biopsy was performed. In the histopathologic examination of the second biopsy specimens, greater differences were seen in the sizes of fibers. Small fibers were type 1, and all fibers had internal nuclei. Muscle injury had deteriorated to muscular dystrophy (Fig. 13.6). The second biopsy was consulted by Professor Caroline Sewry, PhD, FRCpath, and genetic analysis was performed at her suggestion, covering the MTM1, TTN, and RYR1 gene regions. During genetic analyses, a homozygous MTM1 gene mutation (c.731C > T p. R241C) was identified. This variant was previously reported as pathogenic. Finally, the patient was diagnosed with CNM caused by the mutation of the MTM1 gene on Xq28 [41].

CNM is clinically characterized by diffuse weakness that can be predominantly proximal or distal. Serum CK levels are often normal and electrophysiological examinations may be normal or myopathic [42]. AD CNM begins in the adult period, has a slower clinical course, and proximal muscle weakness is likely to occur. There are two subgroups. These are AD CNMs with diffuse muscle hypertrophy, and AD CNMs without diffuse muscle hypertrophy. Those with diffuse muscle hypertrophy have a younger age of onset and a more rapid course. Although ptosis can be

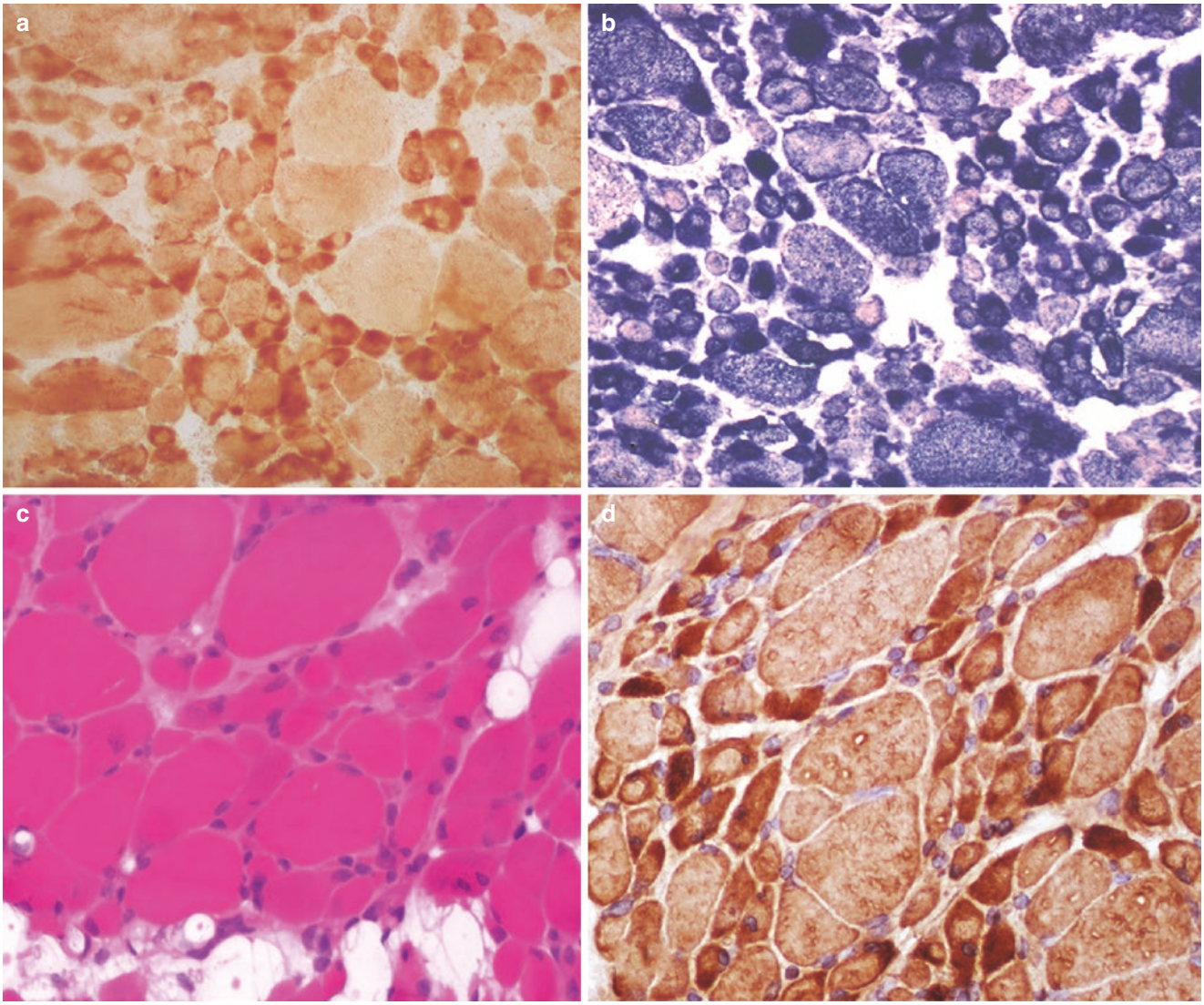

Fig. 13.5 Muscle biopsy of Case 13.6 diagnosed with MTM: (**a**) Necklace fiber appearance in most hypotrophic type 1 fibers (COX ×200), (**b**) Necklace fiber (NADH-TR ×200), (**c**) Muscle biopsy shows variation in fiber size, multiple internalized nuclei, and mildly increased fibrous connective tissue (HE ×200), (**d**) There are prominent central myotilin-unstained areas in the smaller fibers (DAB ×200)

seen, ophthalmoplegia is rare or occurs in the form of restriction in the upward gaze [42]. AR CNMs are divided into three subgroups according to ophthalmoplegia status and the age of onset. These are early-onset with ophthalmoplegia, early-onset without ophthalmoplegia, and late-onset without ophthalmoplegia.Those with ophthalmoplegia are the most severely affected. Dysmorphic facial features of other CMs, such as an elongated face and high palate, may accompany. Late-onset ones are clinically similar to AD CNMs [42].

There is significant fiber size variation with type 1 predominancy in H&E staining, and unlike XLMTM, the fibers are less rounded and more polygonal in shape. The central nuclei are also smaller and more hyperchromatic [4, 5, 42]. It has been reported that a chain appearance occurs in longitudinal sections, especially in *BIN1* mutations [38, 42]. The central dark area contains not only nuclei but also mitochondria and mislocalized organelles. Central reactivity is seen, secondary to perinuclear mitochondrial aggregation and an absence of oxidative enzymes. Poles of central nuclei contain glycogen as shown by PAS staining. The peripheral zone around the central nuclei draws attention [5, 42]. In the biopsies of *DNM2* mutation-associated, late-onset cases, intense stain lines extending radially from the centre to the periphery of fibers can be seen, and they have been rarely reported in *BIN1* mutations [4]. In almost all cases, at least 20% of myofibers have central nuclei. The distribution of centrally located nuclei is variable. In half of AR CNM, and approximately 40% of AD CNM cases, more than 80% of myofibers have central nuclei [40].

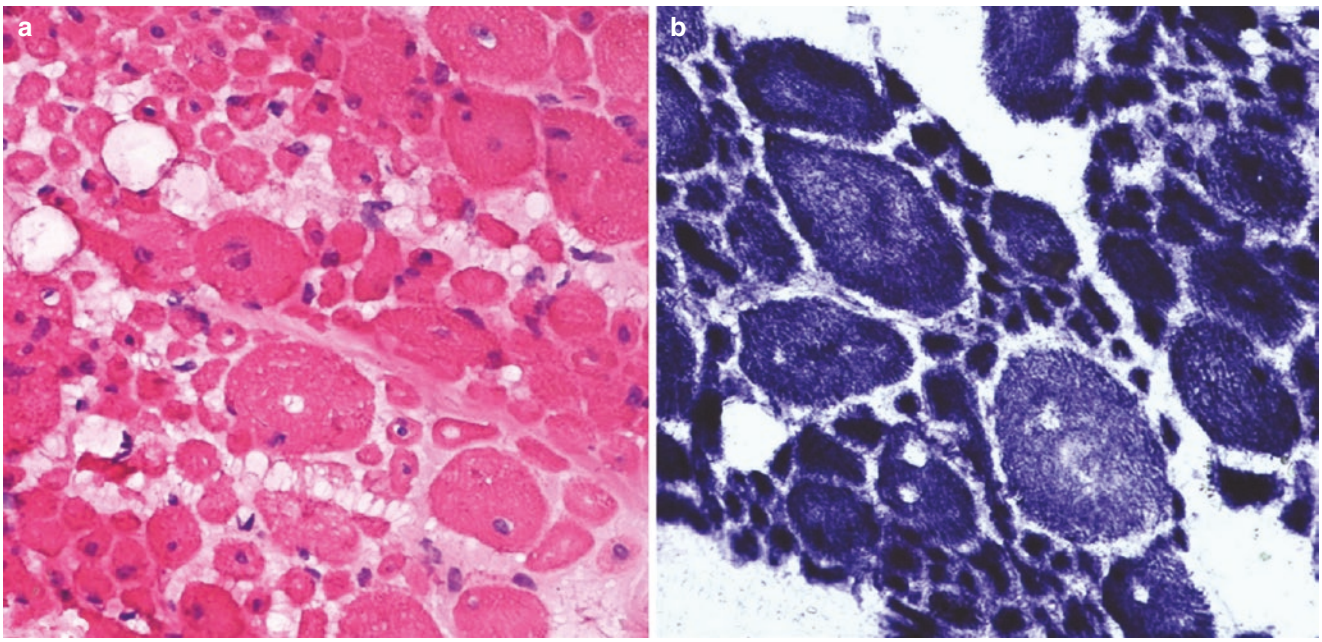

Fig. 13.6 Muscle biopsy of Case 13.7 diagnosed with MTM: (**a**) Muscle biopsy shows a huge variation in fiber size, multiple internalized nuclei, and mildly increased fibrous connective tissue (HE ×200). (**b**) The most hypotrophic are the type 1 fibers (NADH-TR ×200)

The dynamin 2 (DNM2) gene encodes GTPase, which plays a role in many intracellular pathways. AD DNM2 mutations lead to the formation of proteins with higher GTPase activity [24]. It presents with clinical findings ranging from a mild form in adults to a severe form in infants [37]. Clinically predominant distal weakness, distal contractures, ptosis, and involvement of extraocular muscles are seen in some cases. Achilles contracture, pes cavus, atrophy of calf/thenar muscles, and scoliosis may be added [1, 37]. Unlike other CMs, pain is observed during exercise [1]. In adolescents and young adults with a history of motor delay in childhood, difficulty in walking, running, and climbing stairs begins in later ages. Mild ptosis and ophthalmoplegia may accompany [37]. Infants are more severely affected, with generalized weakness, hypotonia, facial weakness, ptosis, and ophthalmoplegia [37]. Muscle biopsies show a triad of the predominance of hypotrophic type 1 fibers, radial strands with oxidative enzyme reactions, and central nuclei [37]. In muscle imaging, distal lower extremity muscles such as the gastrocnemius, soleus, and tibialis anterior are selectively involved in the early period, and the posterior thigh muscles are affected in later stages [1, 37]. Treatment studies with antisense oligonucleotides against DNM2 pre-mRNA are ongoing [24].

In some patients with CNM, homozygous missense mutations of the bridging integrator 1 (*BIN1*) gene were reported [1]. Clinical severity ranges from moderate to severe. There is motor delay, diffuse weakness with muscle atrophy, facial weakness, and ptosis [37]. Some dominant mutations were reported in mild forms with adult-onset [26]. The *BIN1* gene encodes amphiphysin2. It plays a role in membrane tubulationwith *MTM1*[26]. *MTM1* also acts as a desmin binding protein [37]. In immunocytochemical examinations, labeling with *DHPR, RYR*, and desmin are increased in *MTM1* related CNMs, especially in the central area. DNM2 related CNM has more homogenous and direct labeling, while BIN1 related CNM has a significant increase in the central cytoplasm with intense labeling. While labeling with caveolin-3 has a normal distribution in *MTM1* and *DNM2* related CNM, there is increased labeling in the central regions of *BIN1* related CNM [37]. In some cases presenting with proximal/axial predominant weakness, facial weakness, ptosis, ophthalmoplegia, contractures, and restrictive respiratory involvement in the neonatal period, central nuclei and core structures were observed, and AR *RYR1* mutations were reported [1, 37, 38].

Cases 13.8 and 13.9

Two first-degree cousins belonging to a consanguineous family from Turkey, without any ancestral history of NMDs, were admitted to hospital with muscle weakness, developmental delay, and CK elevations (up to 450 U/L). Case 13.8 was an 13-year-old girl and Case 13.9 was a 14-year-old boy. Both were slightly mentally retarded. Case 13.9 was diagnosed with CNM before because 2 years ago, a mild elevation of CK was detected, during preoperative tests due to an undescended testis, and a muscle biopsy, which was performed (Fig. 13.7).*BIN1* sequencing revealed a homozygous

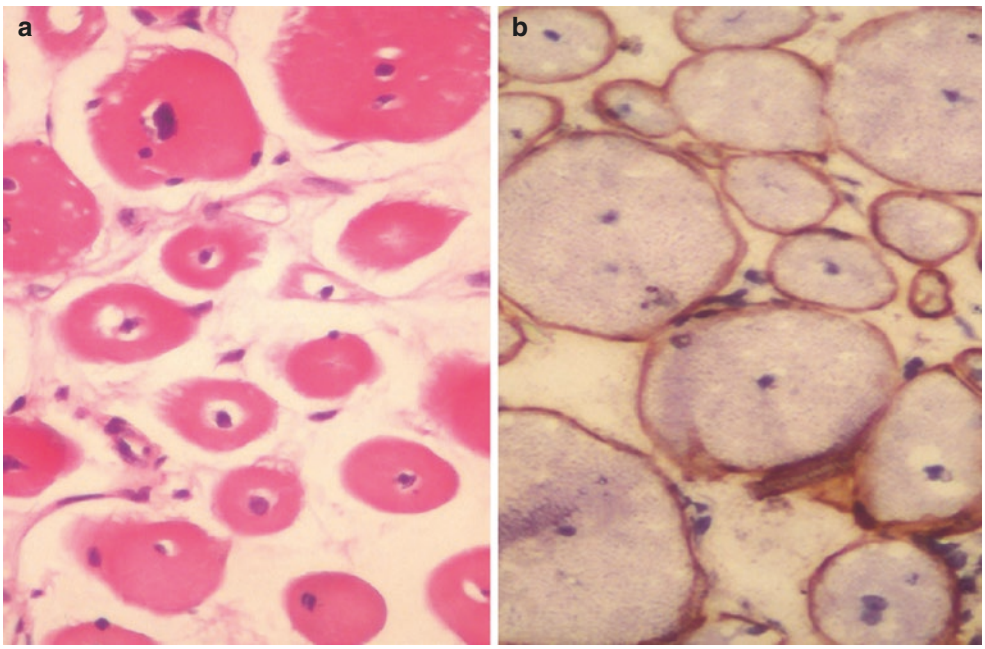

Fig. 13.7 (**a**) Muscle biopsy of Case 13.9 shows a huge variation in fiber size, plenty of internalized nuclei, and increased fibrous connective tissue (HE ×200), (**b**) Sarcolemmal dystrophin expression is normal (DAB ×200)

nonsense mutation in exon 20 in both patients (c.1717C > T; p.Gln573stop). Both patients have healthy parents who are heterozygous for this mutation [38].

In the dominant mutations of the coiled-coil domain-containing protein 78 (*CCDC78*) gene, both core structures and central nuclei were detected during biopsy [26, 28]. The truncating protein, formed with compound heterozygous *TTN* mutations, impairs excitation-contraction coupling by disrupting the triad structure and function of most genes involved in the pathogenesis of CNM, which has been reported in clinically and pathologically diagnosed cases. Titin, on the other hand, provides sarcomere assembly and binding sites for many proteins involved in excitation-contraction coupling. Clinical findings of patients with *TTN*-related CNMs include cardiomyopathy, hypotonia, proximal/distal weakness, a rigid spine, respiratory involvement, and contractures [5, 39].

The striated preferentially expressed gene (*SPEG*) plays a role in the formation of the muscle cell skeleton. It interacts with *MTM1*, and they both regulate calcium homeostasis and play a role in the correct positioning of the nucleus within the cell during the muscle maturation period [43]. In almost all cases with *SPEG* mutations, neonatal/infantile onset generalized weakness with muscle atrophy and motor delay have been reported, in some cases with ptosis, ophthalmoplegia, and respiratory weakness[43]. Dilated cardiomyopathy and tricuspid/mitral valve insufficiency are the signs of cardiac involvement [17, 43].

Myotubularin-related protein 14 (*MTMR14*) has high homology to Drosophila egg-derived tyrosine phosphatase (EDTP). This protein is also called "human JUMPY", because disruption of EDTP protein function in Drosophila causes a phenotype termed "JUMPY". It is characterized by a progressive loss of muscle control together with shaky and slower movements. *MTMR14* (JUMPY) gene expression has been shown to increase during the differentiation of myoblasts to myotubules. It also plays a role in the hydorlysis of membrane inositol rings. Heterozygous mutations have been reported in neonates with neonatal diffuse weakness and ophthalmoplegia [44].

Myogenic factors are members of the basic helix-loop-helix transcription factor family. Myogenic factor 6 (*MYF6*) plays a role in the terminal differentiation of myotubules. The heterozygous mutation was identified in a patient with mild myopathic findings and exercise-induced muscle cramps, with pathology compatible with CNM [45]. Leucine zipper and sterile alpha motif-containing kinase (*ZAK*) gene mutations present with neonatal/infantile-onset generalized weakness, motor delay, and scoliosis. Facial weakness is not expected, but joint hyperlaxity may be present. There are overlapping clinical findings with CM and LGMD, and pathologically with CNM and fiber type 1 dominance. Histopathological examinations showed fiber size variation, type 1 fiber predominancy, central nuclei, and recessive *ZAK* gene mutations, which were reported in cases where electron microscopy found that central nuclei were not surrounded by a halo of membrane and organelles, but by myofibrils, which is a typical finding for CNM. Rimmed vacuoles and an accumulation of subsarcolemmal mitochondria may be a clue. *ZAK* is a serine-threonine kinase, which plays a role in many pathways such as muscle regeneration and myogenesis [46].

Congenital Fiber Type Disproportion (CFTD)

For fiber-type disproportion (FTD), the main diagnostic finding is that the diameters of type 1 fibers are smaller than type 2 fibers [1, 4]. However, since these findings can be seen in other CMs, they should be found alone and with no other histopathological findings[4, 47]. It has been debated for many years whether CFTD is only a pathological finding or a separate disorder. However, since only pure FTD findings were detected in *ACTA1, SELENON, RYR1, TPM2, TPM3, MYH7, MYL2* gene-related CMs, they were grouped under CFTD [4, 47, 48]. It should be kept in mind that concomitant central nuclei, core, rod, or cap structures in the same gene-related CMs, may be detected in the future with age [4, 47]. CFTD is reported at a rate of 0.08–0.56/100,000 in the pediatric age [6]. Pathologically, the term FTD was first defined as the diameter of the type 1 fibers being at least 12% lower than type 2 fibers, but this finding is nonspecific as it can also be seen in other conditions such as myotonic dystrophy, and SMA [47]. Therefore, this ratio was later changed to be at least 25% [4]. As a CM subgroup, there is no pathognomonic finding for CFTD, and it is a diagnosis of exclusion. Firstly, cases should be compatible with typical CM clinical findings. These findings include a static/slowly progressive course, proximal/axial predominant weakness, facial weakness, ophthalmoplegia, dysphagia, and CK levels not five times higher than normal. Respiratory insufficiency has been reported in 30% of cases [49]. It has also been reported that the FTD rate is at least 35–40% in the majority of CFTD cases; the lesser differences are usually encountered in the non-congenital myopathy [47]. In CFTD cases, type 1 fiber predominancy (>55%) is also used in the definition [47].

ACTA1 mutations are detected in up to 5% of CFTD cases [47]. A static course, generalized and severe weakness with respiratory insufficiency are seen. Severe CFTD cases are often associated with the *ACTA1* and *RYR1* genes [47]. Although ophthalmoplegia is seen in half of CFTD cases, the extraocular muscles are spared in *ACTA1*-related patients [47, 50]. With *ACTA1* Asp294Val mutation, the replacement of a negatively charged polar amino acid with a nonpolar-neutral amino acid causes weakness by disrupting the actin-myosin interaction at the protein level. As there is no sarcomericdisorganisation, the rods are not seen in *ACTA1*-related NM [47]. Most missense mutations clustered in hotspot regions from *RYR1* mutations are associated with AD CCD, while other recessive variants have been reported with MmCD, CNM, and CFTD [47]. *RYR1* variants are detected in 20% of CFTD cases [32]. *RYR1*-related CFTD occurs over a broad clinical spectrum. Proximal and axial weakness is predominant. Facial weakness, ophthalmoplegia, and respiratory insufficiency is accompanied by generalized weakness. Unlike other CFTDs, scoliosis is more common [47].

The genetic variants most frequently detected in CFTD cases are related to the *TPM3* gene. AD mutations are rarely detected, and in 25–50% of cases AR mutations are present. CFTD may present with variable symptoms such as severe neonatal hypotonia, motor delay or proximal weakness, and difficulty with exercise [47, 51]. Axial weakness, weakness in ankle dorsiflexion, and mild ptosis with sparing of the extraocular muscles are common [24, 47]. Care should be taken with nocturnal hypoventilation that develops without a loss of ambulation, cases should be monitored for respiratory and sleep problems. Type 1 fiber hypotrophy is very prominent, and diameters can be detected which are 50–77% smaller than type 2 fibers [24, 52]. Most of the mutations are clustered in the fifth of seventh nucleotide-repeat motifs [49]. Most of the pathogenic variants are heterozygous missense variants, and rarely, recessive mutations causing a loss of function have also been reported [49]. In one case, a dropped head with marked axial weakness was reported [49]. Although some improvement with L-carnitine was reported in zebrafish models, studies are still ongoing[24]. Dominant mutation of the *TPM2* gene has been reported in one case [47, 53]. In another case, a diagnosis of *TPM2*-related cap myopathy was made upon detection of cap structures in electron microscopy, although no other pathological abnormality was observed in light microscopy [53].

A heterozygous mutation in the distal rod region of *MYH7* was reported in a patient with infantile-onset proximal weakness, waddling gait, and anterior distal leg involvement, but without cardiac or extraocular involvement, and with CFTD-compatible pathology [47, 54]. In the biopsy of an elderly patient of the same family, findings consistent with myosin storage myopathy suggested that *MYH-7*-related conditions may be a spectrum [54].

Different findings can be detected in the histopathology of *SELENON*-related myopathy. Dystrophic variants, multiminicores, Mallory bodies, and type 1 fiber hypotrophy with type 1 fiber predominancy have been reported. Those whose main pathological findings were small type 1 fibers were included in the CFTD subgroup[47].

Case 13.10

A female patient was born at 35-weeks-gestation and was the second child of consanguineous parents. She had one healthy brother, and the family history was noncontributory. She was hospitalized at 12 hours of age (postnatal) due to bilateral corneal opacity. At her physical examination, she had fair skin and brown hair; a pigment distinctly lighter compared to other family members. Dysmorphic features included a high-arched palate and micrognathia.A neurological examination revealed generalized and truncal hypotonia, with an absence of deep tendon reflexes. An ophthalmologic examination indicated bilateral anterior

sub-capsular cataracts. Her CK levels were elevated (433 U/L). Cranial ultrasonography and MRI revealed agenesis of the corpus callosum. Abdominal ultrasonography found left renal agenesis, but hydronephrosis was not present. Due to the physical and laboratory findings, Vici syndrome was suspected and whole exome sequence analysis was performed, which demonstrated a novel homozygous mutation, p.A1925Vfs*3 (c.5774_5774delC), of the *EPG5* gene. Her muscle enzymes gradually increased during the follow-up. A muscle biopsy of the vastus lateralis muscle was performed. Myofiber shape-size difference, degeneration, and atrophy-like muscle injury were identified. Slightly increased interstitial connective tissue was detected with Gomori trichrome. It was observed that the type 2 fiber ratio decreased with fast myosin and most type 1 myofibers were extremely small (Fig. 13.8). With the histopathological findings, CFTD was considered. She had continuous feeding problems, which necessitated feeding with an orogastric tube. The patient was discharged from the hospital at 5 months of age on room air and with a permanent orogastric tube for enteral feeding. She died from bronchopneumonia at the age of 8 months.

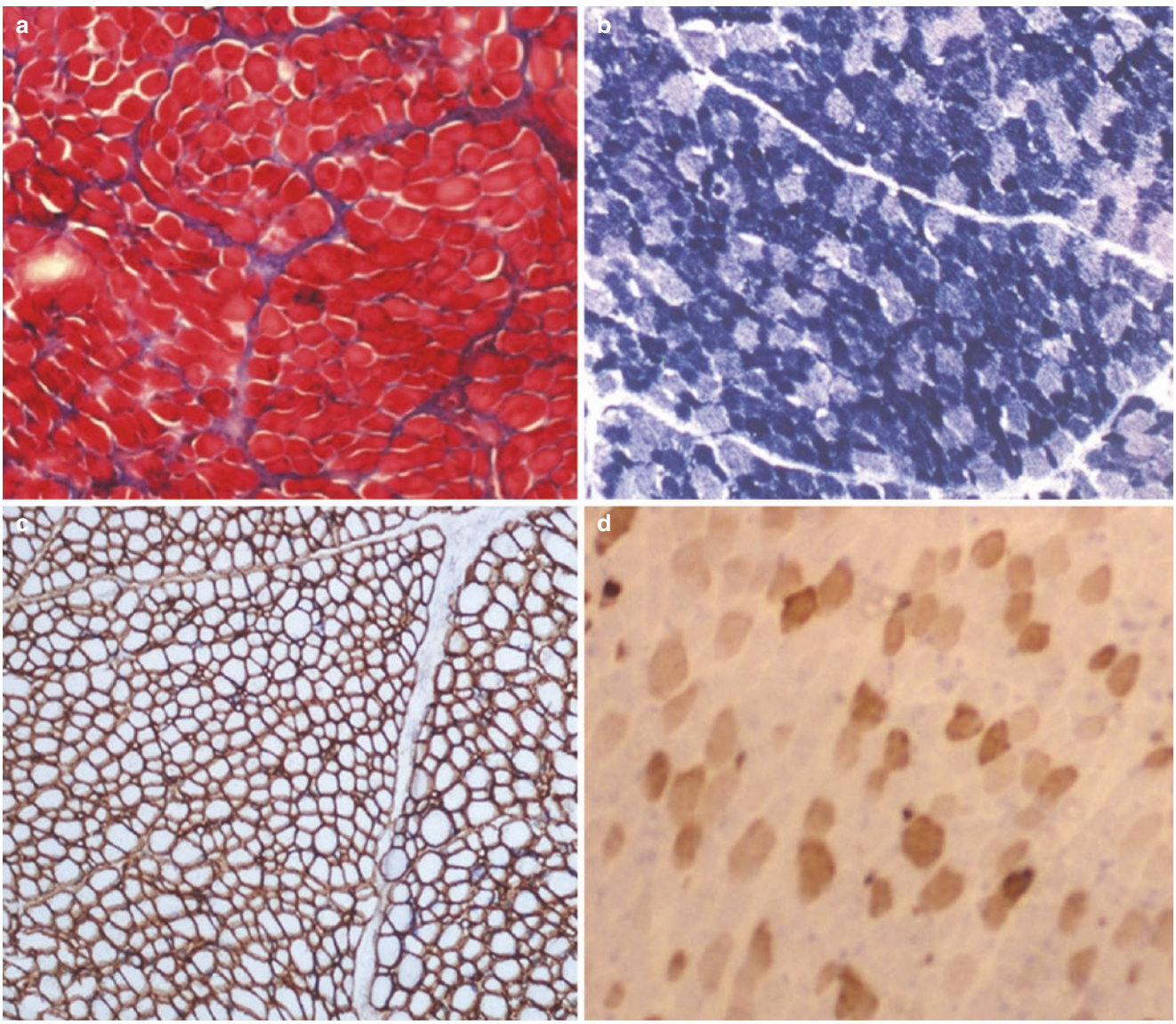

Fig. 13.8 (**a**) Muscle biopsy of Case 13.10 shows a variation in fiber size and increased fibrous connective tissue (Gomori trichrome ×100), (**b**) Numerous small sized type 1 fibers (NADH-TR ×100), (**c**) Normal sarcolemmal merosin expression, (**d**) Presence of pathological fibers with neonatal myosin (DAB ×200)

Other Congenital Myopathies

CMs have a large heterogeneous disease spectrum, and every day, newly identified genes/variants in families or cases associated with histopathological findings are reported. There are many examples of CM reported in the literature as case reports that cannot be classified into a specific CM group:

A case with findings of lamin-A *(LMNA)*gene-related FTD in the pathology was reported, but type 2 fiber hypertrophy was prominent, unlike type 1 fiber hypotrophy typical in CFTD [55]. A nonsense mutation in the neurofilament light polypeptide(*NEFL*) gene was reported in family members with nemaline rods in the biopsy, and with clinically overlapping myopathic and neuropathic findings [56]. Plectin (*PLEC*) is a protein that binds desmin with to outer nuclear membranes, Z discs, and sarcolemma. Recessive loss of function mutations causes epidermolysis bullosa with muscular dystrophy. Cases, in which progressive muscle weakness, ptosis, and ophthalmoplegia are prominent, with mild skin findings, and are classified as limb girdle muscular dystrophies [57]. A case with prominent proximal weakness, FTD, and central nuclei on biopsy was also reported [58]. Honeycomb myonuclei were reported in a case ofspectrin repeat-containing nuclear envelope protein 1 (*SYNE1*) gene-related myopathy [48]. Triadin (*TRDN*) is part of the calcium release complex in cardiac and skeletal muscles. In some cases, cardiac involvement is predominant, and muscle weakness is variable [59]. Adenylosuccinate synthetase like 1 (*ADDSL1*) gene defects causes a myopathy with childhood-to-adulthood onset. It presents with complaints such as easy fatigability, distal weakness, and falling behind in sporting activities from their peers. Nemaline rods can be seen at a rate of <10% less compared to NM in the biopsy. Thin filament structures are not affected, and lipid droplets are visible [24]. However, these cases have not yet been categorized into congenital myopathy subgroups.

Tripartite motif-containing 32(*TRIM32*) gene defects are classified in the group of sarcotubular myopathy and limb girdle muscular dystrophy [10, 11]. Recessive variants of the myosin light chain 2 (*MYL2*) gene have been rarely reported in the case of CFTD, in which both skeletal and cardiac muscles are involved [60]. The heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*) gene is associated with AD inheritance, late adult-onset, and slowly progressive proximal weakness. There is no cognitive deficit, or neuron/ bone involvement in these cases. Histopathology showed rimmed vacuoles in atrophic fibers and isolated inclusion body myopathy-like findings [61]. Myosin binding protein C1–2 (*MYBPC1–2*) is expressed in skeletal muscle and *MYBPC3* in cardiac muscle. A homozygous truncating mutation of the MYBPC3 gene was reported in an infant with skeletal myopathy and cardiomyopathy. However, it has been shown that muscle pathology occurs with the dominant-negative effect of ectopic expression of mutant MYBPC3 [62].

Homozygous frameshift mutations of contactin-1 (*CNTN1*) gene have been associated with severe lethal congenital myopathy with fetal akinesia and nonspecific myopathic changes in muscle [63]. 3-hydroxy acyl-CoA dehydratase 1 (*HACD1*) plays a role in the biosynthesis of very long-chain fatty acids in skeletal and cardiac muscles. Homozygous nonsense mutations and homozygous long interspersed nuclear element insertions have been reported in a small number of patients with CMs. The common feature of these cases is that it presents with severe neonatal weakness, which improves over time, and biopsy shows type 1 fiber hypotrophy, type 1 fiber predominancy, and rare internal nuclei [64]. A homozygous nonsense mutation of beta-IV-spectrin (*SPTBN4*) was reported in a rare form of CM with neuropathy and deafness [65]. In GTPase (*HRAS*) defects, CM with excess muscle spindles was reported. It has a severe clinical picture with severe neonatal hypotonia, contractures, hyperlaxity of the distal joints, respiratory insufficiency, and feeding problems [66].

AR mutations of the myomaker (*MYMK*) gene cause Carey-Fineman-Ziter syndrome with non-progressive CM and facial weakness, additional dysmorphic features, and growth retardation. While the number of muscle fibers decreases due to defective myoblast fusion, compensatory hypertrophy is observed in both types [67]. SH3 and cysteine-rich domain 3 (*STAC3*) are components of excitation-contraction coupling. Recessive mutations cause variable phenotypes from severe neonatal hypotonia to slowly progressive and mild hypotonia. They also cause MHS [68]. Recessive mutations of the fragile X related 1 (*FXR1*) gene in exon 15 have been reported with various clinical presentations from neonatal hypotonia to milder presentations. Muscle biopsies have shown atrophy, type 1 fiber predominancy, multicores, and central nuclei [69].Bi-allelic variants of the transcription factor *PAX7* have been reported as a new genetic cause of myopathy with a different severity, presenting with weakness, ptosis, muscle atrophy, and scoliosis. Muscle atrophy and fibroadipose tissue replacement are detected, but necrosis and muscle fiber instability are absent. Progressive muscle wasting occurs as the survival and regeneration of satellite cells are affected [70]. Homozygous or compound heterozygous mutations in the *SCN4A* gene, causing a loss of function of skeletal muscle voltage-gated sodium channel α-subunits $Na_v1.4$, have been defined as the cause of fetal hypokinesia, severe neonatal hypotonia, and

CM, showing improvement in symptoms over time [71]. Mutations of pyridine nucleotide-disulfide oxidoreductase domain 1 (*PYROXD1*) were reported in cases with clinical early-onset myopathy. During biopsy, fiber size variation, internalized nuclei, fibrosis, core/central nuclei, and myofibrillar irregularities were noted [72]. A frameshift mutation affecting the stop codon in the transportin3 (*TNPO3*) gene has been identified in cases with myopathic abnormalities in biopsy, and slowly progressive proximal axial weakness. As a result of a transcript with an elongated C-terminal component, an accumulation of the protein is observed in the subsarcolemmal and perinuclear areas [73]. Although mucolipidosis type IV, caused by mucolipin1(*MCOLN1*) defects,is a disorder with corneal involvement, and central nervous system involvement, a diagnosis of lysosomal storage disease should be considered in cases with unidentified CK elevation and myopathy [74]. In fast skeletal troponin C (*TNNC2*) gene defects, severe congenital weakness, and respiratory insufficiency occur, and facial weakness, ptosis, and contractures may accompany them. Unlike severe CMs, patients can regain the ability to walk over time and the need for respiratory support may decrease. Myofiber atrophy or ultra-structural variants are not causes of weakness. The calcium sensitivity required for power production is decreased in sarcomeres [75]. Recessive mutations of the myosin light chain 1 (*MYL1*) gene have been reported in cases with severe CM. There is a different form of type 2 fiber atrophy/hypotrophy in biopsy [76]. A variant of the calcium voltage-gated channel subunit alpha1 H (*CACNA1H*) has been reported in a case of infantile-onset severe amyoplasia. Loop mutations, which connect intracellular I–II and II–III domains, cause congenital amyotrophy by affecting the differentiation of myoblasts at an early stage [77].

In addition, although CMs cause muscle-limited pictures, sometimes muscle involvement of various multisystem diseases may demonstrate symptoms and histopathology features similar to those of CMs. Marinesco-Sjögren syndrome (*SIL1* mutations; ataxia, cataracts, microcephaly, myopathy), King-Denborough syndrome (*RYR1* mutations; MHS, dysmorphic features, myopathy, central nuclei/cores on muscle biopsy), Stormorken syndrome (*STIM1* mutations; ichthyosis, thrombocytopathy, myopathy, tubular aggregates on muscle biopsy), Snyder-Robinson syndrome (*SMS* mutations; osteoporosis, skeletal abnormalities, myopathy), Shwachman-Bodian-Diamond syndrome (bone marrow dysfunction, pancreatic insufficiency, hypotonia), and Freeman-Sheldon syndrome (*MYH3* mutations; dysmorphic features, contractures, FTD on muscle biopsy) can be counted among the multisystemic diseases in which myopathy is also present[48].

Case 13.11

An 8-year-old boy who was born to consanguineous parents presented with frequent falls and difficulty in walking/climbing. Neurological examination showed myopathic facies (facial weakness and long-narrow face), nasal speech, dysphagia, axial/proximal muscle weakness, distal laxity, kyphoscoliosis, and reduced muscle bulk (Fig. 13.9). His CK levels were normal (41 IU/L). Nerve conduction studies revealed axonal neuropathy. Muscle biopsy shows severe fatty infiltration of the perimysium, variations in fiber size with a predominance of atrophic fibers, and internal nuclei in most fibers. WES analysis showed two PYROXD1 variants: c.464A > G and c.329_332delTCTG.

Case 13.12

A 12-year-old girl who was born to consanguineous parents was admitted to hospital with newly onset, slowly progressive muscle weakness, and reduced muscle bulk. On examination, generalized muscle atrophy, symmetrical proximal/axial muscle weakness, scapular winging, mild facial weakness, nasal speech, and absent deep tendon reflexes were found. Her CK levels were normal (75 IU/L). Nerve conduction studies revealed normal findings. However, needle EMG identifed myopathic changes. Muscle biopsy showed non-inflammatory myopathic features (Fig. 13.10). WES analysis revealed a pathogenic homozygous mutation in the *PYROXD1* gene, c.464A > G, p.Asn155Ser.

These two patients (Cases 13.11 and 13.12) were diagnosed with CM related to *PYROXD1*. Both cases were reported in the literature [68].

Case 13.13

A male infant, born at 38-weeks-gestation via cesarean section from consanguineous parents, was taken to the neonatal intensive care unit because of severe generalized weakness and respiratory insufficiency. On examination, severe hypotonia, poor sucking, ophthalmoplegia, and contractures were noted (Fig. 13.11). He died at the age of 3 months due to respiratory problems. During histopathological evaluation of the muscle biopsy, mild dystrophic changes such as contraction, regeneration, degeneration, nuclear internalization, and fibrosis were visible. In addition, many pathological immature myofibers were visualized using neonatal myosin staining. Based on immunostaining, dystrophin, merosin, and sarcoglycans were present at normal levels. Interestingly, there were several huge type 1 fibers, which are specific for infantile denervation (Fig. 13.12). WES analysis revealed a homozygous missense variant in the *CACNA1S* gene, c.2366G > A, p.Arg789His.

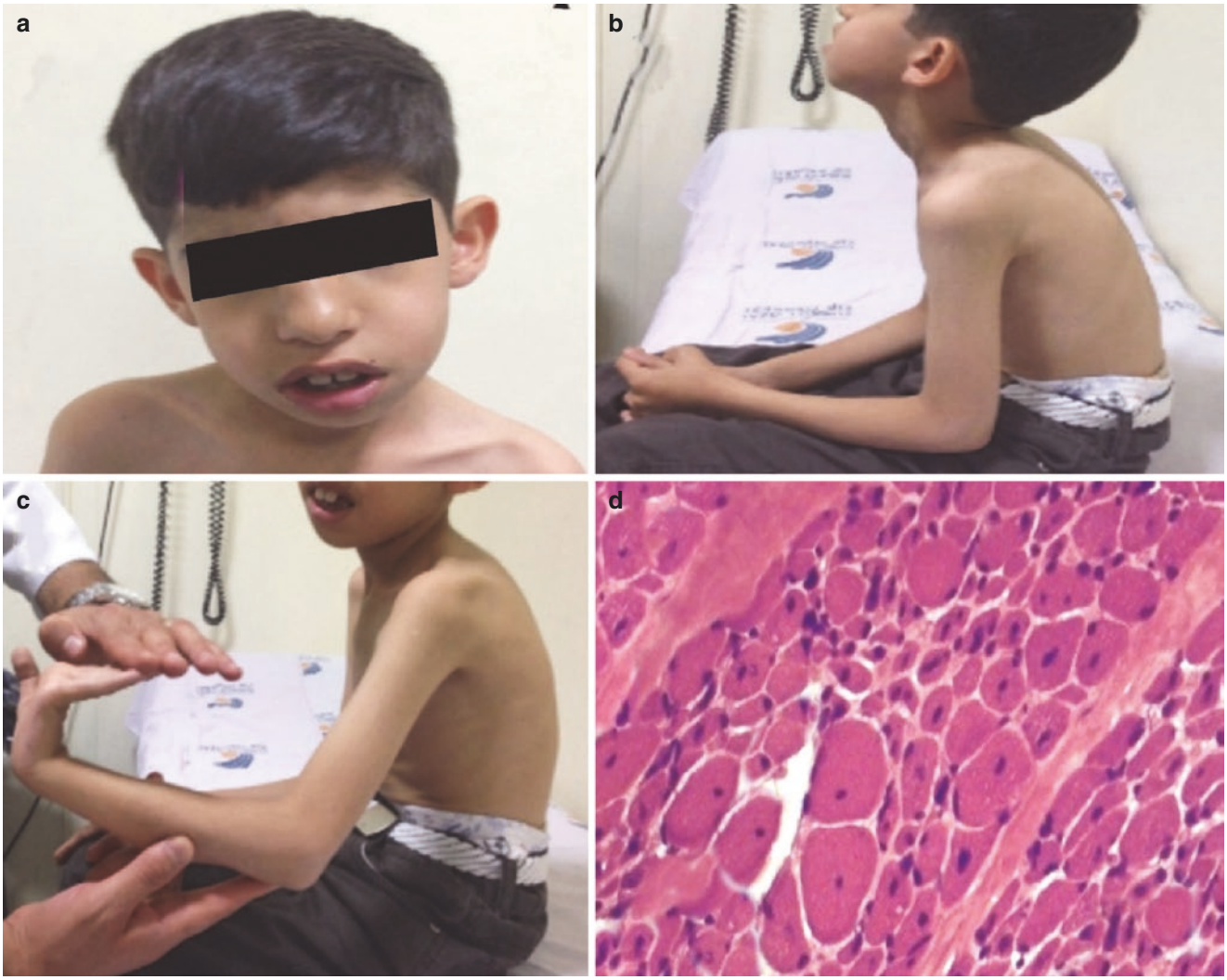

Fig. 13.9 Phenotypic features of Case 11 with *PYROXD1* mutation: (**a**) Long myopathic face and facial weakness, (**b**) Muscle atrophy of shoulder and neck muscles, (**c**) Distal joint laxity, (**d**) Note the huge differences of myofiber sizes (HE ×100)

Case 13.14

A female infant was taken to the neonatal intensive care unit because of severe hypotonia and the need of mechanical ventilation. Her examination revealed severe generalized muscle weakness, distal contractures, absent sucking, and absent deep tendon reflexes. Nerve conduction studies were normal and needle EMG showed myopathic motor unit potentials. Serum CK levels were normal. She was discharged with a tracheostomy and NG feeding tube (Fig. 13.13).During histopathological evaluation of the muscle biopsy, there was a marked variation in fiber size and shape. There was also increased nuclear internalization. There were no myofibrillary irregularities identified with modified trichrome or NADH-TR enzyme staining. Based on immunostaining, dystrophin, merosin, and sarcoglycans were present at normal levels. There were also grouping fascicules of large and small myofibers, which are specific for neuropathies (Fig. 13.14). Neurological examination revealed motor delay with generalized weakness, facial weakness with ophthalmoplegia, scoliosis, and pes equinus deformity at the age of 5. WES analysis revealed a homozygous missense variant in the *CACNA1S* gene, c.2366G > A, p.Arg789His. These two patients (Cases 13.5 and 13.6) were diagnosed with CM with ophthalmoplegia related to *CACNA1S*. These patients were reported in the literature[78].

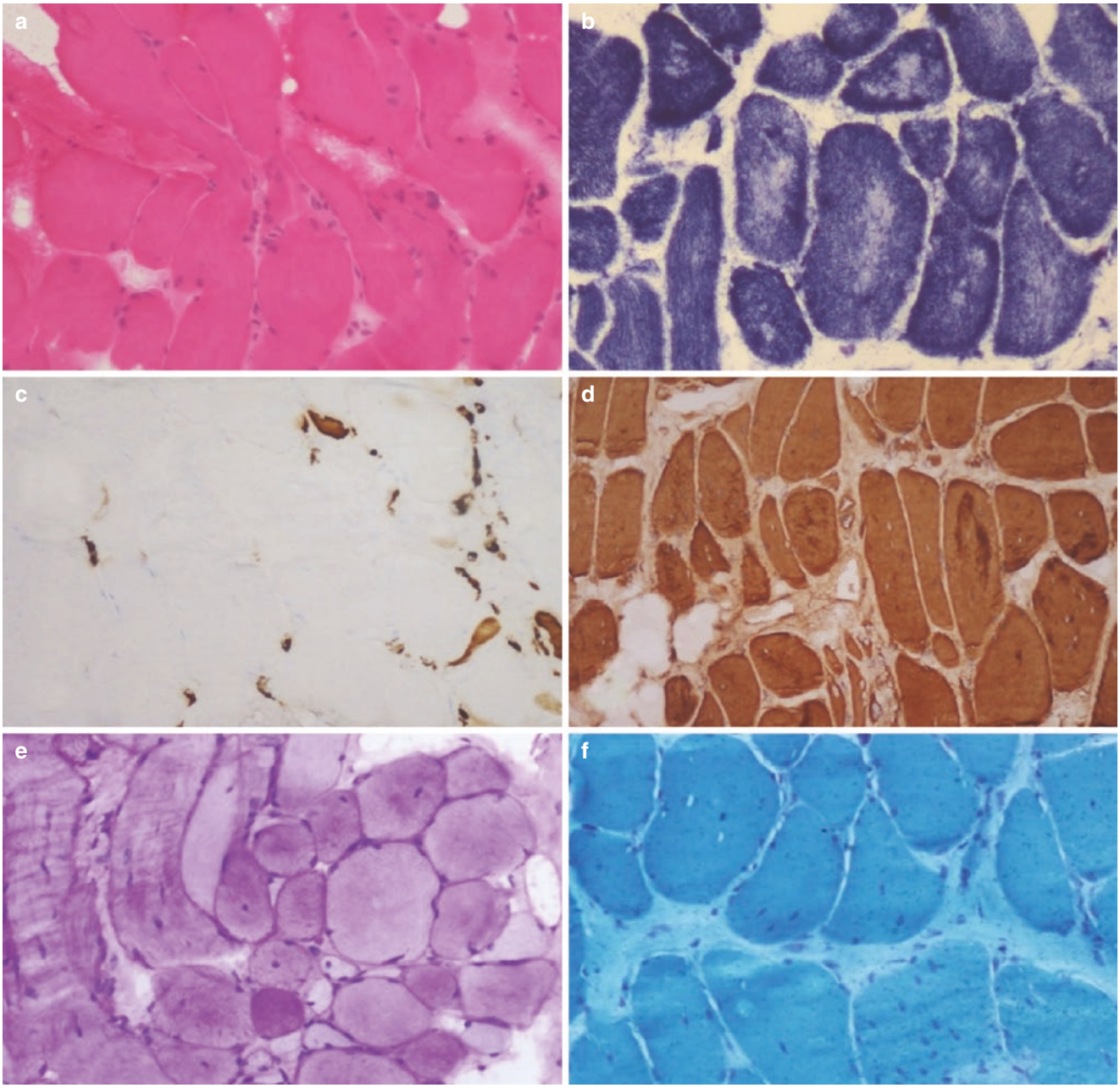

Fig. 13.10 Muscle biopsy of Case 13.12 with *PYROXD1* mutation: (**a**) Muscle biopsy shows variation in fiber size, multiple internalized nuclei, and mildly increased fibrous connective tissue (HE ×200), (**b**) Conspicuous central core formations and myofibrillar irregularity (NADH-TR ×400), (**c**) There are a lot of immature atrophic fibers expressed with neonatal myosin (DAB ×200), (**d**) There are a few central inclusions highly immunoreactive to myotilin (DAB ×200), (**e**) Increased numbers of central nuclei (PAS ×200), (**f**) Note the increased connective tissue. (Modified Trichrome ×200)

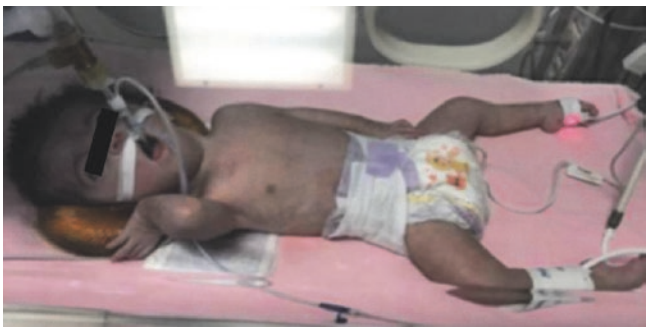

Fig. 13.11 Phenotypic features of Case 13.13 with *CACNA1S* mutation

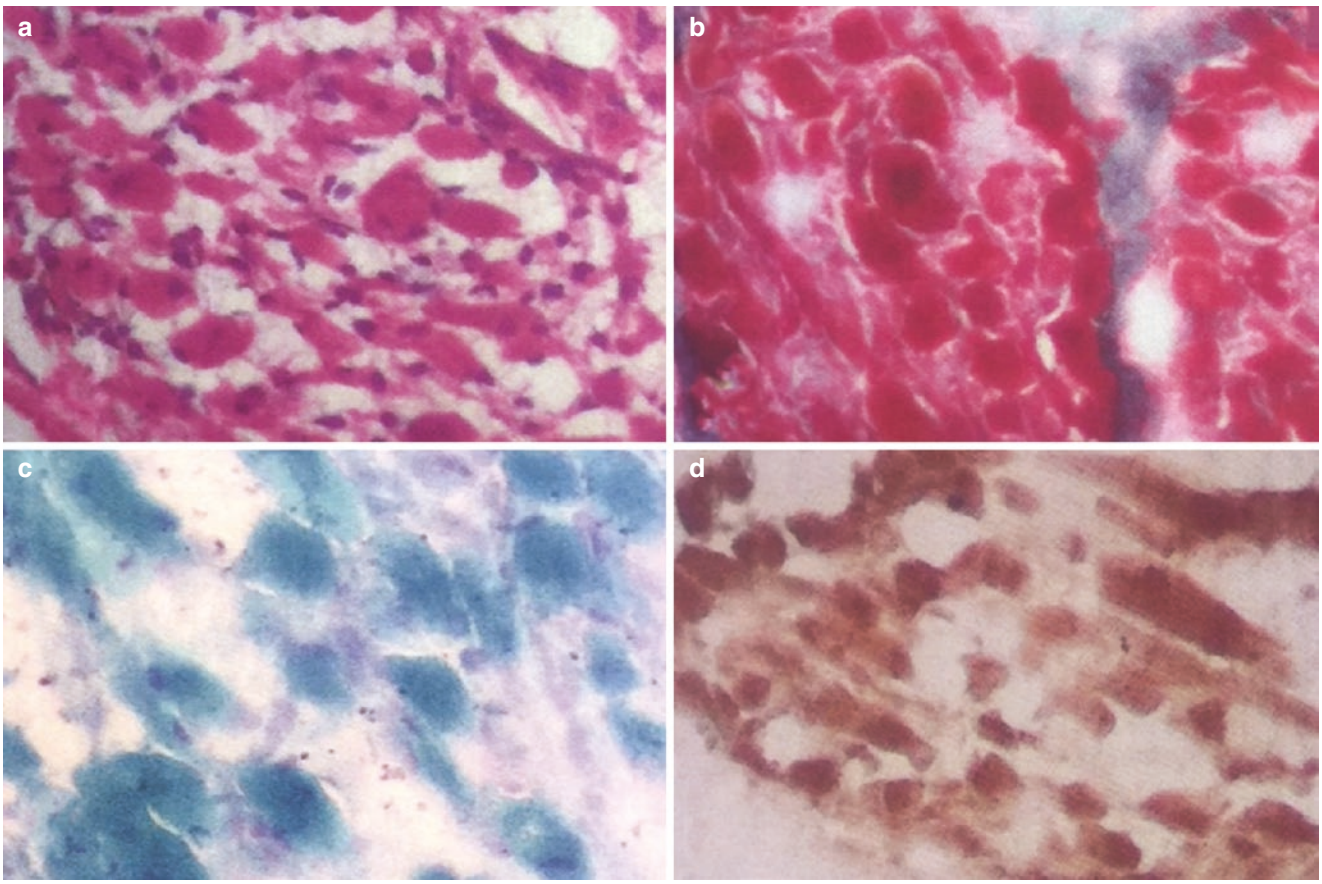

Fig. 13.12 Muscle biopsy of Case 13.13: (**a**) Note the marked variation in fiber size and shape (HE ×400), (**b**) There is marked fibrosis (Gomori Trichrome ×400), (**c**) There are no myofibrillary irregularities or rods (Modified Trichrome ×400), (**d**) Normal mitochondrial function (Combined COX- SDH ×400)

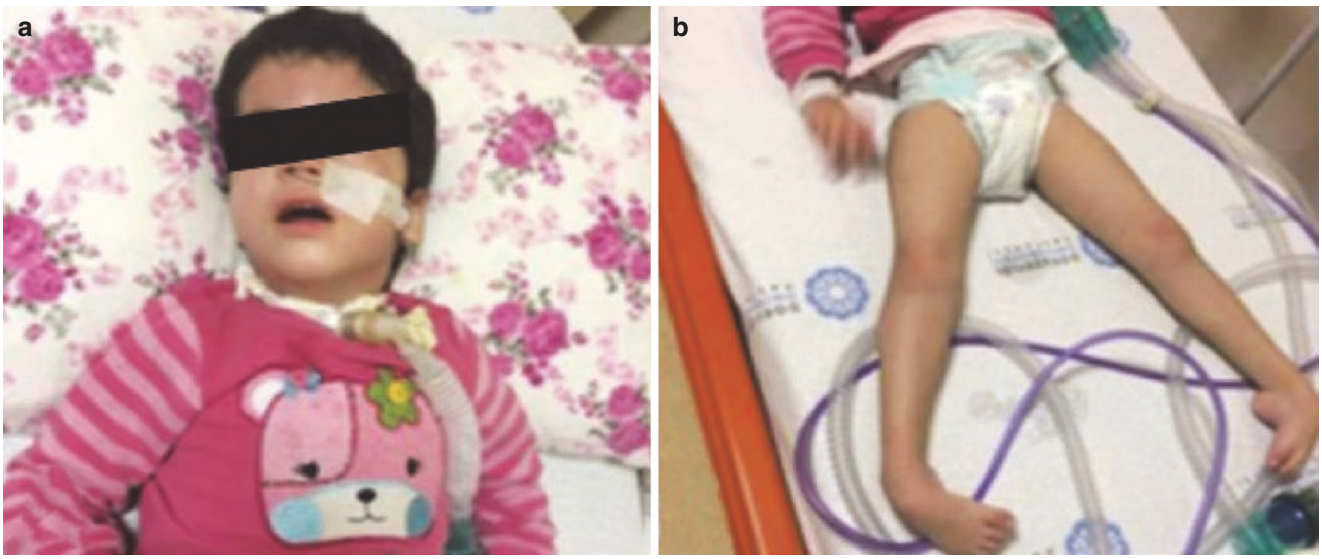

Fig. 13.13 Phenotypic features of Case 13.14 with CACNA1S mutation. (**a**) The patient need tracheostomy for ventialtion and nasogastric tube for feeding (**b**) Muscle weakness and distal contractures

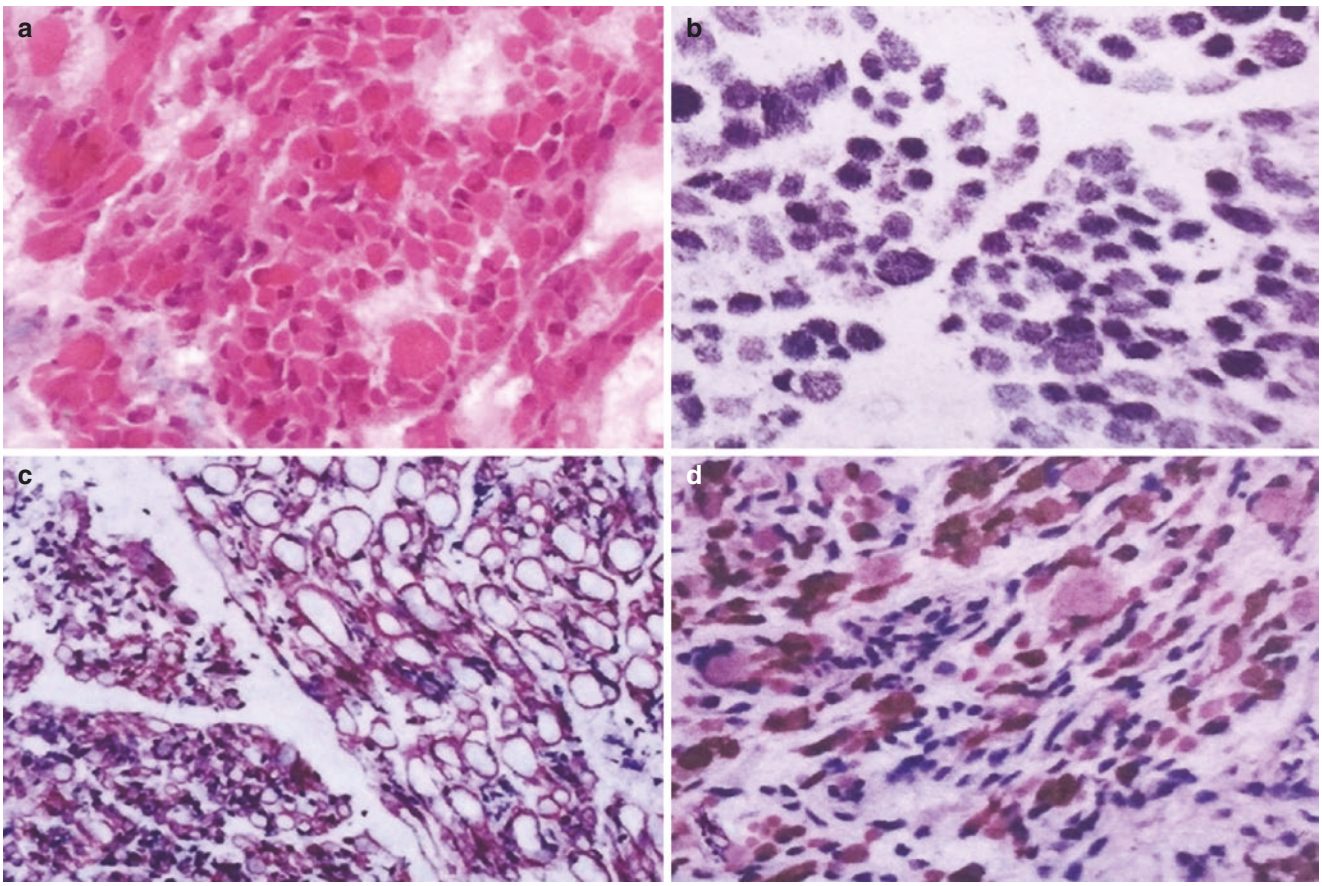

Fig. 13.14 (**a**) Note the marked variation in fiber size and shape (HE ×200), (**b**) There is no marked myofibrillary irregularity (NADH-TR ×200), (**c**) Normal sarcolemmal expression of merosin as well as grouping of large and small myofiber fascicles (DAB ×200), (**d**) Note the presence of huge type 1 fibers with fast myosin antibody (DAB ×100)

All About the Pathology of Congenital Myopathies

CMs are a group of hereditary muscle diseases that typically present at birth or in early infancy. There are multiple modes of inheritance and degrees of severity, which range from a lethal course in the newborn period to milder diseases in older children. Classically, they are defined by skeletal muscle dysfunction, which leads to hypotonia and skeletal muscle weakness. In addition, patients with CMs can have dysmorphic features, a typical facial appearance, impaired growth, and mental retardation, while in other NMDs such as muscular dystrophies or hereditary peripheral neuropathies, patients are generally of a normal phenotype at birth. Histopathologically, CMs are characterized a non-dystrophic muscle appearance with the presence of one or more pathognomonic histological features. Mutations in multiple different genes can cause the same pathology, while mutations in the same gene can cause multiple different pathologies. This has become ever more apparent now with the increasing use of NGS, and so a genetic diagnosis is achieved for a greater number of patients.Thus, the pathophysiological mechanisms underlying CMs are now better understood [79]. However, at the same time, considerable genetic and pathological overlap has emerged, blurring the classically established boundaries. Therefore, histopathological evaluation may become more necessary. For example, a 38-year-old woman was admitted to the hospital with muscle weakness. Her CK levels were normal. Muscle biopsy revealed that type 1 fiber predominance was present, and almost all of the type 1 fibers were small in size (Fig. 13.15). The presence of internal nuclei was mildly increased in both fiber types. A CMFTD diagnosis was based on histopathological findings, but during genetic analyses, two heterozygous variants were detected. One of them was a c.1941 + 8 T > C variant on the *SCN9A* gene, and the other was a c.737C > T p.Ser246Leu variant on the *TPM3* gene. Both variants have been associated with NMs. However, nemaline rods were not seen in the muscle biopsy.

The most important histopathological feature of CMs is the absence of regenerated fibers, which is the hallmark of dystrophies. This is because in the etiopathogenesis of MDs, there is a cycle of cell degeneration, necrosis, and regeneration after severe sarcolemmal damage. If repeated biopsies are performed at intervals of several months or years, the observed picture demonstrates an eventual loss of muscle fibers and their replacement by fibroadipose tissue. Finally, the biopsy will show the appearance of end-stage muscle. However, defects in different components of the sarcomere, which lead the loss of function in CMs, do not cause necrosis of myofibers. Although there are signs of muscle damage such as size and shape differences of myofibers, atrophic/hypotrophic fibers, nuclear internalization, and myofiber type changes, necrosis and regeneration are not observed.

The basic histopathologic findings in CMs are prominent size differences in mostly circular-shaped fibers (Fig. 13.16). There is often an increase in the rate of nuclear internalization, which is a marker of muscle injury. However, in CNMs, almost all myofibers have internal nuclei (Fig. 13.17). Another histopathological feature of CMs is fiber-type disproportion (FTD). This term is used to describe the myofiber hypotrophy, which alters myofiber type (Fig. 13.18). In CFTD, the main diagnostic finding is that the diameters of type 1 fibers are smaller than type 2 fibers [1, 4] However, since these findings can be seen in other CMs, they should be detected without any other histopathological findings to diagnose CFTD. In addition, this feature is also important in

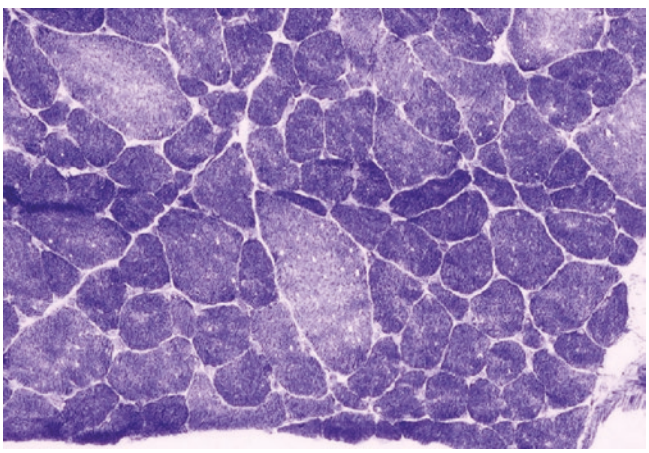

Fig. 13.15 Note the prominence of smaller type 1 fibers (NADH-TR ×200)

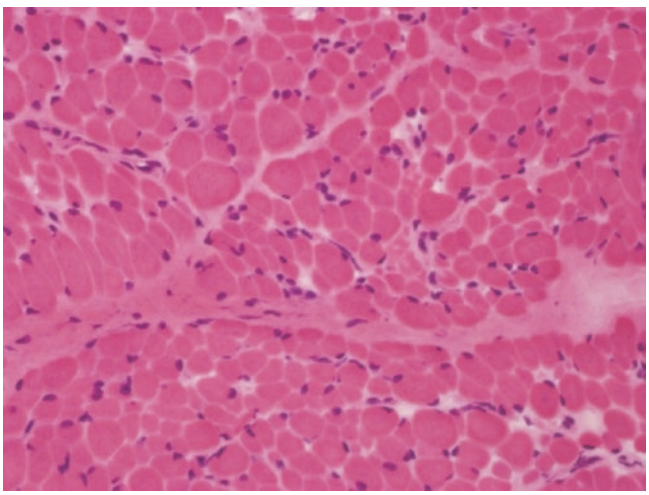

Fig. 13.16 Note the prominent variation of myofiber size in a CM (HE ×100)

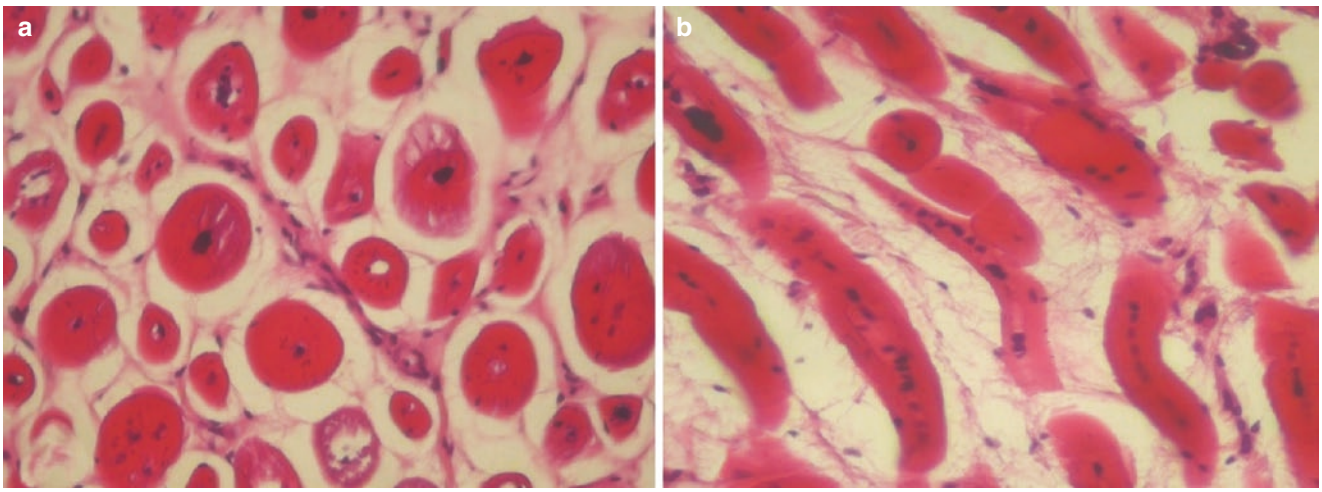

Fig. 13.17 (**a**) In the transverse section, there are centrally placed nuclei with some vacuoles, (**b**) In the longitudinal section, many internal/central nuclei are noticed (HE ×100)

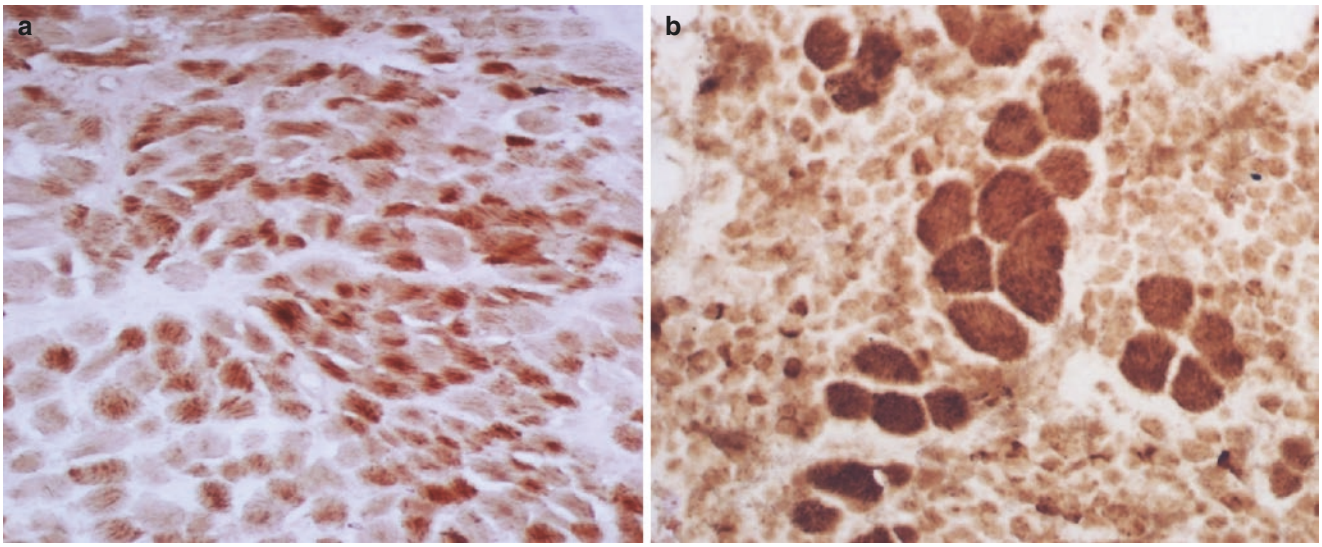

Fig. 13.18 (**a**) Type 1 fibers are smaller in size (COX ×200), (**b**) Type 1 fibers are larger in size (COX ×200)

distinguishing CFTD from infantile spinal muscular atrophy (SMA), which can be clinically confused with CMs, where hypertrophic type 1 fibers are usually observed [4, 43].

Interstitial tissue is mildly increased in CMs, and especially in the later phases, very prominent fibrosis occurs in muscular dystrophies, including the CMDs (Fig. 13.19). In the biopsies of patients with CNM, there can be peculiar sarcoplasmic alterations, which resemble a necklace. These myofibers are called "necklace fibers", which have internalized nuclei aligned in a basophilic ring (necklace) at 3 microns beneath the sarcolemma (Fig. 13.20). Ultrastructurally, it has been demonstrated that the necklace consists of myofibrils of a smaller diameter, in an oblique orientation, surrounded by mitochondria, sarcoplasmic reticulum, and glycogen granules [80].

In addition, the histopathological features that appear in CMs are in most cases indistinguishable with sharp boundaries and they may develop into different patterns over time (Fig. 13.21). For example, two muscle biopsy features were very different in a patient with genetically confirmed MTM. Especially in CNMs, the muscle's nuclei are located peripherally during the infantile period, as they are in normal skeletal muscle, and they shift towards the center during the course of myopathy. In fact, the average age of diagnosis in CNM cases is reported to be around 4 years in the literature because biopsies performed at an earlier age are generally not diagnostic. For this reason, a definitive diagnosis of CMs, especially CNMs, can sometimes be determined by examining biopsy material repeatedly in the following years [41].

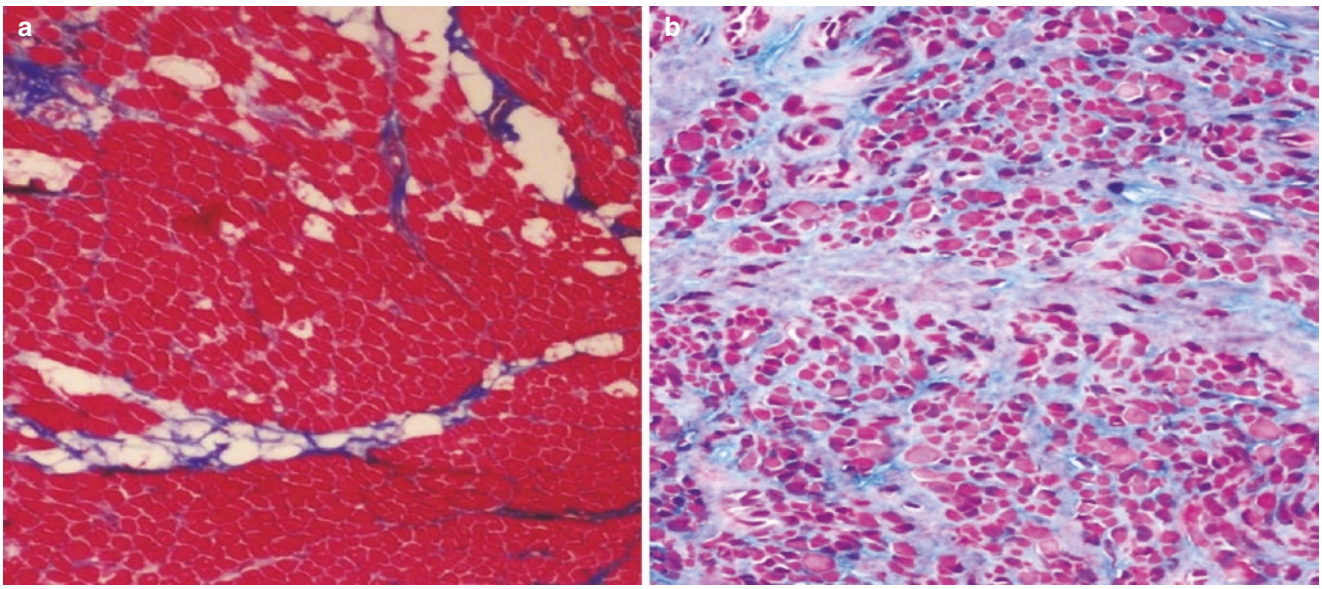

Fig. 13.19 (**a**) Mildly increased interstitial fibrosis (Masson trichrome ×100), (**b**) Prominent interstitial fibrosis in a patient with CMD (Masson trichrome ×100)

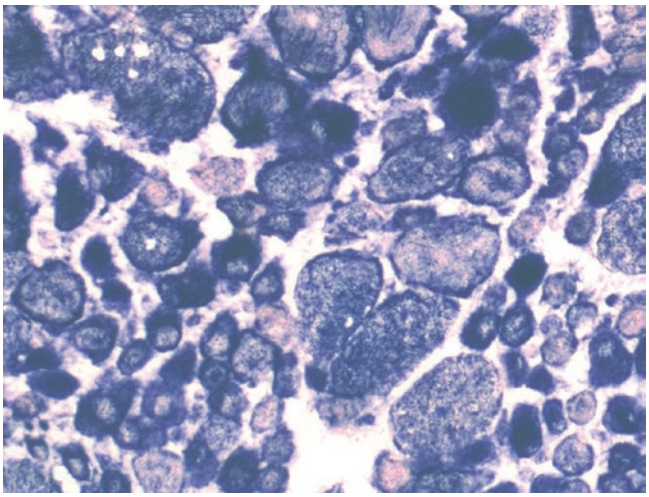

Fig. 13.20 Necklace fibers (NADH-TR ×200)

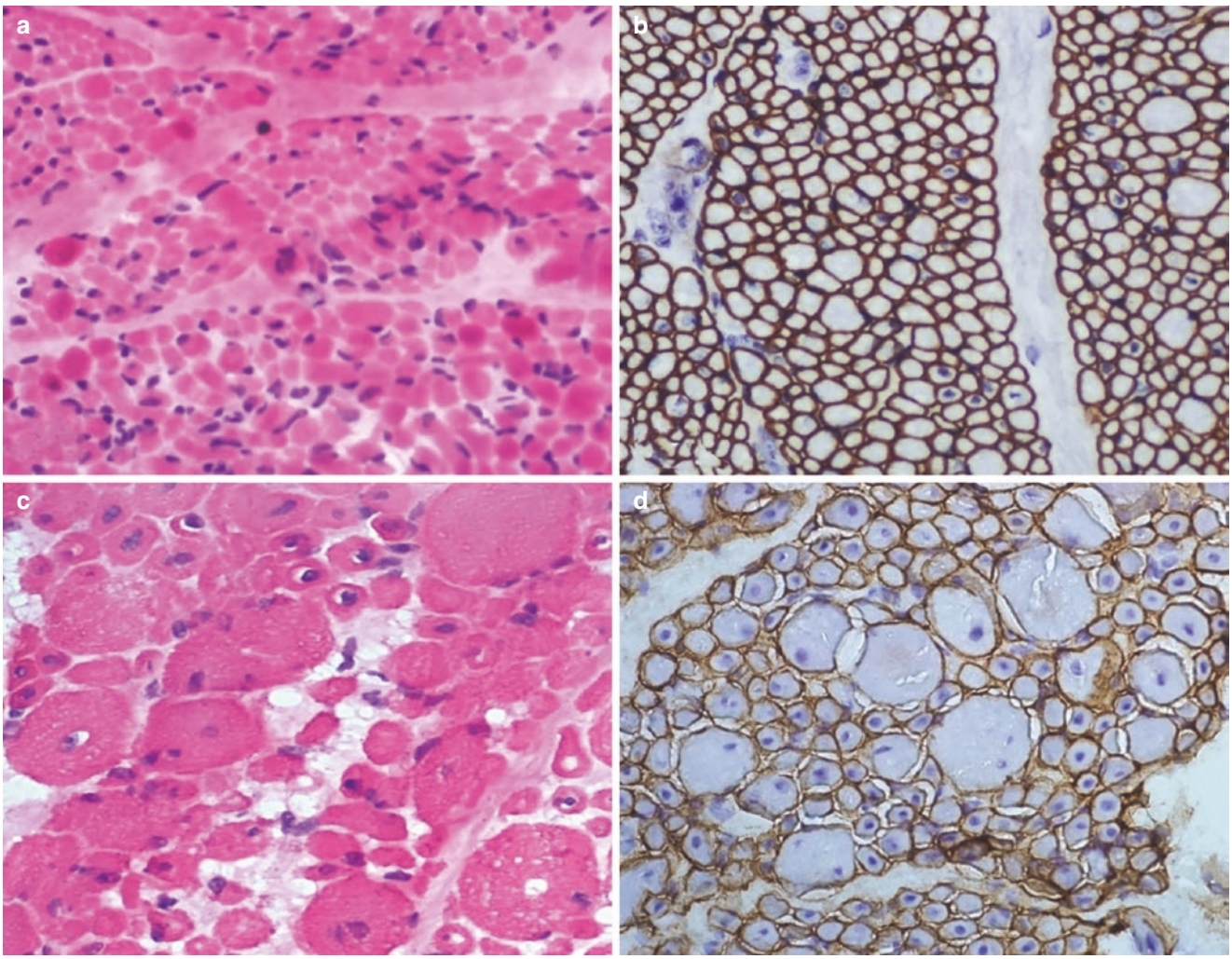

Fig. 13.21 The first biopsy of the patient shows myopathic features such as myofiber shape and size differences and mildly increased amounts of internal nuclei: (**a**) HE ×200, (**b**) Merosin positivity (DAB ×200). The second biopsy performed 2 years later shows huge size differences and prominent increased internal nuclei: (**c**) HE ×200, (**d**) DAB ×200

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Metabolic and Mitochondrial Myopathies

14

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Metabolic myopathies are genetic disorders that cause muscle disease due to a deficiency in energy metabolism [1]. This process involves many enzymatic reactions. Generally, lipid and carbohydrate metabolism affect muscle [1]. In metabolic myopathies, the age at which symptoms can present is a wide range, including infancy, childhood, and adulthood [2]. Most metabolic myopathies are inherited in a recessive manner [1]. An improvement in diagnostic tools and increased awareness has made the diagnostic process easier [3]. There are three main types of metabolic myopathies [2]:

1. Disorders of glycolytic and glycogenolytic metabolism.
2. Disorders of the carnitine cycle and fatty acid oxidation.
3. Disorders of the mitochondrial respiratory chain.

Symptoms of metabolic myopathies are characterized by acute, recurrent attacks of exercise intolerance, rhabdomyolysis, contractures, myalgia, myoglobinuria, and creatine kinase (CK) elevation, as well as chronic, progressive symptoms such as muscle weakness. Often this chronic form is associated with involvement of other organs [4]. Other genetic myopathies can mimic metabolic myopathy, such as Becker's muscular dystrophy, which may be related to fixed weakness [5]. With the acute symptoms, patients are generally asymptomatic between the episodes of myopathy. Also, positive laboratory findings, such as elevated CK levels can be normalized between acute episodes. Key history points such as episodes of rhabdomyolysis, pigmenturia, exercise intolerance, fatigue, symptoms worsening after periods of fasting, prominent myalgia, and cramps need to be evaluated. The presence of affected family members and consanguinity should also be questioned [5].

Disorders of Glycogenolytic and Glycolytic Metabolism

Disorders of glycolysis, gluconeogenesis, and glycogen synthesis are the causes of metabolic myopathies. In glycogen storage diseases (GSDs), glycogen can be stored in many organs but is mainly found in the liver and muscle. In muscle, glycogen is a rapid source of glucose during activity [1]. Some of these disorders can present with fixed myopathies, such as congenital myopathies or muscular dystrophies. Table 14.1 details GSDs and related disorders, which affect skeletal muscle. The symptoms of GSDs affecting muscle may include exercise intolerance and rhabdomyolysis, as seen with McArdle's disease (GSD V) or Tauri disease (GSD VII). Pompe disease (GSD type II) and debrancher defects (GSD IIIa) present with myopathy without rhabdomyolysis [1, 6].

Glycogen Storage Disease Type V (McArdle's Disease)

McArdle's disease was the first described metabolic myopathy in a young man with exercise-induced cramps, reported in 1951. The disease is the most common GSD [7, 8]. The disease caused by deficient muscle phosphorylase activity, results in impaired glycogenolysis due to the mutations in the *PYGM* gene. This gene is located on chromosome 11q13 and is made up of 20 exons. More than 100 mutations in

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Table 14.1 Glycogen storage diseases and related disorders affected skeletal muscle

Disorder	Enzyme	Gene	Clinical findings
GSD 0[a]	Glycogen synthase	GYS1	Exercise intolerance, cardiomyopathy, exercise-induced arrhythmia
GSD II	Alpha-glucosidase	GAA	Muscle weakness, cardiomyopathy, hypotonia
GSD III[a]	Glycogen debrancher	AGL	Hepatomegaly, hypoglycemia
GSD IV[a]	Glycogen brancher	GBE1	Cirrhosis, myopathy, cardiomyopathy, adult polyglucosan body disease
GSD V	Muscle glycogen phosphorylase	PYGM	Skeletal myopathy
GSD VII[b]	Muscle phosphofructokinase	PFKM	Skeletal myopathy
GSD X[b]	Muscle phosphoglycerate mutase	PGAM2	Skeletal myopathy
GSD IXd	Phosphorylase kinase	PHKA1	Skeletal myopathy
GSD XI[b]	Lactate dehydrogenase A	LDHA	Exercise intolerance, rhabdomyolysis
GSD XII	Aldolase A	ALDOA	Skeletal myopathy, hemolytic anemia
GSD XIII[b]	B-enolase	ENO3	Skeletal myopathy
GSD XV[a]	Muscle glycogenin	GYG1	Skeletal myopathy, cardiomyopathy
Phosphoglycerate kinase deficiency	Phosphoglycerate kinase 1	PGK1	Exercise intolerance, rhabdomyolysis, hemolytic anemia, intellectual disability, seizures
Danon disease	Lysosomal associated membrane protein 2 (LAMP2)	LAMP2	Cardiomyopathy, skeletal myopathy, mental retardation

[a] Glycogen synthesis defects
[b] Glycolytic defects, *GSD* glycogen storage disease

PYGM gene have been associated with this autosomal recessive disorder. It encodes the muscle isoform of glycogen phosphorylase, which catalyzes and regulates the breakdown of glycogen to glucose-1-phosphate during glycogenolysis [8–11]. McArdle's disease is a relatively benign disease and rarely, severe myoglobinuria can cause acute renal failure as a complication of widespread rhabdomyolysis.

McArdle's disease occurs in childhood, but it is usually misdiagnosed. Clinical findings include exercise intolerance, muscle weakness, and cramping. The symptoms are revealed by resting. Relief of symptoms after a few minutes of resting is called the "second wind" phenomenon. The breakdown of fatty acids and shift to fatty acid oxidation is the basis of this phenomenon [9]. Moderate exercise, like walking, can be tolerated easily, but brief intense isometric exercise (carrying heavy things) and sustained dynamic exercise (running, climbing) trigger the symptoms [10]. Some patients present with massive CK elevation and rhabdomyolysis with myoglobinuria that may cause kidney failure after exercise [9]. CK can be increased to more than 100,000–1,000,000 UI/L during episodes of rhabdomyolysis [10]. Clinical examination is usually normal between crises, but later in life, there may be persistent and progressive muscle weakness [9]. Two patterns of muscle weakness may be observed: proximal and symmetrical, or scapulohumeral and asymmetrical. Resting serum CK is consistently elevated in McArdle patients [10].

The diagnosis is based on clinical features; during forearm exercise, no lactate elevation, and abnormal ammonia levels are detected. Muscle biopsy reveals sarcolemmal vacuoles, glycogen storage, and absent myophosphorylase activity (Fig. 14.1). However, especially in younger children, there may not be any vacuoles or glycogen accumulation, detected with PAS stain, in biopsies. In these patients, significantly reduced or completely absent enzyme activity, detected in muscle biopsies, is diagnostic (Fig. 14.2). Regular exercise is recommended to increase capacity and functional physical activity by ingesting oral sucrose. Variable results were reported with a ketogenic or high protein diet [9, 12, 13].

Case 14.1

A 36-year-old woman was admitted to the physical medicine and rehabilitation polyclinic of Giresun University Hospital with complaints of progressively worsening weakness and fatigue, dating back to the age of 13. She did not have consanguineous parents, or a notable family history. CK serum levels were determined to be within normal limits. Myogenic abnormalities were identified on electromyography. A muscle biopsy demonstrated multiple glycogen-containing vacuoles and undetectable muscle myophosphorylase activity. Genetic analysis revealed no

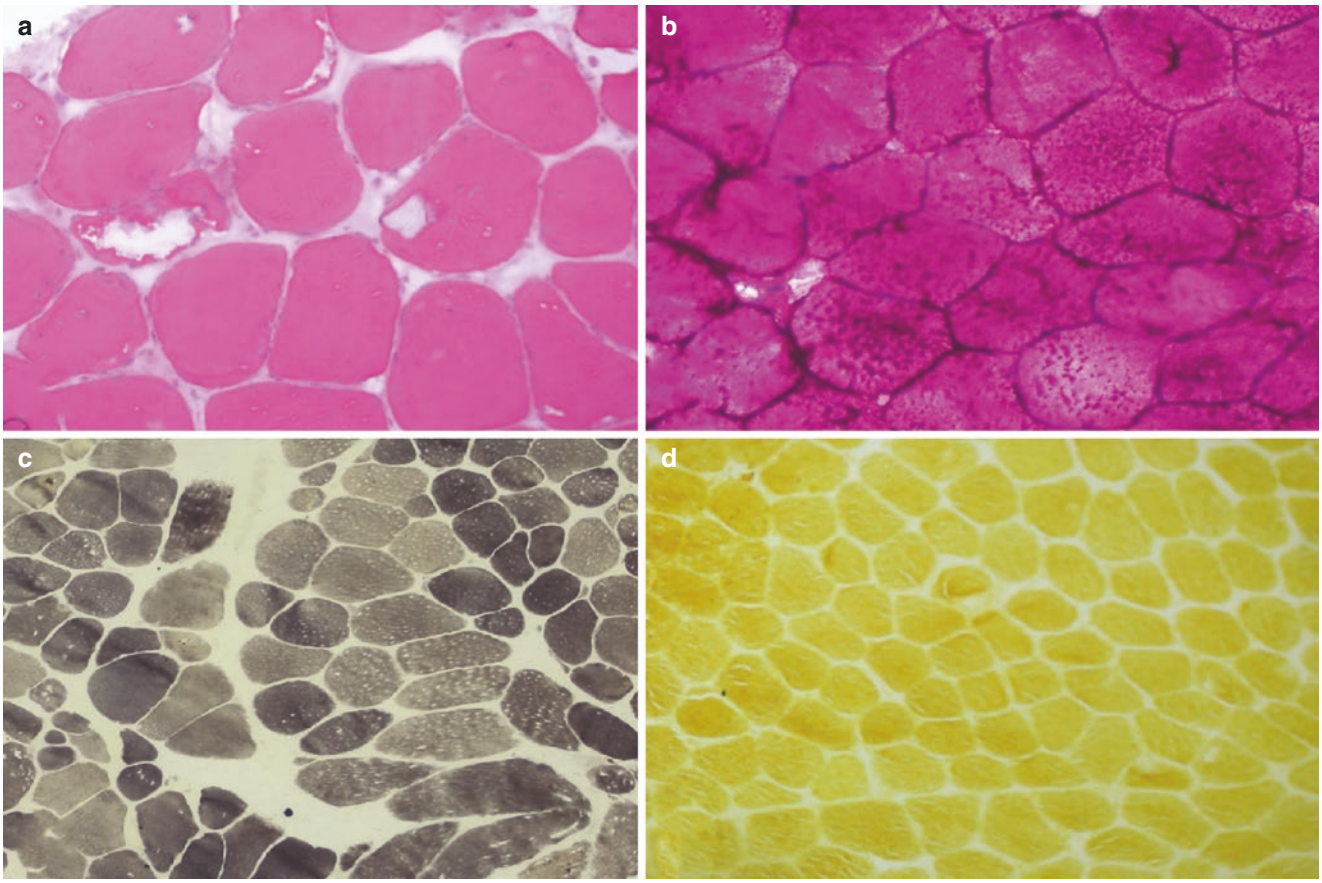

Fig. 14.1 Muscle biopsy of Case 14.1 with a *PYGM* gene mutation: (**a**) presence of small vacuoles (H&E × 400), (**b**) Irregular glycogen accumulation detected with periodic acid-Schiff staining (PAS × 400), (**c**) normal myophosphorylase enzyme activity in a control case (Phosphorylase ×100), (**d**) myophosphorylase enzyme defect in a muscle biopsy from this case (Phosphorylase ×100)

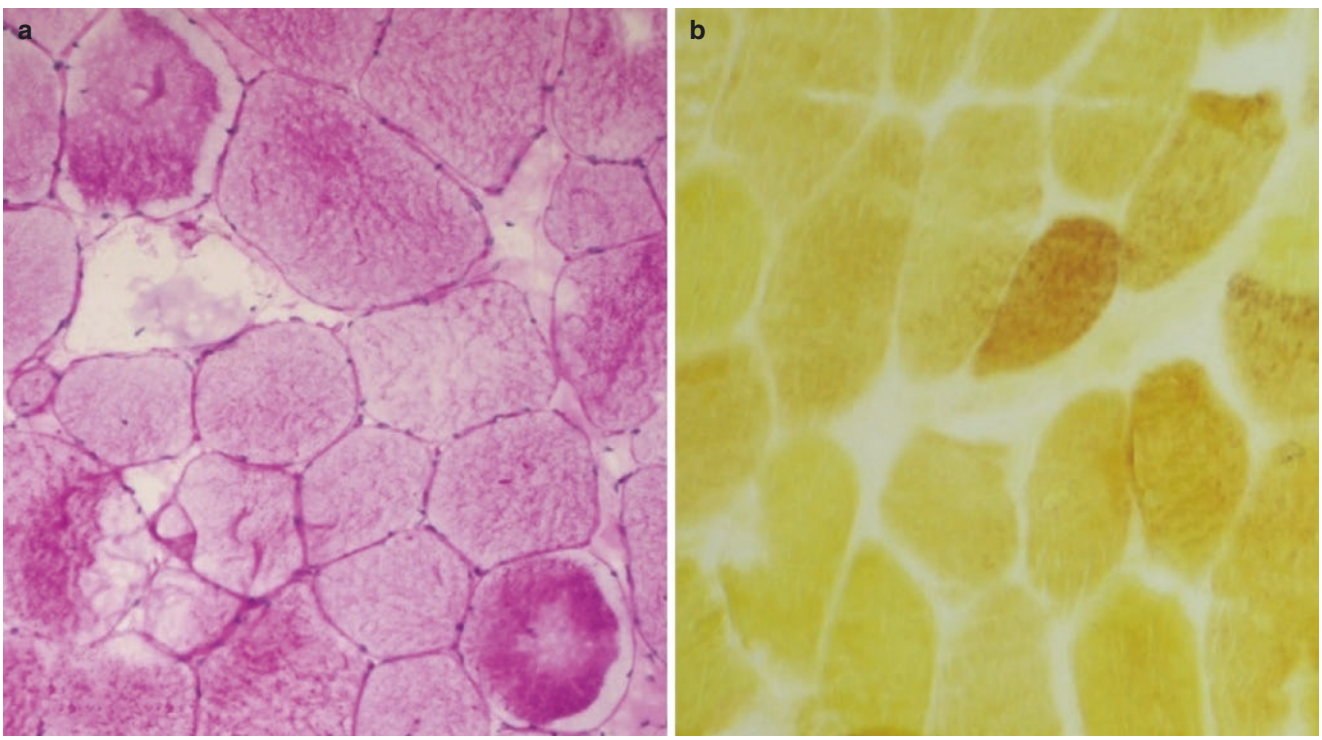

Fig. 14.2 Muscle biopsy of case 14.2: (**a**) Absence of prominent glycogen accumulation detected with periodic acid-schiff staining (PAS × 400), (**b**) myophosphorylase enzyme activity is seen in only a few myofibers (Phosphorylase ×400)

pathogenic mutations in the exonal regions of the *PYGM* gene. It was during sequencing analyses, that rs71049658 insertion/deletion variation, in intron 17, was identified. The patient was diagnosed as McArdle's disease by genetic and histopathological findings [11].

Case 14.2

A 2-year-old boy with no family history or consanguinity between his parents was admitted to hospital with incidentally detected high CK levels (up to 730 U/L). During genetic MLPA analysis, deletion, or duplication in the dystrophin gene was not identified. In a biopsy performed to investigate the etiology of myopathy, changes consistent with non-inflammatory myopathic disease were seen. Vacuole formations and glycogen accumulations were not observed (Fig. 14.2). An enzyme defect was detected with myophosphorylase staining, and the boy was diagnosed with McArdle's disease.

Case 14.3

A 40-year-old female patient with no family history or consanguinity between her parents was admitted to the intensive care unit with a pulmonary infection. High CK levels (up to 1000 U/L) and severe muscle weakness, especially in the intercostal and respiratory muscles, requiring respiratory support, were detected. In a biopsy performed to investigate the etiology of myopathy, changes consistent with non-inflammatory myopathic disease and vacuole formations were observed (Fig. 14.3). An enzyme defect was detected with myophosphorylase staining, and she was diagnosed with McArdle's disease. There are only a few studies in the literature examining the ventilation status of patients with McArdle's disease [14]. This makes it impossible to determine the prevalence of respiratory muscle dysfunction in McArdle's disease. Therefore, it is unclear whether the respiratory distress seen in this case is a rare or common finding. Nonetheless, this case demonstrates that severe inspiratory muscle dysfunction can occur in McArdle's disease.

Glycogen Storage Disease Type II (Pompe Disease)

Pompe disease is an autosomal recessive disorder caused by mutations in the *GAA* gene resulting in a decreased quantity or absence of lysosomal enzyme acid alpha-glucosidase (GAA or acid maltase). It is the second most common GSD. The disease is classified as infantile, juvenile, or adult according to the age of onset, progression rate, and level of organ involvement [15]. Infantile-onset Pompe disease (IOPD) is more severe and begins at the birth or within the first months of life [15]. The classic infantile form usually presents with hypotonia (floppy infant syndrome) and hypertrophic cardiomyopathy. It can also cause death in the first year of life[15]. Late-onset Pompe disease (LOPD) results in

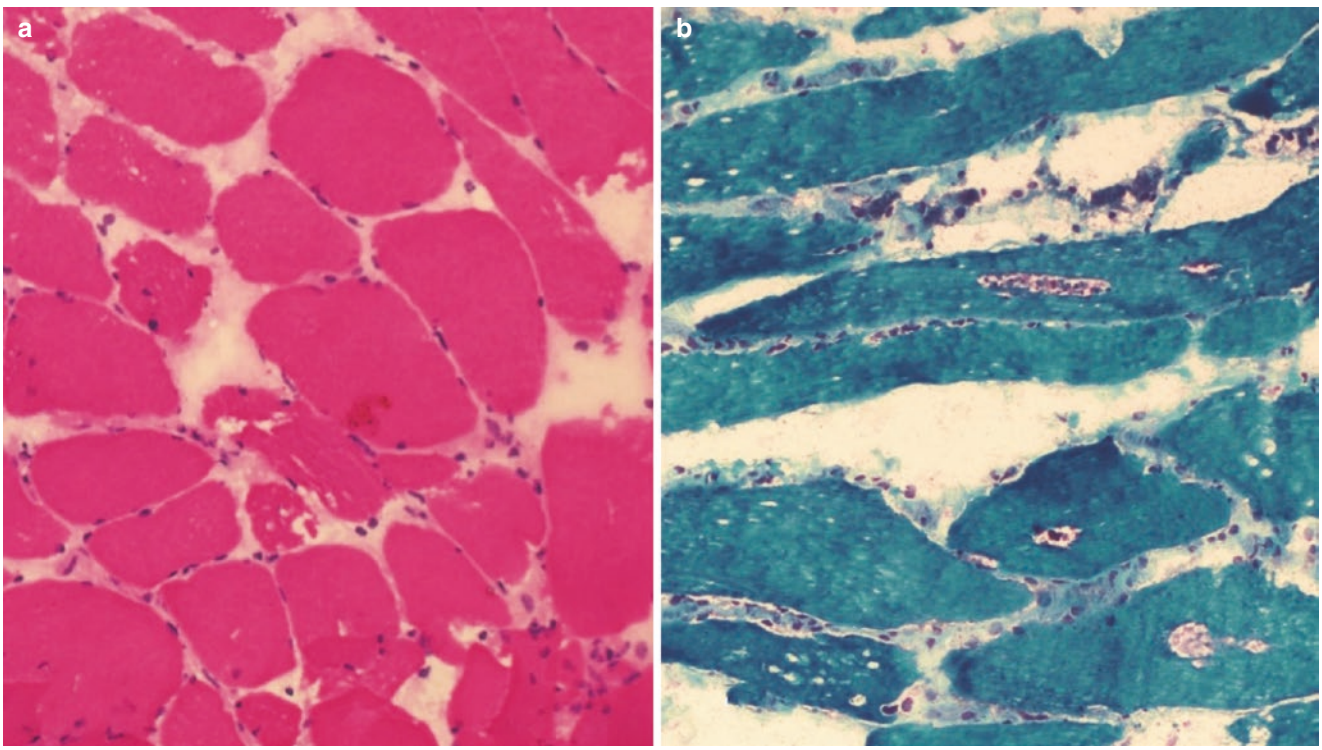

Fig. 14.3 Muscle biopsy of case 14.3: note the presence of vacuole formations seen with (**a**) H&E × 200, and (**b**) modified trichrome

progressive muscle weakness and respiratory failure, which can occur in young or adult patients. The adult form develops in the third or fourth decade, and the trunk and proximal limb muscles are mainly affected. The findings mimic limb girdle muscle dystrophy. In contrast with the infantile form, the heart is generally unaffected in the LOPD form [10, 15].

Enzyme activity in dried blood spot samples is a valuable diagnostic tool for patients with elevated CK levels. Infants with the classic infantile form have less than 1% residual acid alpha-glucosidase activity, whereas children and adults have a residual activity of no more than 30% of the normal values [7, 16]. Muscle biopsy shows a severe vacuolar myopathy with an accumulation of both lysosomal and free glycogen in the infantile form. However, a biopsy is not recommended in babies for the diagnosis of Pompe disease because of the anesthesia risk. A vacuolar myopathy with PAS-positive material is present in adults, but in one-third of cases, the muscle biopsy may be normal or show nonspecific changes [10]. Enzyme replacement therapy (ERT) showed significant beneficial effects on cardiomyopathy and muscle weakness, with increased survival in infants. Long-term follow-up under ERT treatment stabilized the muscle and respiratory involvement function of adults [15].

Case 14.4

A 35-year-old woman was admitted to the hospital with complaints of progressively worsening weakness and fatigue. She did not describe consanguineous parents, or a notable family history. CK serum levels were significantly elevated (CK 4300 U/L). In the physical examination, hepatomegaly was detected, and a liver biopsy was performed. A muscle biopsy was also performed to investigate the etiology of myopathy. Myogenic abnormalities were identified on electromyography. Both biopsies were consistent with glycogen storage disease (Fig. 14.4). The muscle biopsy demonstrated multiple glycogen-containing vacuoles, while muscle myophosphorylase activity was normal. Although no variation known to cause the disease was detected in the sequence analysis in which the GAA gene hotspots were scanned, it was genetically reported that Pompe disease could not be excluded because all regions and deletions of the gene were not examined.

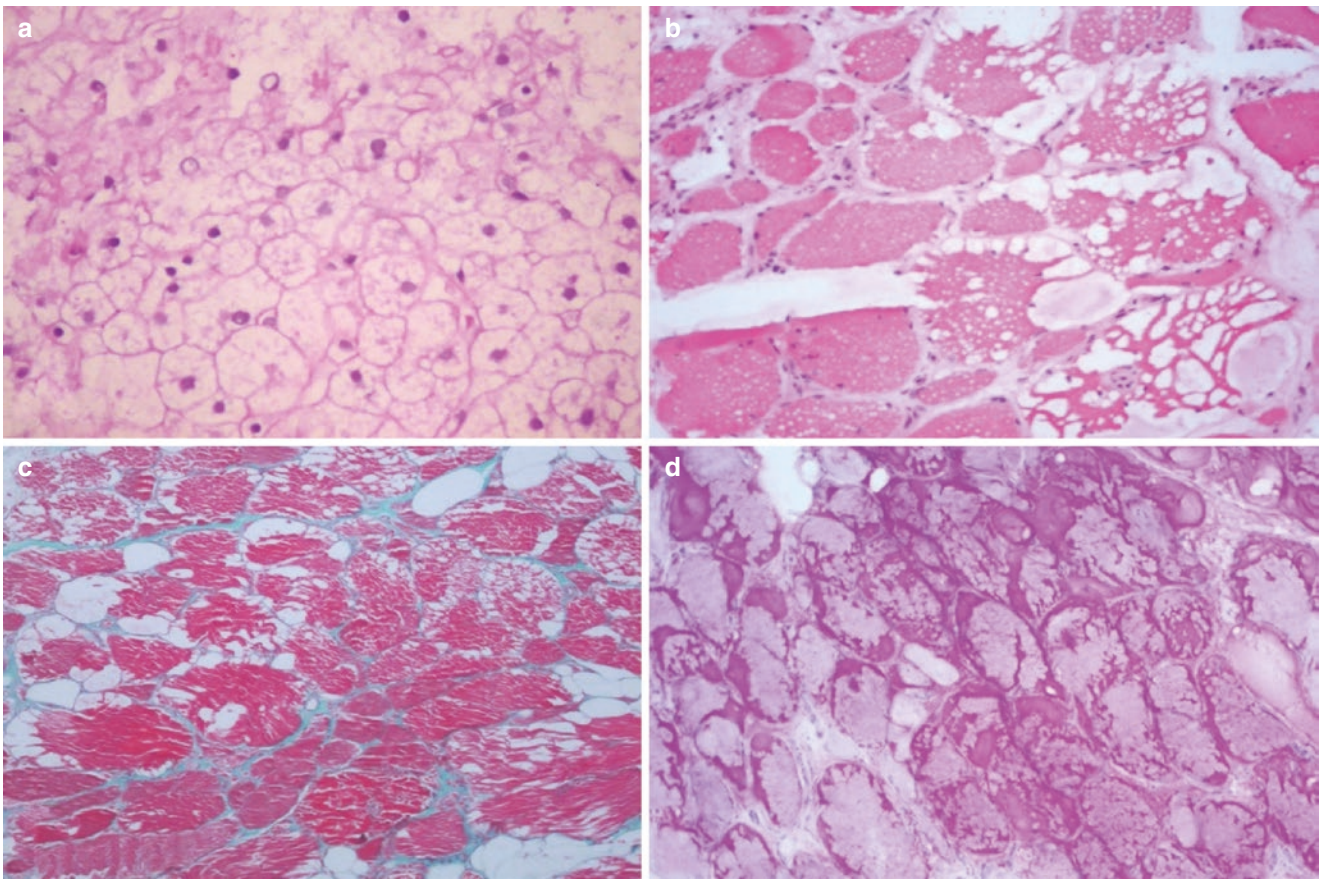

Fig. 14.4 (**a**) Liver biopsy of case 14.4 (H&E × 400), (**b**) presence of large vacuoles in the myofibers (H&E × 400), (**c**) increased interstitial fibrous tissue (Gomori trichrome ×200), (**d**) irregular large glycogen accumulations in the myofibers detected with periodic acid-Schiff staining (PAS × 200)

Glycogen storage disease type IXd (Muscle phosphorylase kinase deficiency) is a rare X-linked disorder caused by mutations in the PHKA1gene. The symptoms of the disease are similar to those of GSD type V. There is a variable lactate response with the forearm exercise test [6].

Glycogen storage disease type IIIa (Cori Forbes disease; debrancher deficiency) is an autosomal recessive disorder, and mostly nonsense mutations in the AGL gene are associated with GSD type IIIa. Clinical findings are those of the conventional GSD type including fasting hypoglycemia, hyperlipidemia, and elevated liver enzyme levels. In the myopathic form, CK elevation can be detected. Skeletal myopathy may worsen with the age. Asymptomatic left ventricular hypertrophy is also common [6].

Disorders of Glycolysis

*GSD type XI (lactate dehydrogenase deficiency) and GSD type XII (aldolase A deficiency)*may affect skeletal muscle. Alodase A (ALDOA) is the predominant isoform of aldolase in skeletal muscle and erythrocytes. ALDOA deficiency is reported very rarely, and the main symptoms include haemolytic anaemia, recurrent episodes of rhabdomyolysis, and mental retardation. CK levels are normal or elevated when resting. Lactate dehydrogenase (LDH) deficiency is also very rare. LDH deficiency should be considered at low LDH levels with CK elevation [6, 17].

*Muscle phosphofructokinase deficiency (GSD type VII, Tarui disease)*is clinically indistinguishable from McArdle's disease. Clinical symptoms include muscle cramps, exercise intolerance, rhabdomyolysis, and myoglobinuria, usually associated with hemolytic anemia. Subtypes of the disease are infantile, late-onset, and hemolytic. Patients with severe the infantile form present with hypotonia early after birth and often die within the first year of life [9]. In some cases, jaundice, myoglobinuria, and myogenic hyperuricemia may also be detected. Clinical findings differ from GSD type V with the absence of the "second wind" phenomenon. The forearm exercise test reveals ammonia elevation with reduced lactate, confirming impaired glycolysis. Strenuous exercise should be avoided. High protein intake during exercise is recommended, and a ketogenic diet has been proposed in patients with the severe form [9].

Muscle phosphoglycerate kinase (PGK) deficiency is a rare X-linked disorder caused by mutations in the *PGK1* gene. The myopathic form of the disease is characterized by recurrent episodes of exercise-induced cramps and myoglobinuria. Strenuous exercise should be avoided and symptoms should be resolved by resting [9].

Glycogen Depletion Syndromes: Muscle Glycogen Synthase Deficiency (Muscle GSD Type 0, GSD 0b) and Glycogenin 1 Deficiency.

Both disorders present with myopathy and cardiomyopathy. Mutations in the *GYS1* gene leads to a deficiency of the glycogen synthase-1 enzyme. Patients can present with exercise-induced arrhythmia, cardiomyopathy, and exercise intolerance. Muscle biopsy reveals the absence of glycogen and polyglucosan bodies with mitochondrial proliferation. It can mimic mitochondrial myopathies [6].

Danon Disease (LAMP2 Deficiency)

Danon disease is an X-linked disorder caused by the primary deficiency of lysosomal-associated membrane protein 2 (LAMP2). Patients present with skeletal myopathy, cardiomyopathy, intellectual disability, retinopathy, or maculopathy. A milder presentation may be observed in hemizygous females [9].

Disorders of Mitochondrial Fatty Acid Oxidation

Lipid storage myopathies are genetic disorders with abnormal lipid storage in different organs, typically in muscle. Patients present with muscle weakness, myalgia, fatigue, and cardiomyopathy. The predominant feature of lipid storage is exercise-induced weakness; rhabdomyolysis patients can have a fixed myopathy and fasting hypoglycemia[1]. The onset of the disease varies depending on residual enzyme activity, the age of the patient, and exposure to stress [18]. The metabolic crisis can be precipitated by stress, fasting, fevers, exercise, and anesthesia. Clinical symptoms can start early in life or remain absent until later decades [1]. Fatty acid oxidation (FAO) disorders are inborn lipid metabolism disorders that cause a deficiency of the enzymes required to break down fats (Table 14.2). The most common lipid myopathies are carnitine palmitoyltransferase II deficiency (CPT II), and very-long-chain-CoA dehydrogenase deficiency (VLCAD) [1].

The evaluation of blood CK, lactate, carnitine, acylcarnitine, and urine organic acid levels is necessary for diagnosing lipid myopathies. However, they can be within the normal ranges between episodes [1, 19]. In the recent past, the evaluation for these disorders consisted of enzyme analysis in fibroblast or muscle biopsies. Nowadays, genetic analysis is given a higher priority. As the clinical and laboratory findings overlap, large-panel genetic sequencing is a valuable method, used to determine the diagnosis [19]. Table 14.1 summarizes the clinical and laboratory findings of fatty acid oxidation disorders presenting with myopathy.

In general, the treatment for these disorders is to avoid fasting, and eat meals rich in complex carbohydrates. Carnitine supplementation can be used, if there is a carnitine deficiency or carnitine transporter deficiency [1, 19].

Table 14.2 Fatty acid oxidation disorders presenting with myopathy

Defect	Gene(s)	Rhabdomyolysis/chronic weakness	Other clinical findings	Laboratory findings plasma acylcarnitine urine organic acid
CTD	*SLC22A5*	–/+	HCM, hypotonia, fatigue	Low carnitine DCA
CPT II	*CPT2*	+/+	CM, hepatomegaly hypoglycemia	C16, C18 elevated
VLCAD	*ACADVL*	+/+	HCM, arrhythmias, sudden death, hypoglycemia, "Reye-like" hepatic syndrome	C16:1, C14:1, C18:1 elevated Variable DCA
LCHAD and MTP	*HADHA* *HADHB*	+/+	CM, sudden death, hypoglycemia, "Reye-like" hepatic syndrome	C18:1-OH, C18-OH, C16:1-OH, C16-OH elevated 3-hydroxydicarboxylic acid, DCA
MAD	*ETFA, ETFB, ETFDH*	+/+	CM, hypoglycemia, hepatopathy, respiratory dysfunction, encephalopathy, acidosis	C6, C8, C10, C12 elevated Ethylmalonic acid, adipic acid, DKA, KB

CTD carnitine transporter deficiency, *CPT II* carnitine palmitoyltransferase II, *VLCAD* very-long-chain-CoA dehydrogenase, *LCHAD* long-chain-3-hydroxyacyl-CoA-dehydrogenase, *MTP* mitochondrial trifunctional protein, *MAD* multiple-acyl-CoA-dehydrogenase, *DCA* dicarboxylic aciduria, *KB* ketone bodies, *HCM* hypertrophic cardiomyopathy, *CM* cardiomyopathy, *DKA* dicarboxylic aciduria

Primary Carnitine Deficiency (Carnitine Transporter Deficiency, CTD)

Carnitine transporter deficiency (CTD) is an autosomal recessive disorder of the carnitine cycle caused by a mutation in the SLC22A5 gene. This gene encodes for organic cation transporter novel 2 (OCTN2). Defective carnitine transport activity of OCTN2 results in increased carnitine urine excretion and low blood carnitine levels. Patients can present with an acute metabolic decompensation, early in life or later in life, with skeletal and cardiac myopathy. Also, sudden death can occur due to arrhythmias [10]. Laboratory investigations usually reveal hypoglycemia, elevated liver function tests, and hyperammonemia. CK levels can be mildly elevated[10, 13]. CTD patients can develop cardiomyopathy, usually between 1 and 4-years-of-age [10, 19]. Patients with CTD respond to carnitine supplementation, if started before irreversible organ damage occurs [20].

Carnitine Palmitoyltransferase II (CPT II) Deficiency

CPT II deficiency generally presents with episodes of rhabdomyolysis. The myopathic form of CPT II deficiency is the most common lipid metabolism disorder affecting skeletal muscle. Prolonged exercise, fasting, infections, and stress usually precipitate episodes. During attacks, muscle pain, and myoglobinuria, which may lead to acute renal failure, can be detected. The severe neonatal form of the disease is usually lethal. Routine laboratory tests should be performed, including measuring blood lactate, pyruvate, creatine kinase, amino acids, and free acylcarnitine levels. Elevations of acylcarnitine, notably C16 and C18:1, can be detected. The diagnosis of CPT II deficiency is established by identifying reduced enzyme activity in muscle biopsy or mutations in the CPT2 gene. Treatment strategies include avoiding trigger factors and reducing the amount of long-chain dietary fat [21].

Case 14.5

A 28-year-old female patient with no family history or consanguinity between her parents was investigated to determine a reason of her muscle weakness in neurology clinics. She had minimally elevated CK levels (up to 300 U/L), and mild muscle weakness, especially of the cervical muscles. She had delivered 18 months before and after that time, her complaints of muscle weakness increased. During her hospitalization period, many biochemical and radiological analyses were performed, and before these analyses, her oral feeding was stopped. Therefore, during her hospitalization, she experienced long fasting times. Afterwards, repetitive vomiting started, and oral feeding was completely stopped. Total parenteral nutrition was started. Her general condition was deteriorating and acidosis developed. At this point, a muscle biopsy was performed. In the biopsy, histopathological changes consistent with non-inflammatory myopathic disease and vacuolar changes were observed (Fig. 14.5). These vacuoles were located mainly in type 1 fibers, stained with oil-red-O dye. The presence of a lipid metabolism disorder was reported to the clinician. At first, parenteral, then oral carbohydrate-rich diet was given to the patient. In the following several days, her general condition rapidly improved. During genetic analysis, a *CPT2* gene defect was identified, and she was diagnosed with carnitine palmitoyl transferase 2 deficiencies.

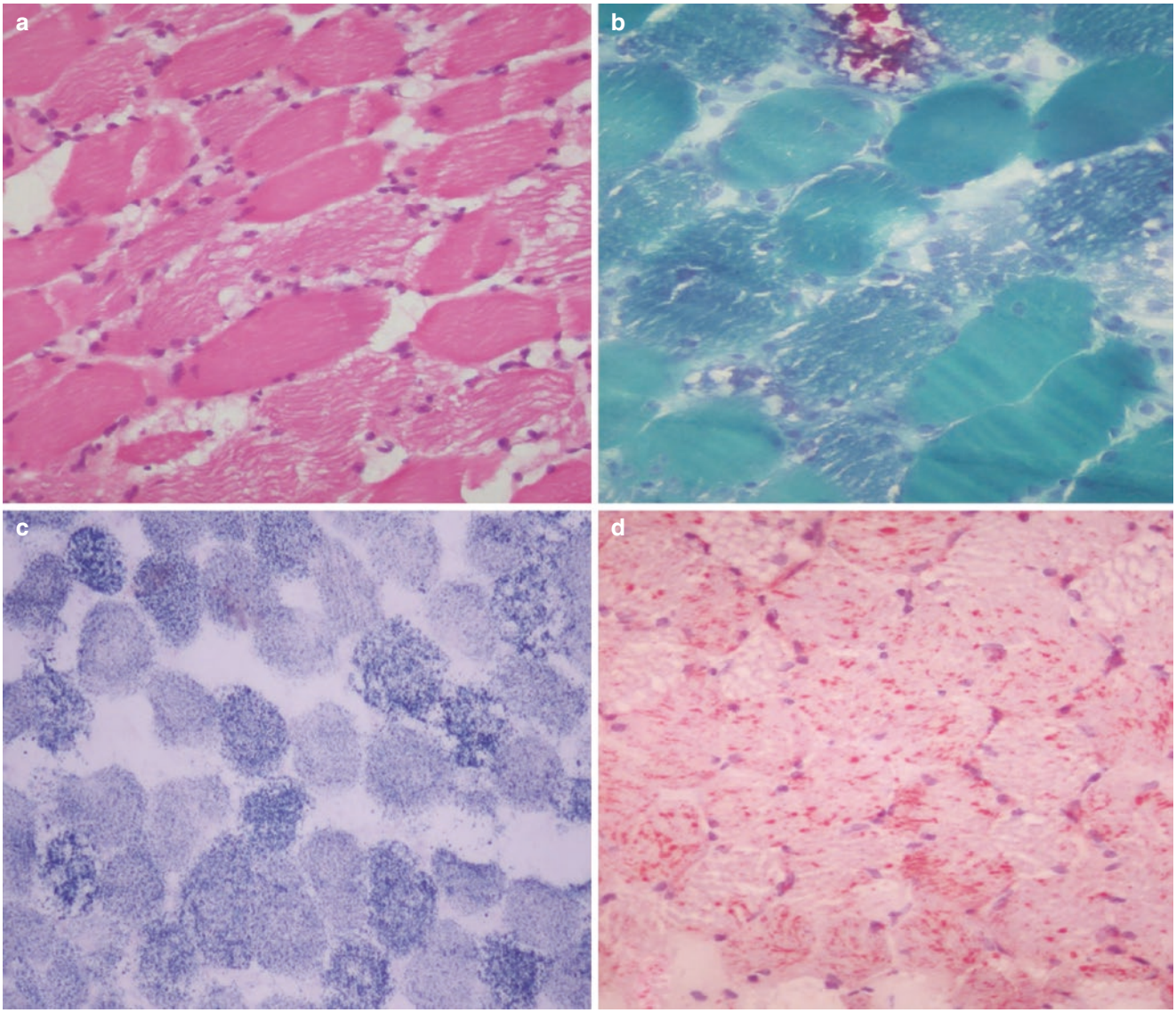

Fig. 14.5 Muscle biopsy of case 14.5 with a CPT2 gene mutation: (**a**) presence of small vacuoles distributed as a checkerboard (H&E × 400), (**b**) irregular small vacuoles (Modified trichrome ×400), (**c**) type 1 fibers show vacuolar changes (SDH × 200), (**d**) lipid accumulations in the muscle cells (Oil red-O × 400)

Very-Long-Chain-CoA Dehydrogenase (VLCAD) Deficiency

VLCAD deficiency is an autosomal recessive disorder of fatty acid oxidation caused by pathogenic mutations in the ACADVL gene. The disease is often categorized into three groups: severe, moderate, and mild. Depending on the severity of the disease, patients present with hypoketotic hypoglycemia, hepatomegaly, myopathy with recurrent rhabdomyolysis, and cardiomyopathy [22]. A diagnosis of VLCAD deficiency should be made with abnormal blood acylcarnitine levels, seen with biochemical testing, and ACADVL gene molecular genetic testing. Low-fat formula or low long-chain fat with high medium-chain triglycerides (MCT) medical food can be used in treatment. Triheptanoin supplementation is also an option for treatment [21].

Long-Chain-3-Hydroxyacyl-CoA Dehydrogenase (LCHAD) and Mitochondrial Trifunctional Protein (MTP) Deficiency

LCHAD and MTP deficiencies are autosomal recessive, fatty acid oxidation disorders. Common signs and symptoms of these diseases include rhabdomyolysis, hypoketotic hypoglycemia, and fatigue. Cardiomyopathy, organ failure, and death are the long-term complications of the diseases. Later-onset forms of the diseases present as myopathic forms [23]. Regarding long-term outcomes, retinopathy may be common in LCHAD deficiency and neuropathy is more common in patients with MTP deficiency. The treatment for LCHAD and MTP deficiencies includes dietary management, which consists of a low-fat diet, eating frequently, high carbohydrate intake, and medium-chain triglyceride supplementation [23].

Multiple Acyl-CoA Dehydrogenase (MAD) Deficiency

Multiple acyl-CoA dehydrogenase (MAD) deficiency represents a clinical spectrum in which presentations can be divided into type I (neonatal-onset with congenital anomalies), type II (neonatal-onset without congenital anomalies), and type III (late-onset). In this disease, there is a loss of activity of seven acyl-CoA dehydrogenase enzymes and two N-methyl enzymes. Together with fatty acid oxidation, the metabolism of branched-chain amino acids, lysine, choline, and sarcosine is also affected. The disease is also known as glutaric acidemia type 2, because the urine of patients has high concentrations of glutaric acid, along with many abnormal amino acids, and fatty acid metabolites. Presenting symptoms for patients with the late-onset form include muscle weakness, exercise intolerance, muscle pain, and episodes of rhabdomyolysis. An elevation of several acylcarnitine species in the blood and abnormal organic acid analyses can be detected. When diagnosing MAD deficiency, pathogenic variants in the ETFA, ETFB, or ETFDH genes should be seen [24]. Muscle biopsy outside an episode of rhabdomyolysis, in the late-onset form with the myopathic presentation, demonstrates extra-mitochondrial lipid accumulation, characteristic of lipid storage myopathy, which may be accompanied by coenzyme Q10 deficiency [25]. Treatment includes limiting fat and protein in the diet and high dose riboflavin (100–300 mg daily), carnitine supplementation (50–100 mg/kg daily) in those with carnitine deficiency, and coenzyme Q10 (60–240 mg daily) supplementation [24].

Case 14.6

A 15-year-old male patient with no family history or consanguinity between his parents was investigated for rhabdomyolysis in the intensive care unit. He had prominent elevated CK levels (25,000 U/L) and muscle weakness. During hospitalization, a muscle biopsy was performed to search for the etiology. In the biopsy, prominent vacuolar changes were observed (Fig. 14.6). These vacuoles were located mainly in type 1 fibers, detected with oil-red-O dye. The presence of a lipid metabolism disorder was reported to the clinician. The patient was diagnosed with MAD deficiency based on clinical, histopathological, and biochemical findings. Genetic analysis revealed a homozygous mutation [c.1130 T > C (p.Leu377Pro)] in the electron transfer flavoprotein dehydrogenase (*ETFDH* gene. This gene defect is associated with multiple acyl-CoA dehydrogenase deficiency (MADD) [11].

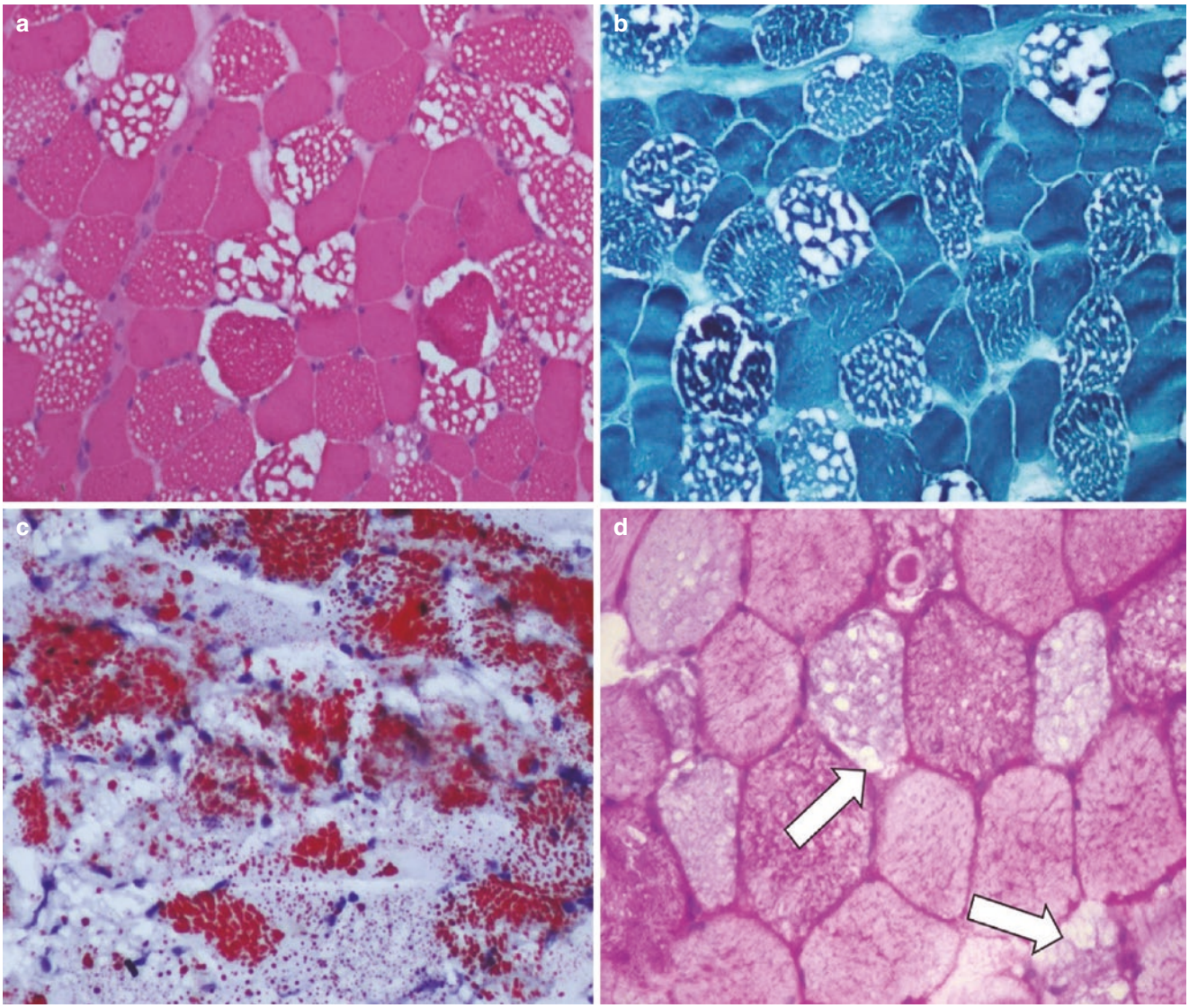

Fig. 14.6 Muscle biopsy of case 14.6 with a MAD gene mutation: (**a**) presence of small vacuoles distributed as a checkerboard (H&E × 200), (**b**) irregular small vacuoles (Modified trichrome ×200), (**c**) huge lipid accumulations in the muscle cells (Oil-red-O × 400), (**d**) absence of glycogen in the vacuoles (PAS × 400)

All About the Pathology of Metabolic Myopathies

The required energy for muscle tissue is provided by the breakdown of stored glycogen. Therefore, defects in any step of the glycolytic pathway can cause a muscle disease, which occurs with several metabolic problems such as fatigue, cramps, or rhabdomyolysis. However, all defects in the glycolytic mechanism do not primarily affect muscles. Herein, histopathological features of GSDs which mainly cause muscle involvement are discussed. Histopathologically, the muscle involvement of GSDs is characterized by excessive glycogen deposition, sometimes as vacuoles. These glycogen accumulations are easily demonstrated by special histological dyes. For instance, the periodic acid-Schiff (PAS) stain is a widely used technique in histopathology for the demonstration of carbohydrates and carbohydrate-rich compounds in tissues. PAS staining demonstrates polysaccharides, mucin, glycogen, certain glycoproteins, glycolipids, basement membranes, and certain fungi in tissues. If staining is completed after the tissue is treated with an enzyme such as diastase, which degrades glycogen before PAS staining, it can be easily understood that the accumulated substance is glycogen due to the absence of staining (Fig. 14.7). However, glycogen can also be easily lost from myofibers during pathological procedures, and this cannot be demonstrated with PAS staining. In this situation, histopathological findings can resemble artifacts, which occur due to improper freezing [26].

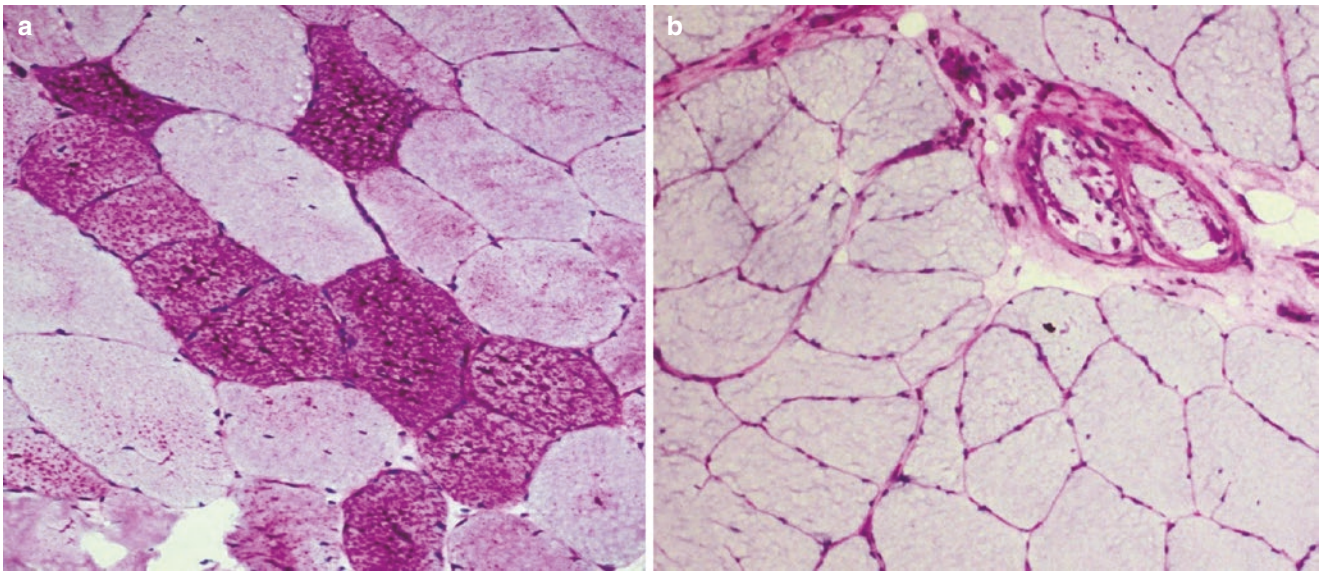

Fig. 14.7 (**a**) Presence of glycogen accumulation in a checkerboard pattern (PAS × 200), (**b**) digestion of glycogen by diastase (dPAS ×200)

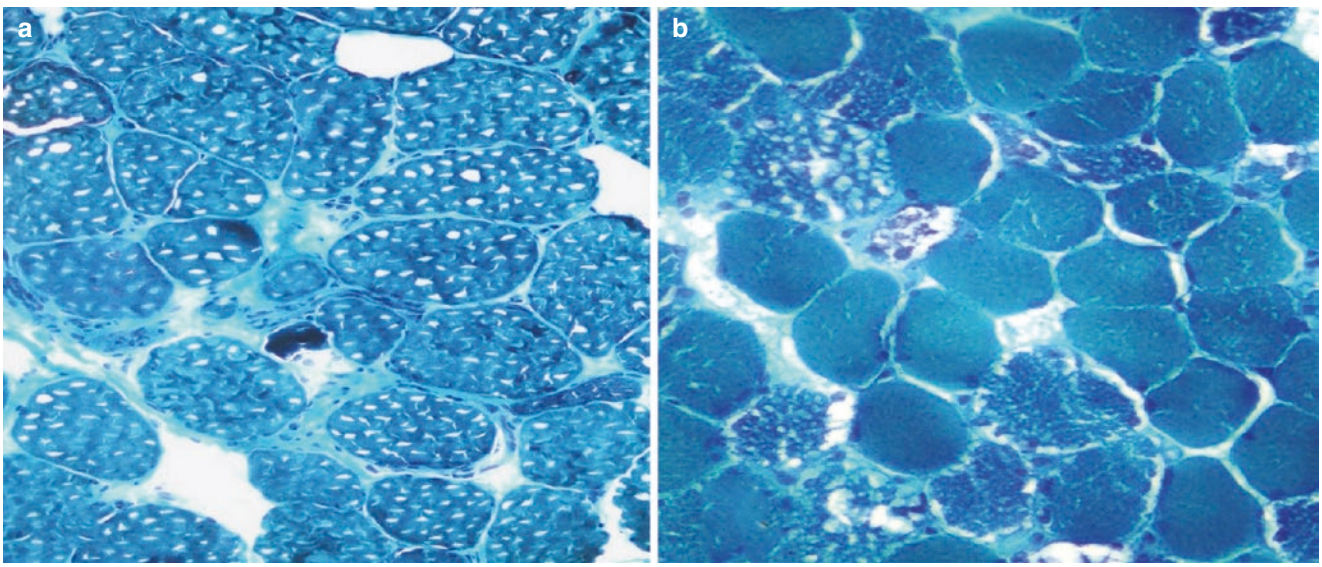

Fig. 14.8 (**a**) Freezing artifacts (modified trichrome ×200), (**b**) distribution of small vacuoles in a checkerboard pattern (modified trichrome ×200)

Vacuoles are common frozen artifacts, made of ice crystals, which can develop due to improper freezing or thawing rates. This kind of artifact may mimic clinical changes, such as metabolic myopathy, or it may distort fibers that have undergone pathological changes, making it impossible to interpret the data correctly. The vacuoles associated with ice crystals, as opposed to pathological vacuoles, are typically found at the tissue's centre or periphery, depending on how slowly the specimen was frozen or thawed. Vacuoles tend to affect every fiber in the region of the artifact, and they are arranged in a gradient according to size. Small vacuoles are abundant, uniform in size, and found in a waffle pattern between the myofibrils of each fiber where artifacts are least present. Larger, transparent vacuoles are seen in areas that are not well frozen. On the other hand, since the vacuoles form due to material accumulation observed in metabolic diseases that may prefer certain muscle fiber types, they can show a checkerboard-like distribution (Fig. 14.8). If such a distribution exists, the diagnosis of metabolic diseases becomes easier. This is because, while forming a vacuole in one of the two myofibers standing side by side, protecting the other cannot be explained by freezing errors. In metabolic disorders with increased lipid storage in the muscle fibers, there are similar vacuolar changes in the frozen sections of muscle and these accumulations are detected with oil-red-O staining. However, lipid accumulations are almost

never shown in the paraffin block sections, because lipids dissolve in the chemicals during tissue processes. Therefore, empty vacuoles are discriminated in the paraffin sections of muscles with lipid storage disorders [26–30].

In the milder form of Pompe disease, vacuolar changes may be minimal or may only be apparent of some fibers, especially type 1 fibers, or both fiber types. Increased glycogen accumulation may be demonstrated with PAS staining. As acid maltase is in lysosome-derived vacuoles, accumulated glycogen is mostly found in lysosomes and there is also increased activity of acid phosphatase, another lysosomal enzyme. If glycogen accumulations are not demonstrated with PAS staining, acid phosphatase enzyme staining must be performed. Increased areas of acid phosphatase activity are always present, and this is one of the most useful applications of the acid phosphatase enzyme stain [26]. As GSD type IV, also known as Andersen's disease, primarily occurs as a liver disease, it will not be mentioned in detail. However, it must be noted that several neuromuscular forms of GSD type IV have been reported (Fig. 14.9). Deficiencies of branching enzyme, encoded by the GBE1 gene, cause this disease [26]. If disease affects the muscles, there are several vacuoles in the myofibers. Materials that accumulate in the vacuoles are PAS-positive, but some of them are amylopectin. Amylopectin is a highly branched polymer of α-glucose units, mainly found in plants. It is more filamentous/amorphous than glycogen. These polyglucosan bodies are resistant to diastase alpha-amylase) digestion (Fig. 14.10) [26–29].

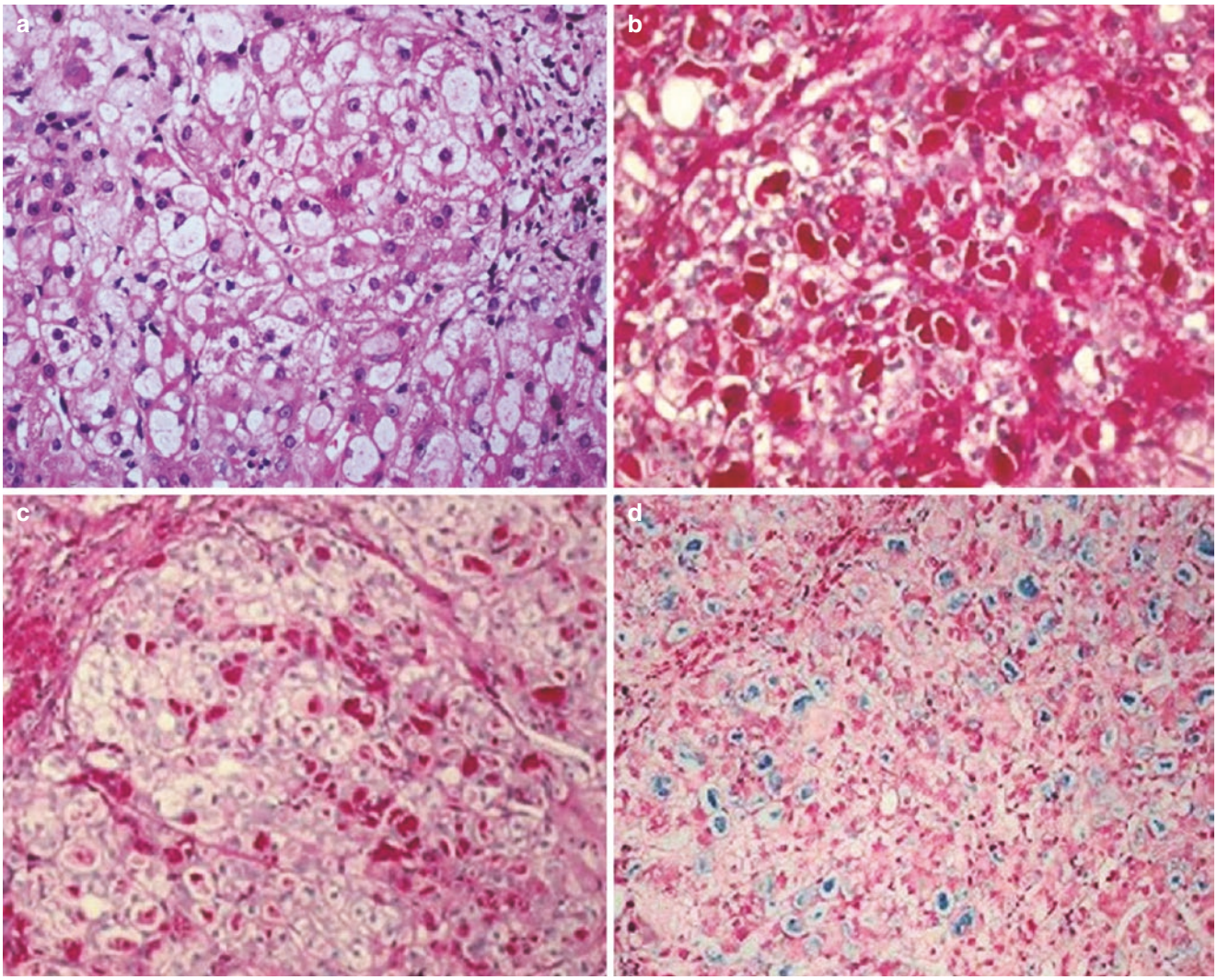

Fig. 14.9 Liver biopsy of a 4-year-old girl with Andersen's disease: (**a**) vacuolar differences in most hepatocytes (H&E × 200), (**b**) PAS-positive large globoid accumulations (PAS × 200), (**c**) some PAS-positive globules are resistant to diastase (dPAS ×200), (**d**) some accumulations are stained with colloidal iron stain (Colloidal iron ×100)

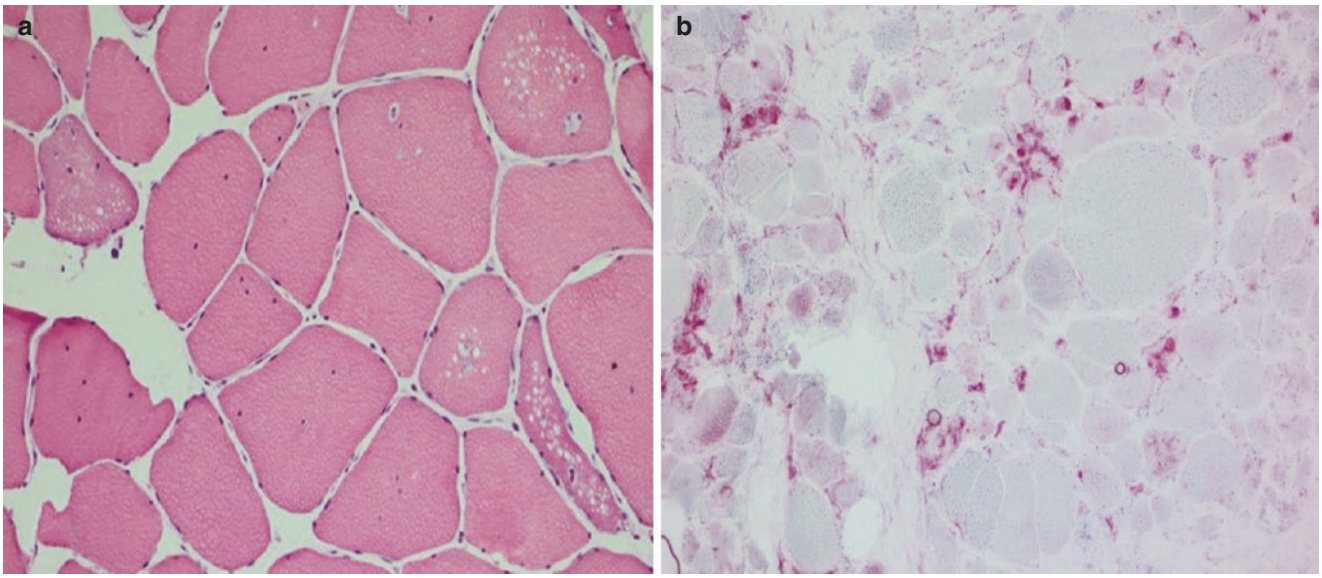

Fig. 14.10 (**a**) Note the presence of only a few fibers containing vacuoles (H&E × 200), (**b**) increased areas of enzyme activity (Acid Phosphatase ×200)

Mitochondrial Diseases

Mitochondria is an organelle found in all eukaryotic cells, originating from a proteobacterium that merged with the host cell because of the evolutionary process called endosymbiosis, approximately 2 billion years ago. It carries out many vital functions, especially energy production [31]. The most important feature that distinguishes mitochondria from other extranuclear intracellular organelles is undoubtedly that it has its own reproducible genetic material (Fig. 14.11). Mitochondrial DNA (mtDNA) is a circular, double-stranded structure consisting of 13 polypeptides, 22 transfer RNAs (tRNAs), 37 genes, and 16,569 base pairs containing two ribosomal RNAs (rRNA) [31]. A significant portion of these proteins are encoded by nuclear DNA (nDNA), and 13 of them are encoded by mtDNA. As a result, mitochondria are under the control of both nDNA and mtDNA, and this is called bigenomic or dual genetic control. Nucleated cells in different tissues in humans contain approximately 100–2000 mitochondria. The number of mitochondria can vary in different tissues, as well as in different cell groups in the same tissue. 10% of the more than 1500 proteins in the mitochondrial proteome (mitoproteome) directly affect oxidative phosphorylation (OXPHOS) and ATP production [32].

The most basic and critical difference between mtDNA and nDNA is that mtDNA is a multi-copy genome. This is very important for understanding the pathophysiology of mitochondrial diseases. The mtDNA copy number can be different in each tissue. A mature oocyte may contain >100,000 copies, whereas sperm may have <100 [33]. Duplications of the mtDNA genome can be homoplasmic (all mtDNA genome copies are identical) or heteroplasmic (combination of mtDNA genome copies with different genetic makeups). While nuclear genes are inherited in accordance with mendelian inheritance; the inheritance of mtDNA is maternal, as paternal mtDNA is actively broken down in the oocyte after fertilization. Although the exact mechanism is not known, there is evidence that mitochondrial endonucleases, which are activated to cleave mtDNA within the paternal mitochondria after fertilization, are responsible for paternal mtDNA degradation [34–39].

Mitochondrial Diseases

Mitochondrial diseases (MDs) are diseases that result in inadequate mitochondrial functions, especially OXPHOS, because of pathogenic variants in nDNA or mtDNA [40]. It is a group of diseases with high clinical heterogeneity, which can affect almost every organ in the organism, at any age, and can show clinical findings in a spectrum. Its frequency varies between 1/2000 and 1/5000 [41]. Since mitochondria are in all cell types except erythrocytes, isolated organ involvement is rarely seen, but it is usually associated with multiple system findings including brain, skeletal muscle, and heart muscle, which have high energy needs. Phenotype-genotype compatibility is very limited [42]. The formation of a phenotype can be caused by pathogenic variants in many

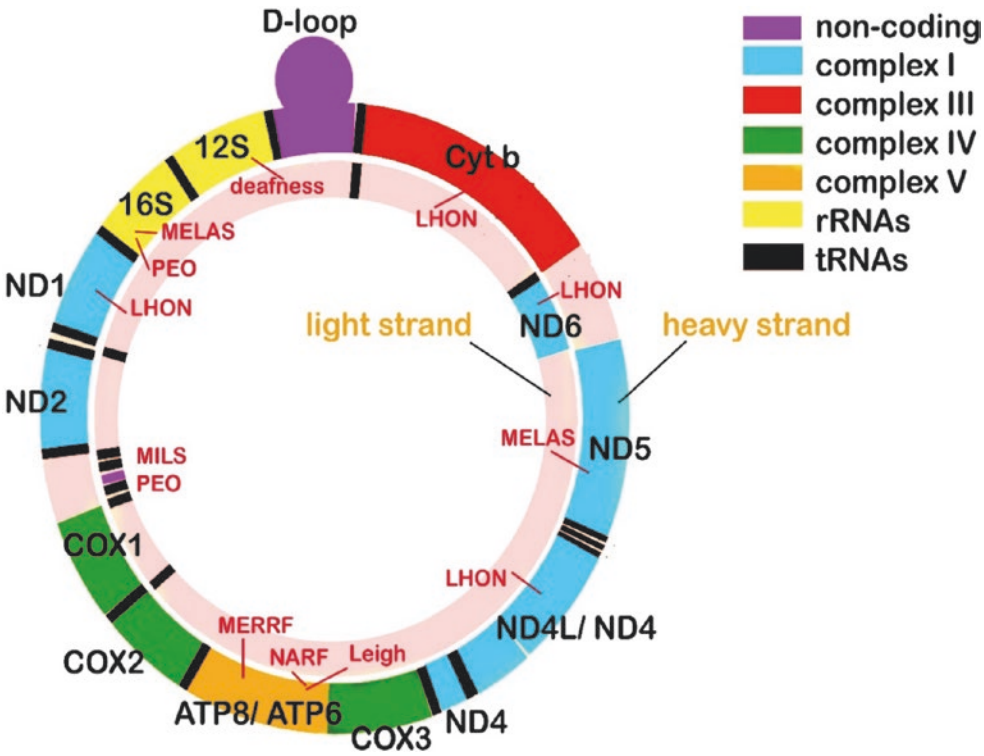

Fig. 14.11 Schematic appearance of mitochondrial DNA

different genes. Different pathogenic variants in the same gene may cause different phenotypes. One of the best examples of this is Leigh syndrome (LS), which is the most common phenotype of pediatric MD. This is a clinical condition characterized by progressive neurodegeneration that usually develops in childhood. As far as is known today, pathogenic variants in more than a hundred genes cause the LS phenotype [43].

The classification of MDs is basically divided into two groups: MDs caused by mtDNA and MDs caused by nDNA (Fig. 14.12). With this, in the historical journey of MDs, there are clinical conditions that are still very valuable due to their diagnostic guidance, which are known as mitochondrial syndromes due to the combination of different systemic findings in the periods when tissue and molecular features were not yet clarified.

Mitochondrial Diseases Caused by mtDNA

mtDNA has a much higher mutation rate of 10–17 times compared to nDNA, which causes increased oxidative stress in OXPHOS [44]. The first disease-associated mtDNA mutations were identified in 1988. Subsequently, MD-associated mtDNA mutations increased exponentially [45, 46]. Today, more than 300 mtDNA mutations have been associated with MDs. These mutations are grouped into three main groups: point mutations in protein-coding genes, point mutations in genes involved in protein synthesis (tRNA or rRNA genes), and mtDNA rearrangement, which includes mtDNA deletions and insertions. While point mutations tend to be maternally inherited; mtDNA rearrangements are mostly non-inheritable and consist of de-novo, large-scale mtDNA deletions [47, 48].

mtDNA shows maternal inheritance characteristics (Fig. 14.13). As a result, variants in mtDNA carried by the mother are passed on to all her children, but only daughters can pass them on to other generations. In the pathophysiology and diagnosis of mtDNA-associated MDs, there are two other concepts that should be understood in addition to the homoplasmy-heteroplasmy concept mentioned above. These are the concepts of 'mitotic segregation' and 'mitochondrial threshold effect'. Patients with mtDNA-associated MDs are usually homoplasmic for mtDNA mutations but may also be heteroplasmic. In the presence of heteroplasmy, the ratio of the mutant mtDNA copy number to the normal mtDNA (wild type) copy number in the tissue is an important factor in the occurrence of clinical findings and the severity of symptoms. This is known as the 'mitochondrial threshold effect'[49]. The mitochondrial threshold effect can be defined as the mutant mtDNA load required to produce a detectable biochemical defect in OXPHOS in each tissue. In many mtDNA

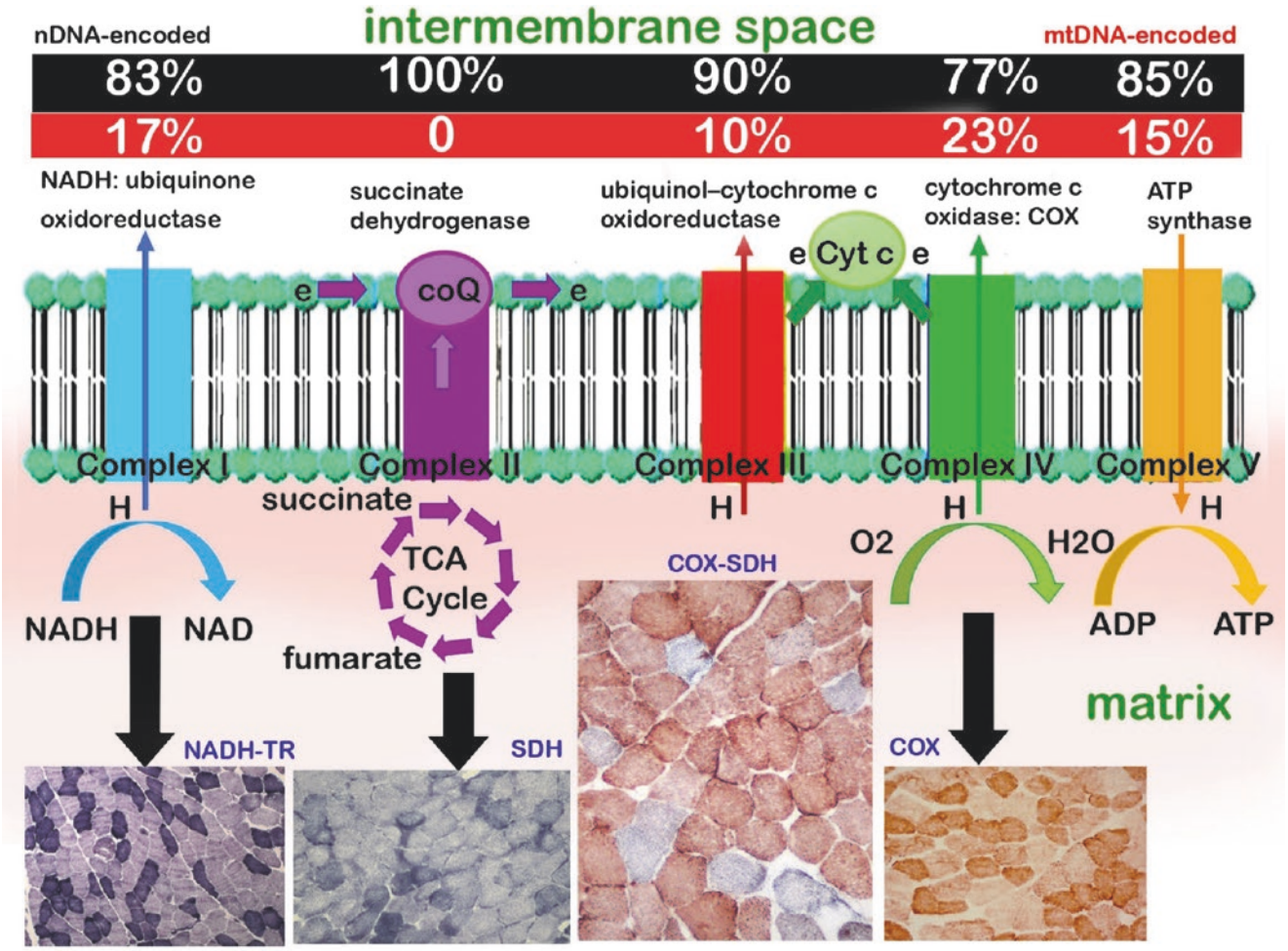

Fig. 14.12 Schematic appearance of the mitochondrial oxidative phosphorylation (OXPHOS) system

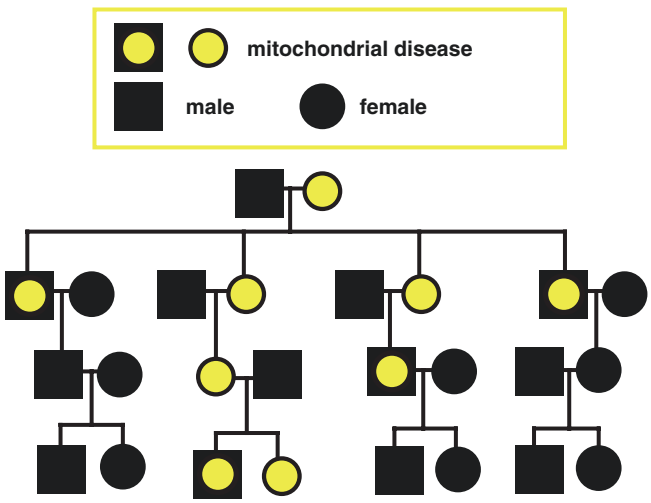

Fig. 14.13 Schematic view of maternal inheritance

inherited MDs, this rate is around 60–80%, but this rate can also vary from person to person. Mitochondrial division is not related to the timing of mtDNA or nDNA cleavage. In dividing cells, the segregation of mitochondria (transfer to daughter cells) is not subject to any rule. Therefore, daughter cells may have varying levels of mutant mtDNA copy numbers. This situation is called mitotic segregation [50].

Clinical findings in mtDNA-associated MDs are related to the energy requirement of the tissues and the mutant mtDNA copy number of the tissues in question, as in all MDs. The most common phenotype is Leber's hereditary optic neuropathy (LHON). LHON is mostly caused by homoplasmic mt.11778G > A (69%), mt.3460G > A (13%), and mt.14484 T > C (14%) changes in mtDNA. Men are affected more often than women. Painless, sudden onset vision loss, due to rapidly developing optic atrophy, are characteristic finding in young adolescents [51]. Another

common phenotype, MELAS, is characterized by mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes, and external ophthalmoplegia. It may progress with diabetes mellitus, hearing loss, early-onset strokes, migraine attacks, and cognitive impairment. It is caused by mt.3243A > G (80%), mt.3271 T > C (7%), and mt.3260A > G (5%) homoplasmic variants in the mitochondrial tRNA Leu gene [31].

Examples of other mtDNA-associated syndromes are MERRF [Myoclonic epilepsy and red ragged fiber (red striped fibers)], NARP (neuropathy, ataxia, retinitis pigmentosa) and Kearns-Sayre syndrome (KSS). MERRF, mtDNA tRNA mt.8344A > G (80%), mt.8356 T > C, mt.8363G > A, and mt.8361G > A (10%), clinical findings include ataxia, dementia, neuropathy, myopathy, multiple lipomas, hearing loss, neuropathy, and intellectual disability [52].

NARP and maternally inherited LS (MILS) occur depending on the ratio of mutant mtDNA copy number to wild-type mtDNA with the mt.8993A > C mutation. If the heteroplasmy rate is between 70 and 90%, NARP syndrome occurs; if the heteroplasmy rate is more than 90%, it presents with MILS [52]. LS is characterized by bilateral basal ganglia involvement, neurodegeneration, movement disorders, and congenital lactic acidosis [49].

KSS is caused by large-scale deletions in mtDNA. Clinical symptoms include the classic triad of progressive external ophthalmoplegia, pigmentary retinopathy, and heart blocks with onset before the age of 20. KSS is also associated with cerebellar ataxia and increased cerebrospinal fluid (CSF) protein levels, although these are minor findings [53]. KSS consists of a very broad clinical spectrum in which tubulopathy, gastrointestinal motility disorders, endocrine disorders, cardiomyopathy, basal ganglia calcification, and secondary leukoencephalopathy due to cerebral folate deficiency are seen [54]. KSS occurs because of large-scale mtDNA deletions, but in the nuclear RRM2B gene. It can also occur with multiple mtDNA deletions resulting from biallelic pathogenic variants [55, 56].

Case 14.7

A 13-year-old boy with no family history or consanguinity between his parents was investigated for a cardiac conduction defect in the pediatric cardiology unit. He had normal CK levels. Chronic progressive external ophthalmoplegia and pigmentary degeneration of the retina were also detected. During hospitalization, a muscle biopsy was performed to search for the etiology. In the muscle biopsy, COX enzyme deficiency and an increased number of mitochondria were detected with electron microscopy (Fig. 14.14). He was diagnosed with KSS based on clinical, histopathological, electron microscopic, and biochemical findings [57].

Pearson Syndrome (PS) is characterized by bone marrow-pancreas syndrome, transfusion-dependent anemia in early infancy, lactic acidosis, feeding difficulties, and growth retardation. A diagnosis is made based the presence of ring sideroblasts and vacuolized myeloid precursors in the bone marrow aspirate, as well as the demonstration of single large-scale deletions in mtDNA in peripheral blood, bone marrow aspirate, urinary epithelial cells, or muscle biopsy. Severe growth retardation, severe metabolic acidosis, and liver dysfunction are seen in some of patients, and mortality is very high in this group. In patients with milder clinical findings and those who survive the early infancy period, anemia is partially resolved, and the 'honeymoon period' may be observed until the signs of multisystem involvement, which are expected to appear later in life, are observed [58–60].

Mitochondrial Disease Caused by nDNA

More than 1500 different nuclear genes encode the mitoproteome. It has been determined that an increasing number of nuclear genes cause MDs. To date, pathogenic variants causing MDs have been identified in 318 different genes [32, 58]. As technological developments in both molecular and tissue level diagnostic methods increase, approximately 10 new nDNA-derived MDs are defined every year [35]. MDs caused by nDNA mutations are classified as: primary OXPHOS deficiency (MDs in this group are usually associated with LS syndrome), mtDNA maintenance defects (causing mtDNA depletion syndromes), mitochondrial translation defects, mitochondrial quality-control defects, metabolic defects, vitamin/cofactor metabolism defects, and other cellular defects which are not fully classified but cause mitochondrial dysfunction [58, 60].

Clinical Findings

Childhood-onset MD is usually quite severe and often fatal [61]. A significant proportion occurs as a result of nDNA mutations or mtDNA point mutations with very high mtDNA heteroplasmy levels. Clinical findings include hypotonicity, generalized weakness, growth retardation, dysautonomia, exercise intolerance, vomiting, seizures, and encephalopathy. The pattern of leukodystrophy and hypomyelination may be clues, helping to distinguish MDs from other white matter diseases, especially due to NDUFS1, DARS2, and ISCA2 gene defects. Renal involvement may result from defects in many different nDNA genes and may coexist with cystic disease (RMND1 gene defects) or proximal tubulopathy (RRM2B gene defects) [62].

Hypertrophic cardiomyopathy (HCM) can be isolated in AARS2 and MTO1 gene defects or can be observed with multisystem involvement in AGK gene-related MD [32].

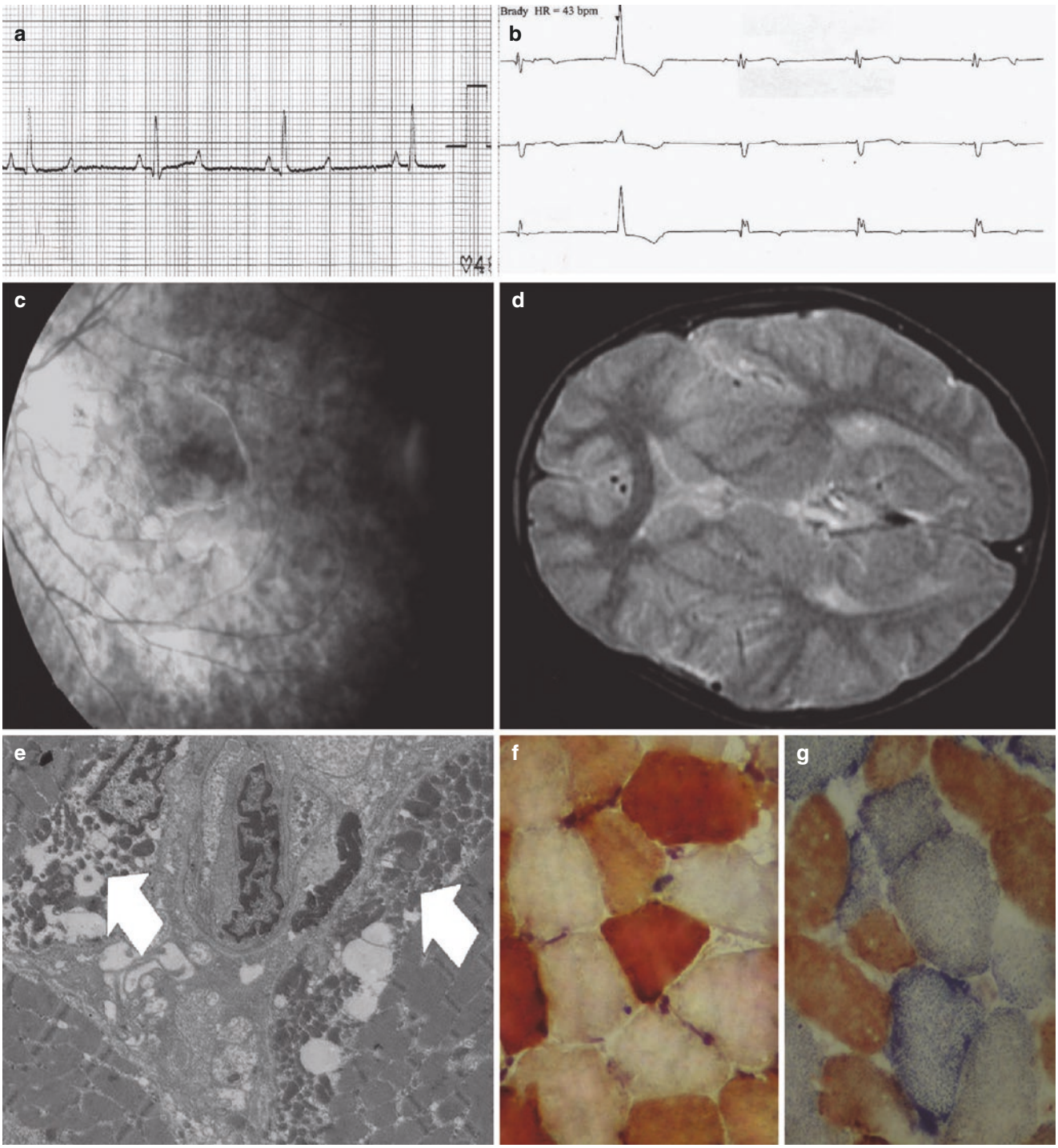

Fig. 14.14 (**a**) Bradycardia and heart block on electrocardiography, (**b**) bradycardia and heart block on holter monitoring, (**c**) pigmentary retinal degeneration at the optic fundus, (**d**) bilateral symmetrical hyperintensities in the basal ganglia, midbrain, and brain stem (magnetic resonance imaging), (**e**) subsarcolemmal mitochondria accumulation (arrows) in electron microscopic examination (×12,000), (**f**) note the staining of type 1 and type 2 fibers with varying intensity, as well as enzyme negative fibers (COX × 100), (**g**) ragged blue fiber appearance resulting from subsarcolemmal non-functional mitochondria (Combined COX-SDH stain ×100)

Hypertrichosis is an important distinguishing feature of SURF1 gene defect-associated LS. Sensorineural hearing loss is seen especially in SUCLA2, SUCLG1, RMND1, and RRM2B gene defects [63–65].

Leigh Syndrome (LS) is a subacute, necrotising encephalomyelopathy which was defined by Denis Leigh, a neuropathologist, in 1951 as bilateral symmetrical spongy lesions with demyelination and vacuolization in the basal ganglia

and brain stem, in the autopsy study of a 7-month-old baby who died with vision-hearing loss, growth retardation and lactic acidosis [64]. It has been identified as the most common phenotype of childhood MDs in the sixth decade after it was first described. It usually starts between the ages of 3 months and 2 years, but it can also be seen in older ages. More than hundred genes are known to cause LS. Although LS is a neuropathological diagnosis, the diagnosis of LS is made by clinical and brain imaging with T2 hyperintense lesions in the basal ganglia, striatum, and brain stem [65–67].

Mitochondrial DNA Depletion Syndrome

MtDNA depletion syndromes (MDDSs) were defined as a marked reduction in tissue mtDNA copy number compared to controls in the same age group. Although 30% is generally accepted as the limit, it is seen that the mtDNA copy number is below 10% in all affected tissues in severely affected infants. MDDSs are grouped according to the clinical presentation (myopathic, encephalomyopathic, hepatocerebral, and multisystem disease) or the underlying genetic etiology (mtDNA replication defects, mtDNA repair defects, nucleotide metabolism, and mitochondrial dynamics defects) [66]. Types of MDDS include:

- Early-onset MDDS with progressive myopathy occurring shortly after birth.
- RRM2B deficiency characterized by a loss of hearing, renal tubulopathy, and seizures.
- DGUOK and MPV17 deficiency with progressive liver failure, lactic acidosis, and encephalopathy.
- Alpers-Huttenlocher Syndrome (AHS) is characterized by progressive myopathy.
- TK2 deficiency is characterized by clinical findings including lactate and CK elevation, childhood-onset resistant epilepsy, neurodegeneration, and liver disease.

Although pathogenic mutations in the POLG gene are responsible for the etiology in a significant number of AHS cases, defects in the FARS2, NARS2, and PARS2 genes, encoding mitochondrial tRNA synthetase, can also cause AHS [67, 68]. One of the most important clinical presentations of MDDSs is mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). MNGIE arises due to biallelic pathogenic variants in the TYMP gene. It is characterized by progressive external ophthalmoplegia, gastrointestinal motility disorder causing recurrent ileus-like acute abdominal attacks, peripheral neuropathy, and diffuse leukoencephalopathy. It starts in childhood and death is usually observed in the fourth decade [69].

Congenital Lactic Acidosis

Lactic acidosis detected shortly after birth, may occur due to reasons such as hypovolemia, hypoxia, or sepsis, but hereditary metabolic diseases (HMDs) should be considered in the differential diagnosis. Congenital lactic acidosis is an important manifestation of MDs, such as pyruvate dehydrogenase deficiency (PDHD), pyruvate carboxylase deficiency (PCD), and primary OXPHOS defects [58].

When Should We Consider MD?

The diagnosis of mitochondrial myopathies involves a multidisciplinary approach. History and physical examination are crucial for recognizing that mitochondrial myopathy is a potential diagnosis, but also to suggest the most appropriate diagnostic studies. The diagnostic investigations include histological and immunohistochemical studies, enzymatic analysis of the OXPHOS complexes, and the genetic analysis of mtDNA. Additionally, if a nuclear genetic diagnosis is suspected, a targeted nDNA sequencing approach may be used. If no pathogenic mutation is identified, whole genome and whole exome screens are now commonly used to search for potential genetic diagnoses, with new disease genes constantly being identified. By integrating the information from these diagnostic tests, it allows for a diagnosis in most patients.

There is usually no pathognomonic finding for MDs. However, in the presence of early-onset hypotonicity, or in the presence of a loss of acquired motor functions, multiorgan involvement, hearing loss, vision loss, cardiomyopathy, heart blocks, renal tubulopathy, stroke-like attacks, acquired ptosis and/or ophthalmoplegia, sideroblastic anemia, or a combination of these findings, one should consider MDs (Fig. 14.15). The most important step in the diagnostic process of MDs is clinical suspicion, as in other inherited metabolic disorders. The most important data that can create this clinical suspicion is the history of the patient. One of the most important stages of MD suspicion is taking a complete patient history. The findings of a significant portion of MDs manifest themselves in the first few months of life. During this period, the patient's nutritional history, weight-gain rate, speed of gaining or loss of motor developmental steps compared to other children in the same age group, whether there is a vision problem in children who can start school, history of cataract operations, and history of tiring easily compared to peers should be questioned. Patients who describe exercise intolerance or have external ophthalmopathy, should be especially questioned whether their mother or siblings have similar findings [65–68].

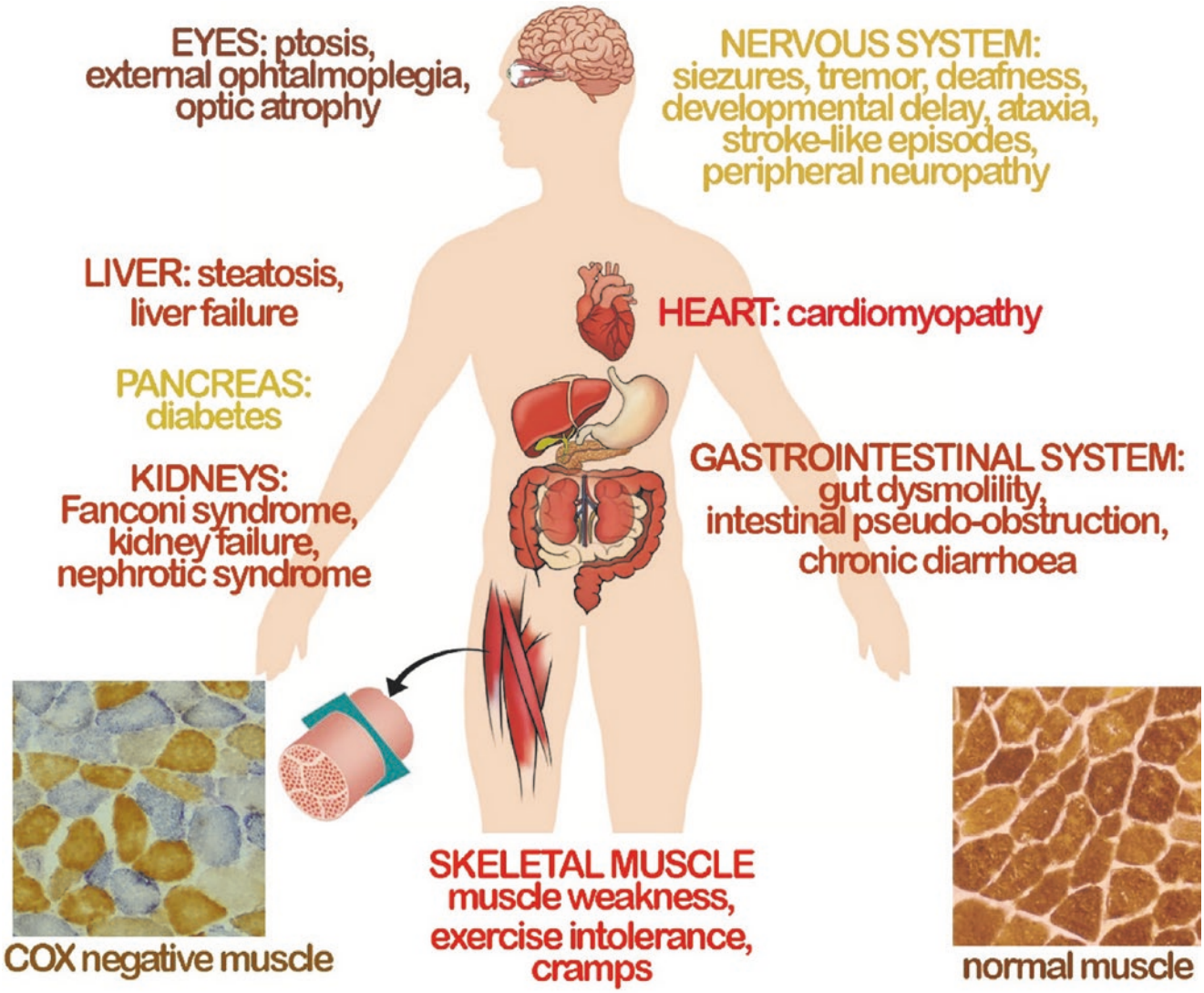

Fig. 14.15 Mitochondrial disease symptoms and skeletal muscle biopsies

Biochemical Studies

Spectrophotometric evaluation of individual respiratory chain complex activities is an important approach in the biochemical investigation and diagnosis of mitochondrial myopathies. It can be performed in either fresh or frozen muscle homogenate; however, the latter is more common in diagnostic centers due to cross-continental or national referral of patients. Each complex can be analyzed in isolation following the oxidation/reduction of specific substrates or substrate analogues. The spectrophotometric enzyme assays are as follows: NADH:ubiquinone oxidoreductase for complex I, succinate:cytochrome c oxidase oxidoreductase for complex II, ubiquinol cytochrome c oxidoreductase for complex III, and cytochrome c oxidase for complex IV. The measurement of complex V (oligomycin-sensitive ATP synthase) is more challenging and requires the use of fresh material and is often measured in cultured skin fibroblasts. It is recommended 20–30% of normal complex activities as a criterion for the diagnosis of mitochondrial myopathies. However, a normal respiratory chain enzyme activity does not exclude the diagnosis of MD as a small percentage of respiratory chain deficiency cells may not be detectable by enzyme measurements on tissue homogenates. Another biochemical study, which proves to be a helpful step in the diagnosis of mitochondrial myopathy is the blue native acrylamide page (BN-PAGE) assay, which is used to assess the relative abundance of fully assembled respiratory chain enzyme complexes. Like the spectrophotometric assay, a deficiency of the complexes can be seen as an isolated single complex or a combined deficiency [69–71].

Neuroimaging

The most common changes observed in the radiological evaluation of MDs are gray matter involvement, leukodystrophy, and atrophy. Magnetic resonance imaging findings observed in LS include brain stem, thalamus, and basal ganglia symmetrical hypodensities, bilateral striatal necrosis, tegmental tract involvement, diffuse cavitation, and leukoencephalopathy [71].

All About the Pathology of Mitochondrial Diseases

Unfortunately, the definitive pathological diagnosis of mitochondrial diseases, which present with a wide variety of findings and different mutations, is very difficult. Whereas, especially in diseases presenting as myopathic disease, diagnosis is very easy with the help of enzyme staining [57]. In the presence of defective mitochondria due to any mutation in the mitochondrial or nuclear genome, mitochondria constantly divide and multiply to meet the energy needs of the cell. However, this proliferation does not provide any benefit other than the formation of new defective mitochondria. As a result, while different clinical findings develop due to the inability to meet the energy requirements of the cell, defective mitochondria accumulate in clusters. This accumulation is especially dense in the subsarcolemmal area and is easily observed in electron microscopic examination (Fig. 14.16). However, the utility of electron microscopic examination in mitochondrial diseases is still limited because non-functional defective mitochondria can accumulate due to aging and oxidant stresses. It is reported that the muscle fiber ratio with mitochondrial accumulations in advanced age can be around 5%. Therefore, the increase in the ratio of these fibers needs to be studied in larger areas. On the other hand, subsarcolemmal mitochondrial accumulation may be more exaggerated in children than in adults, and it is extremely difficult to detect whether these are non-functioning defective mitochondria on electron microscopic examination [26–30, 57].

Just as mitochondria are concentrated in tissues with high energy needs, they also concentrate in areas of the cell with high energy needs. In striated muscle, type 1 fibers are denser and are found in large numbers between myofibrils, adjacent to the Z disc, under the sarcolemma, at the nucleus margin, and in the motor endplate. When the accumulated defective mitochondria reach a certain density, they form hematoxyphilic deposits that can be distinguished even in hematoxylin-eosin sections (Fig. 14.17) The same accumulation is seen as red deposits with the modified Gomori trichrome dye applied to the frozen sections, and such muscle fibers are called "ragged red" fibers (Fig. 14.18). However, the formation of histochemically visible red lumps requires a much greater accumulation of mitochondria and may not be seen, especially in mitochondrial myopathies in early childhood [57].

The cytochrome oxidase (COX) enzyme, tested in the enzyme staining panel applied to muscle biopsies, plays a role in the fourth stage of the respiratory chain and is the most concrete indicator of oxidative phosphorylation in mitochondria. It is encoded by both nuclear and mitochondrial DNA. Succinate dehydrogenase (SDH) enzyme is encoded by pure nuclear DNA. It is an enzyme that plays a role both in the second stage of the respiratory chain and in the citric acid cycle. Therefore, SDH activity is almost always present in mitochondria. In other words, SDH enzyme activity only shows the presence of mitochondria. However,

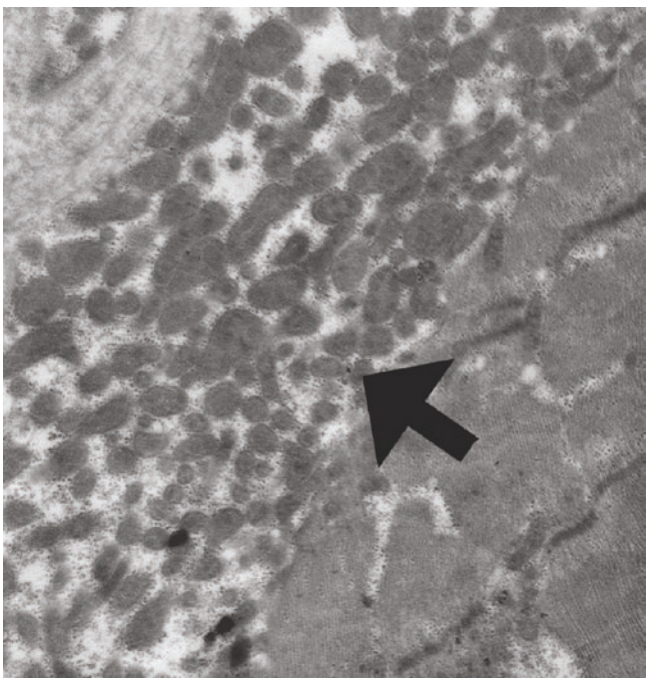

Fig. 14.16 Increased subsarcolemmal mitochondrial storages (TEM × 15,000)

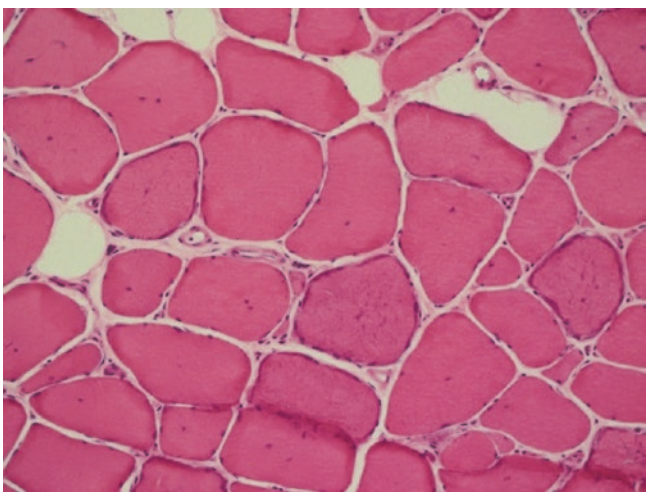

Fig. 14.17 Basophilic subsarcolemmal mitochondrial accumulation (H&E × 200)

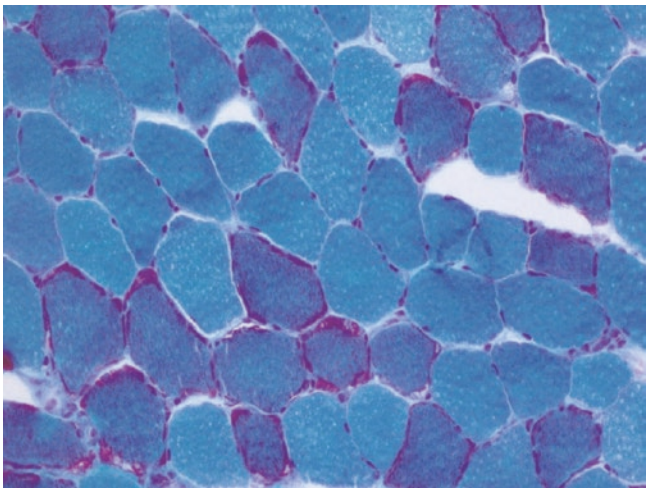

Fig. 14.18 Ragged red fibers (Modified trichrome ×200)

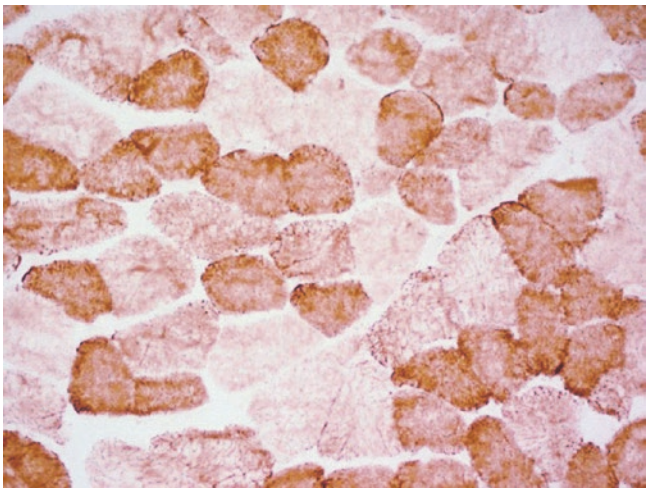

Fig. 14.19 Note the presence of COX-negative fibers (COX × 200)

if there is no COX enzyme activity, these mitochondria are defective, in other words non-functional (Fig. 14.19). Even more limited mitochondrial deposits can be demonstrated with the SDH enzyme stain, which is much more sensitive than histochemical staining. Mitochondrial accumulation, which can be demonstrated by SDH, leads to the formation of "ragged blue" fibers [57].

An absence of COX activity in fibers with a "pathological blue fiber" appearance in combined COX-SDH enzyme staining, shows that mitochondria in that cell are not capable of oxidative phosphorylation and provides a definitive diagnosis of mitochondrial myopathies (Fig. 14.20). In summary, in diseases where the accumulation of defective mitochondria is not exaggerated and in early childhood, revealing COX and SDH enzyme activity in muscle fibers with enzyme dyes is the only way to definitively diagnose mitochondrial diseases. It has been reported that even a genetic diagnosis may be insufficient due to organ specificity and heterogeneous distribution of defective mitochondria to tissues, especially in childhood mitochondrial diseases manifested by different presentations defined in recent years [57].

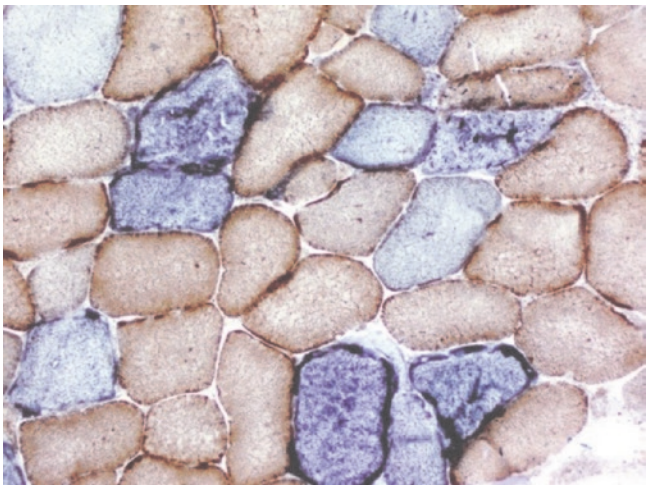

Fig. 14.20 Pathological blue fibers (combined COX-SDH × 200)

In summary, it must be kept in mind that the mitochondria, as the "power plants" of the cells, may cause all kinds of disorders. For example, diabetes mellitus and cardiovascular diseases, the most prevalent diseases worldwide, are closely associated with abnormalities in the structure and function of mitochondria [72, 73]. Similarly, a relationship between Alzheimer's disease and mitochondrial dysfunction has been suspected for a long time [74]. Recently, the association of inflammatory processes with mitochondrial dysfunction has also been reported. In some research, mitochondrial dysfunction is accused of being a reason for the vicious circle in neurodegenerative disorders [75–77].

Therefore, evaluations of the enzyme activities, which play roles of OXPHOS in the mitochondria, are the most important steps of the muscle biopsy evaluation. Sometimes, during muscle biopsy evaluation, like in the reported case (Case 14.8), very surprising findings can be observed.

Case 14.8

A 10-year-old girl presented to clinic with complaints of pain in her arms, shoulders, legs, and knees with a gait disorder and weakness in the arms and legs, which began 6 months ago and progressed in the last month. She was the second born baby of non-consanguineous parents, delivered at term with no significant perinatal problems. There is a history of celiac disease and diabetes in her 14-year-old sister, goiter in her mother, and multiple sclerosis in her paternal uncle. Neurological examination revealed that her bilateral proximal upper and lower extremity muscle powers were decreased. Her CK levels were 4694 U/L (5–130), CK-MB was 106 U/L (0–2), C-reactive protein was 1.46 mg/dL (0.0–0.8), and erythrocyte sedimentation rate was 32 mm/h (0–20). Electromyography was also consistent with myopathy. Magnetic resonance imaging revealed an inflammatory process around the proximal muscles of the lower extremi-

ties. The patient was diagnosed with juvenile polymyositis, and thereafter, oral prednisolone (2 mg/kg/day) treatment was started without muscle biopsy due to consent being withheld by the caregivers. The patient encountered no response to steroid treatment. After the second week of prednisolone treatment, pneumonia, urinary tract infection, and oral candidiasis were observed, as complications of immunosuppression, due to multi-systemic infection, were treated properly. Due to no response to 18 days of prednisolone treatment, pulse steroid therapy was given for 3 days. However, the patient showed no clinical response. Thereafter, muscle biopsy was considered with her family and performed uneventfully. Biopsy revealed only mild inflammatory infiltration that may have been suppressed with corticosteroid treatment. In addition, there was inconspicuous perifascicular atrophy and numerous COX negative myofibers (Fig. 14.21). The patient was diagnosed with COX-negative inflammatory myopathy, a subtype of inflammatory myopathies, which is generally resistant to therapy and occurs in older patients. Her prednisolone dose was

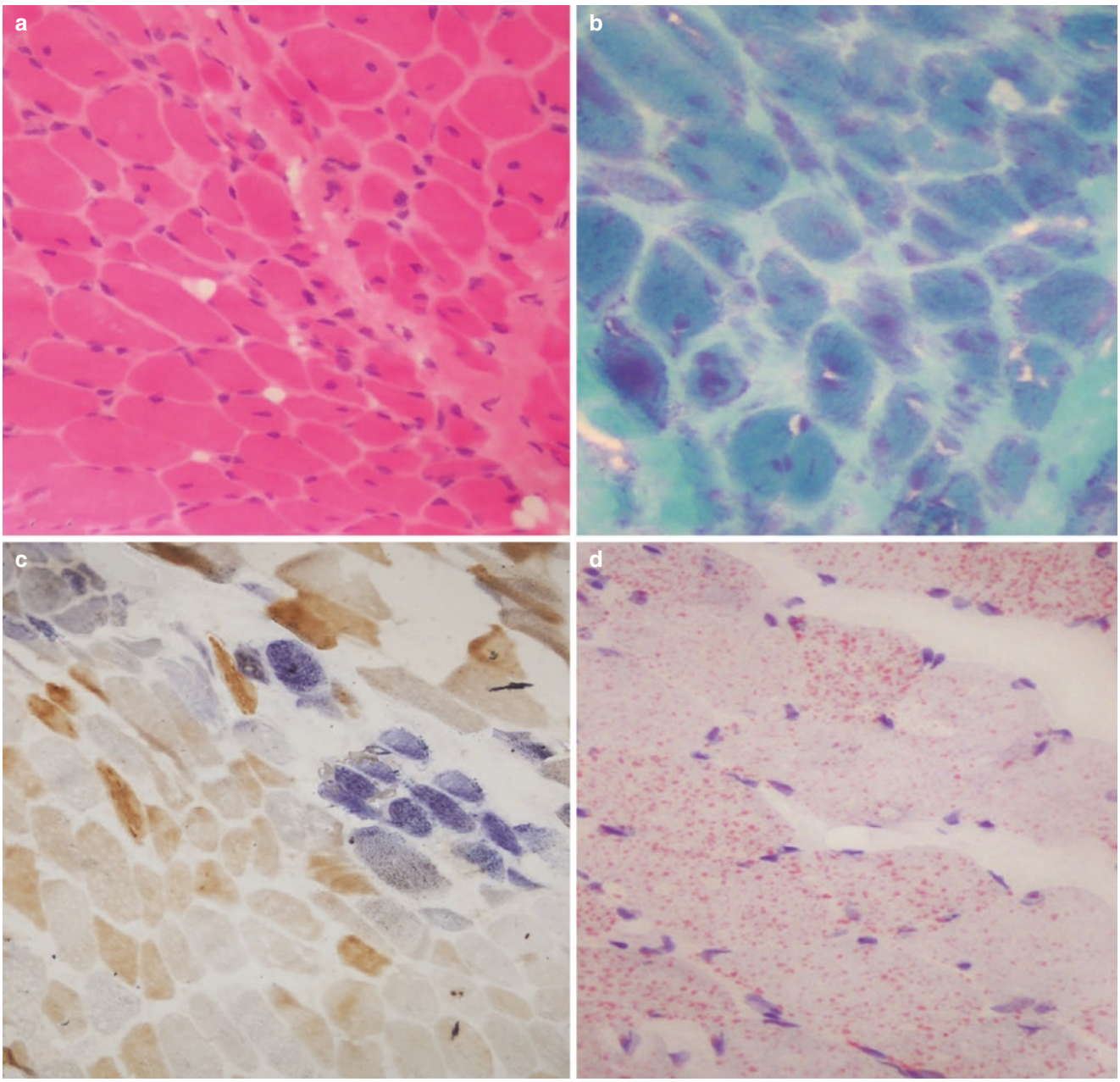

Fig. 14.21 Muscle biopsy of case 14.8 with inflammatory myopathy: (**a**) perifascicular atrophy (H&E × 100), (**b**) myofibers reminiscent to ragged red fibers (Modified trichrome ×200), (**c**) pathological blue fibers (Combined COX-SDH stain ×100), (**d**) mild lipid accumulations in the muscle cells (Oil red-O × 400)

decreased, and methotrexate (250 μg/kg/week) was added to the treatment. With recent therapy, her complaints decreased over several months and outpatient follow-up continues at the pediatric immunology clinic. She has been well now for 10 years after her diagnosis. Additionally, during that time, mtDNA sequence analyses identified 19 mutations in the rRNA, ND2, ND4, ND5, COX1, COX3, and CytB genes of the mitochondrial DNA of the patient and her mother. These mutations generally caused the production of synonym amino acids (silence mutations). However, m.8860A > G, Thr112Ala and m.9070 T > G, Ser182Ala variations at the ATP6 gene, m.10907 T > G, Phe50Leu variation at the ND4 gene, and m.15326A > G, Thr194Ala variation at the CytB gene were missense mutations and they have caused structural changes in amino acids. None of these mutations have been previously reported as pathogenic variants. We have thought that these variations in such essential genes might destabilize mitochondrial DNA and could probably affect the ATP synthesis in our patient. This development reminded us of the analogy of whether the chicken comes out of the egg, or the egg comes out of the chicken. Our final diagnosis was myositis due to an abnormal inflammatory response, induced by a hereditary mtDNA defect, rather than an inflammatory myopathy with mitochondrial dysfunction.

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Approach to the Differential Diagnosis of Hypotonia in Infancy

15

Atilla Ersen and Gulden Diniz

In this chapter, diseases that cause motor neuron dysfunction, such as spinal muscular atrophy (SMA), will be explained. They are characterised by hypotonia mostly in early infancy, which may cause difficulty in differentiating motor neuron diseases from congenital myopathies (Fig. 15.1). At the end of this chapter, under the title "All about the pathology of motor neuron diseases", case examples, surprising cases, short clinical histories, and histopathological pictures will be presented.

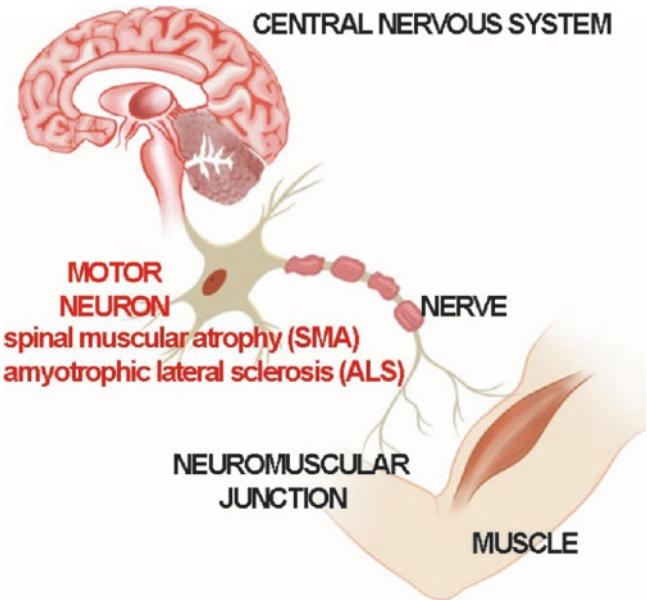

Fig. 15.1 Origination of spinal muscular atrophy and amyotrophic lateral sclerosis

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Motor Neuron Diseases of Childhood

Spinal Muscular Atrophy

Spinal muscular atrophies (SMAs) are a group of neurodegenerative disorders that cause progressive muscle weakness and atrophy characterised by the loss of anterior horn cells in the spinal cord and lower brainstem. Compared to the first description of SMA by Werdnig and Hofmann in the early 1890s, the understanding of the clinical and genetic spectrum of the disease has changed dramatically due to rapid molecular and genetic advances over the past decades [1–3]. Electromyography and muscle biopsies indicating denervation were once used as the basis for diagnosis; however, molecular testing has since replaced these techniques as the core diagnostic method for SMA [4, 5]. The spectrum of SMAs can be classified based on the age of onset, the pattern of muscle involvement, and inheritance pattern. SMA is also categorised according to "survival of motor neuron" (SMN) gene-related SMAs, termed 5q SMAs and non-5q SMAs, which are based on molecular genetic findings [3–5]. SMA often refers to the most common form, which accounts for 95% of patients, caused by homozygous deletions or mutations involving the SMN1 gene located on chromosome 5q13. However, a small portion of disorders termed SMA-like motor neuron disorders or SMA plus syndromes that affect motor neurons are not related to SMN1 gene mutations and may present with additional clinical features, other than flaccid weakness, hypotonia, decreased reflexes, and varying degrees of atrophy [3–6].

Although the most common form is 5q SMA, numerous gene mutations and significant phenotypic variability have been identified with current genetic and further comprehensive investigations. A new approach to diagnosis and increased awareness about SMA enables earlier diagnoses and improves access to treatment options through the implementation of newborn screening programs and the development of disease-modifying treatments. However, challenges still remain to overcome the disease course [6–8].

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Clinical Presentations

SMN1-Related SMA (5q-SMA)

It is an autosomal recessive motor neuron disease with an incidence of 1 in 6000–11,000 live births and a carrier frequency of 1 in 40–60 adults [3, 6, 9]. The clinical features of SMA are progressive muscle weakness and atrophy, which are caused by the degeneration of α motor neurons in the anterior horn of the spinal cord. The disease typically begins in infancy or childhood with the core clinical features of muscle weakness, atrophy, profound hypotonia, and hypo- or areflexia. The weakness is usually symmetrical and predominantly proximal, and there is relative sparing of the diaphragm, extraocular, and facial muscles. Lower limbs are more involved than the upper limbs, and deep tendon reflexes are generally absent or diminished. Despite the relative sparing of the diaphragm, respiratory insufficiency is an important complication of 5q SMA [3, 6, 7].

Genetic studies showed that two nearly identical survival motor neuron (SMN) genes (SMN1 and SMN2) are present on each chromosome 5, forming an inverted duplication at locus 5q13 [6]. SMN1 is the telomeric copy of the SMN gene and SMN2 is the centromeric copy, differentiated from SMN1 by 5 nucleotide changes. The critical difference between SMN1 and SMN2 is a C to T transition at the sixth position in exon 7 of the SMN2 gene, leading to an alternatively spliced mRNA isoform that lacks exon 7. This C > T change within the coding sequence affects splicing and creates an exonic splicing suppressor in exon 7, leading to the exclusion of exon 7 in most transcripts. Consequently, ~90% of transcripts from SMN2 lack exon7. The exclusion of exon 7 in most transcripts reduces functional SMN proteins production [4, 6, 10–13]. Although the SMN2 gene predominantly produces a shortened, unstable, and rapidly degraded isoform, alternative splicing events in the SMN2 gene cause ~10% of SMN2 transcripts to include exon7 and produce some full-length SMN proteins [14–18]. Consequently, the number of SMN2 copies and the level of the full-length SMN proteins created by SMN2 transcripts directly impact disease severity. Severe forms of SMA typically have one to two copies of SMN2, whereas milder forms are mostly associated with three to four copies of SMN2 (Fig. 15.2) [19].

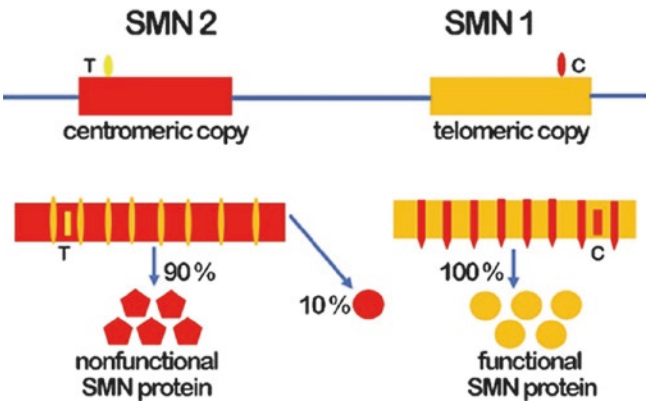

Fig. 15.2 The SMN1 and SMN2 genes are responsible for production of SMN protein. The C > T transition in exon 7 of the SMN2 gene causes the production of nonfunctional SMN protein

The spectrum of clinical severity is classified into clinical subtypes based on symptom severity, determined principally by motor milestones attained and age of onset (Table 15.1). Motor milestones are categorised as non-sitters for type I, sitters for type 2, and walkers for type 3. The disease represents a phenotypic continuum with a spectrum of severity from the very severe form, with onset in utero, to the very mild, with onset during adulthood [6, 20, 21]. Age at onset is mostly used in SMA classification, but it is not sufficient as a sole determinant of disease subtype due to overlapping subtypes, genetic variabilities, and treatment effects on the clinical picture [6, 19, 22].

Type 0 SMA, which is occasionally subclassified as SMA type 1A, is the most severe phenotype. Prenatally, patients may display decreased intrauterine movements and contractures. They cannot achieve any motor milestones with generalised weakness and hypotonia, respiratory distress, and poor feeding at birth [6, 19, 20].

Type 1 SMA is the most common subtype and accounts for approximately 45% of cases. Most patients with type 1 SMA have one or two copies of the SMN2 gene. The disease onset is usually before 6 months of age, they do not achieve independent sitting, and without respiratory support, respiratory

Table 15.1 Classification of spinal muscular atrophy (SMA)

SMA type	Copies SMN2	Onset	Motor milestones	Clinical features	Natural history prior to disease-modifying therapy
0	1	Prenatal, at birth	Non-sitter, No head control	Generalised weakness, hypotonia, respiratory failure, poor feeding, contractures	Death within weeks of birth
1	1–2	0–6 months	Non-sitter	Proximal predominant weakness, respiratory insufficiency, poor feeding, tongue fasciculations	Death by age 2
2	3	6–18 months	Sits independently, never stands or ambulates	Proximal predominant weakness, tongue fasciculations, minipolymyoclonus, scoliosis	Most alive at 25 years
3	3–4	A:1.5–3 years B: 3–30 years	Ambulates independently	Proximal, lower extremity predominant weakness, abnormal gait	Normal lifespan
4	4 or more	>30 years	Ambulates independently	Maintain ability to ambulate	Normal lifespan

failure usually leads to death within the first 2 years. They have poor head control, hypotonia with predominantly proximal limb weakness, areflexia, and tongue fasciculations, and they eventually have difficulty swallowing and poor feeding. Respiratory insufficiency, weakness of the intercostal muscles with relative sparing of the diaphragm, and a risk of aspiration are also life-threatening processes. Prominent findings in examinations include a bell-shaped chest, a pattern of paradoxical or "belly"-breathing, "frog-leg" posture, and "slip through" on vertical suspension [3, 6, 19]. Treatment modalities for nutritional and respiratory support facilitate some improvements in the progression of SMA with a 30% decrease in mortality rate at 2 years. About half of the survivors beyond age 2 are fully dependent on noninvasive ventilation. SMA was thought to be a purely motor neuron disorder, manifestations affecting various organs, such as the brain, cardiac, vascular, and even sensory nerves have been noted. Congenital heart disorders, autonomic dysfunction, vasculopathy, sensory nerve pathology, and axonal degeneration have been reported to be affecting SMA 1 patients [23–27].

Type 2 SMA, an intermediate form of SMA, accounts for 30% of cases and patients most often have three SMN2 copies. Progressive proximal weakness, hypotonia, and areflexia appear between 6 and 18 months. The patients can sit unsupported (sitters), often by 9 months, but are never able to stand alone or walk independently. Progressive scoliosis, due to weak intercostal musculature, may worsen respiratory insufficiency in the disease course. Joint contractures, tremors, polyminimyoclonus of the hands, and tongue atrophy/fasciculations may be prominent features on examination [6, 19]. Proper supportive treatments prior to the onset of disease-modifying therapy lead to an increased lifespan and patients are expected to survive till adulthood [3, 22].

Type 3 SMA, also called Kugelberg-Welander disease, represents the milder spectrum of patients known as "walkers" with onset occurring from 18 months to adulthood. These patients may be subdivided into type 3A with symptom onset between 18 months-3 years, and type 3B with onset after 3–30 years. These patients may able to stand and walk without support at some point. Progressive proximal weakness with an abnormal gait and difficulty climbing stairs is a major concern and patients may need a wheelchair during the disease course. *Type 4 SMA* can be subclassified with symptom onset occurring at 30 years or more. The milder type 3 and 4 patients usually present with 3 and 4 copies of SMN2 [6, 19, 22].

Genetics

SMN protein is essential for every cell, playing a role in small nuclear ribonucleoprotein biogenesis, translation, transcription, microRNA metabolism, stress granule formation, cell survival, ubiquitin homeostasis, DNA damage response, actin cytoskeleton dynamics, endocytosis, vesicular transport, vesicle trafficking along neurons, and energy homeostasis [22, 28, 29]. Humans are the only species with two SMN genes, SMN1 and SMN2. SMN1 differs from SMN2 with a C-to-T transition that creates an exon splicing suppressor in exon 7 of SMN2. This disrupts an exonic splice enhancer in exon 7 and because of this change, most SMN2 transcripts lack exon 7, creating an incomplete and degraded SMN protein. An estimated 10% of the protein produced by each SMN2 copy is functional, making it a modified gene [19, 22]. Regardless of disease severity, the majority of SMA cases are caused by an absence of the SMN1 gene, with 96% showing homozygous deletions in SMN1 and 4% carrying point mutations. De novo mutations occur at a rate of about 2%, which is relatively high [6, 22, 30]. Although disease severity inversely correlates with the SMN2 copy number, additional variants within the SMN2 gene or independent modifiers, such as increased expression of plastin 3 or downregulation of the neuronal calcium sensor, neurocalcin delta, can further influence disease severity [31].

Diagnostic Testing

Since a homozygous deletion in SMN1 exon 7 is responsible for 95% of SMA cases, deletion analysis of the SMN1 and SMN2 genes with multiplex ligation probe amplification (MLPA) is currently the most appropriate genetic test for establishing the initial diagnosis [19, 22]. The test is convenient, highly sensitive, and capable of determining both SMN1 and SMN2 copy numbers. MLPA with sequence analyses can detect compound heterozygotes and an intragenic point mutation in the other allele. However, it will not detect exonic deletions or duplications. Polymerase chain reaction-based targeted mutation analysis can also be used to detect homozygous deletions or carriers (i.e., heterozygous deletion) [32–34].

The SMN1 exon 7 deletion tests are reliable and accurate confirmatory tests for the majority of SMA patients with 95% sensitivity and nearly 100% specificity. The first step in the diagnostic evaluation of patients with suspected proximal SMA is tested for homozygous deletions of the SMN1 gene. In patients without homozygous SMN1 deletions with features of proximal type SMA, a measurement of the SMN1 copy number will guide further investigations. A single SMN1 copy may suggest compound heterozygosity, with a deletion on 1 allele and a point mutation on the other, and SMN1 sequencing is indicated [3, 30]. When 2 SMN1 copies are identified, then a review of clinical features, measurement of creatinine kinase, and neurophysiological studies with repetitive stimulation may help to differentiate alternative diagnoses such as another motor neuron disease, myopathy, muscular dystrophies, meta-

bolic disorders, and neuromuscular junction disorders. Although biopsies are no longer the first-line diagnostic approach and are unnecessary in most cases, muscle or nerve biopsy may be required in accordance with characteristic patterns of neurophysiological studies. Additional investigations including magnetic resonance imaging of the brain and spinal cord, as well as metabolic and genetic studies may be undertaken [3, 19]. The differential diagnosis of SMA is detailed in Table 15.2 [34].

Treatment Modalities

Many clinical trials are in progress, investigating small-molecule SMN enhancers, antisense oligonucleotides to correct SMN2 splicing, neuroprotectants, stem cell and gene therapies, and regulators of muscle function. However, there is currently no effective treatment for SMA [3, 6]. Treatment mainly consists of controling the symptoms, preventing complications, and supportive interventions which remain crucial for SMA patients. Recent research, especially in the molecular genetic field, have led to the development of novel disease-modifying therapies. Three different disease-modifying treatments were introduced over the past decade: nusinersen, onasemnogene abeparvovec, and risdiplam. These agents function to increase functional SMN protein production, by altering SMN2 RNA to produce full-length SMN protein, or to deliver the SMN1 gene via a viral vector [7, 19].

Table 15.2 Differential diagnosis of 5q spinal muscular atrophy

Spinal cord disorders
Neoplasms (SMA types I, II, and III)
Other myelopathies (SMA types I, II, and III)
Other motor neuron disorders
SMARD1 (SMA type I)
Other non-5q SMAs (SMA types I, II, and III)
Neuropathies
Congenital hypomyelinating or axonal neuropathies (SMA types I and II)
Hereditary motor and sensory neuropathies (SMA types I, II, and III)
CIDP (SMA types II and III)
Neuromuscular junction disorders
Botulism (SMA type I)
Congenital myasthenic syndromes (SMA types I, II, and III)
Lambert-Eaton myasthenic syndrome (SMA type III)
Autoimmune myasthenia gravis (SMA types II and III)
Myopathies
Congenital myopathies (SMA types I, II, and III)
Congenital myotonic dystrophy (SMA type I)
Congenital muscular dystrophies (SMA types I and II)
Muscular dystrophies (DMD/BMD or LGMD) (SMA type III)
Mitochondrial myopathies (SMA types I, II, and III)
Acid maltase/Pompe disease (SMA types I, II, and III)
Other metabolic myopathies (SMA types I, II, and III)
Inflammatory myopathies (SMA type III)
Channelopathies (SMA type III)

BMD becker muscular dystrophy, *CIDP* chronic inflammatory polyneuropathy, *DMD* duchenne muscular dystrophy, *LGMD* limb girdle muscular dystrophy, *SMA* spinal muscular atrophy, *SMARD1* spinal muscular atrophy with respiratory distress 1

Nusinersen (Spinraza) is an antisense oligonucleotide (ASO) that increases the expression levels of SMN protein by altering the splicing of the SMN2 transcript. It hybridises to a target in SMN2 intron 7, blocking the binding of heterogeneous nuclear ribonucleoproteins, thereby preventing the assembly of the spliceosome. The net result is the retention of exon 7 and expression of full-length SMN protein [7, 35–37]. It requires intrathecal administration and is given as a series of 4 loading doses over 2 months, followed by maintenance doses every 4 months.

Risdiplam (Evrysdi) is a small molecule that alters SMN2 splicing, with the ease of oral administration. Compared to ASOs, small molecules also have the advantage of having significant activity outside the central nervous system. The Food and Drug Administration (FDA) approved the drug in August 2020 [7].

Onasemnogene abeparvovec (Zolgensma) is a gene therapy that uses an adeno-associated virus capsid to deliver a copy of the SMN1 gene, via an adeno-associated virus serotype 9 vector, to increase low functional SMN protein levels. It was approved for the treatment of SMA by the FDA in May 2019 for children less than 2 years old and it offers a one-time treatment for SMA [7, 19].

Non-SMN1-Related SMA (Non-5q SMAs)

An autosomal recessive condition, caused by loss-of-function SMN1 mutations on 5q13, is the most common form of SMA, but less than 5% of infantile SMA cases are not related to the SMN gene; they are called non-5q forms of SMA. Although very rare, the number of causal genes associated with non-5q SMA has been identified due to the advent of next-generation sequencing technologies [3, 6].

They are usually classified based on their inheritance pattern (autosomal dominant, autosomal recessive, or X-linked), distribution of muscle weakness (proximal, distal, or bulbar), and other associated manifestations [6, 38]. The diagnosis of SMA can be challenging, as these forms comprise a clinically and genetically heterogeneous group of diseases. The main features include hypotonia, progressive postnatal weakness, and areflexia suggestive of motor neuron disease. However, the evaluation of an infant presenting with hypotonia and weakness consists of a broad spectrum of disorders such as congenital myopathies, muscular dystrophies, congenital myotonic dystrophy, congenital myasthenic syndromes, metabolic myopathies, and congenital disorders of the motor neuron/peripheral nerve. Non-neuromuscular conditions including genetic syndromes such as Prader-Willi syndrome, acute hypoxic ischaemic encephalopathy, neonatal sepsis, and dyskinetic or

metabolic conditions should also be evaluated [38, 39]. In the case of early-onset anterior horn impairment, and after the exclusion of SMN1 deletions or point mutations, in consideration of the above-mentioned diseases, non-5q SMA should be investigated. The history and neurological examination should focus on additional features, such as arthrogryposis, myoclonic epilepsy, sensorineural deafness, or pontocerebellar hypoplasia [3, 38, 39].

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is probably the second most encountered paediatric form of SMA, caused by mutations in the IGHMBP2 gene which encodes immunoglobulin μ-binding protein 2, leading to progressive spinal motor neuron degeneration [3]. It typically presents with respiratory failure due to diaphragmatic paralysis with sparing of the intercostal muscles and distal muscular atrophy, particularly of the lower extremities. The clinical features of diaphragm eventration or preterm birth, combined with the onset of respiratory distress requiring mechanical ventilation between the ages of 6 weeks and 6 months, play a core role in the diagnostic pathway. Evidence suggests three different forms of SMARD1 vary based on the age of onset: an early-onset form, which usually appears before 3 months of age and is very severe; a classical onset form; and a very rare late-onset form [40]. Butterfiled et al., [41] also described an overlapping phenotype in a single patient who also developed early diaphragmatic weakness with distal weakness and contractures due to a mutation in LAS1L, which is known as SMARD2 [6].

Scapuloperoneal SMA is another early-onset form of non-5q SMA with TRPV4 mutated, passed on via autosomal dominant inheritance. Progressive weakness of the extremities beginning at birth or early childhood is mostly proximal in the upper limbs and distal in the lower limbs. Distinctive features of this subtype include progressive scapuloperoneal atrophy, and laryngeal palsy with a hoarse voice and respiratory stridor [38, 42]. Scapuloperoneal SMA, distal SMA, and hereditary motor and sensory neuropathy type 2C are reported as overlapping phenotypes related to TRPV4 mutations [38].

Lethal arthrogryposis with anterior horn cell diseases and X-linked SMA (SMAX2) are among the most severe forms of motor neuron disease. The clinical phenotype includes hypotonia, areflexia, chest deformities, facial dysmorphism, hydrops fetalis, arthrogryposis multiplex congenita, and pulmonary hypoplasia. Death occurs in the early neonatal period due to respiratory insufficiency. Electromyography and muscle biopsy findings are consistent with the loss of anterior horn cells [6, 38]. Lethal congenital contracture syndromes 1 and 2 are associated with recessive mutations in the GLE1 and ERBB3 genes, respectively. SMAX2 is an X-linked recessive disorder caused by point mutations in the UBE1 gene [43, 44].

Spinal muscular atrophy with lower limb predominance (SMALED) is an early-onset static or slowly progressive disorder. It is best characterised by proximal muscle weakness and atrophy, which predominantly affects the lower extremities. SMALED type I and II are autosomal dominant SMAs, accounting for <2% of cases, caused by heterozygous mutations in DYNC1H1 and BICD2, respectively [45–47].

Pontocerebellar hypoplasias (PCHs) represent a heterogeneous group of exceptionally rare disorders characterised by hypoplasia of the cerebellum and pons, variable cerebral involvement, microcephaly, severe delay in cognitive and motor development, and seizures. Currently, 13 different types are listed and among them 3 types of PCH have been associated with an SMA-like phenotype: PCH1A, associated with mutations of VRK1; PCH1B, associated with mutations of EXOSC3; and PCH1C, associated with mutations of EXOSC8 [6, 48–51]. Ataxia, nystagmus, or oculomotor apraxia in infants with motor neuron disease should prompt an evaluation for PCH and additional features such as microcephaly, severe hypotonia, tendon areflexia, muscle weakness, joint contractures, and in the case of prenatal onset, arthrogryposis, could be helpful in determining the diagnosis [6, 38].

Spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) is an early-onset disorder characterised by progressive lower motor neuron degeneration followed by progressive myoclonic epilepsy. Early development milestones are usually normal, progressive symmetrical weakness, and muscle atrophy of the lower and then upper limbs usually occur between 2.5 and 6 years of age, and later, myoclonic epilepsy is observed from 3 to 12 years of age. As the disease progresses, it leads to the inability to stand up and sit unsupported, a lack of head control, difficulty swallowing, fasciculations of the tongue, severe scoliosis, and subsequent respiratory insufficiency. The peripheral motor deficit, with a decrease in/absence of deep tendon reflexes, could be detailed with electromyography showing chronic denervation, while muscle biopsy may show neurogenic atrophy [52, 53]. SMA-PME is an autosomal recessive condition caused ASAH1 gene mutations [53].

Brown Vialetto-Van Laere (BVVL) syndrome and Fazio-Londe (FL) syndrome belong to a spectrum of progressive motor neuron diseases with predominant bulbar weakness. BVVL is characterised by onset in late childhood/early adulthood with sensorineural hearing loss, followed by additional lower cranial nerve involvement, lower motor neuron signs in the limbs, and respiratory insufficiency. FL patients do not have hearing loss and are earlier-onset cases. It shows a increased rapidly progressive course, presenting with pontobulbar palsy, muscle weakness, and respiratory insufficiency [6, 54, 55]. These are autosomal recessive diseases caused by mutations of the SLC52A2 and SLC52A3 genes which encode riboflavin transporters. Since riboflavin transport may be affected in these patients, a high dose of riboflavin is recommended as soon as possible when the

diagnosis is suspected [55, 56]. Kennedy disease is an X-linked recessive form of spinobulbar muscular atrophy which represents the most common adult-onset SMA. Patients show progressive limb and bulbar weakness, chin and perioral fasciculations, dysarthria, dysphagia, proximal, and occasional distal muscle wasting, with additional distinctive clinical features including gynecomastia and some endocrine manifestations. The disease is caused by a CAG-repeat expansion in the first exon of the androgen receptor gene [3, 57, 58].

Distal hereditary motor neuropathies (dHMNs), also known as distal SMA, are a clinically and genetically heterogeneous group of disorders characterised by various phenotypes related to individual genes. Until now, more than 30 causative genes implicated in dHMN have been identified [59]. This group is characterised by a slowly progressive symmetrical, length-dependent, motor neuropathy that causes distal muscle weakness and minimal/no sensory involvement. Besides these classical clinical presentations, congenital forms and additional features such as severe generalised weakness, predominantly upper limb onset, vocal cord paresis, diaphragmatic palsy, or pyramidal features have been reported [3, 59, 60]. This group was classified into seven clinical subgroups based on inherited pattern, age of onset, severity, and distinguishing clinical features. Autosomal dominant dHMNs include typical dHMN with childhood-onset (dHMN-I), and dHMN with adulthood-onset (dHMN-II) which present with distal leg and subsequent distal arm weakness and are associated with mutations in HSPB1, HSPB8, HSPB3, GARS, FBXO38, and DYNC1H1. dHMN with upper limb predominance (dHMN-V) is distinguished by the onset of weakness in the hand muscles and is associated with dominant mutations in BSCL2, GARS, or REEP1. Distal HMN type VII is distinguished by vocal cord paresis and may be caused by dominant mutations in SLC5A7, or DCTN1. Autosomal recessive dHMN comprises chronic dHMN (dHMN-III), chronic forms with diaphragmatic palsy (dHMN- IV) that are linked to 11q13 mutations, and spinal muscular atrophy with respiratory failure (dHMN-VI) associated with IGHMBP2 [3, 59, 60].

All About the Pathology of Motor Neuron Diseases

Regardless of the location of the pathology, changes in muscle that occur due to a denervation process are very similar. In most cases, thorough clinical and electrophysiological analyses point to the defective gene, and genetic analyses are carried out to further investigate that gene. Therefore, muscle biopsy is rarely performed for the differential diagnoses of neurogenic disorders. Disorders affecting the motor neurons exhibit some characteristic muscle changes [15].

Denervated myofibers become smaller, but their internal structure remains relatively unchanged. Even the myofibril cross-striations are retained until late in the atrophic phase. At the periphery of myofibres, the basal lamina that covers the atrophic fibers becomes extensively folded. However, these folds can only be identified with electron microscopy [15].

In chronic conditions such as ALS, the atrophic fibers become angular in shape, and are often of the same muscle fiber type, in contrast to the round shape of the atrophic fibers in SMA (Fig. 15.3). With chronic denervation, some atrophic fibers may appear as a clump of pycnotic nuclei (Fig. 15.4). Atrophic fibers typically form groups, and the number of fibers within these groups increases with increasing severity of denervation until entire fascicles become atrophic. The presence of small or large groups of atrophic fibers is pathognomonic for denervation (Fig. 15.5) [61–63].

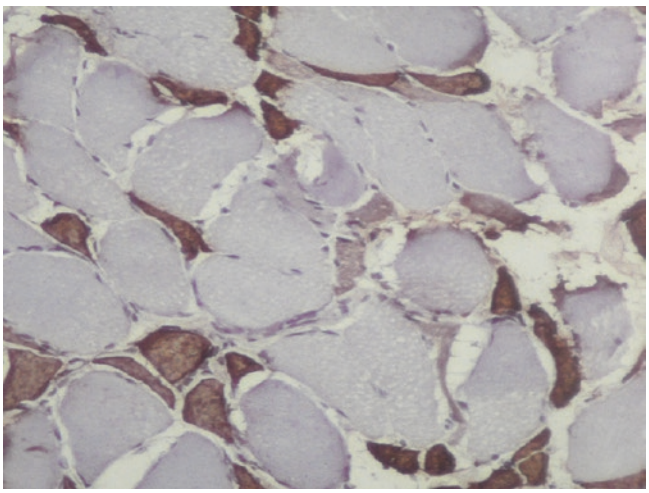

Fig. 15.3 Note all angular atrophic fibers are of type 2 nature seen with fast myosin immunohistochemistry (DAB ×200)

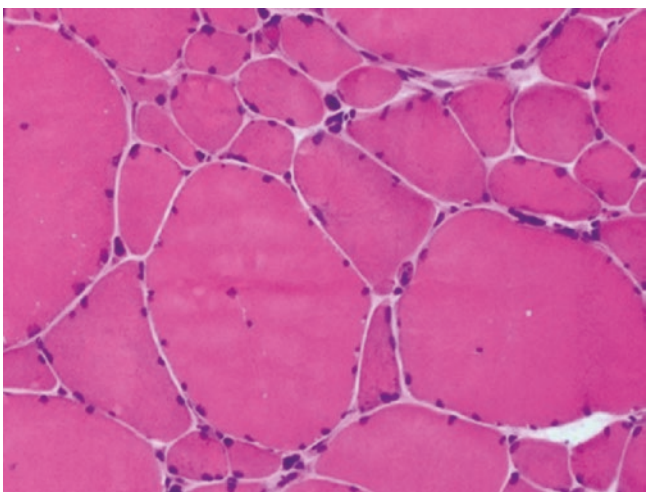

Fig. 15.4 Angulated fibers and clumps of pycnotic nuclei between the more rounded hypertrophied fibers (H&E × 200)

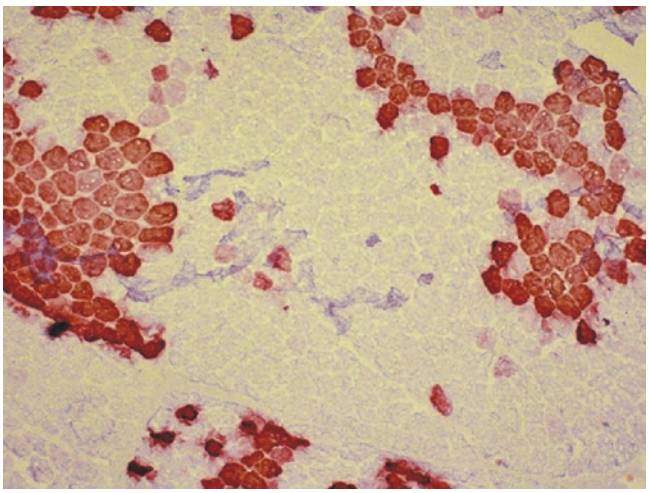

Fig. 15.5 Groups of type 2 fibers expressing fast myosin surrounded by unstained type 1 fibers in a patient with chronic denervation (DAB ×100)

Myofibrillar changes called core or target structures, and angular-shaped atrophic fibers can be seen in any disorder that results due to chronic denervation. Histopathological findings in SMA type III, the mildlest form of SMA, are like those seen in ALS or peripheral neuropathies (Fig. 15.5). Immunolabelled muscle fiber types help to discriminate group atrophy, which is an important clue for neurological disorders (Fig. 15.6). Similarly, merosin expression must be examined to differentiate congenital muscular dystrophy from SMA, and this staining, as well as enzyme stains which discriminate fiber types, are very helpful for demonstrating large and small fiber groups (Fig. 15.7). Atrophic fibers occur in all biopsies, generally in large groups, and they are interspersed with hypertrophied fibers fascicles. Atrophic fibers are usually round, contrary to other forms of neurogenic atrophies. However, it must be kept in mind that in neonatal patients with SMA type I, pathognomonic group atrophy can be absent.

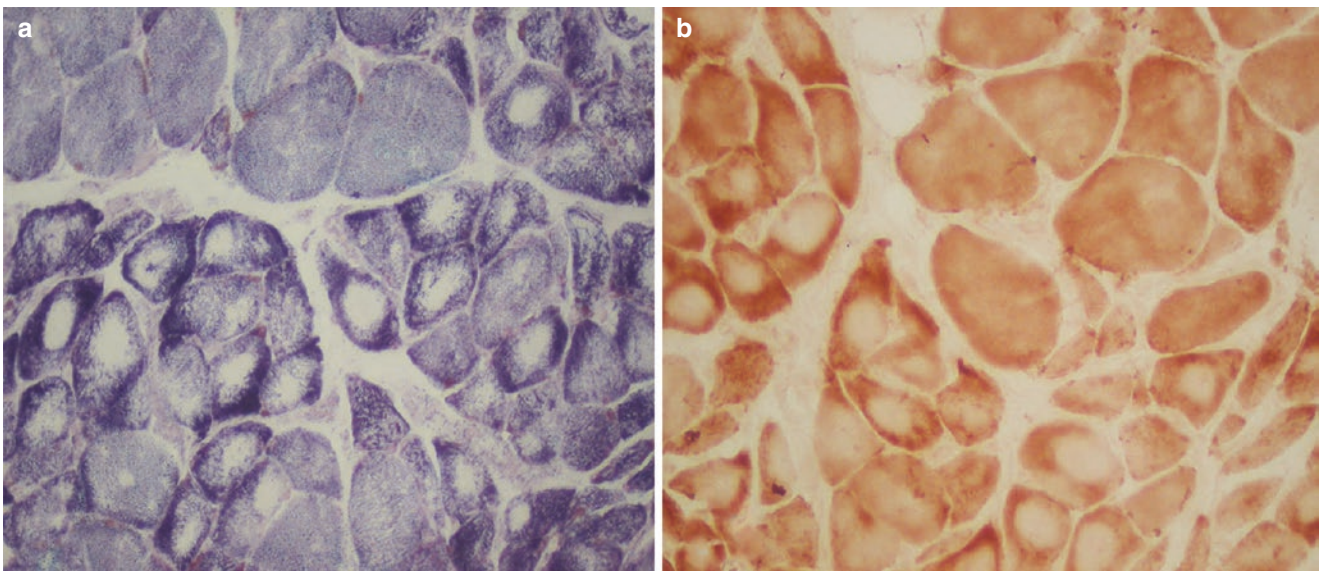

Fig. 15.6 A 7-year-old girl with spinal muscular atrophy type III showing many intensely stained angulated atrophic fibers with core and target structures within them: (**a**) NADH-TR × 200, (**b**) COX × 200

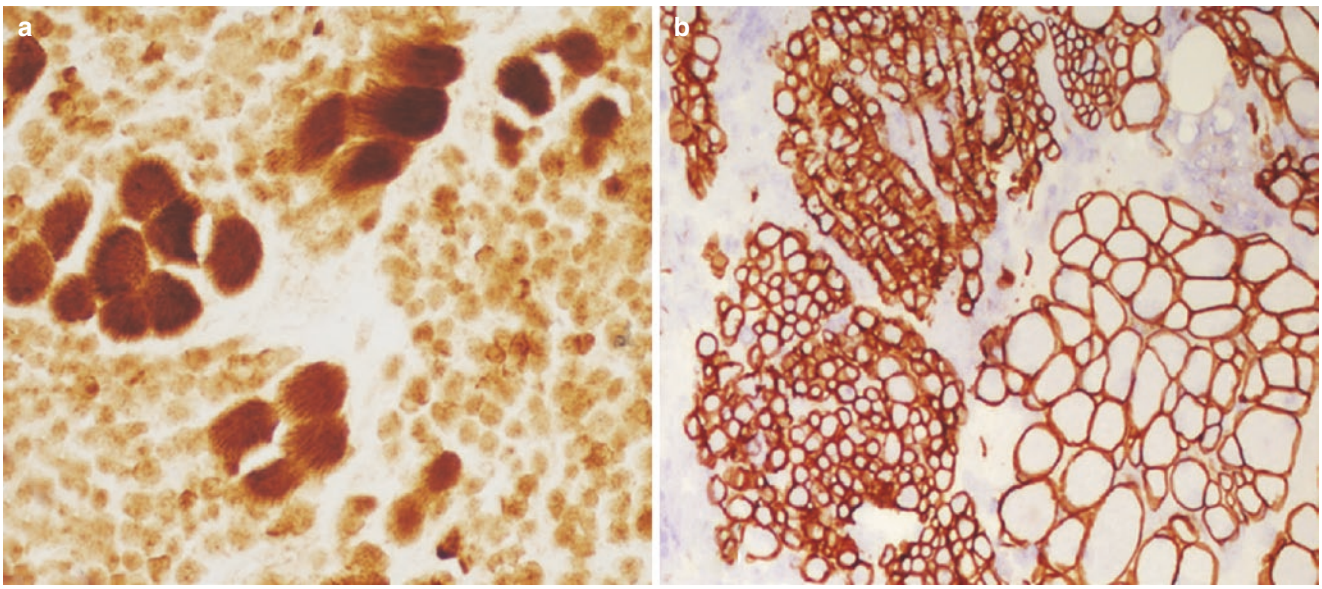

Fig. 15.7 Note the small and large groups that are pathognomonic for denervation: (**a**) hypertrophic fibers are type 1 fibers (COX × 200), (**b**) merosin immunolabelling highlights the presence of group atrophy (DAB ×200)

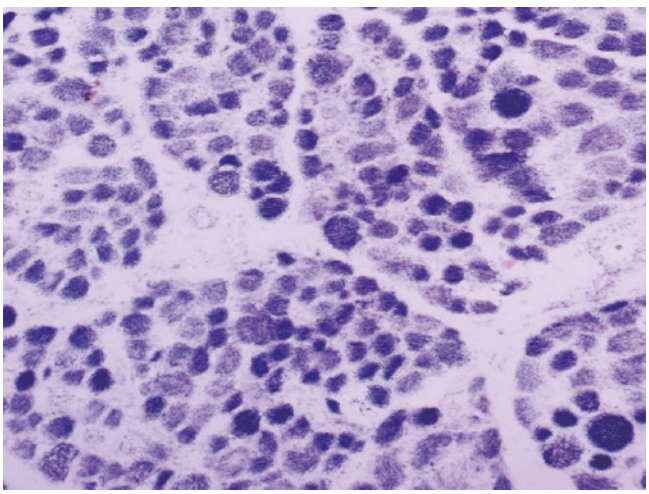

Fig. 15.8 Note the few hypertrophied, round, type 1 fibers in an infantile case of spinal muscular atrophy type I (NADH-TR × 100)

Histopathologically mixed-type small and large round fibers and few hypertrophied round-type 1 fibers are also observed (Fig. 15.8) [15, 61–63].

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Inflammatory Myopathies

16

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Introduction

Idiopathic inflammatory myopathies (IIMs) are a group of rare, heterogenous disorders. Although IIM is mainly characterised by muscle inflammation, the condition may also affect the skin, joints, gastrointestinal system, lung, and heart. IIMs were historically defined by proximal skeletal muscle weakness, muscle enzyme elevations, myopathic patterns on EMG, typical histology of muscle biopsy specimens, myositis-specific/myositis-associated autoantibodies, and typical skin lesions in dermatomyositis. However, IIMs have evolved into conditions defined by complex multiorgan involvement and fatal subgroups. IIM subtypes are usually defined based on classification criteria. Although the development of classification criteria may present some challenges as IIMs are rare conditions, several classification criteria have been developed for IIMs. Criteria are based either on clinical manifestations or histopathological features. As a result of a recent discovery of new myositis-specific autoantibodies, various subtypes of myositis have been defined. Classification criteria are required to gain insight into disease mechanisms, develop novel therapies, define patients with IIMs, differentiate IIM patients from other patients with myopathies, and to cover patients with amyopathic dermatomyositis (ADM) and anti-synthetase syndrome (ASyS), which are mainly characterised by extramuscular manifestations and mild skeletal muscle weakness. IIMs are mainly divided into three types: dermatomyositis (DM), polymyositis (PM), and inclusion body myositis (IBM). Moreover, overlap with other connective tissue disorders such as systemic sclerosis, Sjogren's syndrome, systemic lupus erythematosus (SLE), mixed connective tissue disease, or rheumatoid arthritis is not rare [1, 2].

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Epidemiology

Data on the prevalence and incidence of IIMs are heterogeneous. The prevalence and annual incidence of DM and PM are 2.9–34 individuals per 100,000 people and 2–11 new cases/1,000,000, whereas the estimated prevalence and annual incidence of IBM are 3.3 individuals per 100,000 and 1–2 new cases/1,000,000, respectively. Factors affecting the incidence of IIMs include age, sex, and ethnicity. Black people and women are at a greater risk for developing DM/PM, whereas IBM is more common in men [3, 4].

Aetiology and Pathogenesis

The interaction between genetic and environmental factors plays a role in the variability of clinical presentation. Strong associations have been defined between IIMs and human leucocyte antigen (HLA) genes, particularly HLA class II alleles. Associations were found between IIMs and both DRB1*08:03 and DPB1*05:01 haplotypes, in a study conducted by Furukawa et al. [5]. The haplotypes HLA DRB1*09:01 and DPB1*04:01 appear to be protective against PM. Single nucleotide polymorphisms (SNPs) involving the interleukin-1β gene have been associated with an increased risk for developing ASyS. No significant association was found between ASyS and MUC5B polymorphism rs35705950, although there is an established association between this polymorphism and idiopathic interstitial lung disease, indicating that different pathways may be involved in the pathogenesis. Recently, several studies have focused on transcriptome analysis in tissue biopsy

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specimens from patients with IIMs. Clinical phenotypes including DM and IBM have been investigated to detect whether there are differences in pathways involved in the pathogenesis, based on innate and adaptive immune responses. In general, the expression of certain genes has been associated with type 1 interferon in DM, cell-mediated immunity in IBM, structural proteins of myocytes in immune-mediated necrotizing myopathy (IMNM), and both interferon (IFN) pathways and structural proteins of muscle cells in ASyS. Unlike other skin disorders, interferon signature has been detected in skin lesions of patients with DM [6]. As for underlying cellular mechanisms in the pathogenesis of IIMs, mitochondrial pyroptosis has been implicated in perifascicular atrophy in DM, and muscle ischaemia has been implicated in perimysial and arteriolar disease such as wall thickening, thrombosis, and endothelial lipid accumulation in anti-NXP2 DM [7, 8]. As for the role of immune cells in the pathogenesis of IIMs, the contribution of CD8+ T cells to muscle injury through apoptosis has been demonstrated in PM and IBM [9, 10]. Proinflammatory effects of blood monocytes, via increased TLR2 and TLR4 expression, have been detected in sera from patients with IIMs. Reductions observed in muscle monocytes after 1 year of treatment predict long-term outcomes, regardless of disease activity and steroid therapy [11, 12]. Dysregulated neutrophils/low-density granulocytes (LDGs) and neutrophil extracellular traps (NETs) play a key role in the pathogenesis of IIMs. Anti-MDA5 auto-antibodies can directly induce NET formation, but these autoantibodies are also associated with increased NET formation [13].

The role of protein misfolding has been investigated in the pathogenesis of IBM, and it has been found to be associated with the overexpression of T complex protein-1 (TCP-1) and sodium/myoinositol cotransporter 1 (SLC5A3) [14]. Possible underlying mechanisms may include a reduced clearance and overexpression of misfolded proteins, followed by the induction of inflammation through a compensatory mechanism [15]. Another piece of evidence suggesting mitochondrial dysfunction was that in vitro treatment with mitochonic acid-5 (MA-5) improved myocyte survival by enhancing adenosine triphosphate (ATP) production in IBM specimens. Chaperone-assisted selective autophagia (CASA) leads to the degradation of misfolded proteins via protein p62. Although p62 accumulation has been suggested as a marker of IBM, this protein was detected in other forms of myositis. Therefore, its specificity is controversial. The CASA pathway may be involved in muscle necrosis in IMNM and may be associated with endoplasmic reticulum stress induced by anti-SRP and anti-HMGCR antibodies, and inflammation related to MHC-I overexpression [16]. Furthermore, infiltration by Bcl-2 and beta-chemokine receptor 4 (CCR4) positive lymphocytes is a marker of anti-HMGCR-positive IMNM [17]. As for environmental risk factors, ultraviolet exposure has been associated with an increased risk for developing DM rather than other forms of IIMs [18]. Anti-MDA5 disease follows a seasonal pattern (from October to March) and is more prevalent in coastal areas [19].

Outline of Pathogenesis

- Associations have been found between specific HLA haplotypes and the development of IIMs, as well as the development of specific IIM subtypes.
- A consistent number of LDG is not only associated with clinical phenotypes or specific myositis-specific autoantibodies, but it may also help to differentiate IIMs from other pathological conditions with similar clinical presentations.
- Micro RNA may play a role in the pathogenesis of IIMs.
- Cellular mechanisms such as a mitochondrial pyroptosis and perimysial and arteriolar disease.
- Immune cells involved in IIMs include monocytes, neutrophils, and CD8+ T cells.
- Misfolded proteins and mitochondrial dysfunction play a major role in the pathogenesis of IBM.
- Infiltration by Bcl-2- and CCR4-positive lymphocytes is a marker of anti-HMGCR-positive IMNM.

The T cell-mediated immune response plays a major role in the pathogenesis of IIMs (Fig. 16.1). Acute and self-limiting forms of myositis may occur in association with viral infections including influenza, echovirus, and coxsackie virus, particularly in children; however, the role of these viruses in chronic myositis has remained undetermined. Clinical and histopathological features of retroviral infections (e.g., human T cell leukaemia/lymphoma virus and human immunodeficiency virus) may be like those of PM. In addition, *Trypanosoma cruzi* infections have been linked to myositis.

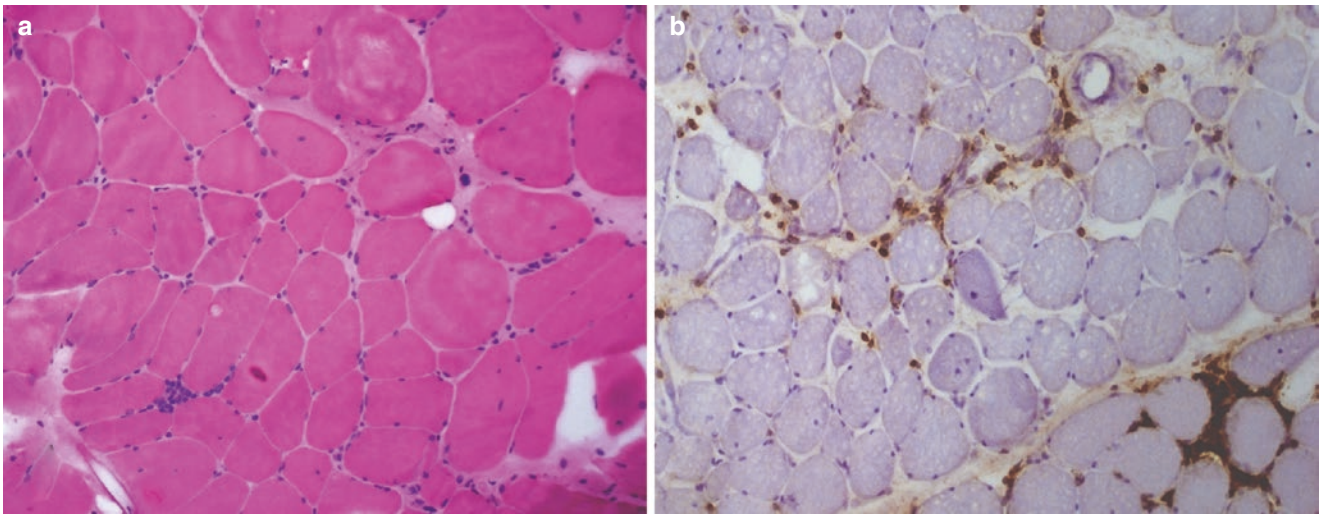

Fig. 16.1 (**a**) Note the small lymphocyte groups in the endomysium in the muscle of a case with polymyositis (H&E × 200), (**b**) infiltration mainly consists of CD3 positive T lymphocytes (DAB × 200)

Historical Evolution of Classification Criteria

The first set of modern criteria defined for IIMs was the Medsgers criteria [20]. Later, Bohan and Peter's criteria were published in 1975 and were widely used for a long time [21]. Bohan and Peter's criteria were suggested as a classification criterion as well as a diagnostic criterion. Bohan and Peter's criteria were developed based on the expert opinion from the data of a single institution. A characteristic of this criteria set is the requirement to rule out other myopathies. Patients should present with symptoms of myopathy. The classification includes PM, DM, overlap syndrome, cancer-associated myositis subtypes, but not the IBM subtype. The criteria did not include serologic features or extramuscular manifestations such as interstitial lung disease (ILD). About 20 years later, Tanimato et al. developed a new criterion for PM and DM in 1995 [22]. In addition to Bohan and Peter's criteria, this new criterion included clinical symptoms such as myalgia, nonerosive arthritis, and positive serology for anti-Jo1 antibodies. Targoff et al. included anti-SRP and anti-Mi2 antibodies, as well as inflammation on MRI scans in their criteria published in 1997 [23]. In 2004, the European Neuromuscular Centre published a classification criterion focused on histopathology [24]. This classification defined IMNM and nonspecific myositis subtypes. In 2005, Troyanov et al. defined clinicoserological overlap myositis, extended the auto-antibody profile, and proposed the term "cancer-associated myositis" [25]. The EULAR-ACR criteria published in 2017 address the shortcomings of prior classification criteria by encompassing a wider spectrum of inflammatory myopathies and subgroups, including a better defined and accessible clinical and laboratory criterion [26]. Criteria are based on data from 976 patients (74% adults) with idiopathic inflammatory myopathy and 624 patients (82% adults, 18% paediatric patients) with non-IIM mimicking conditions. This classification includes two separate scoring systems depending on whether muscle biopsy data are available. The classification defines the probability of the disease based on a score that corresponds to a "definite," "probable," or "possible" IIM and contains a subgroup system. Paediatric patients are grouped into juvenile dermatomyositis (JDM) or non-JDM, and adult patients are grouped into PM, DM, ADM, or IBM. A web-based calculator is also available to facilitate scoring. However, the EULAR–ACR criteria have some limitations. The cohort included very few patients who presented with clinical characteristics of IMNM, as this condition was newly defined in 2004 when the project was started. Therefore, the polymyositis subgroup may also include patients with IMNM. Furthermore, some patients with muscular dystrophy, presenting with inflammatory infiltrates like IIM, may have been misclassified. The criteria do not include variables such as MRI findings, which were available in less than 38% of patients in the study cohort, as well as myositis-specific antibodies which were not commercially available (e.g., IMNM-associated anti-HMGCR antibodies). In a recently published study, Barsotti et al. compared the performance of the Bohan and Peter criteria to that of the EULAR-ACR criteria in 439 patients. The sensitivity of the EULAR-ACR criteria was higher than the Bohan and Peter criteria, and the specificity of the EULAR-ACR criteria was found to be as high as 98%, particularly in 3 conditions (PM, DM, and IBM) [27].

Considering all classification criteria, IIMs can be classified as follows:

- Dermatomyositis.
- Immune-mediated necrotizing myopathy.
- Overlap myositis (including anti-synthetase syndrome).
- Sporadic inclusion body myositis.
- Amyopathic dermatomyositis.
- Polymyositis.
- Cancer-associated myositis.

Dermatomyositis (DM)

DM is characterised by muscle weakness and typical skin manifestations which develop within weeks or months. Elevations of muscle enzymes, a myopathic pattern on EMG with myopathic motor unit potentials, fibrillation, and spontaneous sharp waves can be observed. Pruritus is among the most common symptoms. Other typical symptoms include a heliotrope rash, Gottron's papules, V-shawl sign, and calcinosis. A blue or violate rash over the eyelids (heliotrope rash) and violate desquamating eruption over the interphalangeal joints (Gottron's rash) are common symptoms. An erythematous rash may be seen over the knees, malleoli, and neck, and a rash over the anterior chest wall (frequently V-shaped), back, and shoulders (shawl sign) and may be aggravated by sunlight (Fig. 16.2). Poikiloderma refers to a skin condition characterised by hypopigmentation/hyperpigmentation, telangiectasis, and epidermal atrophy. Enlarged nailbed capillaries are typical. Cuticles may be irregular, thickened, and abnormal. A skin biopsy may reveal findings specific to interface dermatitis and membrane attack complex (MAC) deposition. Interface dermatitis may also be seen in skin disorders such as cutaneous lupus or lichen planus. A skin biopsy is required to confirm a diagnosis of DM and rule out other conditions such as psoriasis, eczema, or multicentric reticulohistiocytosis. Perifascicular atrophy in muscle biopsy specimens is quite specific (>90%) of dermatitis (Fig. 16.3). Especially in DM children, the number of inflammatory cells in the muscle biopsy can be very scarce. Therefore, the presence of perifascicular atrophy, perivascular immunoglobulin, or complement deposition, detected with direct immunofluorescence staining, is very important for diagnosis. The sensitivity of muscle biopsy ranges from 25 to 50% in the absence of perifascicular atrophy [28]. Plasmacytoid dendritic cells, CD4+ T-cells, B-cells, and macrophage infiltration can be seen around the perimysium and medium-sized vessels. In 16% of cases, necrosis may be so prominent that a muscle biopsy may not differentiate DM from IMNM, in the absence of cell infiltration [28].

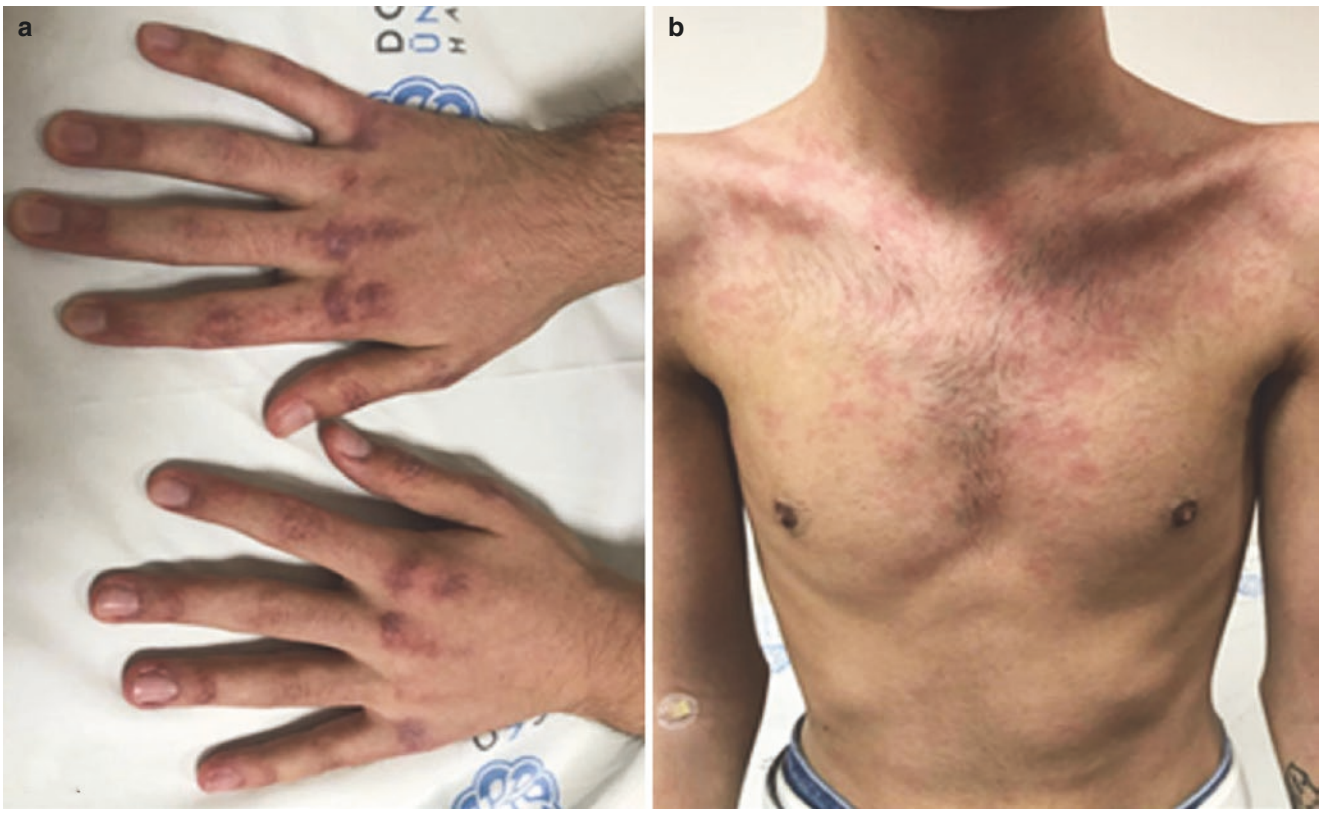

Fig. 16.2 (**a**) Gottron's sign; macules covering the extensor surfaces of the joints, (**b**) erythema on the lower neck and upper chest (V-sign)

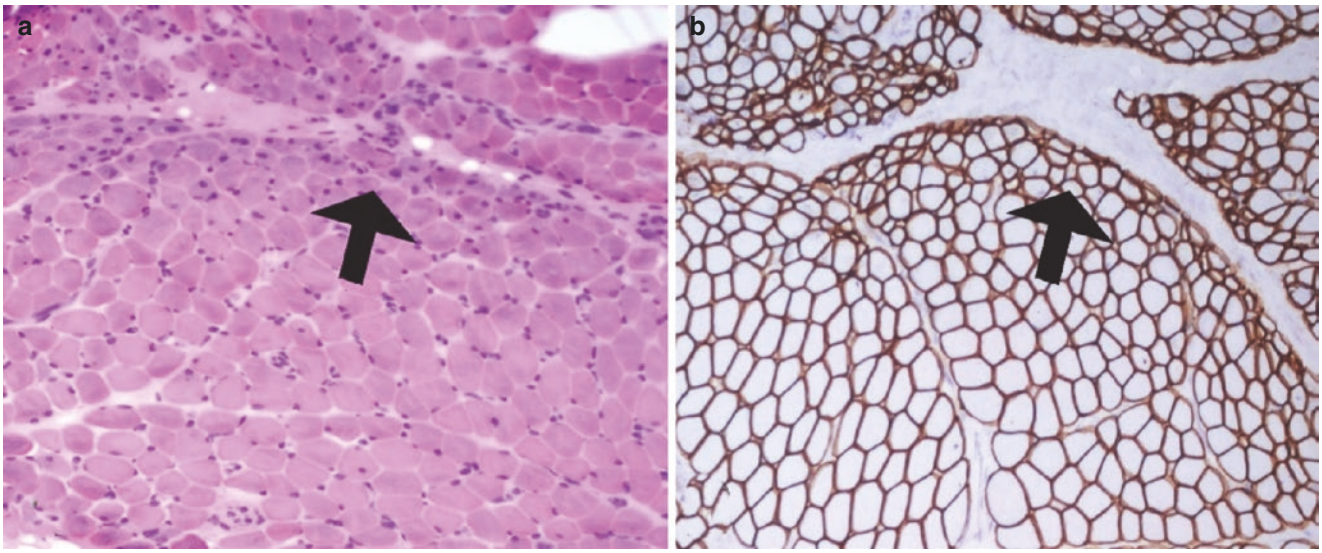

Fig. 16.3 A 3-year-old girl with DM: (**a**) note the presence of perifascicular atrophy (H&E × 100), (**b**) atrophic myofibres are highlighted with sarcolemmal merosin expression (DAB × 100)

Amyopathic Dermatomyositis and Hypomyopathic Dermatomyositis

New DM subtypes have been increasingly recognised in the last 10 years. Some patients may present with mild skin symptoms, whereas muscle involvement may be absent (normal EMG, muscle MRI, creatine kinase levels, and muscle biopsy) in some cases. These entities are characterised by the absence of evidence of proximal muscle weakness and normal muscle enzyme levels for 6 months or longer, normal muscle function based on laboratory and imaging studies, and common skin lesions of classical DM. Skin involvement should be confirmed by a biopsy. MDA5 antibodies may be present in ADM, and MDA5 antibody positivity may be associated with progressive disease and ILD [29]. Mortality rates are high in patients with rapidly progressive ILD and ADM; therefore, early detection and treatment are important.

Immune-Mediated Necrotizing Myopathy (IMNM)

Proximal muscle weakness, very high levels of muscle enzymes, and a myopathic pattern on EMG are predominant findings in IMNM. Extramuscular findings are rare and generally mild. Two-thirds of patients are positive for anti-signal recognition particle (SRP) or anti-HMGCR auto-antibodies. Mechanisms underlying myofibre necrosis remain unclear in immune-mediated necrotizing myopathy, although there are some theories. For instance, anti-SRP and anti-HMGCR have been suggested to play a pathogenic role due to MAC deposition in non-necrotic fibres. Minimal lymphocytic infiltrates, necrosis, and regeneration without perifascicular atrophy are important histological findings in muscle biopsy specimens. However, despite the presence of lymphocytic infiltrates, IMNM may not be differentiated from other necrotizing conditions based on histological findings in 20% of patients who test positive for anti-SRP and anti-HMGCR. Based on this classification, specific biopsy features are not required to classify patients with antibody-positive IMNM, but biopsy is required to classify patients with antibody-negative IMNM. Anti-SRP and anti-HMGCR myopathies share several characteristics including muscle biopsy findings, high CK levels, and mild extra muscular manifestations. Both myopathies tend to be more aggressive and resistant in younger patients [30]. However, there are some differences:

1. Anti-HMGCR myopathy is associated with statin exposure.
2. Muscle weakness tends to be more severe in anti-SRP myopathy compared to anti-HMGCR myopathy.
3. Muscle biopsy reveals a higher number of necrotic fibres in anti-SRP myopathy compared to anti-HMGCR myopathy.
4. Although ILD is rare in both conditions, the rate of ILD is slightly higher in anti-SRP myopathy compared to anti-HMGCR myopathy.
5. Anti-HMGCR myopathy and autoantibody negative IMNM are associated with an increased risk of malignancy, whereas the risk for increased malignancy is not increased in anti-SRP myopathy.
6. Cardiac involvement is rare in anti-HMGCR myopathy.

Several cross-sectional studies reported an increased rate of cardiac involvement in patients with anti-SRP myopathy, although this finding was not supported by cohort studies [31]. Electrocardiogram and echocardiogram tests should be performed if cardiac involvement is suspected in patients with IMNM. MRI scans with gadolinium-based contrast agents may reveal muscle inflammation in selected patients. The diagnosis may be confirmed by endomyocardial biopsy.

Inclusion Body Myositis (IBM)

IBM is the most common inflammatory myopathy in patients aged ≥50 years. IBM is usually misdiagnosed as PM, and IBM is considered when patients do not benefit from PM therapy. Some patients present with knee buckling and falls due to early involvement of the quadriceps muscles, whereas others present with difficulty gripping objects such as a golf club or performing activities such as turning a key or knot tying, due to the loss of strength in the small muscles of the hand (finger flexors, in particular). Sensory examination is usually normal other than a reduced sensation of vibration in the ankles that is associated with old age in some patients. Distal muscle involvement such as motor neuron disease or peripheral nerve disease is the result of the myopathic process that selectively affects distal muscles. The presence of rimmed vacuoles and other histopathological features are required to diagnose IBM based on criteria defined by Griggs [32] in 1995. However, the sensitivity of these criteria is inadequate to classify IBM. Endomysial infiltration by CD8+ T cells has been demonstrated in non-necrotic muscle fibres without rimmed vacuoles in patients showing clinical characteristics of the IBM phenotype. No differences were found between patients who meet all histopathological criteria and those who do not, in terms of the course of the disease or treatment response [33]. Muscle weakness, increased CK levels that are present in most cases, and myopathy findings on EMG are the common characteristics. However, there are some differences:

1. IBM is two times more common in males.
2. IBM usually affects people over the age of fifty.
3. The condition slowly progresses over years.
4. Muscle weakness shows an asymmetrical pattern.
5. Muscle weakness usually involves not only distal muscles including finger and wrist flexors, ankle dorsiflexors, and knee extensors but also arm abductors and hip flexors, although the strength of these muscles is maintained better than distal muscles.
6. Progressive dysphagia may occur in 60% of patients and may result in choking attacks.
7. Sporadic IBM is not associated with any autoantibody specific to myositis. Although autoantibodies against cytosolic 5′-nucleotidase 1A (NT5C1a) are present in 30–60% of patients, these autoantibodies are not specific to IBM.
8. There is no evidence indicating the benefit of immunosuppression in IBM.
9. MRI scans show a characteristic pattern indicating heavy involvement of the anterior compartment of the thigh.

Rimmed vacuoles, the characteristic histopathological finding of sporadic IBM, are best detected with the Modified Gomori trichrome stain. This feature may be important in differentiating sporadic IBM from other inflammatory myopathies without rimmed vacuoles [34]. The way in which rimmed vacuoles form remains unknown. The detection of nuclear membrane proteins within vacuoles suggests that vacuoles may be degenerated myonuclear remnants and that they may develop due to protein folding and autophagic dysfunction. Rimmed vacuoles may also be seen in some patients with hereditary myopathy. The term hereditary inclusion body myopathy (HIBM) is used to describe the muscle disorders characterised by autosomal inheritance and the presence of muscle fibers with rimmed vacuoles, revealed with muscle biopsy. The GNE gene, which codes for a rate-limiting enzyme in the sialic acid biosynthesis pathway, is mutated in the most prevalent form of HIBM. As a result, glycoproteins are sialylated abnormally, perhaps causing the degeneration of muscle fibers. GNE myopathy (GNEM) or Nonaka myopathy is an adult-onset autosomal recessive myopathy that is clinically characterised by progressive distal leg atrophy and weakness, particularly involving the lower limbs but sparing the quadriceps group. Therefore, it is also known as distal myopathy with rimmed vacuoles. The first symptom of GNEM is often foot drop, and progressive weakness causes difficulties in walking, climbing stairs, or getting up from a sitting position. Patients with GNEM generally become lifetime wheelchair users within 10–15 years after disease onset [35–37].

Case 16.1

A 38-year-old female presented with difficulty in climbing stairs, episodes of falling, and shortness of breath. She was 30 years old when her first symptoms of steppage walking began. Neurological evaluation revealed proximal 4/5 and distal 4/5 muscle power bilaterally in the upper extremities and proximal 3/5 and distal 3/5 muscle power bilaterally in the lower extremities, while the strength of the quadriceps femoris muscle was relatively spared. Deep tendon reflexes were hypoactive in all extremities. Her full blood count, biochemical and thyroid function test results, sedimentation rate, C-reactive protein (CRP), and vitamin B12/D values were within normal limits. The CK value was 230 U/L (29-168 U/L). Results of electromyography were consistent with myopathy. Her vital capacity was low as revealed with respi-

ratory function tests. Electrocardiography (ECG) and echocardiography findings were within normal ranges. The histopathological examination of a muscle biopsy specimen taken from her gastrocnemius muscle revealed myopathic features with several rimmed vacuoles (Fig. 16.4). The family history revealed that her sibling also had similar problems.

Case 16.2

A 36-year-old female presented with difficulty in going upstairs, sitting, and standing with frequent episodes of falling. She was 34 years old when she experienced her first symptoms of foot drop. Neurological examination revealed similar findings to her old sister (Case 16.1). Blood tests showed high CK levels (480 U/L), while other results were normal. EMG results were consistent with myopathy. Respiratory function tests and blood gas evaluation results were normal. ECG and echocardiography results were within normal limits on cardiological evaluation. A biopsy specimen obtained from the gastrocnemius muscle revealed end-stage muscle disease. In addition, the presence of group atrophy and angular fibres lead to suspicion of neurogenic myopathy. A second biopsy from the deltoid muscle led to a diagnosis of hereditary inclusion body myopathy. Five years after the histopathological diagnosis, genetic tests were performed in both sisters and their families. All exons and splice regions of the GNE gene were sequenced and a novel homozygous mutation in the kinase domain of the GNE gene in both sisters was determined. This mutation (c.2152G > A/p.A718T) was a G-to-A transition (c.2152G > A), which changes an amino acid at codon 718 from alanine (A) to threonine (T). Similar heterozygous

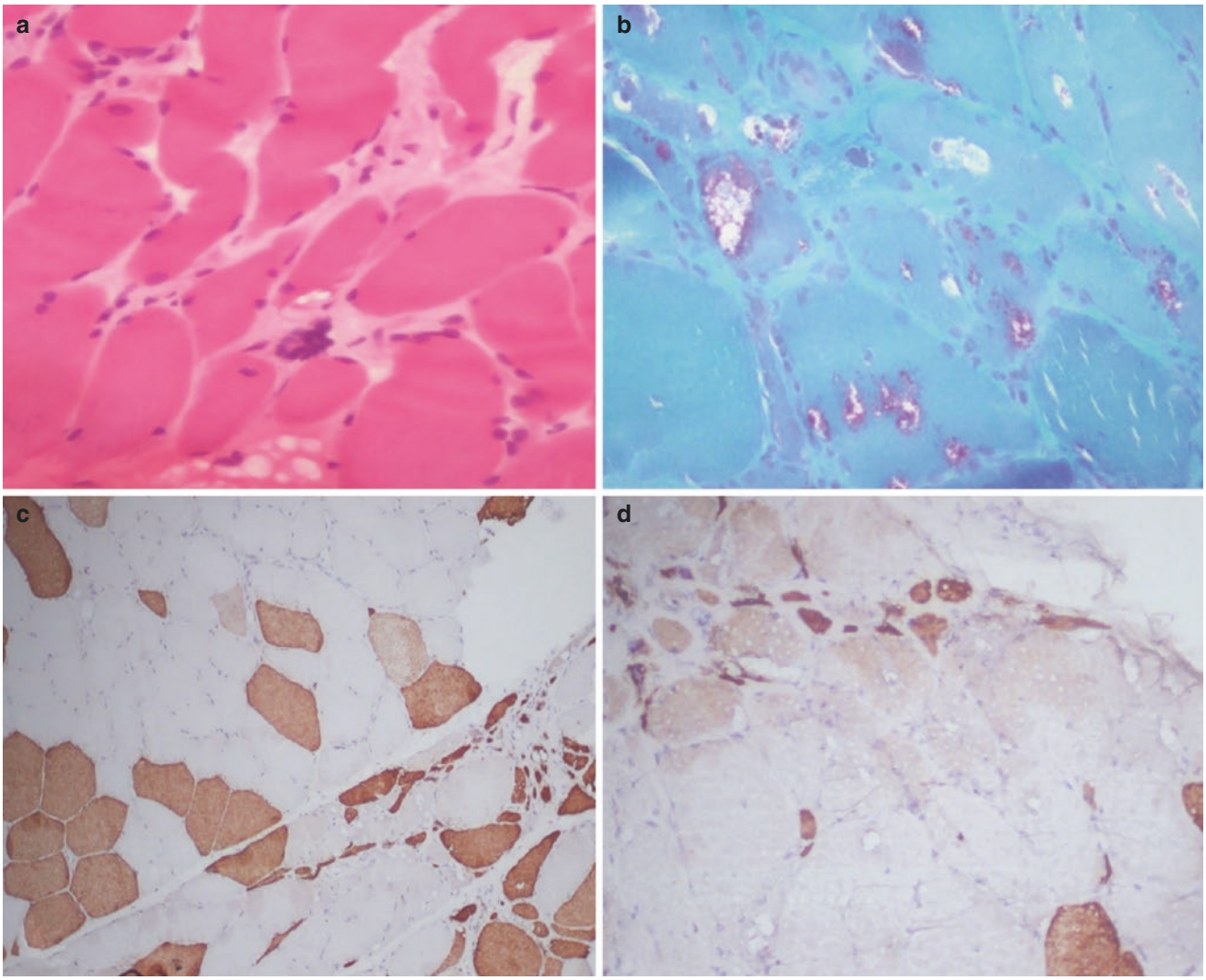

Fig. 16.4 Muscle biopsies from two sisters who were diagnosed with GNE myopathy: (**a**) note the nuclear clumping in the first biopsy of the younger sister (H&E × 200), (**b**) prominent rimmed vacuoles in both sisters (Modified trichrome ×200), (**c**) presence of atrophic angular type 2 fibers detected with fast myosin (DAB ×200), (**d**) increasing numbers of immature fibres detected with neonatal myosin (DAB × 200)

mutations were found in their father and mother, and in two daughters of the older sister, but the GNE genes were normal in the other family members [37].

Overlap Myositis

Overlap myositis is characterised by the presence of connective tissue disorders such as scleroderma or systemic lupus erythematosus, in addition to inflammatory myopathy. The most common presentation of overlap myositis is anti-synthetase syndrome. Scleroderma is the most common connective tissue disorder associated with inflammatory myopathies. In patients with scleroderma, anti-PM-Scl, anti-Ku, or anti-U1RNP autoantibodies are associated with inflammatory myopathies.

Anti-Synthetase Syndrome

Typically, in addition to one or more clinical characteristics, patients are positive for one of the autoantibodies that target amino acid tRNA synthetases. Patients with this syndrome may be positive for antibodies against histidyl tRNA synthetase (anti-Jo1), threonyl tRNA synthetase (anti-PL7), or alanyl tRNA synthetase (anti-PL12) [35, 36]. Clinical features include inflammatory myopathies, ILD, Raynaud's syndrome, fever, and mechanic's hand characterised by hyperkeratotic lesions on the radial aspects of the fingers. A skin rash like that of DM may be seen in patients with ASyS, and muscle weakness may not be present in all patients with ASyS. Ninety percent of patients with Anti-Jo-1 autoantibodies have an inflammatory myopathy, whereas 50% of patients with anti-PL12 autoantibodies have ILD but no muscle involvement. Muscle weakness is more severe in anti-Jo-1-positive patients, while lung involvement is more severe in patients positive for anti-PL7 and anti-PL12 autoantibodies [38]. Mechanic's hand may also be seen in patients without anti-ARS antibodies. It is important to know that there is overlap between IIM subgroups in terms of skin manifestations.

Polymyositis

Polymyositis is characterised by muscle weakness, high CK levels, myopathic findings on EMG, CD8+ T cell infiltration in muscle biopsy, and a lack of findings unique to other myopathies (DM, IMNM, IBM). Some patients previously classified as PM may be classified as anti-synthetase syndrome without a rash, IMNM, or IBM based on clinical characteristics, serology, and muscle biopsy findings. Other possible diagnoses should be ruled out, even in patients with PM and these patients should be closely monitored for new clinical characteristics suggestive of alternative diagnoses. Rashes, extraocular/facial muscle involvement, a family history of neuromuscular disease, a history of exposure to myotoxic pharmaceuticals or toxins endocrinopathy, neurogenic disorder, muscular dystrophy, and muscle enzyme deficiency should be excluded to make a diagnosis of PM. PM may be associated with a systemic autoimmune disorder or viral/bacterial infections. Furthermore, D-penicillamine, statins, and zidovudine may trigger an inflammatory myopathy like PM. In some patients, there are increased numbers of fibres devoid of COX activity. This situation may lead to a suspicion of mitochondrial myopathy. However, it must be kept in mind that the number of these fibres increases with age (Figs. 16.5 and 16.6).

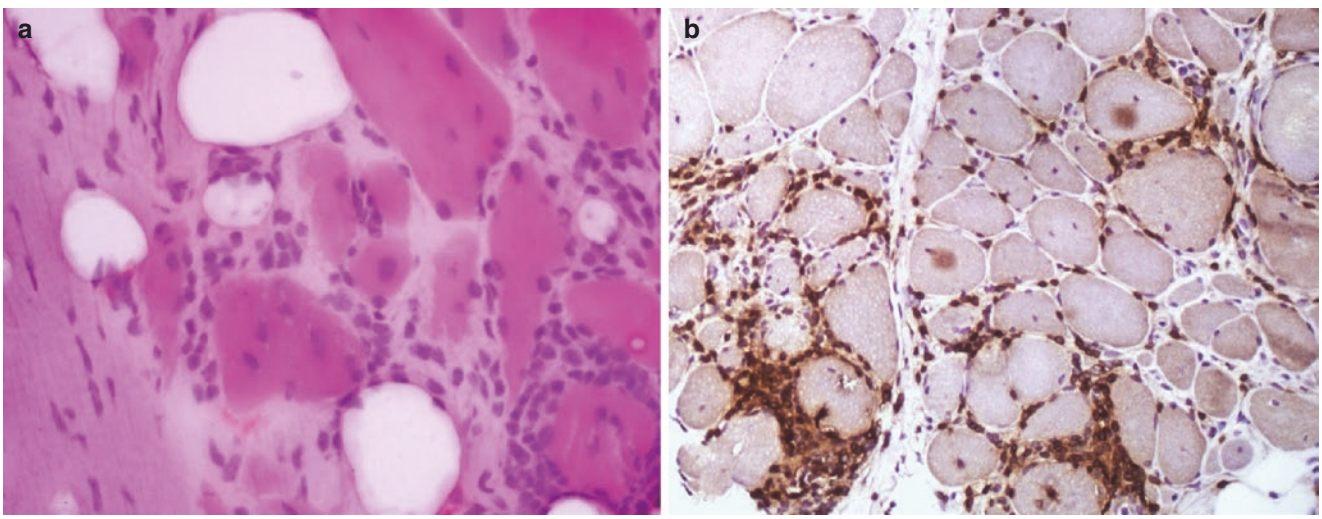

Fig. 16.5 (**a**) Polymyositis which developed in a 62-year-old male with rheumatoid arthritis (H&E × 200), (**b**) predominant inflammation with CD3 T lymphocytes in the endomysium (DAB × 100)

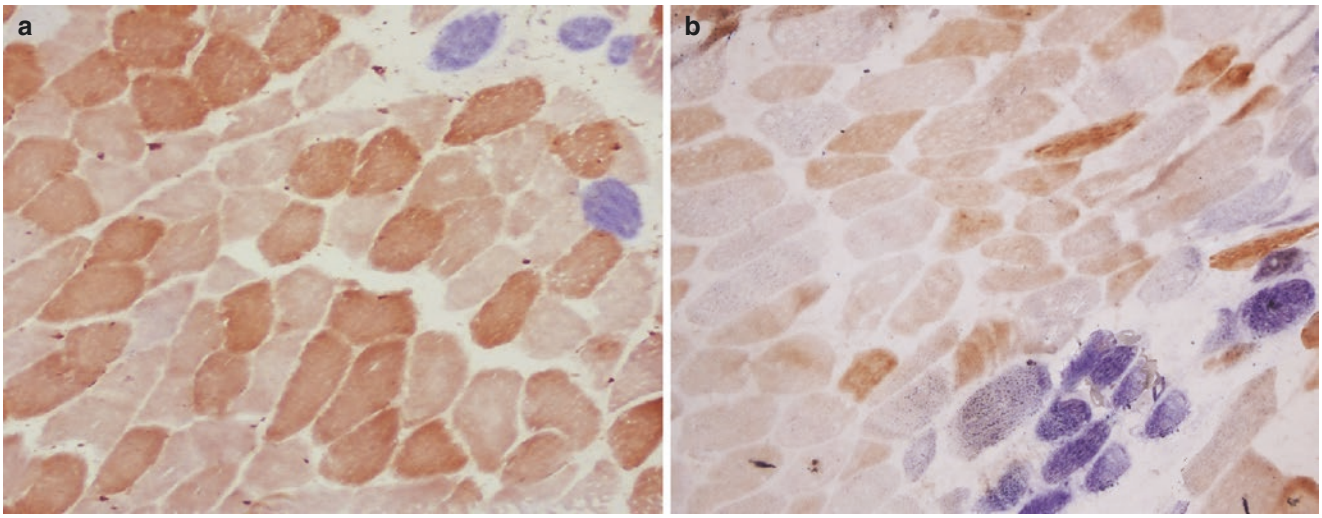

Fig. 16.6 (**a**) Note the pathological COX negative blue fibers in a 61-yeare in a 13-year-old girl with a COX gene mutation (COX-SDH × 100)

Cancer-Associated Myositis

Auto-immune myositis is strongly associated with malignancies and global malignity rates range from 3 to 40% [39]. In a meta-analysis, underlying neoplasms were found in 24% of patients with DM. Overall standardised incidence rates (SIR) for cancer vary between 3.8 and 7.7 in patients with DM, while the association between cancer and PM is rather rare (SIR: 1.7 to 2.2) [40]. The prevalence of cancer was reported to be 12% in 2017, based on data from two large DM cohorts. Forty percent of patients were diagnosed with cancer within 1 year of the onset of DM symptoms and old age was determined as the single risk factor for cancer. There was no clear predominance of a specific malignancy and the distribution of breast, lung, colorectal, gallbladder, and rare gastric and thymic cancers was comparable to that of the overall population [39]. The risk is further increased for Hodgkin lymphoma, lung, and bladder cancers. A multitude of reports suggest that the clinical course of myositis reflects that of cancer and the short interval between the onset of myositis and the onset of cancer supports the concept that actual myositis may be a paraneoplastic disorder. Therefore, it has been suggested that the development of malignancy may be the cause of cancer-associated myositis, rather than developing because of it [39–43]. DM, and less frequently IMNMs, are the most documented forms of cancer-associated myositis. Recent findings indicate that the autoantibodies anti-TIF1γ, anti-NXP2, and anti-HMGCR are associated with cancer, particularly within the first 3 years of the disease [43]. The targets of these three autoantibodies include cellular pathways associated with tumorigenesis. Anti-TIF1γ is the leading antibody associated with the risk of malignancy and malignancy rates have been reported to vary between 68 and 80% in anti-TIF1γ-positive patients, and the association between anti-TIF1γ and HLADQA1*0301 has been underlined. Anti-NXP2 autoantibody is associated with an increased risk of malignancy and malignancies are detected in about 30% of patients positive for anti-NXP2. The prevalence of malignancy varies between 13 and 36% in anti-HMGCR-positive patients with IMNM (associated with HLADRB1*1101), and the risk for malignancies is increased in seronegative IMNM. The prevalence of anti-SAE ranges from 1 to 4%, and this antibody is associated with an increased risk of malignancy, whereas anti-Mi-2 antibodies seem to be protective against malignancy [44]. Patients with cancer-associated autoantibody profiles should be screened carefully and a positron emission tomography (PET) scan may be recommended, considering that these patients may be asymptomatic [45].

Diagnosis of IIMs

Clinical Characteristics

Except for IBM, which is characterised by asymmetrical involvement, patients with IIMs typically present with symmetrical and progressive muscle weakness. Patients have difficulty performing basic daily activities requiring muscle strength such as getting up from a chair, going up/down the stairs, or combing their hair. Motor skills such as writing, knitting, or tying shoelaces, which reflect distal muscle strength, are affected in the later stages of DM and PM, but in early stages of IBM. The ocular muscles are not affected in the later stages, even in untreated patients. The facial muscles are not affected in PM and DM, but mild facial muscle

weakness occurs in IBM. The neck flexors and pharyngeal muscles are involved in any form of inflammatory myopathy, leading to swallowing difficulties and head drop. Sensory nerves are spared, and deep tendon reflexes are preserved, but a severe loss of muscle strength and muscle atrophy may lead to abolished deep tendon reflexes (particularly in IBM with marked atrophy in the quadriceps and distal muscles). The loss of muscle strength occurs subacutely, within weeks or months in DM/PM, whereas in IBM, the loss of muscle strength progresses slowly over years.

Extramuscular Manifestations

Skin Involvement

Skin lesions unique to or characteristic of DM include a periorbital violaceous rash and oedema (heliotrope rash), erythematous violaceous papules (Gottron's papules), and macules over the extensor aspect of the joints (Gottron's sign). Cuticular punctuate haemorrhage and periungual nailbed erythema are common. Erythema over the lower neck, upper chest (V sign), and the back, neck, and shoulder (shawl sign) is also characteristic of DM. Skin changes may result in poikiloderma characterised by hyperpigmentation, hypopigmentation, telangiectasis, and superficial atrophy. Itching is a significant symptom. Patients with anti-MDA5 DM frequently develop ulcerations over joint surfaces and palmar papules. Anti-TIF1 DM and anti-SAE DM are associated with extensive skin lesions. Non-itchy, hyperkeratotic lesions over the lateral aspects of the fingers, known as "mechanic's hand," are distinctive skin characteristics of anti-synthetase syndrome, although mechanic's hand may also be seen in patients with DM and in those who test negative for anti-PM-Scl antibodies. Abnormal, insoluble calcium may be deposited in the skin and subcutaneous tissue in patients with DM, and more frequently in juvenile DM. Calcinosis occurs more frequently in areas exposed to microtrauma and in patients with anti-NXP2 DM. Histological characteristics of DM include dyskeratotic interface dermatitis, dermal mucin deposition, perivascular inflammatory infiltrates, and vascular dilation and/or damage in specimens from the skin (Table 16.1). However, these findings cannot be distinguished from those seen in SLE. A skin biopsy is recommended in patients with clinically amyopathic dermatomyositis (CADM) without muscle involvement.

Pulmonary Involvement

ILD is present in 78% of IIM cases and typically in patients with ASyS and anti-MDA5 DM. The most severe clinical form is the acute, rapidly progressing ILD leading to adult respiratory distress syndrome and respiratory failure. This form is associated with anti-MDA5 autoantibodies which indicate a poor prognosis. A chest CT may reveal rapidly progressing ground-glass consolidations, perilobular opacities, and in advanced stages, traction bronchiectasis. Manifestations tends to occur in the lung bases. Some patients with anti-synthetase syndrome, who have organising pneumonia and non-specific pneumonia, may develop rapidly progressive ILD. Rapidly progressive ILD occurs less frequently in patients with ASyS compared to those who are positive for anti-MDA5 autoantibodies. In general, a nonspecific interstitial pneumonia pattern on chest CT is a favourable prognostic factor in patients with IIMs. ILD mostly follows a subacute or chronic pattern in patients with IIMs. ILD may be asymptomatic and may respond well to treatment in patients positive for anti-PM-Scl or anti-SAE autoantibodies.

Cardiac Involvement

Cardiac involvement refers to myocarditis, inflammatory inflammation in the cardiac conduction system, and fibrosis. Clinically, overt heart diseases are present in only roughly 10% of all patients with IIMs; however, subclinical involvement in IIMs is more frequent (up to 75% of patients). Historically, cardiac involvement was believed to occur more frequently in anti-SRP autoantibody-positive patients, although this belief has changed recently: cardiac involvement occurs more frequently in patients with overlap myositis and systemic sclerosis. Cardiac MRI has a high sensitivity to detect myocardial inflammation at very early stages. Serum cardiac troponin levels provide sensitive and specific measurements of cardiac involvement, whereas troponin levels may also increase with inflammation of non-cardiac striated muscle tissue.

Joint Involvement

Arthritis is common, particularly in patients with ASyS. Arthritis may be the presenting symptom of IIMs, involving the small joints, in a symmetrical pattern. Consequently, arthritis associated with IMMs may be misdiagnosed as rheumatoid arthritis.

Table 16.1 Skin manifestations characterised by myositis-specific autoantibodies

	NXP2	TIF1	MDA5	SAE	Mi-2
Gottron's papules/ Rash/heliotrope rash	May be Absent	Frequently	Frequently violaceous	Frequently	Frequently
Additional findings	Calcinosis	Photosensitivity, Erythroderma, calcinosis	Palmar violaceous macule/papule and skin ulceration associated with vasculopathy	Erythroderma, Angel wing sign	Rare

Oesophageal Involvement

Whether dysphagia is a muscular or extramuscular manifestation is subject to debate. Dysphagia is present in 60% of patients and is particularly common in patients with IBM. Dysphagia results from weakness of the pharyngeal and upper oesophageal muscles and may be so severe that patients may have trouble swallowing liquids. Dry mouth may be present in patients with secondary Sjogren's syndrome, and its presence may aggravate dysphagia. Dysphagia is a risk factor for developing aspiration pneumonia.

Physical Examination

Symmetrical weakness of the proximal muscles is the most consistent physical finding in PM. Occasionally, muscle tenderness may be present, but deep tendon reflexes are preserved. Sensory examination is usually normal. A loss of muscle mass or atrophy may occur in advanced cases of IIMs. Lung auscultation may reveal inspiratory rales. Detailed manual strength testing is critical (Fig. 16.7). The Medical Research Council's grading system is widely used to test muscle strength. According to this scoring system, mainly the strength of neck, shoulder, elbow, wrist, hip, knee, and ankle muscles are evaluated (Table 16.2).

Muscle strength levels are as follows:

Grade 0 = No muscle contraction.

Grade 1 = Muscle contraction may be seen, limb movement is impossible.

Table 16.2 IMACS test for the evaluation of muscle strength

IMACS manual muscle testing			
Muscle Groups	Anti-Gravity	Gravity-eliminated	Score
Axial muscles			*(0–20)*
Neck flexors	Supine	Sidelying	(0–10)
Neck extensors	Prone	Sidelying	(0–10)
Proximal muscles			*(0–160)*
Trapezius (shoulder elevators)	Sitting	Supine	R (0–10), L (0–10)
Deltoid middle (shoulder abductors)	Sitting	Supine	R (0–10), L (0–10)
Biceps brachii (elbow flexors)	Sitting	Sidelying	R (0–10), L (0–10)
Gluteus maximus (hip extensors)	Prone	Sidelying	R (0–10), L (0–10)
Gluteus medius (hip abductors)	Sidelying	Supine	R (0–10), L (0–10)
Iliopsoas (hip flexors)	Sitting	Sidelying	R (0–10), L (0–10)
Hamstrings (knee flexors)	Prone	Sidelying	R (0–10), L (0–10)
Quadriceps femoris (knee extensors)	Sitting	Sidelying	R (0–10), L (0–10)
Distal muscles			*(0–80)*
Wrist extensors	Sitting (pronation)	Sitting (neutral)	R (0–10), L (0–10)
Wrist flexors	Sitting (supination)	Sitting (neutral)	R (0–10), L (0–10)
Ankle dorsiflexors	Sitting	Sidelying	R (0–10), L (0–10)
Ankle plantarflexors	Prone/ standing	Sidelying	R (0–10), L (0–10)

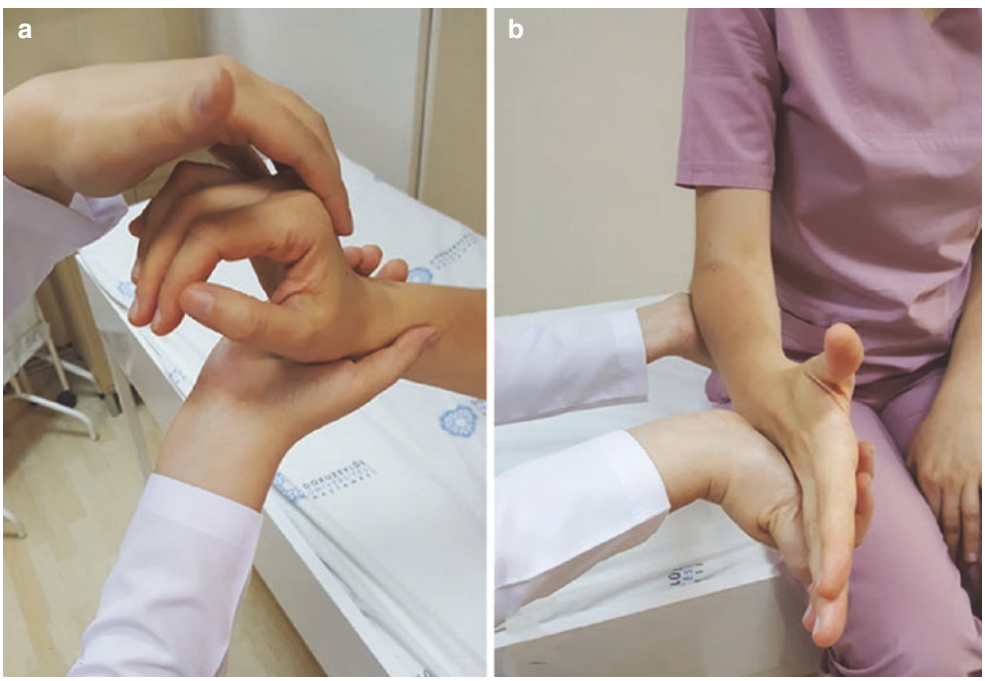

Fig. 16.7 Evaluation of wrist extensors: (**a**) Anti-gravity position, (**b**) gravity-eliminated position

Grade 2 = Limb movement can only occur if gravity is eliminated.

Grade 3 = Movement against gravity, without resistance coming from the examiner.

Grade 4 = Movement against moderate resistance.

Grade 5 = Full strength against resistance.

Laboratory Testing

Serum levels of muscle-derived enzymes are elevated in most patients with active muscle disease. CK is the most sensitive marker and is used for the diagnosis and follow-up of IIMs, although CK levels may fall within the normal range in some patients with active DM, ASyS, or IBM. Occasionally, serum aldolase levels may be selectively elevated without increased CK levels.

Indirect immunofluorescence using Hep2 cells substrate, which is the standard screening method for antinuclear antibodies, has limited value for detecting myositis-specific antibodies (MSAs) and myositis-associated antibodies (MAAs). As the targets of several MSAs and MAAs are cytoplasmic antigens, an indirect model results in a weak positive or negative nuclear staining pattern. Immunoprecipitation is the gold standard technique to define most MSAs and MAAs, but it is time consuming and inappropriate for routine testing. Enzyme-linked immunosorbent assays and the line blot assay are used for a few MSAs and MAAs, although their validation processes have not been completed yet.

Myositis-Specific Autoantibodies (MSAs): The frequency in which DM-specific autoantibodies are found in 70% patients who present with a typical phenotype of DM [46]. Anti-Mi2 antibody was the first MSA to be associated with skin rash. Anti-Mi2 antibody is associated with classical DM, characterised by proximal muscle involvement and severe skin involvement. In DM, anti-Mi2-positive patients develop a classical skin rash in skin areas exposed to sunlight [47]. Certain MSAs are associated with different skin symptoms in DM. In contrast with classical DM, nuclear matrix protein 2 (NXP2 = MORC3) antibody correlates with proximal and distal muscle weakness, subcutaneous tissue oedema, dysphagia, calcinosis, milder skin lesions compared to classical DM and a decrease in the frequency of Gottron's sign [48]. Patients positive for anti-transcription intermediary factor 1γ (TIF1γ = TRIM33) antibodies usually have more extensive skin involvement. The presence of this antibody is associated with palmar hyperkeratotic papules, psoriasis-like lesions, hypopigmented/telangiectatic areas, and a decreased risk of calcinosis. Furthermore, the presence of anti-TIF1γ antibodies is associated with an increased risk for cancer [43]. Another two important antibodies, associated with skin manifestations rather than muscle involvement, are anti-small ubiquitin-like modifier activating enzyme (SAE) antibodies and melanoma differentiation-associated gene 5 (MDA5) antibodies. Anti-SAE antibodies are associated with an initial presentation of amyopathic dermatomyositis. Sometimes, progression to IIM occurs along with a very severe rash and severe dysphagia. Anti-MDA5-positive patients often present with ulcerations over the interphalangeal joints, palmar erythematous macules/papules over the interphalangeal joint creases/ and palmar/fingertip mottled erythema [29]. As with anti-SAE antibody-positive patients, anti-MDA5-positive patients also have hypomyopathic or amyopathic DM. Unlike other DM autoantibodies, anti-MDA5 autoantibody is associated with a progressive course and ILD, in some cases [49]. In a study conducted with anti-MDA-5-positive Japanese patients, ILD and ADM were reported in 93% and 77% of all patients, respectively [50]. In general, mortality rates in patients with ADM associated with ILD are higher than in patients with DM associated with ILD, highlighting the requirement for early detection of IIM subgroup. Every patient with myositis suspected of having ILD should be screened and monitored with PFT- DLCO and HRCT. A recently published study assessed the prevalence of specific autoantibodies and the most common three autoantibodies were anti-Jo-1, anti-TIF1γ, and anti-MDA5 autoantibodies, respectively (Fig. 16.8).

Imaging: Magnetic resonance imaging (MRI) studies have a significant role in identifying areas to be targeted for muscle biopsy and are used as a supplementary test in the diagnosis and follow-up of myositis. Meyer et al. [51] demonstrated that histogram parameters could predict which muscles may have pathological spontaneous activity on EMG and, therefore, they could help in deciding the site of muscle biopsy. Whole-body MRI demonstrated that the muscle damage in IMNM was in the lumbar and pelvic-femoral area and correlated with disease duration [52]. Fatty infiltration in tight muscles is prominent in IBM, and a strong negative correlation was found between a proximal-to-distal gradient and disease activity [53]. Fatty infiltration on MRI scans is associated with histopathological damage in IBM, and muscle oedema is associated with the severity of inflammation [54]. In a study using CT, patients with IBM had significantly more severe degeneration in the rectus femoris, vastus, sartorius, adductor, anterior calf, and medial gastrocnemius muscles, compared to patients with PM or amyotrophic lateral sclerosis [55]. Furthermore, patients with IBM exhibited specific ultrasonographic features such as higher echogenicity and reduced muscle thickness compared to patients with PM/DM [56].

Electromyography: The triad of EMG changes in myopathy as follow:

- Polyphasic, short, small, low-amplitude motor unit potentials (MUPs).
- Fibrillation potentials at rest.
- Bizarre high-frequency repetitive discharges.

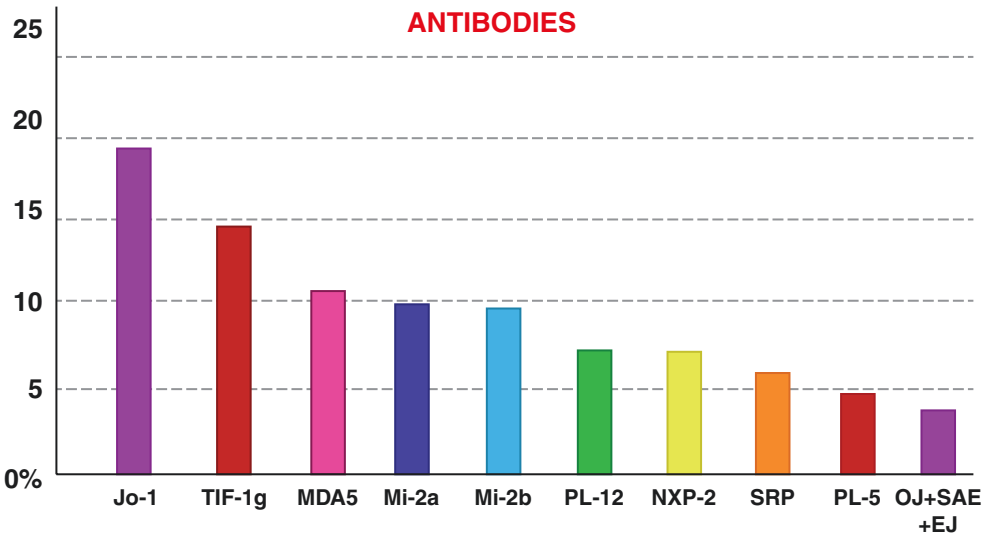

Fig. 16.8 Prevalence of specific autoantibodies in IIMs

In *PM* and *DM*, prominent membrane irritability (fibrillations, positive sharp waves, and even myotonic discharges), particularly in proximal muscles, is seen with needle EMG. MUPs recruit early and are small, short, and polyphasic. These abnormal characteristics cannot differentiate inflammatory myopathies from other myopathies showing muscle membrane instability. Small, mixed MUPs of long duration can be seen in long-standing disease. The degree of abnormal membrane irritability is considered to reflect ongoing disease activity. Abnormal spontaneous activity may be expected in active myositis.

EMG in *IBM:* EMG studies are not specific and may confound the diagnosis. Fibrillations and positive sharp waves are common and associated with small, short, polyphasic MUPs. However, large, and long MUPs may also be observed in many patients, and they reflect the chronic nature of the disease. Nerve conduction studies may reveal a mild axonal sensory polyneuropathy in a third of patients.

EMG in *toxic necrotic myositis:* MUPs of short duration with early recruitment, fibrillations, positive sharp waves, and even myotonic discharges may occur and are more prominent in weak muscles. Nerve conduction studies reveal low CMAP amplitudes without any evidence of demyelination. Sensory nerve action potential amplitudes may be reduced if a concomitant polyneuropathy is present or if there is fluid leakage into the third space (which is not rare in critically ill patients) or due to technical reasons. Needle EMG reveals muscle membrane irritability, in addition to MUPs of short duration with early recruitment.

It must be noted that several diseases mentioned in Table 16.3 may cause difficulty in the differential diagnosis of IMs.

Table 16.3 Major causes of myopathy

Drugs and toxins
• HMG-CoA reductase inhibitors
• Glucocorticoids
• Alcohol
• Colchicine, antimalarial drugs, penicillamine, daptomycin, zidovudine
• Illicit drugs (cocaine, heroin)
Endocrine disorders
• Cushing syndrome
• Hypothyroidism, hyperthyroidism
• Hyperparathyroidism
Electrolyte disorders
• Hypokalaemia, hypophosphatemia, hypocalcaemia, hyponatraemia
Infections
• Viral (HIV, echovirus, influenza, Coxsackie, Epstein-Barr virus, cytomegalovirus, adenovirus)
• Bacterial (Pyomyositis, Lyme)
• Parasitic (trichinosis, toxoplasmosis)
• Fungal
Inherited myopathies
• Acid maltase deficiency
• Muscular dystrophy
Metabolic myopathies
• Disorders of carbohydrate, lipid, and purine metabolism
Neuromuscular junction diseases
• Myasthenia gravis, Lambert-Eaton
Degenerative diseases
• Amyotrophic lateral sclerosis
Rhabdomyolysis
• Crush trauma
• Seizures
• Hyperkinetic state with delirium tremens
• Malignant hyperthermia
• Vascular surgery

Infectious pathogens including viruses, bacteria, fungi, and parasites may cause myositis. Myositis secondary to influenza usually presents with calf pain and difficulty walking, approximately 3 days (range: 0–18 days) after the onset of infection symptoms. Myositis secondary to influenza is relatively more common among children and in infections with Influenza type B, although type A is predominantly associated with rhabdomyolysis. Muscle enzyme levels are elevated, and myopathic changes are seen on EMG. Muscle histology reveals small inflammatory cell infiltrates, degeneration, and necrosis like IMNM. The disease is self-limited and resolves within 3 days (range: 1–30 days). No treatment has proved effective, other than symptomatic treatment. Similar cases of myositis have been reported with Coxsackie A/B viruses, ECHO viruses, adenoviruses, and respiratory syncytial viruses. Human immunodeficiency virus (HIV) can cause nemaline rod myopathy and may be a triggering factor for amyotrophic lateral sclerosis, brachial amyotrophic diplegia, and inflammatory muscle diseases such as IMNM, at any point in its course. Late-onset, HIV-related, sporadic nemaline myopathy is characterised by subacute proximal muscle weakness of the limbs. The condition often affects the neck, pharyngeal, and respiratory muscles. Progressive external ophthalmoplegia has been reported. Deep tendon reflexes are diminished and fasciculations may be seen. CK levels are normal. Histological examination of muscle biopsy specimens stained with modified Gomori trichrome reveals characteristic red actin rods.

All About the Pathology of Inflammatory Myopathies

It should not be forgotten that the leading actors of inflammatory myopathies are lymphocytes. In addition, there are regenerated fibres which are indicators of myofibre damage and necrosis. The classical pattern of lymphocyte subtypes in IIMs can be summarised as follows [57]: CD4+ T cells and B cells are prevalent in the perimysium in dermatomyositis, with CD4+ Th cells and B cells infiltrating endomysial capillaries as well (Fig. 16.9). In contrast, CD8+ T cells predominantly populate the endomysium in polymyositis (Fig. 16.10). Inflammatory infiltration may be very mild, especially in childhood dermatomyositis. In this case, the presence of perifascicular atrophy, depositions of immunoglobulin/complement on vascular walls detected by direct immunofluorescence microscopy, and the upregulation of sarcolemmal MHL-class 1 are diagnostic for dermatomyositis.

The diagnosis of inflammatory myopathy is suspected if there are only macrophages in addition to regenerated fibres in the muscle biopsy. In this situation, primary non-inflammatory muscle diseases, usually muscular dystrophies rather than inflammatory myopathies, are considered (Fig. 16.11). However, lymphocyte infiltration may be present in a polymyositis pattern in some MDs, especially FSHD and dysferlinopathy. In addition, mostly macrophage infiltration in inflammatory myopathies, such as inflammatory myopathy with abundant macrophages (IMAM), has been reported. Similarly, the presence of only macrophages in addition to abundant regenerated fibres, may be seen during a rhabdomyolysis attack of another non-inflammatory myopathy such metabolic, toxic, or drug-related disease (Fig. 16.12).

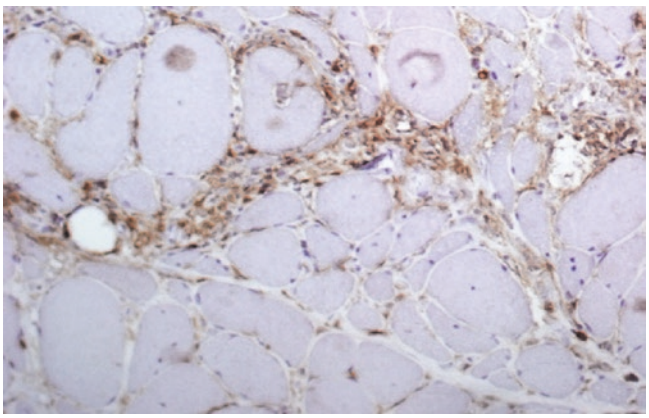

Fig. 16.9 CD4-positive T cells are prevalent in the perimysium (DAB ×100)

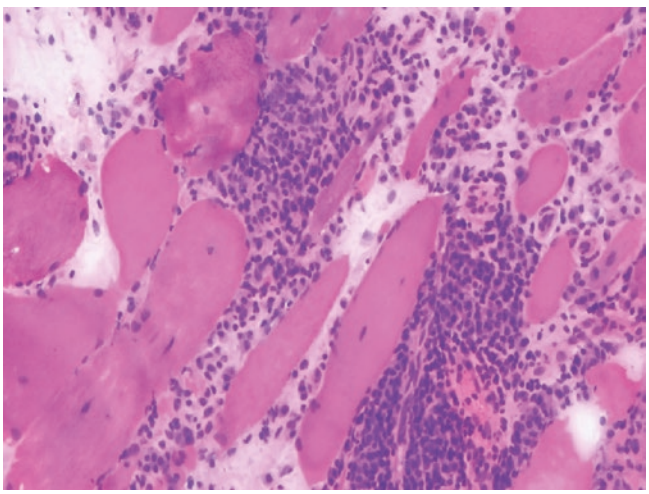

Fig. 16.10 Inflammation mainly in the endomysium (HE ×200)

Case 16.3

A 42-year-old female, who presented with pain and swelling in the small joints of her hands, skin hardening, fingers turning blue with exposure to cold/stress, was seen in the rheu-

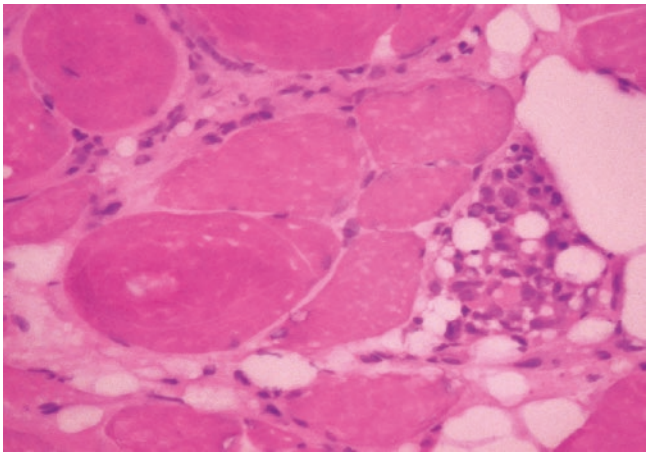

Fig. 16.11 Lymphocyte infiltration is present in a polymyositis pattern in a patient with dysferlinopathy (HE ×400)

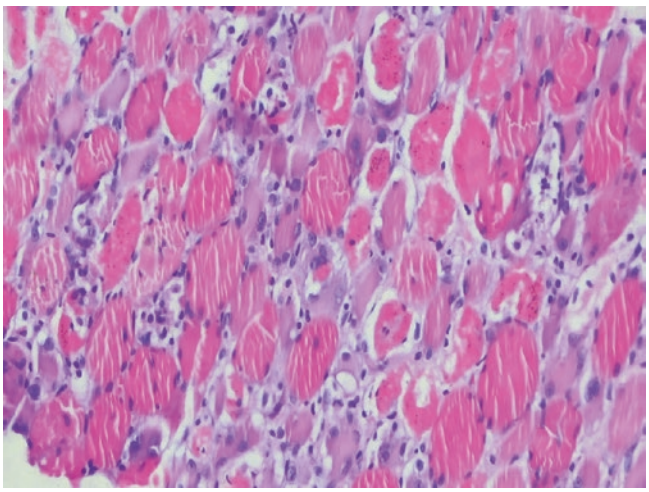

Fig. 16.12 Note the abundant regenerated fibres with a few macrophages in a patient with rhabdomyolysis (HE ×100)

matology outpatient clinic. She had accompanying constitutional symptoms and proximal muscle weakness. In her physical exam, lung auscultation revealed fine rales at the base of both lungs. Arthritis was detected in the bilateral metacarpophalangeal, proximal interphalangeal, and wrist joints. Proximal muscles showed 4/5 strength in both upper limbs and 5/5 in both lower limbs. Capillaroscopy was consistent with an early systemic sclerosis pattern. Anti-nuclear antibodies were directed to the centromere, with a cytoplasmic reticular (AMA)-like pattern and were positive at titres of >1/3200. Other test results were as follows: anti-CENP-B: +2 positive, anti-Jo1: +3 positive, AMA: +3 positive, CK: 1860 U/L, AST: 149 U/L, ALT: 123 U/L, LDH: 609 U/L, CRP: 50 mg/L, erythrocyte sedimentation rate: 53 mm/h. She was complaining about a non-productive cough and a high-resolution computed tomography scan of the lung revealed increased peripheral subpleural reticular opacities, predominantly in the lower lobes of both lungs, with ground-glass opacities and areas showing a crazy paving pattern. These findings were consistent with non-specific interstitial pneumonia. Needle EMG revealed findings of active denervation in the bilateral vastus medialis, right vastus lateralis, and bilateral deltoid muscles with neurogenic MUP activities in these muscles along with polyphasic MUPs of short duration. Histological examination of muscle showed inflammatory cell infiltration consisting mostly of T lymphocytes. Several B lymphocytes, plasmacytes, neutrophils, and histiocytes were also present among the T cells. Inflammatory cells predominantly populate the endomysium, like polymyositis. Muscle cell damage and regenerated fibres were also noticed. Although inflammation extended to perivascular areas in surrounding tissues, vascular damage (vasculitis) was absent (Fig. 16.13). Malignancy screenings were negative, and the patient was diagnosed with polymyositis. She is on prednisone at a dose of 1 mg/kg daily and azathioprine and is still on follow-up.

Case 16.4

A 54-year-old female presented with difficulty combing her hair and going up the stairs, an extensive itchy rash all over her body, and periorbital oedema which was present for the last 2 months (Fig. 16.14). She had constitutional symptoms and had difficulty swallowing which began a week ago, but she had no accompanying dyspnoea. In her physical exam, her bilateral proximal muscles showed 4/5 strength in the upper and lower limbs, elbow expansion showed 4/5 strength, and full strength was maintained in the remaining muscles. She had erythema all over her face, periorbital oedema, erythematous, mildly desquamated, guttate-size papules over the metacarpophalangeal joints in both hands, erythema, and areas of excoriation over the medial aspect of both femurs. Anti-nuclear antibodies were positive in a speckled, lysosome-like pattern at titres of 1/1000–1/3200. Anti-Sm/RNP and anti-Jo1 were positive, and other tests were as follows: CK: 284 U/L, LDH: 796 U/L, ALT: 85 U/L, AST: 36 U/L. Skin biopsy was consistent with lichenoid interface dermatitis. An EMG study was performed according to the myopathy protocol, and myogenic MUP activity and an early recruitment pattern were detected along with marked active denervation in axial muscles and proximal extremity muscles. Muscle MRI scans revealed signal increases consistent with oedema in proximal muscle groups around the hip joints, particularly in the anterior and posterior thigh muscles and in the pelvic and gluteal muscles. Histological examination of muscle biopsy specimens revealed that there were myopathic changes, such as nuclear internalisation, fibre

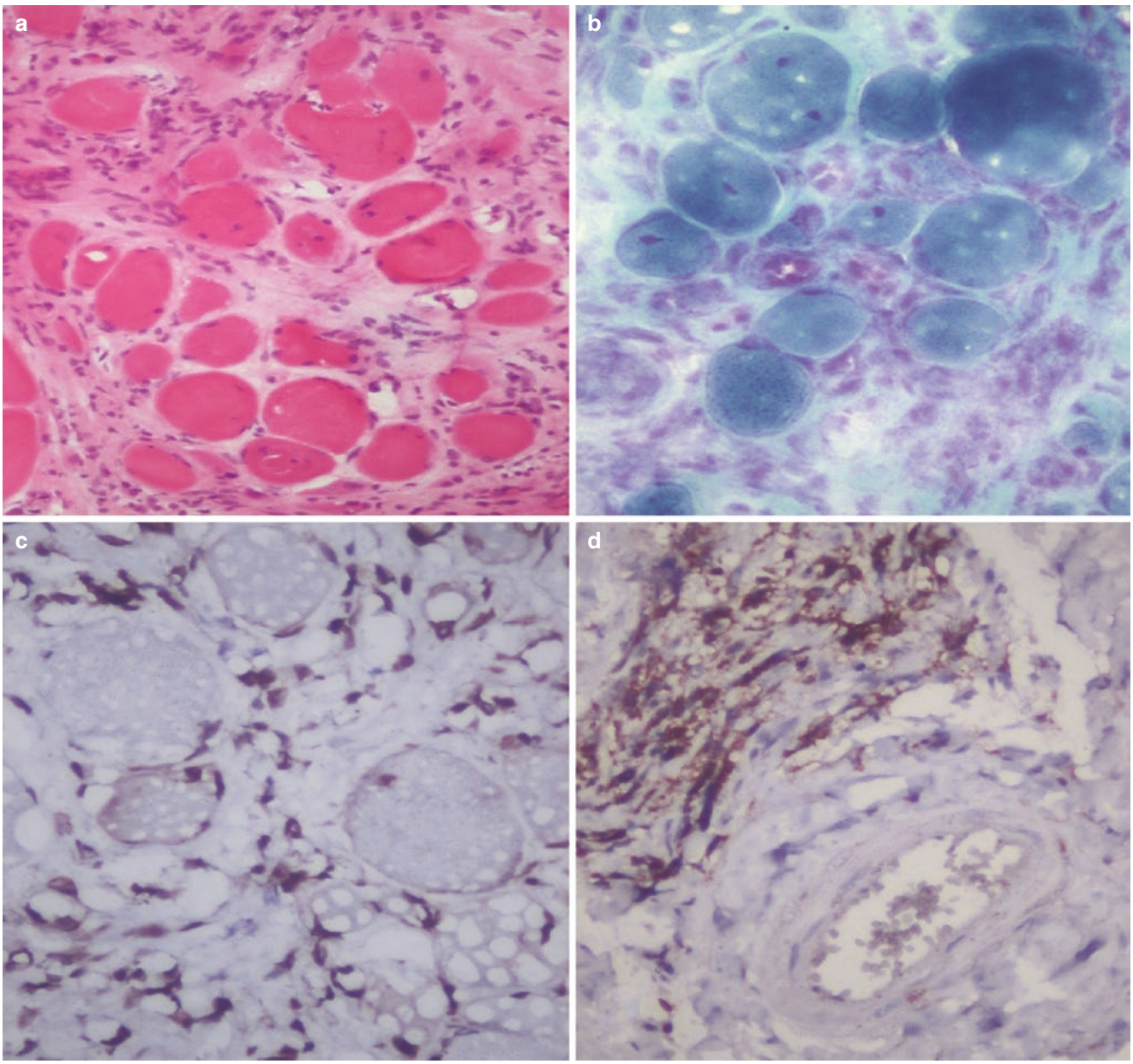

Fig. 16.13 (**a**) Inflammation mainly in the endomysium (HE ×40), (**b**) Increased nuclear internalisation (modified Gomori Trichrome ×100), (**c**) Endomysial CD3-positive T cells (DAB ×200), (**d**) CD3-positive T cells adjacent to a normal vessel (DAB ×200)

necrosis, and regeneration. In perimysial area, inflammatory infiltrates were observed in vessel walls. There was a CD4-positive lymphocyte predominance in the CD4+/CD8+ cell ratio. Direct immune fluorescence (DIF) was positive for C3 and IgG in the vessel walls (Fig. 16.15). A high-resolution computed tomography scan of the lung revealed subpleural reticular opacities in the lower lobes of both lungs. A preliminary diagnosis of dermatomyositis was made, and the patient was started on prednisone at a dose of 0.5 mg/kg daily and azathioprine. A significant improvement was observed with this treatment. During follow-up, a BI-RADS 4 lesion was detected in the breast during cancer screening and the skin rash did not improve. Therefore, she was switched to intravenous immunoglobulin therapy. A clinical and biochemical response was obtained with the current treatment and breast biopsy did not reveal any malignancy.

Case 16.5

A16-year-old male patient presented with pain and weakness in the arms, legs, and knees. These symptoms were present

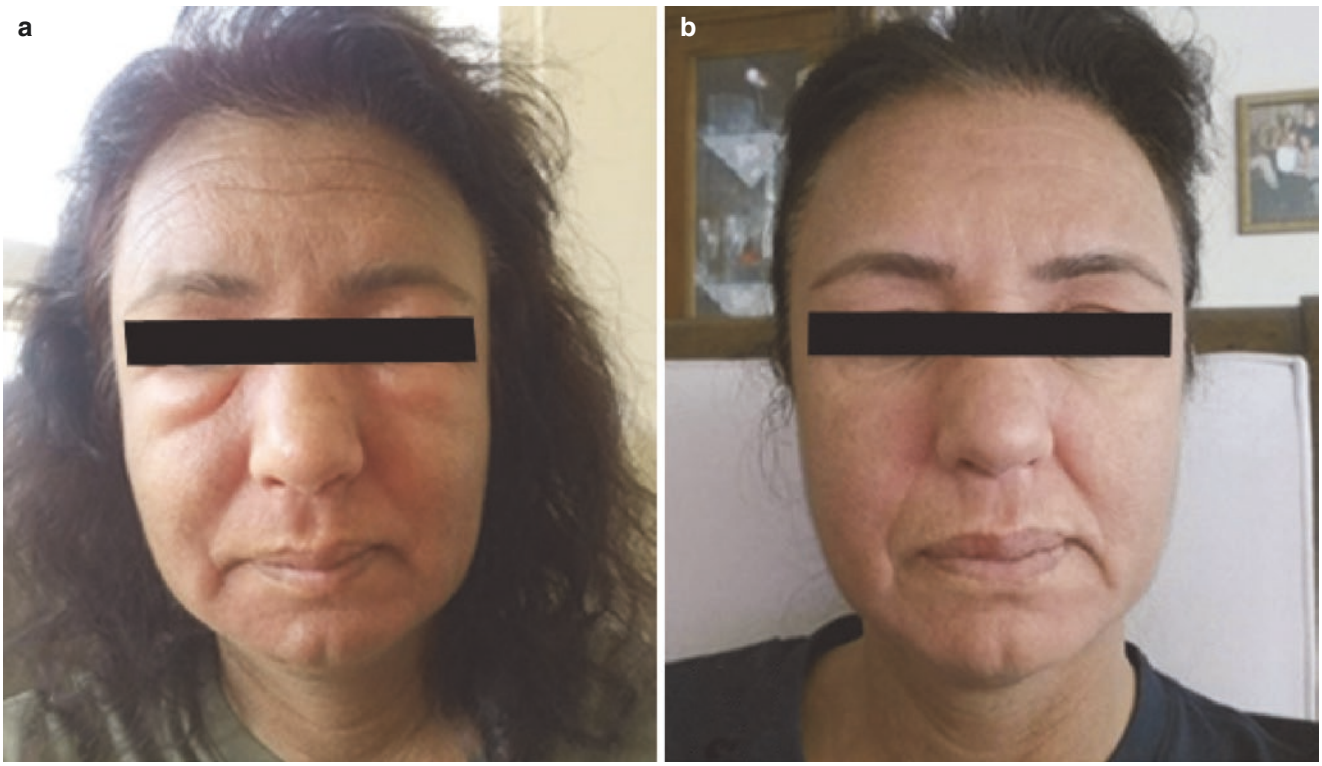

Fig. 16.14 Periorbital erythema (**a**) and after treatment (**b**)

for the last 5 years and gradually worsened over the previous 5–6 months. He had no history of morning stiffness, photosensitivity, malar rash, Raynaud phenomenon, skin rash, joint swelling, or serositis. He had no history of systemic symptoms such as fever or weight loss. He had no arthritis and muscle strength was normal except for the deltoid and thigh muscles which showed 4/5 strength. He had no muscle atrophy and deep tendon reflexes were normal. At that time, laboratory test results were as follows: CK: 1327 U/L, LDH: 270 U/L, AST: 50 U/L, ALT: 107 U/L, ESH: 9 mm/h, CRP: 0.57 mg/dL, TSH: 2.19 mIU/mL, and ANA/ENA were negative. Electrophysiological studies showed myogenic and chronic neurogenic MUP alterations. A muscle MRI showed atrophy of the posterior muscle groups in both thighs compared to the anterior muscle groups, whereas mild atrophy was observed in the bilateral subscapularis, infraspinatus, and deltoid muscle groups. Histological examination of specimens from the left deltoid muscle revealed a difference between muscle fibres in size and shape, a presence of regenerated fibers using neonatal myosin, and a defect in sarcolemmal dysferlin expression. Direct immune fluorescence (DIF) was negative. Interestingly, there was endomysial mild inflammatory infiltration, too (Fig. 16.16). Genetic analysis identified a c.334 C > T (p.Gln112Ter) homozygous pathogenic variant in the dysferlin (DYSF) gene. The patient was diagnosed with dysferlin deficiency based on histopathological and genetic findings.

Case 16.6

A 30-year-old female patient who had presented with fatigue, difficulty going up the stairs, and weakness of the proximal muscles of her lower limbs had been diagnosed with polymyositis based on elevated CK levels, EMG, and MRI results and was initiated on methylprednisolone and azathioprine. The patient was referred to our rheumatology outpatient clinic as no clinical or biochemical response had been obtained. In her physical exam, Gower's sign was positive. No muscle weakness was found in the upper extremities, whereas the proximal muscles of her lower limbs showed 3–4/5 strength. She had no contractures and deep tendon reflexes were normal. Lately, she began to suffer from exercise dyspnoea. Laboratory test results were as follows: CK: 1182 U/L, AST: 47 U/L, ALT: 66 U/L, LDH: 391 U/L, CRP: 4 mg/L, ESH: 27 mm/h, and PTH: 53 pg/mL. She tested negative for ANA, myositis-specific, and myositis-associated antibodies. Thyroid function tests were within normal limits. Nerve conduction studies revealed electrophysiological findings consistent with myopathy and positive sharp waves

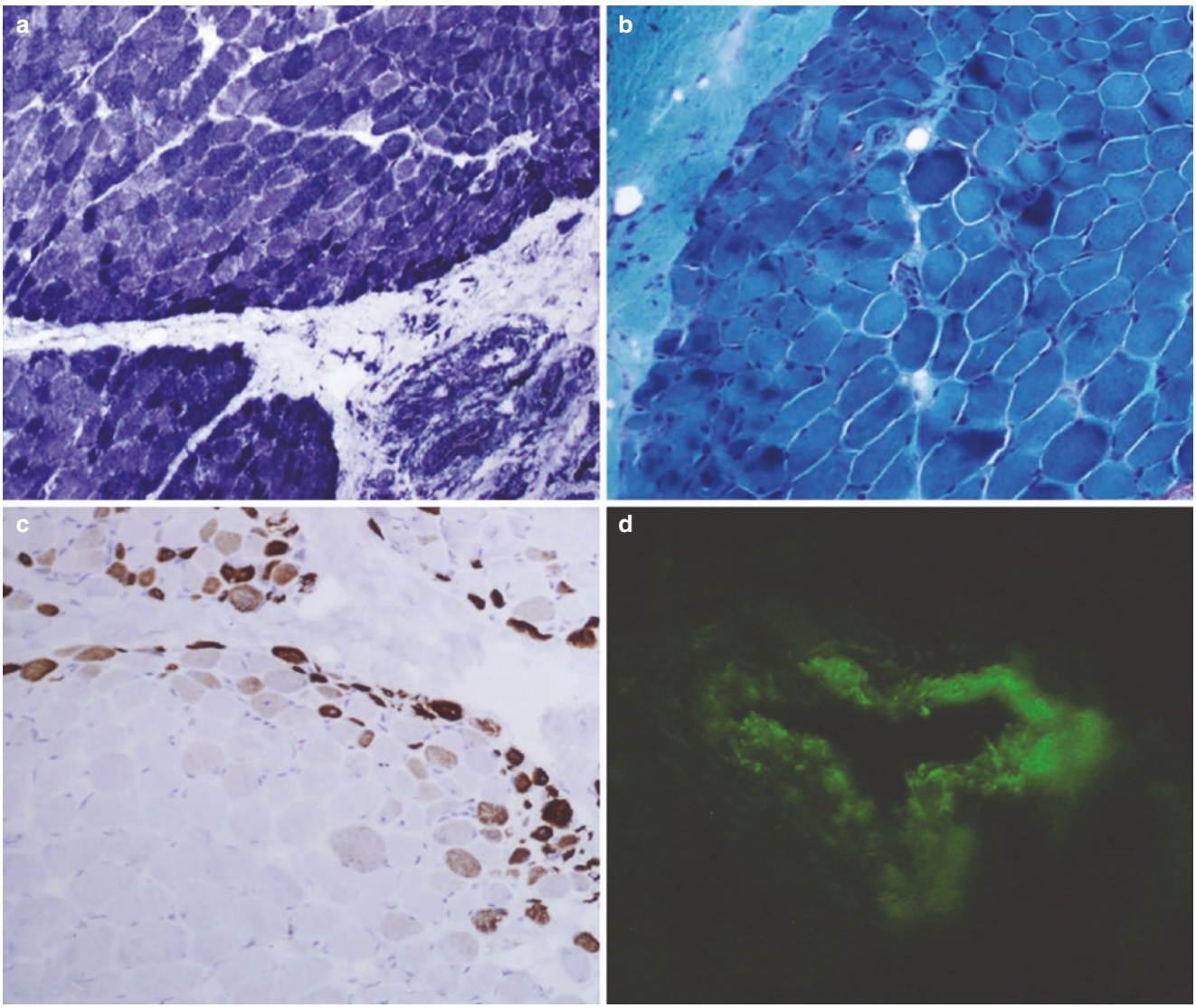

Fig. 16.15 (**a**) Note the presence of perifascicular atrophy (NADH-TR × 100), (**b**) normal intercellular tissue (Modified trichrome ×200), (**c**) atrophic myofibres are highlighted with neonatal myosin expression (DAB ×100), (**d**) IgG storage on a vessel wall (DIF × 400)

in the proximal muscles. Extremity MRI revealed fatty atrophic areas and patchy areas of oedema of the bilateral thigh muscles. A muscle biopsy from the rectus femoris revealed cytoplasmic vacuolation in certain muscle fibers, and this finding was consistent with noninflammatory myopathy with vacuoles (Fig. 16.17). Periodic Acid Schiff (PAS) and oil red O stains staining did not show glycogen or lipid accumulations because accumulated materials may be dispersed during tissue processing. The alpha glucosidase enzyme level was 0.2 nmol/mL/h (low). The patient was compound heterozygous for two variants in the GAA gene [GSD2, c.-32-13 T > G and c.1061delA (p. Tyr354Serfs)]. The patient was diagnosed with late-onset Pompe disease. She was receiving immunosuppressive agents and this therapy was discontinued. She was started on enzyme replacement therapy. She had no systemic organ involvement and enzyme replacement therapy provided significant reductions in enzyme levels and a significant improvement in muscle strength.

In terms of last two mentioned patients (Cases 16.5 and 16.6), it is necessary to emphasise again; muscle biopsy is sometimes essential for the differential diagnosis, especially in hereditary myopathies that can mimic inflammatory myopathy. Thus, patients do not have to use unnecessary immune suppressor or immune modulator therapies. Another important point is that a multidisciplinary approach, in which clinical, electrophysiological, genetic, radiological, and pathological findings are considered together, is essential in determining the differential diagnosis of neuromuscular disorders. The presence of inflammatory cells in the muscle biopsy alone may not be meaningful because, as we encountered in Case 16.5, some hereditary diseases may be accompanied by inflammatory cells.

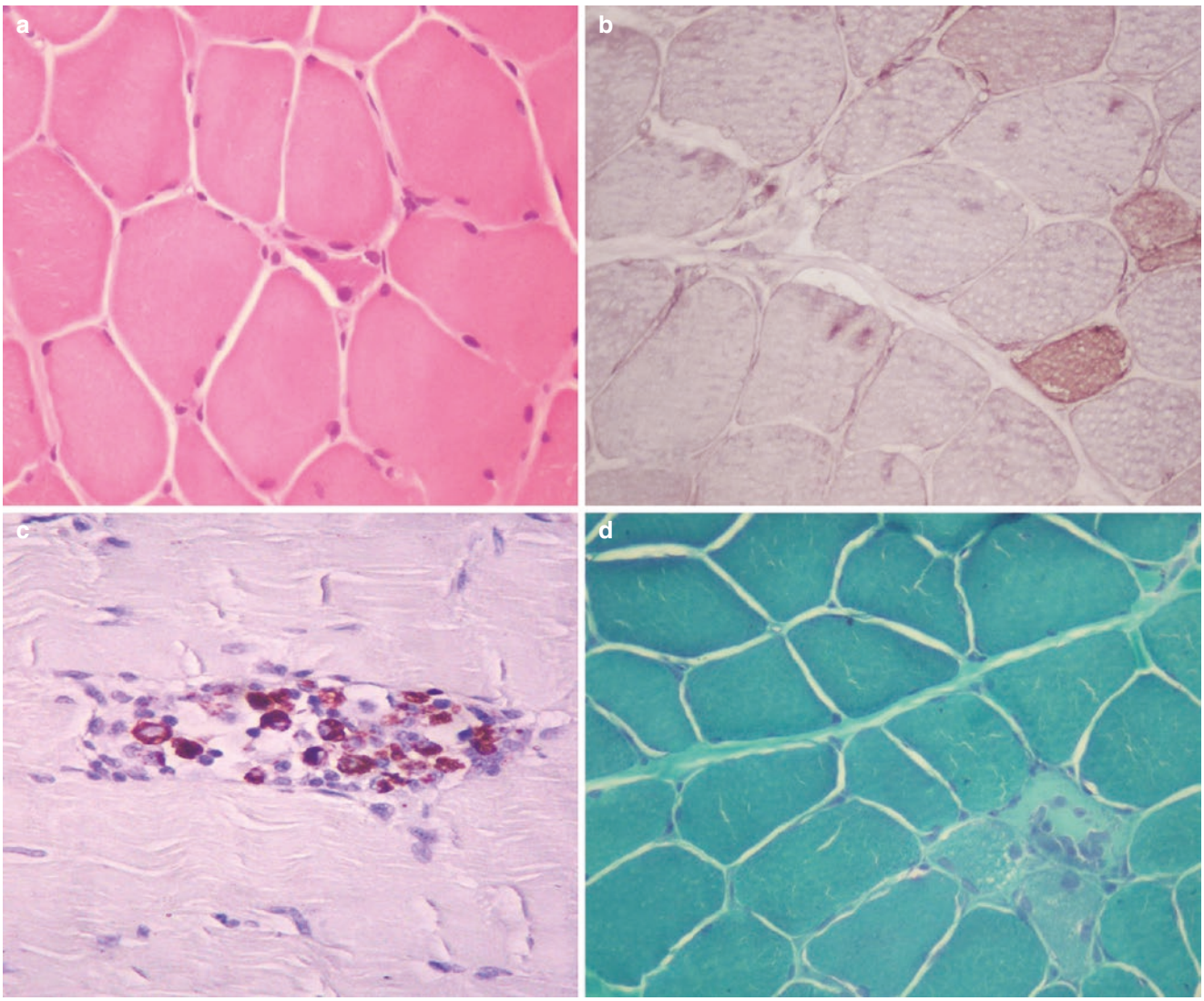

Fig. 16.16 (**a**) Myofibre shape and size changes with a regenerated fiber (H&E × 200), (**b**) sarcolemmal dysferlin defect (DAB ×200), (**c**) endomysial CD3-positive T cells (DAB ×200), (**d**) increased interstitial fibrosis (modified Gomori Trichrome ×200)

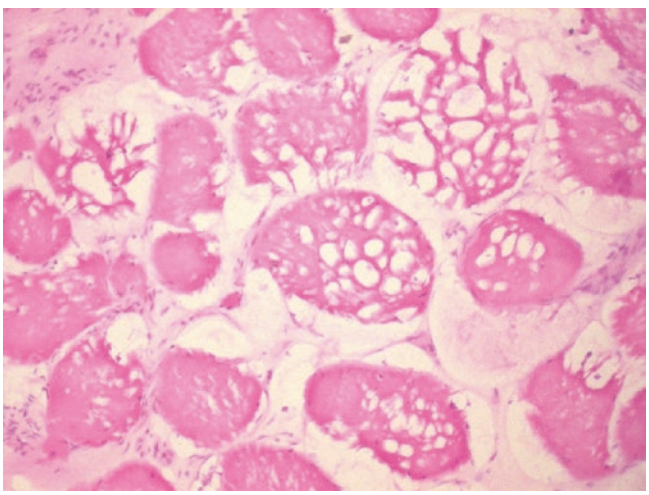

Fig. 16.17 Note the vacuoles in muscle fibres of a patient with Pompe disease (H&E × 200)

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Toxic and Drug-Induced Myopathies

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Introduction

Many medications, even commonly used ones, from various branches of medicine may cause potentially reversible (when the causative agents are withdrawn) muscle damage [1]. This clinical presentation is one of toxic, or drug-induced myopathy. These myopathies generally present with proximal muscle weakness, myalgia, cramps, creatinine kinase (CK) changes (hyperCKemia), and myoglobinuria caused by rhabdomyolysis. They may be confused with inflammatory myopathies because of the proximal muscle involvement in subacute stages. The pathophysiology of muscle damage in skeletal muscle has many underlying mechanisms including necrotizing, mitochondrial, inflammatory, microtubular, myofibrillar, and autophagic processes. In this chapter, we will discuss all forms of toxic and drug-induced myopathies with histopathological samples. Types of toxic myopathies can be classified as follows:

Forms of Toxic Myopathies

- Necrotizing myopathies.
- Amphiphilic drug myopathies.
- Inflammatory myopathies.
- Colchicine-related antimicrotubular myopathy.
- Antiretroviral-related mitochondrial myopathy.
- Protein synthesis impairment or increased catabolism-associated myopathies.
- Toxic myopathies due to other pathogenic mechanisms.
- Anesthetic agents and centrally acting drug-related myopathies.
- Myopathies secondary to drug abuse.

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Necrotizing Myopathies

Statins: Cholesterol-lowering agents including the hydroxy-3 methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors may cause toxic myopathy. HMG-CoA reductase is a rate-limiting enzyme in cholesterol synthesis. Statins block the production of mevalonate from HMG-CoA by inhibiting the HMG-CoA reductase enzyme. Statins can be the cause of statin-induced neuromuscular diseases which include necrotizing myopathy/rhabdomyolysis, immune-inflammatory myopathies, necrotizing autoimmune myopathy (NAM), pol ymyositis/dermatomyositis, mitochondrial myopathy, unmasking of pre-existing metabolic myopathy, myasthenia gravis, and axonal polyneuropathy [1].

All approved statins can cause asymptomatic hyperCKemia, myalgia, proximal muscle weakness, and rarely, myoglobinuria [2]. Myalgia and cramps are more frequent symptoms (20%) than asymptomatic CK elevation, occurring in 5% of patients taking statins [3]. Generally, symptoms begin between 1 and 6 months after the start of statin treatment. Myalgia is a very common symptom even in patients without statin use. It is very important to differentiate that statin use caused the myalgia symptom. Generally, statin-related myalgia occurs symmetrically in the large proximal muscle groups. Cramps are more prominent in the small muscles of the hands and feet [3]. Severe myopathy risk is lower than 1%. An increased risk of toxic myopathy is expected with concurrent use of other medications affecting statin metabolism, such as fibric acid derivatives, niacin, ezetimibe, amiodarone, calcium channel blockers, colchicine, azole antifungals, cyclosporine, protease inhibitors, rapamycin, and sirolimus. Excessive use of grapefruit juice has the same effect as medications. If statins are combined

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with either gemfibrozil or cyclosporin, rhabdomyolysis risk increases more than normal estimated percentages (more than 2–3/100,000 persons per year). Rhabdomyolysis is expected to occur in the early period of statin use. Statin-associated muscle symptoms (SAMS) can be evaluated in five subgroups according to the severity of disease [4]. These groups are myalgia, myopathy, myositis, myonecrosis, and rhabdomyolysis. Although there is no special finding in myalgia in the muscle pathology, other groups have some histopathological findings including variable inflammation, atrophy, the presence of T/B cells, the presence of macrophages, and dead tissue. Phagocytic muscle fiber necrosis and regenerating myofibers are common. Myofibers including vacuoles with lipids, and myofibers with COX (cytochrome oxidase) negative staining are not persistent pathological findings. Muscle biopsy frequently demonstrates increased expression of major histocompatibility antigen-1 (MHA1) and membrane attack complex in the sarcolemma of non-necrotic muscles in necrotizing myopathy patients. Although the pathogenesis of statin-induced myopathy is not clear, interruption downstream in the HMG-CoA pathway may be a potential causative pathogenetic mechanism [2]. Low cholesterol levels in myofibers, depletion of metabolites in the cholesterol pathway, and low levels of coenzyme Q, which is decreased because of HMG-CoA reductase inhibitors, are other possible pathogenetic mechanisms.

Female gender, old age, lower body mass index, Asian ethnicity, hypothyroidism, low vitamin D levels, diabetes mellitus, renal, hepatic/muscle diseases, carnitine palmitoyl transferase II deficiency, frailty, alcohol consumption, heavy exercise, and major surgery are risk factors of SAMS onset [5]. Also, genetic factors play an important role. A genome-wide association study found that 60% of patients with statin myopathy have a single nucleotide polymorphism located within the SLCO1B1 gene encoding a protein regulating hepatic processing of statins [6]. In addition to this gene, the CYP2D6, CYP3A4, and CYP3A5 genes controlling the alteration of cytochrome P450 expression, and vitamin D receptor have been identified to cause SAMS [4]. As a rare clinical entity, immune necrotizing myopathy can occur during statin use. Its clinical form is indistinguishable from toxic necrotizing myopathy. The avoidance of statins does not improve the symptoms; additionally, immunomodulatory treatment is required. 60% of patients have HMG-CoA reductase antibodies.

CK levels are very high, reaching 5000-10,000 U/L in both immune and toxic necrotizing myopathy patients. Even in asymptomatic patients, CK levels can be found to be as high. In electrophysiological evaluations, routine nerve conduction studies are normal. Electromyographic (EMG) examinations performed in weak muscles reveal myopathic motor unit action potentials (MUAPs) and irritable muscle findings, including fibrillation potentials, positive sharp waves, and sometimes myotonic discharges [2]. EMG in asymptomatic patients with high CK levels is generally normal. As for treatment, it is first suggested to stop statins in both asymptomatic and symptomatic hyperCKemia patients. After stopping the medication, it is advised to test for HMG-CoA reductase antibodies. If patients have toxic myopathy, CK levels decrease after the cessation of statins. If they do not, immune-mediated diseases should be suspected, and immune therapies are required.

Other lipid-lowering agents such as clofibrate and gemfibrozil are fibric acid derivatives used in hyperlipidemia treatment either alone or with statins [7]. The toxic effect of these drugs may result from destabilization of the lipophilic membrane. These fibric acid derivatives can cause toxic muscle damage in 2/3 months-2 years after treatment is commenced [8]. Like statin myotoxicity, muscle ache, weakness, and cramps are the main symptoms. Myoglobinuria is not a frequently present symptom. The combined use of gemfibrozil and lovastatin causes severe toxic myopathy in 5% of patients [9]. Although nerve conduction studies are normal, denervation potentials, myopathic MUAPs, and myotonic discharges can be recorded by needle EMG. Serum CK levels are elevated. In muscle biopsy, dispersed non-inflammatory necrotic fibers with groups of atrophic myofibers can be seen [2]. Ezetimibe and niacin can cause toxic myopathy, usually in patients that are already using statins.

Other drugs causing necrotizing myopathy: The immunophilins (cyclosporin and tacrolimus) generally used in transplant surgery, labetalol, propofol, and alcohol can cause necrotizing myopathy. Necrosis and vacuoles can be seen during muscle biopsy. Electrophysiological findings are the same with other necrotizing myopathies. Serum CK levels are high. If patient are on statins or colchicine, the development of toxic myopathy is more probable. High dose and concomitant use of corticosteroids in intensive care units is a risk factor for toxic myopathy in patients using propofol.

Amphiphilic Drug Myopathies

Chloroquine, hydroxychloroquine, and amiodarone have both hydrophilic and hydrophobic parts. Therefore, they are named amphiphilic molecules. These agents cause both neuropathy and myopathy. Generally, neuropathy with neuropathic symptoms in distal parts is more prominent than myopathy. CK levels are normal or slightly elevated. If patients are using statins, they are more likely to develop toxic myopathy with the use of these molecules [10, 11]. Unlike amiodarone, patients can recover after the cessation of chloroquine and hydroxychloroquine. After the cessation of amiodarone, partial recovery or a long-term recovery time is expected. In electrophysiological examinations,

neuropathy is detected in nerve conduction studies. Fibrillation potentials, positive sharp waves, rare myotonic discharges, and myopathic potentials are observed in needle EMG. Decreased recruitment with neurogenic MUAPs are seen in distal parts of the extremities [3]. Muscle biopsy shows autophagic vacuoles with curvilinear structures stained positive with acid phosphatase and myeloid debris [3]. Type 1 muscle fibers are more affected than type 2 fibers. Similar autophagic vacuoles are detected in nerve biopsies. A possible pathogenetic mechanism behind the development of autophagic vacuoles in amphiphilic molecules is lysosomal enzyme resistant drug–lipid complex formation after interaction with lipid membranes.

Inflammatory Myopathies

Lipid-Lowering Agents: HMG-CoA reductase inhibitors (statins) and other cholesterol lowering agents can cause dermatomyositis, polymyositis, and immune-necrotizing myositis; all requiring immune therapy to improve. Clinical findings do not improve by stopping the treatment. Statin associated autoimmune necrotizing myopathy (NAM) is one of the immune related myopathies, which has many causative factors such as HMG-CoAR antibodies, antisignal recognition antibodies, antisynthetase antibodies, malignancy, and viral infections [1]. In statin-induced NAM, CK levels are quite high and the before-mentioned muscle symptoms appear. Histopathological myonecrosis and regeneration are detected in muscle samples with very few or no inflammatory cells.

Immune Check Point Inhibitors (ICIs) are agents that are increasingly used in the treatment of numerous cancers such as renal cell carcinoma (RCC), urothelial carcinoma (UC), metastatic melanoma, nonsmall cell lung cancer (NSCLC), metastatic head/neck cancers, and Hodgkin's lymphoma (HL). They act by targeting certain cell surface proteins responsible for balancing the T cell response such as cytotoxic T lymphocyte associated protein-4 (CTLA-4), programmed cell death protein-1 (PD-1), and/or programmed death ligand 1 (PD-L1). These proteins can be used by tumor cells to create a microenvironment conducive to their growth. ICIs disrupt the binding of these receptors and their ligands to hinder this process. Although this direct intervention to the cancer biomechanics is effective, it carries a great potential for autoimmune adverse events by shifting the balance in favor of an excessive T cell response.

Ipilimumab (Yervoy), which is a CTLA-4 monoclonal antibody (mAb), was the first ICI to be approved for use in cancer treatment and was followed by pembrolizumab (Keytruda), and nivolumab (Opdivo) for PD-1 [12–14]. Atezolizumab (Tecentriq), avelumab (Bavencio), and durvalumab (Imfinzi) for PD-L1 ligands are relatively new ICIs. Neurological ICI associated immune-related adverse events (IrAEs) include toxic myopathies, neuromuscular junction disorders, peripheral neuropathies, ataxia, retinopathy, bilateral internuclear ophthalmoplegia, and headache. Although rare (estimated incidence is 0.7%) [15], neuromuscular complications are considered as serious side effects as they can be lethal [16].

ICI-related toxic myopathies (IrTMs) are mostly seen as necrotizing autoimmune myositis (NAM) [17] and are particulary associated with pembrolizumab, ipilimumab, and nivolumab [18]. Male patients seem to be more vulnerable to developing IrTM [19]. Although IrTMs generally affect junctional muscles, they can present as external ophthalmoplegia, orbital myositis, and cardiomyopathy [20] or they can resemble myasthenia gravis (MG) [19], usually occurring within 3 months after the initial ICI dose [18]. Overlap with MG and/or myocarditis is not rare [20]. IrTMs are mostly negative for autoantibodies or extramuscular manifestations, while high CK levels are a common feature [21]. The histopathological features can differ between patients and vary from nonspecific myopathy to inflammatory myopathies [15]. A specific pattern that is characterized by clusters of necrotic myofibers was observed in muscles biopsies [22].

When findings suggestive of myopathy develop in a patient receiving ICI treatment, paraneoplastic inflammatory myopathies such as dermatomyositis must be excluded before the patient is diagnosed with IrTMs. Systemic oral corticosteroids are recommended as the first-line treatment in grade 1–2 patients after discontinuation of ICI [21]. Intravenous corticosteroids, immunoglobulins, or plasma exchange therapy should be administered if the patient's clinical findings are severe or not responsive to oral corticosteroids after a week. Immunomodulatory agents such as cyclophosphamide, rituximab, azathioprine, or methotrexate should be considered if the patient has not fully recovered. Corticosteroids should be tapered off after waiting for the half-life of the associated ICI [23].

In addition, cytokine-based therapies such as type 1 interferons [24], TNF alpha inhibitors such as etanercept, infliximab, adalimumab, and lenercept [25], and VEGF inhibitors such as bevacizumab [26] can cause inflammatory myopathy. Case reports reveal that d-penicillamine, cimetidine, procainamide, and phenytoin can cause inflammatory myopathy [2].

Colchicine-Related Antimicrotubular Myopathy

Colchicine is an antigout agent. Like other amphiphilic drugs, it has minimal amphiphilic features and affects microtubular structures by blocking tubular polymerization and preventing the formation of tubules. Due to this mechanism,

it causes neuromyopathy and leads to vacuolar histopathology in muscle. After daily colchicine use of 0.6–4 mg, progressive proximal muscle weakness occurs. CK levels are elevated. If patients are elderly, have renal insufficiency, use statins, or use cyclosporine, and myopathy risk increases. Sometimes myotonic discharges were recorded in electrophysiological examinations, and clinical myotonia has been observed. Axonal neuropathy and muscle irritation findings can be seen in needle EMG [27].

Antiretroviral-Related Mitochondrial Myopathy

Azidothymidine (AZT), in other words zidovudine (ZDV), is a nucleoside analog known as a reverse transcriptase inhibitor used in human immunodeficiency virus (HIV) treatment [28]. The incidence of ZDV myopathy ranges from 8 to 50% in studies [28]. HIV itself is a cause for myopathy, which is called HIV-related myopathy. The activity of mitochondrial DNA polymerase is inhibited by ZDV during treatment of HIV. ZDV myopathy causes symptoms of myalgia and proximal muscle weakness, which are very similar to HIV-related myopathy. Chronic inflammatory neuropathy and myasthenia gravis are also other diseases that can occur with HIV infection, which have the same clinical symptoms and should be considered in the differential diagnosis. CK levels are mildly elevated or normal, as also seen in HIV-related myopathy. CK levels are nearly five times higher in HIV-related myositis. Muscle weakness can be multifactorial in HIV-positive patients. In EMG, positive sharp waves, fibrillation potentials, and myopathic MUAPs are seen. Early recruitment patterns are also recorded in needle EMG [2]. As seen in other mitochondrial myopathies, ragged red fibers are remarkable for ZDV myopathy in histopathology. When the cumulative dose of ZDV increases, the number of ragged red fibers also increases in muscle specimens [29]. Nemaline rods, necrotic fibers, microvacuolated fibers, and cytoplasmic bodies are other histopathological findings in muscle biopsies [29]. Treatment involves the discontinuation of treatment.

Protein Synthesis Impairment or Increased Catabolism-Associated Myopathies

Steroid Myopathy: Steroid-associated myopathy was first described in Cushing's syndrome as proximal weakness and atrophy related to excess endogenous glucocorticoids. Since then, it has become the most common cause of drug-induced myopathies due to the use of steroids in the treatment of many diseases [30–32]. Steroid-associated myopathy can occur acute or chronically. The chronic form of steroid-induced myopathy is characterized by a myopathy in which the proximal muscles of the lower extremities and pelvic girdle muscles are more prominently affected, while the oculobulbar and facial muscles are generally spared [31, 33]. In the chronic form of steroid-related myopathy, slow progressing proximal muscle weakness is usually painless [34]. Proximal weakness due to this toxic non-inflammatory steroid-induced myopathy typically occurs after prolonged treatment with the equivalent of 30 mg of prednisone daily. Fluorinated combinations of steroids are more likely to cause myopathy than nonfluorinated steroids. However, all steroids have a risk of causing myopathy [2, 3]. In patients using high-dose intravenous corticosteroids, acute onset generalized weakness may develop, whether with concomitant neuromuscular blocking agent use or sepsis [33]. Steroids act on protein metabolism, reducing the rate of protein synthesis, resulting in muscle atrophy. However, the most obvious effects are the induction of protein catabolism [31]. The activation of major cellular proteolytic systems such as the ubiquitin-proteasome system, the lysosomal system (cathepsins), and the calcium-dependent system (calpains) of steroids results in a catabolic effect [35].

Steroids inhibit the transport of amino acids into the muscle. Steroids inhibit the stimulating effect of insulin, insulin-like growth factor-1, and amino acids (especially leucine) on the phosphorylation of two basic factors (4E-BP1 and S6K1), which have an important role in mRNA translation. Additionally, there is evidence that glucocorticoids cause muscle atrophy by inhibiting myogenesis through the downregulation of myogenin [32, 36, 37]. In steroid-associated myopathy, fast-twitch glycolytic type 2b muscle fibers are primarily affected, and muscle biopsy shows the presence of atrophy in type 2 muscle fibers, particularly fast-twitch glycolytic type 2b muscle fibers, while the effect on type 1 muscle fibers is less [38, 39].

Serum CK levels are normal [2, 3]. Sensory and motor nerve conduction studies are within normal limits. Needle EMG examinations are usually normal. If myopathy is severe, myopathic MUAP changes (low amplitude, short duration) may be seen, but spontaneous activity is not observed [40]. Clinical worsening of inflammatory myopathies during steroid therapy may be due to the inflammatory myopathy itself or due to newly developed steroid-associated myopathy, which sometimes confuses the diagnosis. In such a clinical picture, abundant abnormal spontaneous activity on needle EMG has great importance in the differential diagnosis, as it suggests that the clinical worsening is associated with myositis [40]. Reducing the dose of steroids, switching to an alternate-day treatment regimen, using nonfluorinated steroid types, following a low-carbohydrate diet, and exercise programs are the main steps of treatment [3].

Emetine (Ipecac) is an alkaloid derivative of ipecac, known to be abused by patients with anorexia nervosa and bulimia to provoke vomiting. The use of 500-600 mg/day for more than 10 days may cause severe proximal myopathy and cardiomyopathy. There may be pain, tenderness, and stiffness in the affected muscles [41]. A mild to moderate elevation of serum CK levels may be detected. Nerve conduction studies are within normal limits, but needle EMG usually shows myopathic MUAP changes (short duration, low amplitude, and early recruitment), and spontaneous activity can be found together [2]. Muscle biopsy shows scattered necrotic fibers, small atrophic/regenerative fibers, and cytoplasmic bodies. Oxidative enzyme stains show targetoid or moth-eaten-like structures. Electron microscopy shows myofibrillary degeneration and compacted myofibrillar remnants (cytoplasmic bodies) [2, 33]. Although the exact pathogenic basis of the disease is unknown, it is hypothesized that emetine may inhibit the synthesis of important muscle proteins [33]. Myopathy resolves with the discontinuation of emetine [33].

Finasteride: The 5 alpha-reductase inhibitors (5ARI), finasteride and dutasteride, are commonly used in the treatment of benign prostatic hyperplasia [42]. There are case reports in the literature reporting myopathy associated with finasteride treatment. It was mentioned that predominantly proximal myopathy developed in one case, with the coexistence of myalgia and serum CK elevation in another case [33, 43]. In a study investigating the effect of 5-alpha reductase inhibitors on the risk of rhabdomyolysis, it was mentioned that there was no increase in the risk of rhabdomyolysis, but that there was an increase in the risk of myopathy and myositis [42]. Serum CK levels may be within normal limits or elevated. Nerve conduction studies are within normal limits, and needle EMG shows small polyphasic MUAPs. Muscle biopsy shows mild variability in fiber size, type 2 muscle fiber atrophy, and signs of increased central nuclei [33]. The mechanism by which 5 alpha-reductase inhibitors causes muscle complications is not well defined. Due to their structural similarity to steroids, they are thought to cause a steroid myopathy-like picture [33]. With the discontinuation of finasteride treatment, a normalization of muscle strength and improvement in EMG abnormalities are observed.

Toxic Myopathies Due to Other Pathogenic Mechanisms

Acute quadriplegic myopathy/critical illness myopathy: In intensive care unit (ICU) patients, neuromuscular weakness often occurs due to critical illness myopathy (CIM), critical illness polyneuropathy (CIP), or a combination of the two. High-dose corticosteroid use may trigger critical illness myopathy in patients with prolonged intensive care hospitalization and concomitant use of neuromuscular blocking agents [33]. Patients who are critically ill in ICU are at risk for CIM/CIP, but those with sepsis, multiorgan failure, and systemic inflammatory response syndrome are at a particularly high risk [3]. Previous studies have emphasized that the main risk factor for CIM is intravenous glucocorticoid use in the ICU. The glucocorticoid relationship is controversial, and CIM can develop in critically ill patients with no exposure to intravenous glucocorticoids [44, 45].

The distribution of muscle weakness in CIM includes proximal weighted flaccid quadriparesis and respiratory muscle weakness. Although the facial muscles are usually spared, some patients may be affected, and ophthalmoplegia is extremely rare [46]. Serum CK levels may be within normal limits or mildly elevated. Nerve conduction studies show that low CMAP amplitudes, conduction velocities, and distal latencies are within normal limits. Sensory conduction studies are normal, unless there is a coexistent condition resulting in abnormal sensory potentials, such as CIP and/or other polyneuropathies. Needle EMG demonstrates myopathic MUAP patterns such as short-duration, low-amplitude MUAPs often with denervation potentials, especially in the early clinical period with a normal or early recruitment pattern [40].

Muscle biopsies show type 2 fiber atrophy, the presence of necrotic muscle fibers, and/or a loss of myosin thick filaments [33]. The pathogenesis of CIM is thought to be multifactorial. In some patients, widespread muscle fiber necrosis is observed in proportion to weakness in muscle biopsies, while in other patients this finding is not detected [2]. The mortality is high in CIM due to sepsis and organ failure. During the treatment of the underlying systemic condition, if possible, corticosteroid agents and neuromuscular blocking agents should be discontinued [33].

Isotretinoin is a synthetic vitamin A derivative used in the treatment of resistant acne vulgaris. In the literature, there are some case reports detailing various toxic myopathy presentations such as exercise-related myalgia cases, a case with prominent proximal pelvic girdle involvement, a case with extraocular myopathy, and another case with rhabdomyolysis in a patient treated with this indication [47–49]. Serum CK levels may be within normal limits or elevated. Nerve conduction studies are within normal limits, and needle EMG shows small polyphasic MUAPs. Muscle biopsy shows the presence of muscle fiber atrophy [33]. The precise mechanism of isotretinoin-associated myopathy has not yet been clarified. However, there is evidence that isotretinoin causes catabolic events induced by forkhead box class O (FoxO) transcription factors in muscle cells, which presents with increased CK levels, as well as muscle signs and symptoms [47, 50]. Complaints and symptoms improve with discontinuation of drugs [33].

Omeprazole is a proton pump inhibitor (PPI), which effectively blocks gastric acid secretion by inhibiting the hydrogen-potassium ATPase pump located on the luminal surface of the parietal cell membrane. Cases with proximal dominant myopathy, myalgia, and elevated CK levels have been reported during the use of omeprazole in the literature [51]. Nerve conduction studies may be within normal limits or reveal an axonal sensory motor neuropathy [52]. EMG can be normal or can show small polyphasic MUAPs. Muscle biopsy shows type 2 muscle fiber atrophy. Findings improve after the discontinuation of the drug causing the clinical picture [33].

Anesthetic Agents and Centrally Acting Drug-Related Myopathies

Malignant Hyperthermia is an exaggerated hypermetabolic response to volatile anesthetic agents and depolarizing muscle relaxants, such as succinylcholine, because of a pharmacogenetic defect in skeletal muscles [53]. An increase in body temperature is characteristic of malignant hyperthermia. Severe hyperthermia (a core temperature above 44 °C) causes a significant increase in carbon dioxide production, oxygen consumption, dysfunction of vital organs, and disseminated intravascular coagulation [54]. The earliest manifestations of malignant hyperthermia are tachycardia, increased end-expiratory carbon dioxide concentrations despite increased minute ventilation, muscle rigidity, rhabdomyolysis, acidosis, and hyperkalaemia [54]. Unexplained increased end-tidal carbon dioxide concentrations, elevated CK levels, combined metabolic/respiratory acidosis, hyperkalemia, cardiac arrhythmia, and renal failure are the main clinical and laboratory findings of malignant hyperthermia [55]. In the period between attacks, EMG examination is within normal limits. Spontaneous activity and early recruitment of small polyphasic MUAP morphology can be observed in EMG performed shortly after the attack [2].

The uncontrolled flow of calcium ions from the sarcoplasmic reticulum of skeletal muscle is involved in the pathophysiology of malignant hyperthermia. This uncontrolled release causes an increase in myoplasmic calcium and continuous myofibrillar contractions. Due to contractions, ATP stores are reduced, which causes muscle cell damage and rhabdomyolysis [54, 56]. The presence of defective ryanodine receptors 1 (RYR1) in the sarcoplasmic reticulum is the most common cause of calcium dysregulation in malignant hyperthermia. Other proteins that can cause this disorder are DHPR, FK506, and triadin [54]. Malignant hyperthermia is an emergency and treatment should be commenced immediately. First, the causative agent should be discontinued (convert to nontriggering iv. agents if necessary). Hyperventilation is achieved with 100% oxygen, the body is cooled (cooling of the body surface, washing the body cavities with 4 °C i.v. serum physiological), and 2.5 mg/kg/dose iv. of push dantrolene is applied via the widest vein (central venous catheter, if possible) [53].

Myopathies Secondary to Drug Abuse

Alcoholic Myopathy: Chronic alcohol abuse often causes polyneuropathy. In addition, various types of alcohol abuse-related myopathy have been described in the literature, such as acute necrotizing myopathy, acute hypokalemic myopathy, chronic alcoholic myopathy, asymptomatic alcoholic myopathy, and alcoholic cardiomyopathy [33]. Clinically, acute alcoholic myopathy is characterized by pain, tenderness, weakness, and oedema in the affected muscles. It can also manifest with rhabdomyolysis associated with the breakdown of muscle tissue. During this period, serum CK levels increase. In severe cases, myoglobinuria can cause renal failure [57].

Chronic alcoholic myopathy, the most common type of alcohol abuse myopathy, is characterized by slowly progressive mild proximal muscle weakness [3]. In asymptomatic alcoholic myopathy, serum CK levels are elevated, but clinically, there is no muscle weakness [57]. Muscle biopsy in acute necrotizing myopathy reveals extensive muscle fiber necrosis, and occasionally, fibers with tubular aggregates. In slowly progressive proximal chronic alcoholic myopathy, muscle biopsy may reveal scattered muscle fiber atrophy, necrosis, and regeneration [2]. Early muscle biopsy reveals muscle fibers with vacuoles. Myopathy improves with the correction of the serum potassium level [2].

Illicit drugs/Controlled Narcotic Agents: The muscle-damaging effect of controlled narcotics and illicit drugs (e.g., heroin, meperidine, cocaine, pentazocine, piritramide, amphetamines, etc.) can take place in several forms. These effects can be in the form of direct trauma to muscle tissue, muscle infarction secondary to a vasoconstrictive effect, or rhabdomyolysis. Inhalation of volatile substances such as toluene may also cause general muscle weakness and myoglobinuria. In addition, toluene causes distal renal tubular acidosis, severe hypokalemia, hypophosphatemia, and mild hypocalcemia. Muscle strength returns after the correction of electrolyte abnormalities and avoidance of further exposure [33].

All about the Pathology of Toxic and Drug-Induced Myopathies

Many kinds of drugs used in the treatment of various diseases may have myotoxic effects on the muscle through direct, indirect, or immunological mechanisms. Drug-

associated toxic myopathies can develop with an acute or chronic course, showing a clinical spectrum ranging from clinically asymptomatic elevated CK levels or mild myalgia to very severe myopathy and even difficulty in weaning from mechanical ventilation in the ICU. A good medical history should be obtained from patients with suspected toxic myopathy. All medical drugs used for any disease should be carefully questioned and noted, and alcohol consumption and/or illegal drug use should be questioned. Taking a good medical history becomes more important given that the myopathy symptoms will largely resolve with the discontinuation of the offending drug.

It should be kept in mind that multidisciplinary evaluation may be required due to the variability of the severity of the clinical pictures of the patients. Some toxic myopathies caused by alcohol, steroids, and statins are common, while other toxic- or drug-related myopathies are very scarce. For example, the widespread use of statins has resulted in many patients presenting with myalgia and hyperCKemia in clinical practice. It is important that the clinician and pathologist are aware of these conditions and can diagnose them. Therefore, the withdrawal of the hazardous agent usually allows full recovery, otherwise, damage can be serious, potentially becoming fatal. Myotoxicity of drugs and toxins can result from several mechanisms, such as direct harmful effects of toxins on muscle cells, through immunological processes, or because of secondary systemic effects like ischemia or electrolyte disturbances [58].

Histopathologically, toxic or drug-induced myopathies can show different pattens like focal myopathy, inflammation, necrosis/rhabdomyolysis, mitochondrial damage, type 2 fiber atrophy, or vacuolar myopathy [58]. Focal myopathy may be caused from repetitive intramuscular injections both of antibiotics and even abused drugs (Fig. 17.1). Several drugs and toxins can cause muscle fiber necrosis (Fig. 17.2), regeneration (Fig. 17.3), and even widespread muscle fiber necroses called rhabdomyolysis (Fig. 17.4). In this situation, CK levels increase over 1000 times the normal limits due to massive leakage of cellular contents and even acute renal failure can occur. Alcohol, opiates, and statins also may cause rhabdomyolysis. In some toxic myopathies, inflammation may be more prominent that can be diagnosed as inflammatory myopathy (Fig. 17.5). Mitochondrial damage may occur during some treatment regimens with special drugs (Fig. 17.6). Similarly, type 2 fiber atrophy is often associated with several myopathies. Two common causes of chronic myopathies with type 2 fiber atrophy are alcohol and steroid use (Fig. 17.7). Drug-induced vacuolar myopathy may occur due to two mechanisms. One of them is the development of autophagic vacuoles of lysosomal origin. The second is the development of spheric bodies derived from the sarcoplasmic reticulum (Fig. 17.8).

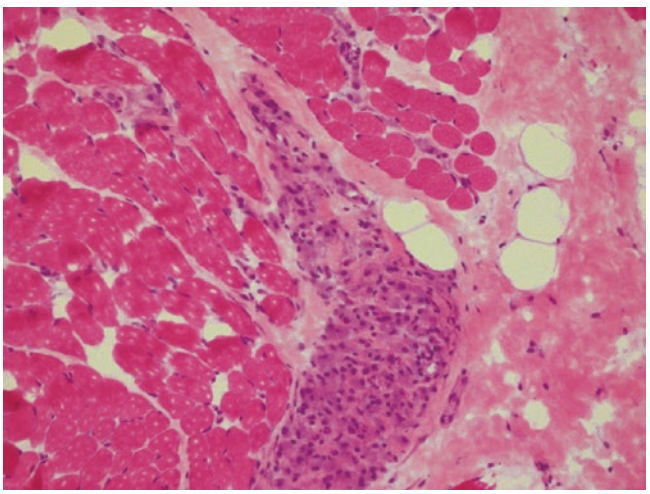

Fig. 17.1 Trophic myofibers shown in biopsy material taken at the injection site characterized by focal myopathy with fibrosis (H&E × 100)

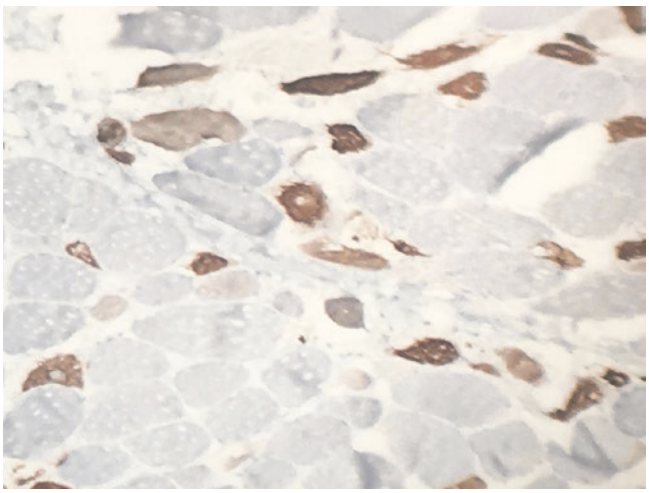

Fig. 17.2 Presence of several necrotic fibers detected with neonatal myosin (DAB × 100)

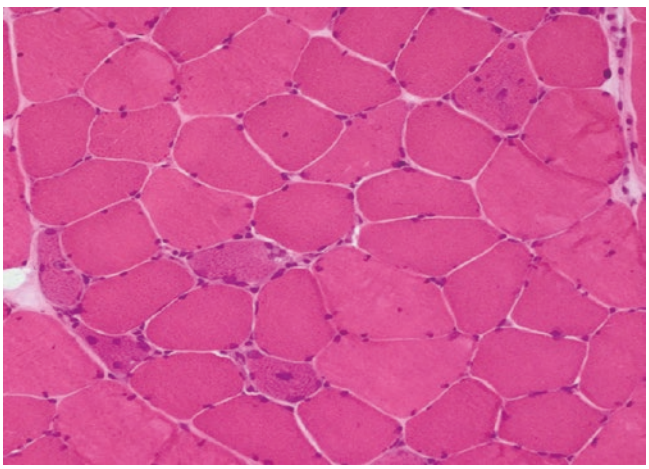

Fig. 17.3 Note the presence of regenerated fibers (H&E × 200)

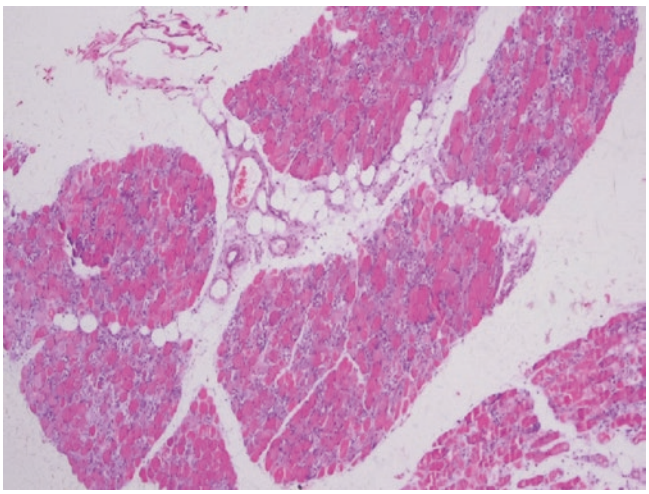

Fig. 17.4 Widespread rhabdomyolysis (H&E × 100)

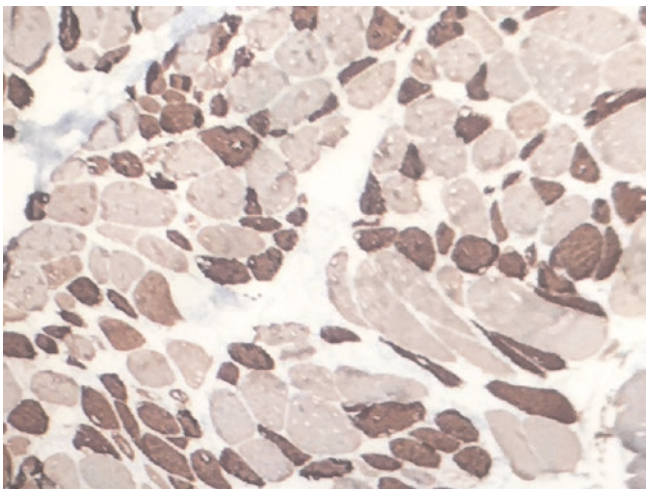

Fig. 17.7 Type 2 fiber atrophy detected with fast myosin staining (DAB × 100)

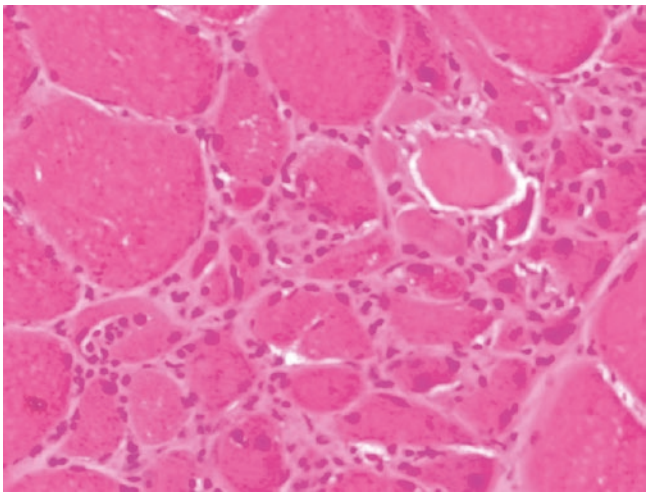

Fig. 17.5 Note the inflammatory cells, mostly of which are macrophages, in a patient with statin-related myopathy (H&E × 200)

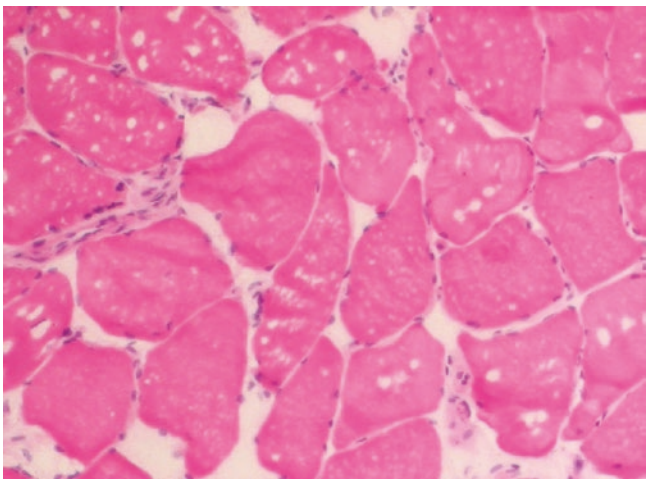

Fig. 17.8 Vacuolar myopathy associated with colchicine use (H&E × 200)

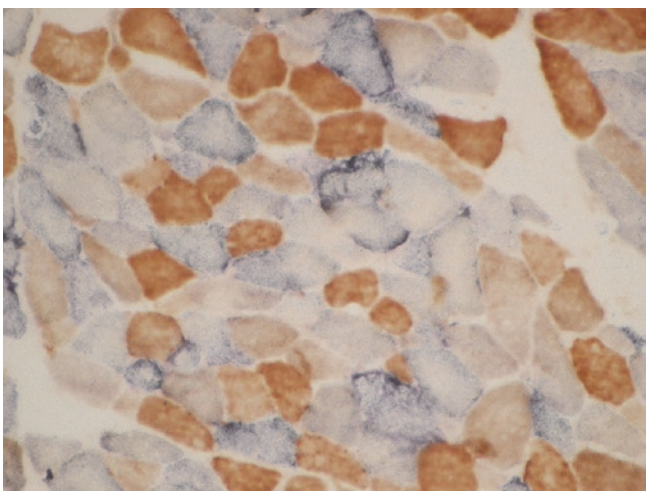

Fig. 17.6 Mitochondrial enzyme dysfunction due to cyclosporin-related myopathy (Combined COX-SDH × 100)

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Myopathies Associated with Systemic Disorders and Aging

18

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Many systemic diseases can cause muscle damage through various inflammatory and non-inflammatory pathways. Proximal muscle weakness is usually seen in systemic diseases associated with myopathies. A myopathic pattern detected during electromyography examination and an increase in serum creatine kinase can be seen. In this chapter, we will discuss the endocrine myopathies, malignancy/inflammatory myopathies, cancer-associated myositis, myopathies associated with connective tissue diseases, overlap syndromes, amyloidosis, and aging-related myopathies. The myopathic process known as sarcopenia, where muscle strength and mass decrease with aging, will also be discussed in this section.

Endocrine Myopathies

Myopathy refers to any abnormal condition of striated muscle. Skeletal muscle dysfunction is related to pathologies in muscle fibers, as well as neuromuscular junction and neuronal processes [1]. Therefore, disruption of the hormonal balance in endocrine system diseases may affect any step in these stages and cause muscular dysfunction. Endocrine disease effects are usually mediated through protein and carbohydrate metabolism [2]. However, the pathogenesis is not well defined [3]. Clinical presentations vary. In general, patients have muscle weakness, pain, and spasms that may occur with varying frequency and severity. The true prevalence of endocrine myopathies is not clear, as histopathological confirmation is not always used in the diagnosis of myopathy. Its prognosis depends on the underlying endocrine pathology. Imaging findings are nonspecific and are not used to make a direct diagnosis but can identify the affected muscle and guide biopsy. At the same time, imaging has an important place in the diagnosis of endocrine pathology. The myopathic pattern can be detected in EMG, but normal findings do not exclude the diagnosis. EMG findings may be confused as some endocrine diseases can also cause neuropathy. Histopathological changes in endocrine myopathies are variable and nonspecific. However, a biopsy is important for the exclusion of other processes. Maintaining hormonal balance is essential in the treatment of most endocrine myopathies. In this section, myopathies caused by hormone excess and deficiencies originating from the thyroid, parathyroid, adrenal gland, pituitary gland, and pancreas are reviewed.

Thyroid Disorders

Thyroid hormones, due to their effects on carbohydrate, protein, and lipid metabolism, can lead to muscle dysfunction by impairing protein turnover and carbohydrate utilization in muscle cells, particularly affecting calorigenesis [4].

Hypothyroidism: Hypothyroid myopathy generally causes proximal muscle weakness, especially in the shoulder and pelvic girdle. In addition, cramps, muscle pain, and slow reflexes may occur. Neuromuscular symptoms have been reported in 30–80% of hypothyroid patients [5]. The duration and severity of hypothyroidism were related to the severity of myopathy [6]. Serum CK levels are frequently elevated, but CK levels do not correlate with the severity of myopathy [7]. The presence of myxoedema may help differentiate hypothyroid myopathy from other endocrine myopathies. Rarely, it may present with Hoffman's syndrome or rhabdomyolysis [8, 9]. Hoffman's syndrome is characterized by proximal muscle enlargement and accompanying muscle weakness in a hypothyroid patient. It is mostly seen in primary hypothyroidism. A normal serum

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aspartate aminotransferase value and response to hormone replacement are useful in distinguishing the muscular enlargement in Hoffman's syndrome from pseudohypertrophy in muscular dystrophy [6]. Kocher–Debre–Semelaigne syndrome is a specific hypothyroid myopathy seen in childhood and is associated with muscular enlargement, myxoedema, and cretinism [10–12]. Hypothyroid myopathy can be diagnosed with low serum thyroid hormones, elevated CK levels, and the presence of a myopathic low/small-amplitude potential pattern in EMG [13]. EMG helps differentiate delayed muscle relaxation from myotonia. Muscle symptoms are expected to improve with hormone replacement.

Case 1

A five-year-old girl with complaints of weakness, difficulty climbing stairs, and difficulty walking was admitted to hospital. She was investigated for myopathy due to the high level of muscle enzymes (CK: 6000 U/L) and was examined in the outpatient clinic. A defect in thyroid function tests was seen, which was first observed at another hospital, due to late walking and the inability to speak, where she had been diagnosed with hypothyroidism. It was discovered that L-thyroxine treatment had already begun. In the physical examination, it was determined that she had no growth or mental retardation. A herculean appearance due to significant hypertrophy in the shoulder, back, leg, and calf muscles was noted in the patient. In a muscle biopsy, interstitial edema was prominent. There were also mild size and shape differences in myofibers. Additionally, there was a reduction in the amount of type 2 myofibers (Fig. 18.1). She was diagnosed with Kocher–Debre–Semelaigne syndrome [11].

Hyperthyroidism: In the pathogenesis of hyperthyroid myopathy, the effect of thyroid hormones and the increase in cellular metabolism and catabolism are at the forefront. Like hypothyroid myopathy, there is proximal muscle weakness. It is also accompanied by muscle atrophy. Unlike hypothyroid myopathy, reflexes are brisk. 80% of thyrotoxicosis cases have been reported to have muscle involvement [14]. The respiratory, pharyngeal, and esophageal muscles may also be affected, and dysphagia, dysphonia, and respiratory distress may develop. Unlike hypothyroidism, no increase in the serum CK levels is expected. Serum myoglobin concentrations are also normal. Patients with Graves' disease may also have specific ocular myopathy. This is characterized by an increase in volume in the extraocular muscle bodies and an increase in retro-orbital adipose tissue due to an immune-mediated reaction (Fig. 18.2). Commonly, the inferior rectus muscle is involved. In rare cases, thyrotoxic periodic paralysis may develop in hyperthyroid patients. This is characterized by sudden onset paralysis resulting from the deterioration of the intracellular balance of potassium and hypokalemia. Speech is spared [15]. Its genetic basis has been defined, and it is generally expected in Asian populations due to its racial distribution [6].

Elevated serum thyroid hormones, generally normal CK levels, and the presence of a myopathic pattern in EMG are helpful in the diagnosis of hyperthyroid myopathy. It is important to ensure a euthyroid state with its treatment. In addition, beta-blockers can be used to help gain muscle strength [5].

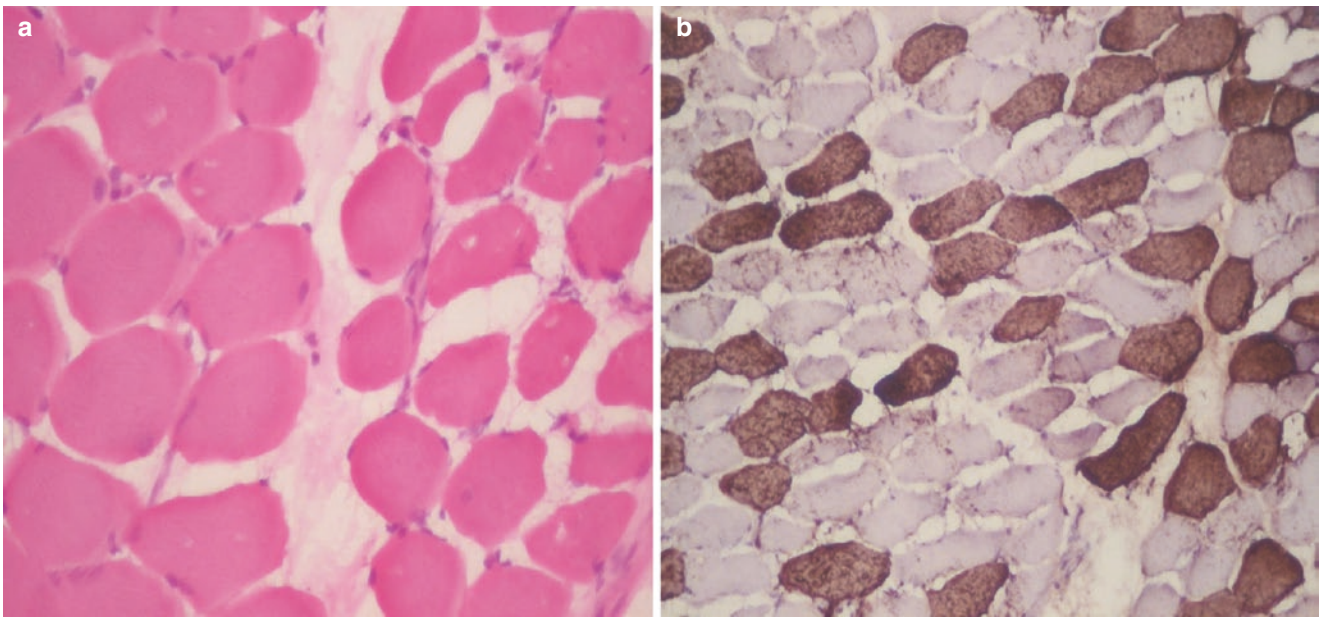

Fig. 18.1 Muscle biopsy of Case 1: (**a**) note the prominent intercellular oedema (HE ×200), (**b**) atrophy of type 2 fibers detected with fast myosin (DAB x 100)

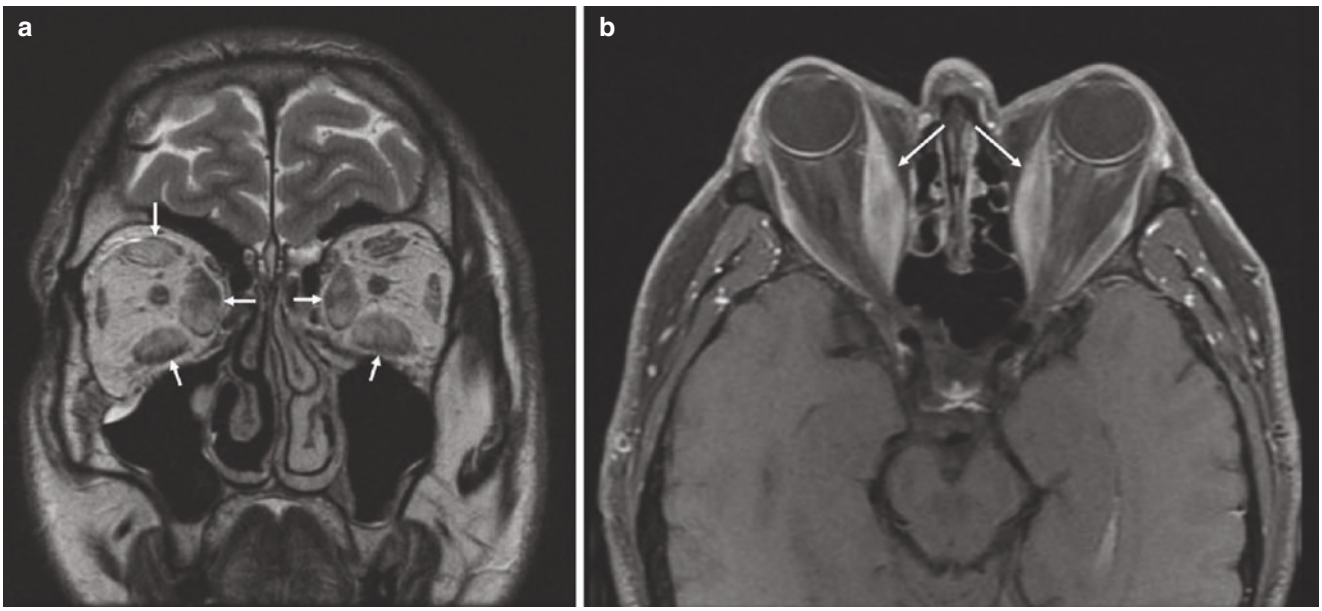

Fig. 18.2 Orbital magnetic resonance imaging of a 46-year-old female patient diagnosed with Graves' disease: (**a**) coronal plane T2-weighted image shows increased volume and signal (arrows) in the extraocular rectus muscles, (**b**) axial plane post-contrast T1-weighted image reveals that the volume increase in the medial rectus muscle is prominent in the muscle body. This appearance is typical for thyroid orbitopathy. There is also diffuse enhancement of the muscle

Parathyroid Disorders

Parathyroid hormone affects calcium metabolism. It affects neuromuscular functions with changes in blood calcium levels.

Hypoparathyroidism: Hypoparathyroidism rarely causes myopathy, but when it does, muscle weakness is usually present. Neuromuscular symptoms mainly include neuromuscular hyperexcitability and tetany due to hypocalcemia. With muscle damage caused by tetany, serum CK levels may increase. Reflexes are weakened [16]. EMG and muscle biopsy are unremarkable. In its treatment, calcium and magnesium levels should be brought to normal levels.

Hyperparathyroidism: In 25% of patients with primary hyperparathyroidism, symptoms such as proximal muscle weakness and hyperreflexia can be seen [17]. Increased intracellular calcium, protein degradation, and reduced calcium sensitivity predispose patients to hyperparathyroid myopathy [18]. Serum CK and transaminase levels are usually within normal limits. EMG findings are normal or nonspecific. No relationship has been demonstrated between serum calcium and phosphorus levels and symptom severity [6]. However, it is important to bring these to normal levels to relieve symptoms.

Adrenal Gland Disorders

Adrenal gland-related myopathies are generally caused by the effects of cortisol hormone on homeostasis and a deterioration in metabolism (especially with protein and carbohydrate) in muscle. At the same time, myopathy may occur due to the effects of cortisol and aldosterone hormones at any stage in the hypothalamus–pituitary–adrenal gland axis.

Hypercortisolism: It can be endogenous or iatrogenic. Iatrogenic steroid myopathy is the most common endocrine myopathy [19]. Muscle degradation accelerates and protein synthesis decreases due to hypercortisolism. It is characterized by subacute onset proximal muscle weakness and atrophy [19]. Serum CK levels are normal. There is a myopathic pattern in which low-amplitude and short-duration potentials are observed in EMG. Reducing the serum cortisol level is essential in its treatment.

Hyperaldosteronism: The cause of neuromuscular symptoms in hyperaldosteronism is low serum potassium. Aldosterone hormone does not directly affect muscle. There is generalized muscle weakness, but the proximal muscles are more severely affected. As a result of hypokalemia, cell membrane depolarization is impaired, and muscles become unexcitable. In addition, disruption of cellular metabolism and necrosis may develop due to its place in the cell energy cycle. Therefore, serum CK levels may be elevated, and myoglobin can be detected in the urine. As in thyrotoxicosis, hypokalemic periodic paralysis may be seen rarely, although it is more common in Asian populations [6]. Symptoms regress with the provision of hormone and electrolyte balance.

Adrenal insufficiency: Primary adrenal insufficiency can be autoimmune, infectious, neoplastic, traumatic, or iatrogenic. Generalized muscle weakness is present in most

cases because of hypernatremia, hypokalemia, and hypocortisolemia secondary to hormone deficiency originating from the adrenal gland. Serum CK levels are normal. It occurs due to disturbances in carbohydrate metabolism and electrolytes rather than occurring as a true myopathy [19, 20].

Hypothalamus and Pituitary Gland Disorders

The previously mentioned thyroid and adrenal gland endocrine myopathies may develop due to secretory and stimulating hormones originating from the pituitary–hypothalamus axis. At the same time, growth hormone disorders originating from the pituitary may also effect muscles. Proximal muscle weakness can be seen in acromegaly without muscle atrophy. While the decrease in cellular excitability and muscle ATPase activity causes weakness, the anabolic effect of growth hormone prevents muscle atrophy. Serum CK levels may be slightly increased [18].

Proximal muscle weakness may develop because of hypernatremia in antidiuretic hormone deficiency. The reason for this may be intracellular energy reduction and rhabdomyolysis due to overworking of the sodium/potassium pump [21]. Serum CK levels increase, and there are myopathic findings in EMG. With the correction of serum sodium levels, muscle functions are restored.

Pancreatic Islet Cell Disorder (Diabetes Mellitus)

The neuromuscular effects of diabetes usually occur secondary to neuropathy and correlate with the severity and duration of diabetes. The myopathic effect is diabetic muscle infarction, which is extremely rare. Although its pathogenesis is not known clearly, it is thought to develop as a result of vascular complications [22]. It is usually seen in the thigh muscles, and there is a sudden onset of pain and swelling in the infarct area.

Malignancy and Inflammatory Myopathies/ Cancer-Associated Myositis

Cancer-associated myositis refers to malignancy that develops within 3 years of the diagnosis of myositis [23]. The prognosis is worse in patients with cancer-associated myositis compared to patients with primary myositis. Associated with malignancy, several subtypes of inflammatory myopathies can be distinguished, such as dermatomyositis, polymyositis, inclusion body myositis, and immune-mediated necrotizing myopathy [24]. Patients with idiopathic inflammatory myopathies experience progressive proximal muscle weakness and produce autoantibodies that are associated with unique clinical features. Muscle biopsy and histological analysis remain crucial, particularly in the absence of autoantibodies, to accurately specify the diagnosis and rule out the other disorders which mimic findings [25].

Dermatomyositis and Polymyositis: Dermatomyositis (DM) and polymyositis (PM) are characterized by proximal skeletal muscle weakness and the evidence of muscle inflammation. Extramuscular manifestations, including arthritis, dysphagia, and interstitial lung disease, may be found in malignancy patients with DM and PM [26]. DM has an increased risk for malignancy, superior to other forms of idiopathic inflammatory myopathies. The incidence of malignancies appears to be specifically increased in patients with DM. Patients with DM have a 4.5-fold higher risk of developing malignancy compared to the general population [27]. DM can develop at the same time as, before, or after the diagnosis of cancer. Most cases develop cancer in the first year after diagnosis [28]. The most common types of cancer in DM are ovarian adenocarcinoma, breast cancer, melanoma, colon adenocarcinoma, and non-Hodgkin's lymphoma. However, in Asians, nasopharyngeal cancer is common too [2].

Every patient should be thoroughly examined, when diagnosed, for risk factors and undergo appropriate screening at baseline and follow-up if needed. Cancer screening is recommended even at younger ages followed by long-term surveillance. Annual examination for early cancer detection should include pelvic, breast, and colorectal examinations [3]. MRI is the imaging modality of choice for evaluating patients with myopathy [29]. In patients with inflammatory myopathy, areas of oedema, atrophy, fatty replacement, and calcification can be detected within the muscles on MR images (Fig. 18.3).

Edema is the most common pattern of muscle abnormality seen on MRI. However, it is not specific and may not distinguish inflammatory myopathy changes from changes in other myopathies and muscular dystrophies. MRI can be particularly beneficial to help determine whether muscle disease is active in a patient with known DM or PM [26]. Both initially involve the proximal lower limb muscles. In the early stages, T2-weighted images show patchy or diffuse increased signal in the proximal muscles suggesting edema. MR imaging shows symmetrical edema with preserved muscle architecture. Subcutaneous and perifascial thickening/fluid may be detected. This usually selectively involves the hamstrings. Other muscles that can be involved in patients with DM and PM are the proximal upper extremities, neck flexors, and pharyngeal muscles [30, 31]. After a few months, T1-weighted images may show symmetrical muscle atrophy without inflammation and fatty transformation of the buttocks, proximal upper limbs, neck flexors, and pharyngeal muscles [32].

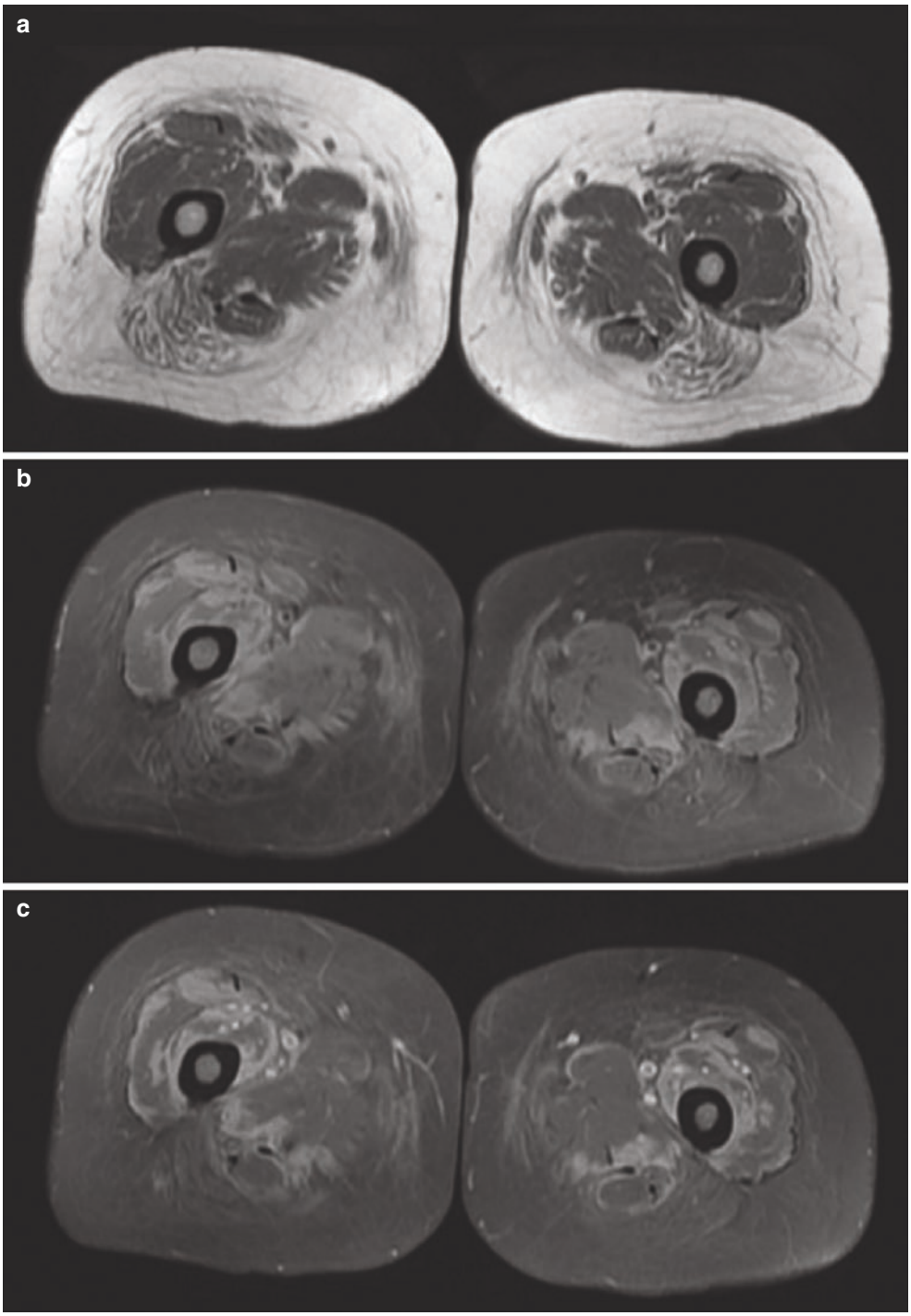

Fig. 18.3 Thigh magnetic resonance imaging of a 42-year-old female patient diagnosed with polymyositis: (**a**) on the axial plane T1-weighted image, there is atrophy and fatty infiltration in the muscles, especially in the biceps femoris muscle, (**b**) the axial plane fat-suppressed proton density image shows edema-like signal increases in the muscles, and (**c**) axial plane post-contrast fat-suppressed T1-weighted image reveals their enhancement

Muscle ultrasonography is an alternative imaging method to MRI used to establish the presence of muscle disease. Ultrasonography is also beneficial in helping to locate an appropriate site for muscle biopsy. The muscle becomes hypoechoic due to edema and fatty tissue infiltration in patients with inflammatory myopathy. Hypervascular changes on power Doppler are seen in active early disease [33]. These sonographic changes are not specific to inflammatory myopathies.

DM and PM are typically diagnosed with muscle biopsy findings. DM can also be diagnosed by skin biopsy or by the combination of proximal muscle weakness and the presence of a characteristic rash. EMG and auto-antibodies may support the diagnosis of PM or DM but are not required for diagnosis [34].

There is a 30% incidence of associated malignancies in DM and therefore it remains practical to examine a muscle biopsy for appropriate, diagnosis, management, and counseling. Muscle biopsy is also essential to distinguish PM from

DM. Muscle biopsy is occasionally required to differentiate critical illness myopathy from other myopathies or from critical illness polyneuropathy. It is also helpful for determining the prognosis as severe cases, especially with necrotizing myopathy, are associated with a high mortality rate [35].

Histopathologically, the presence of inflammatory cells mainly consisting of lymphocytes, which is the hallmark of DM and PM. In DM, B or helper T-lymphocytes invade the perimysium and vessels, while in PM, mostly CD8-positive T-lymphocytes invade the endomysium. A pathognomonic finding, perifascicular atrophy, is seen only in DM. In many muscle biopsies, inflammation may be mild. In these situations, the evaluation of MHC-1 expression is very useful for the differential diagnosis of inflammatory myopathies (Fig. 18.4). In normal muscle, MHC-1 can only be detected on the small capillaries in the endomysium or blood vessels in the perimysium. However, in all kinds of inflammatory myopathies, MLH-1 is densely expressed on the sarcolemma, as well as the sarcoplasm.

Immune-mediated Necrotizing Myopathy: Immune-mediated necrotizing myopathy (IMNM) associated with cancer is a rare condition reported in limited case reports [35]. Reported types of cancer associated with necrotizing myopathy are gastrointestinal malignities, lung cancer, cholangiocarcinoma, breast cancer, and prostate cancer [36]. IMNM typically presents as a progressive disease with subacute symmetrical involvement of the proximal muscles. Muscle abnormalities are common in the rotator and gluteal muscle groups in patients with IMNM. Fatty replacement of muscle tissue may be detected early during the disease [37]. Patients with IMNM and antisignal recognition particle antibodies have more fatty replacement and atrophy than those with anti-HMGCR antibodies. Recent studies have reported that cancer is as frequent in IMNM patients as it is in the general population [38]. Unlike DM, cancer screening in patients with IMNM is recommended when necessary, considering the patient's family history, age, and predisposing factors.

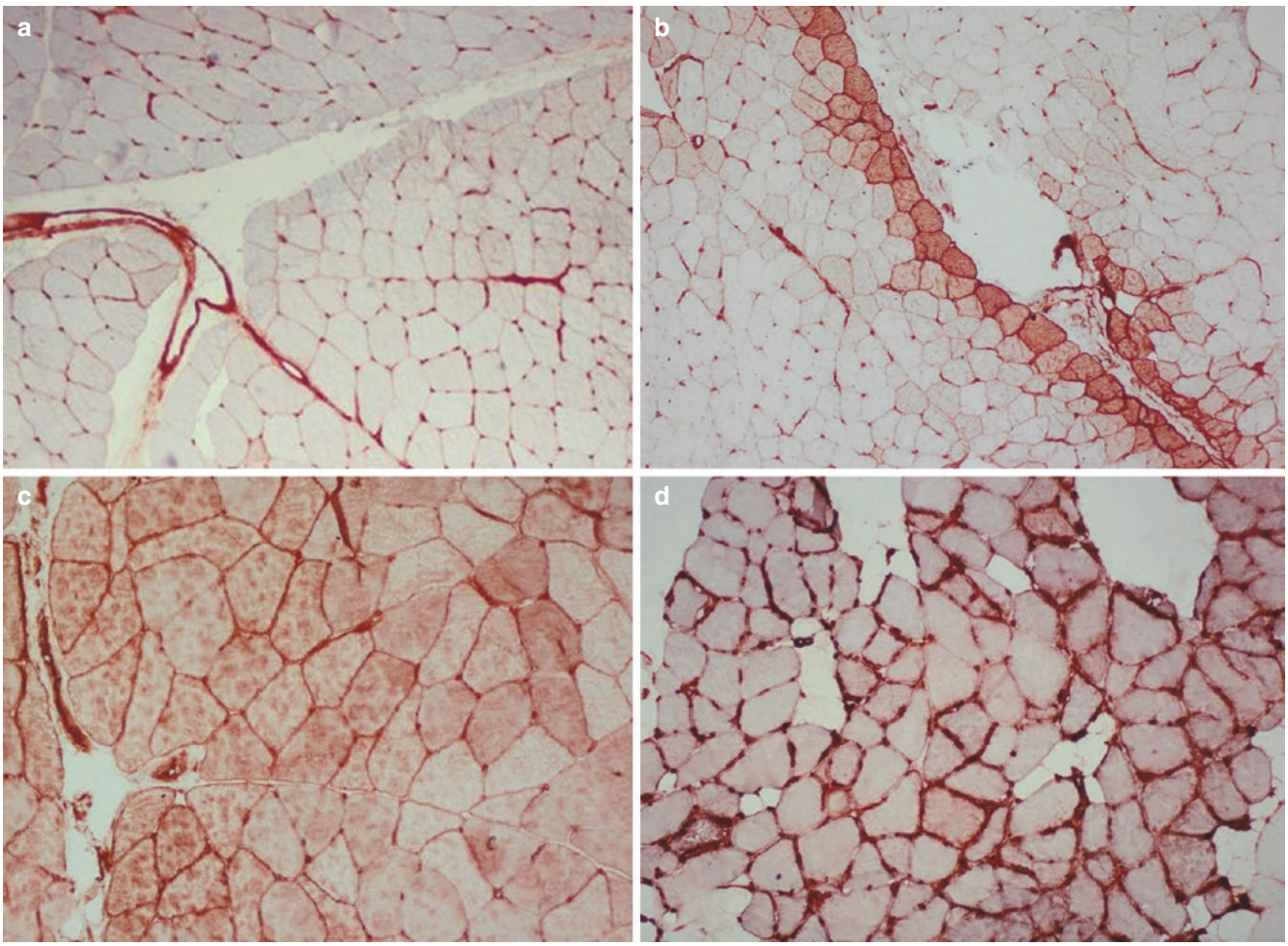

Fig. 18.4 MHC-1 expression in different conditions: (**a**) normal muscle (DAB x 100), (**b**) perifascicular myofibers show increased expression in a patient with DM (DAB ×100), (**c**) increased sarcoplasmic MLH-1 expression in a patient with PM (DAB ×200), (**d**) increased sarcolemmal MLH-1 expression in a patient with PM (DAB ×200)

Inclusion Body Myositis: In patients with tumor-associated myositis, CK and lactate dehydrogenase elevations are lower compared to primary myositis [39]. After surgical treatment of the malignancy, these values are usually significantly reduced. Patients with inflammatory myopathy, especially DM and PM, should be examined for risk factors and undergo appropriate screening for malignancy. Cases of remission of muscle symptoms have been reported after tumor treatment, but a recurrence of malignancy may trigger an exacerbation of myopathy.

Myopathies Associated with Connective Tissue Diseases (Overlap Syndromes)

Inflammatory myopathy may develop in patients with autoimmune connective tissue disorders such as scleroderma, rheumatoid arthritis, mixed connective tissue disease, and systemic lupus erythematosus. The association of inflammatory myopathies with connective tissue disease defines the overlap syndromes. Overlap syndrome is mostly seen in cases of scleroderma. The other two common connective tissue disorders associated with the overlap syndrome are rheumatoid arthritis and systemic lupus erythematosus. PM and DM are the most frequent inflammatory myopathies in patients with overlap syndromes.

Scleroderma: Between 5 and 10% of patients with scleroderma also have myositis. The clinical manifestations of dermatomyositis and polymyositis are identified simultaneously with scleroderma in most cases [40]. There is a higher frequency of vascular and pulmonary involvement in scleroderma compared to rheumatoid arthritis and systemic lupus erythematosus. Raynaud's phenomenon and interstitial lung disease are the most common vascular and pulmonary manifestations. Fibrosis is the predominant pathological finding in scleroderma and can be seen in other organs, including the gastrointestinal tract, salivary glands, and thyroid gland.

Usually, in patients with scleroderma, there is muscle weakness due to malnutrition and skin fibrosis. Fibrosing myopathy may also develop due to muscle involvement in scleroderma. In these cases, muscle fibrosis and atrophy are detected [41]. These patients may have mildly elevated CK levels. Inflammatory myositis can be seen in 5–10% of patients with scleroderma. Patients with scleroderma-myositis have one of two forms of scleroderma: progressive systemic sclerosis or CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal motility disorder, sclerodactyly, and cutaneous telangiectasia). Scleroderma myositis is associated with rapidly progressive proximal muscle weakness, biopsies with inflammatory myositis, and elevated blood CK levels [40].

Patients with scleroderma may be positive for antinuclear antibodies and scleroderma myositis may be associated with anti-PM-scleroderma (PM-1 or PMScl) antibodies, but these antibodies are also found in idiopathic myositis and rheumatoid arthritis [41]. Muscle biopsy features include perimysial inflammation/fibrosis, atrophy of type 2 fibers, and vasculopathy typical of scleroderma.

Skeletal muscle from patients with rheumatoid arthritis shows at least 3 patterns of infiltration by inflammatory cells: polymyositis, focal nodular myositis, and arteritis at the light microscopy and ultrastructural levels. Typically, in arterial inflammation, there is a predominance of mononuclear cells. The smaller arterial vessels tend to be the most severely affected; there is frequent luminal narrowing, and fibrinoid necrosis may be present. Polymyositis is known to occur in the setting of rheumatoid disease, and in many cases, it is morphologically indistinguishable from primary polymyositis; changes include the infiltration by mononuclear inflammatory cells, widespread fiber necrosis/regeneration, and endomysial connective tissue proliferation which occurs later on. Focal nodular myositis may occur in several disorders, but is especially prominent in patients with active, chronic rheumatoid disease. The characteristic lesion in focal nodular myositis is a small, nodular 1-2 mm accumulation of plasma cells and lymphocytes adjacent to an arteriole, with or without coincident damage to regional muscle fibers. It should be emphasized that significant muscle weakness in patients with rheumatoid arthritis may be secondary to arthritis, pain, medications (e.g., steroids), inactivity, or peripheral neuropathy; a recent quantitative controlled EMG study found an increased prevalence of neurogenic but not myogenic changes in patients with rheumatoid arthritis compared with controls. Corticosteroids are used in the treatment of the inflammatory myositis component of scleroderma. Methotrexate and hydroxychloroquine may be helpful in treating myositis in people with scleroderma [42]. Adult patients with scleroderma-myositis have more esophageal, lung, and heart involvement than pediatric patients. Immunosuppressive therapy is usually effective in these situations [41].

Systemic Lupus Erythematosus: Myositis is a rare complication of systemic lupus erythematosus (SLE). The prevalence of muscle involvement in SLE is between 4 and 16% of adult patients and as high as 30% of pediatric patients [43]. Specific rashes, arthralgias, arthritis, systemic sclerosis, and cytopenia are common clinical and laboratory features in patients with SLE. Raynaud's phenomenon and dysphagia may be present. People with childhood-onset SLE disease and black people have a higher prevalence of myositis [41]. During SLE, DM, or PM may occur. Patients with SLE-myositis generally have proximal symmetrical muscle weakness and myalgia. SLE-myositis usually shows moderate to severe muscle involvement [43]. SLE can be associated with idiopathic thrombocytopenia, hemolytic anemia, autoimmune neutropenia, progressive systemic sclerosis, and myasthenia gravis. The SLE Disease Activity Score

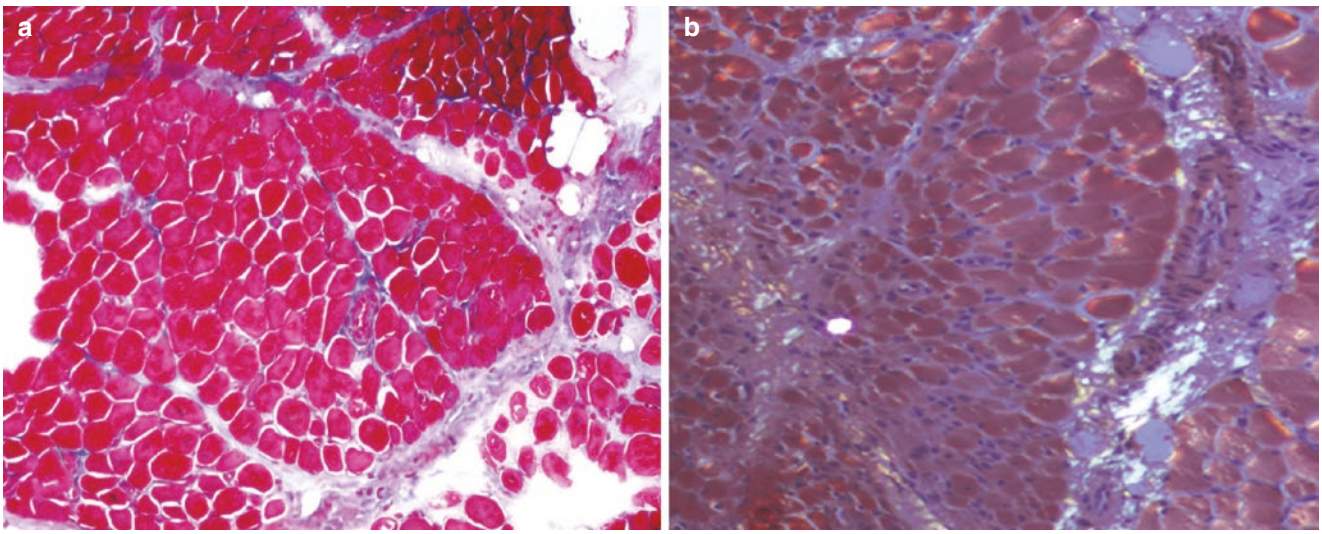

Fig. 18.5 (**a**) A muscle biopsy, which may be interpreted as non-inflammatory myopathy if amyloid deposition is not observed (Masson trichrome ×40), (**b**) the amyloid shows green birefringence under polarized light (Congo red ×40)

(SLE_DAS) has improved sensitivity to changes with more items to include rare manifestations such as myositis, hemolytic anemia, and cardiopulmonary, and gastrointestinal manifestations [44]. This new score system also has a high specificity. Antibodies to native DNA are highly specific in SLE. Up to 50% of SLE patients have anti-RNP antibodies, including anti-Sm antibodies, and anti-U1 snRNP antibodies [45]. Myositis is rare in patients with SLE who are positive for anti-Ku antibodies [46]. The most common clinical course for patients with lupus myositis is a relapsing and remitting one. Corticosteroids are used as a first-line therapeutic agent, often with an additional immunosuppressive agent such as azathioprine.

Rheumatoid Arthritis: Extra-articular manifestations can be seen in rheumatoid arthritis. Muscular involvement is a lesser-known condition that begins in the early period and is relatively common. Muscle weakness and atrophy are seen in most patients with rheumatoid arthritis [47]. Intracellular contractile dysfunction plays an important role in muscle weakness in rheumatoid arthritis [48]. Muscle atrophy is usually of the type 2 fibers. In addition, myositis- or vasculitis-based inflammatory myopathic changes may occur [49]. Medication-related toxic myopathy and denervation myopathy have also been reported in rheumatoid arthritis [47]. The catabolic effects of inflammatory cytokines on muscle tissue are another cause of rheumatoid arthritis-associated myopathy. As a result of myopathic changes seen in rheumatoid arthritis, a decrease in muscle mass/strength, increase in inflammatory markers, and fatty infiltration of the muscle are defined as rheumatoid cachexia. Rheumatoid cachexia was found to be associated with disease severity, quality of life, morbidity, and mortality [50].

Like inflammatory myopathies, serum CK levels increase and a myopathic pattern is seen on EMG in rheumatoid myopathy. The extent and severity of muscle tissue involvement can be examined with MRI. The recovery of muscle tissue can also be evaluated during the treatment process. Myopathic changes are nonspecific, and collagen vascular disorder-related myopathy is generally indistinguishable from polymyositis on MRI. Depending on the acute, subacute, or chronic periods, edema, atrophy, and fatty infiltration can be seen in the muscle. Keeping the loss of muscle tissue to a minimum and regaining the loss have positive effects on the course of the disease.

Amyloidosis

Amyloid is a pathologic proteinaceous substance that stains with Congo red and shows green birefringence with polarizing microscopy. Amyloidosis refers to the extracellular depositions of amyloid and may be secondary to malignancies, chronic inflammatory disorders, and even aging [12]. Muscle biopsy demonstrates perivascular or interstitial amyloid deposition (Fig. 18.5).

Aging-Related Myopathies

Myopathies are not uncommon in elderly people. Contrary to popular belief, the loss of muscle strength that develops with aging is usually caused by a type of myopathy called "sarcopenia" [51]. Sarcopenia refers to the loss of muscle mass and function [52]. Although its prevalence varies according to ethnicity, it is up to 30% in the elderly population, and this rate increases in the presence of comorbid chronic diseases [53, 54]. It is more prominent in the extremity muscles than in the trunk muscles. A relative loss of muscle strength was found in elderly men and women at similar rates of 20–40% [53]. In addition, sarcopenia may develop in systemic inflam-

matory diseases such as malignancy or organ failure, regardless of age [51]. Elderly patients with sarcopenia are vulnerable to frailty and physical disability. This can result in various morbidities and ultimately mortality [52]. Therefore, prevention and treatment of sarcopenia in geriatric patients may reduce the negative outcomes. With the increasing rate of the elderly population in society, the importance of understanding sarcopenia will increase even more.

Although the presence of sarcopenia can be detected by various imaging methods, these are not included in the accepted criterion. Low muscle strength is the main diagnostic criterion in the consensus decision of the European Working Group on Sarcopenia in Older People [51]. In addition, low muscle mass and low muscle quality also contribute to the diagnosis. The term muscle quality describes muscle architecture and composition. The decrease in body skeletal muscle mass starts in the third decade but does not become substantial until the fifth decade [55, 56]. With ultrasonography, computed tomography, magnetic resonance imaging, and dual-energy X-ray absorptiometry, the change in muscle volume and composition can be evaluated qualitatively and quantitatively (Fig. 18.4). It has been reported that muscle mass measurement at the level of the lumbar third vertebrae or at the mid-thigh level reflects the body muscle mass. In addition, in these methods, muscle quality can be evaluated based on muscle fat infiltration [51]. However, globally accepted cutoff values for all these measurements are not yet available.

Sarcopenia may occur due to neurological, muscular, and behavioral factors. Among the muscular causes, anabolic resistance, predominant muscle fiber-type change, mitochondrial dysfunction, nicotinamide adenine dinucleotide (NAD) metabolism dysfunction, various catabolic cytokines, gut microbiota diversity, and cerebral atrophy have been implicated [53, 57]. Anabolic resistance refers to the resistance of muscle tissue to anabolic hormones such as insulin-like growth factor-1 with aging. In addition, testosterone and estrogen hormones, which are anabolic for muscle tissue, are reduced. It has been shown that there is a decrease in the number and size of type 2 (fast-twitch) muscle fibers with aging, and type 1 (slow-twitch) fibers become relatively dominant [58]. Neurologically, the decrease in the number and size of alpha motor neurons in the anterior horn with age has been held responsible [58]. Decreased physical activity with age and a sedentary lifestyle are behavioral factors.

Myalgia and elevated serum CK levels are not expected in sarcopenia, and if they are accompanied, the differential diagnosis should be considered. The differential diagnosis includes idiopathic inflammatory myopathy, late-onset muscular dystrophies (facioscapulohumeral dystrophy, oculopharyngeal dystrophy, late-onset limb girdle dystrophy, myotonic dystrophy type) and 2), late-onset mitochondrial myopathy, axial myopathies, endocrine/metabolic myopathies, amyloid myopathy, and drug-induced myopathies, which can be more common in elderly patients [59]. In late-onset limb-girdle dystrophy, proximal limb weakness slowly progresses, and a waddling gait is typical. Exercise-induced myalgia is an initial symptom in myotonic dystrophy type 2, and proximal muscle weakness and myotonia may also be present [60] (Fig. 18.6).

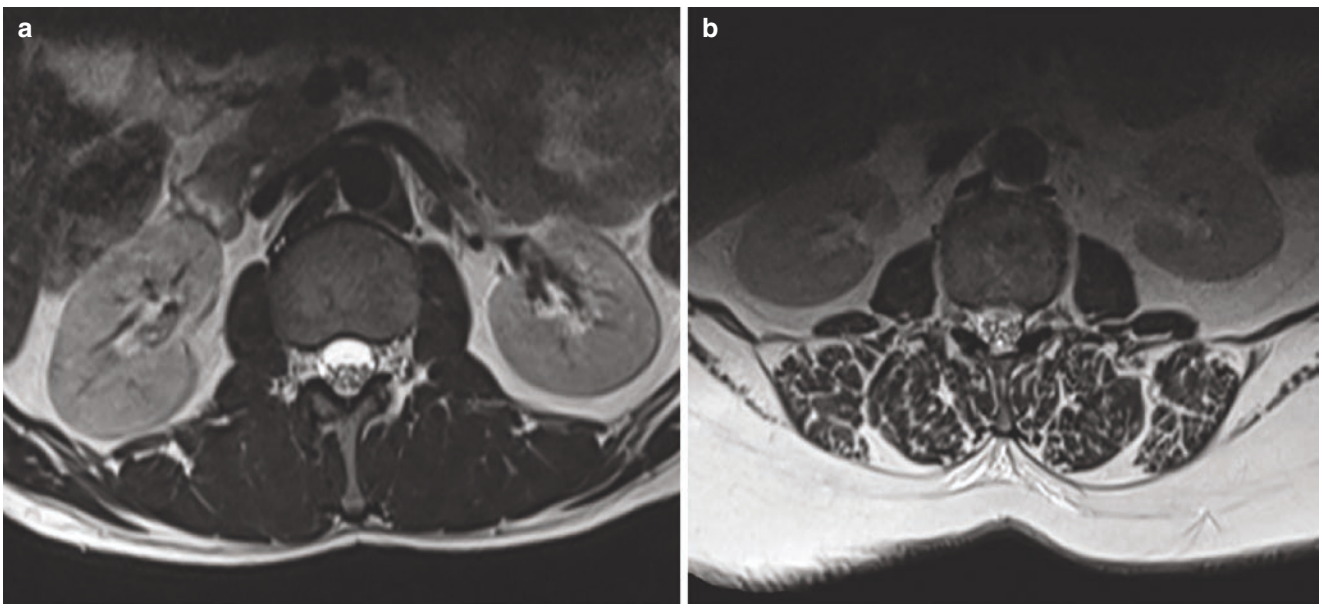

Fig. 18.6 Axial plane T2-weighted magnetic resonance images of the lumbar vertebrae of a 32-year-old female patient (**a**) and a 71-year-old female patient (**b**). (**a**) The volume and signal characteristics (hypointense = mostly dark) of the young patient's paravertebral muscles are normal, (**b**) elderly patient has a decreased volume of the paravertebral muscles and muscular fatty infiltration (hyperintensities = bright areas in the muscles)

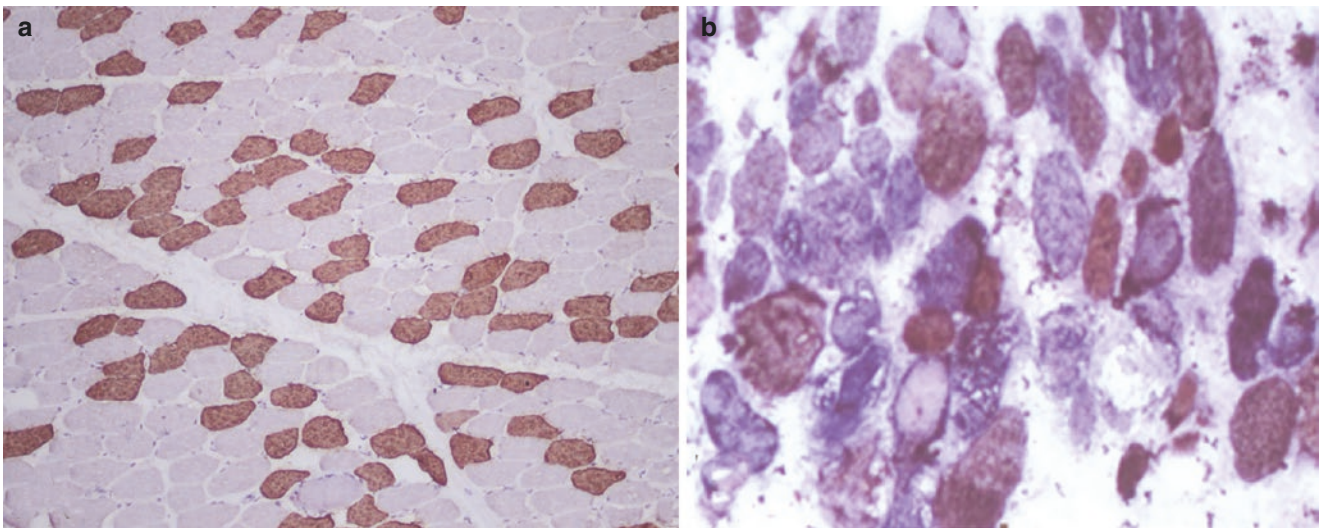

Fig. 18.7 The two most common changes in skeletal muscle due to aging: (**a**) decrease in type 2 myofiber ratio with fast myosin (DAB ×100), (**b**) increase in pathological blue fiber ratio showing mitochondrial dysfunction (COX-SDH ×200)

All about the Pathology of Myopathies Associated with Systemic Disorders and Aging

Various diseases and even healthy aging have the capacity to effect muscle through different mechanisms. It should be kept in mind that multidisciplinary evaluation is required for the differential diagnosis due to the variability of clinical pictures. Although different findings can be observed histopathologically, all of them are nonspecific. For example, in overlap syndromes, findings in muscle biopsies are like those found in inflammatory myopathies or vasculitis. Particularly, systemic diseases that cause vascular damage and ischemia can also cause necrosis or rhabdomyolysis.

One of the most frequently observed changes, especially in sarcopenia due to aging, is type 2 myofiber atrophy (Fig. 18.7). Similarly, the presence of myofibers with mitochondrial dysfunction increases with aging. Therefore, the evaluation of myofiber type and distribution with oxidative enzyme stains is a very important step in muscle biopsy examination [12].

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A Quick Glance at the Therapeutic Approaches for Neuromuscular Disorders

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Neuromuscular disorders affect many individuals of all ages and have a substantial burden on patients, caregivers, and healthcare systems. Huge progress has been made in the last decade in terms of elucidating the mechanism of various neuromuscular disorders, identifying potential therapeutic targets, and developing novel agents. In this chapter, novel therapeutic approaches that have been lately implemented in clinical practice, and the candidate drugs that are still at early stages of clinical trials regarding neuromuscular disorders will be discussed.

Motor Neuron Diseases

This section includes novel therapeutic approaches and the efforts to develop new treatment methods for motor neuron diseases, of which at least one novel therapeutic agent has been approved by the Food and Drug Administration (FDA) since 2015.

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Spinal Muscular Atrophies

Spinal muscular atrophy (SMA) is an inherited disease that is characterized by progressive and irreversible degeneration of the motor neurons in the spinal cord anterior horn and the motor nuclei in the lower portion of the brainstem. The disease gradually leads to loss of muscle strength and muscular atrophy. Survival motor neuron (SMN) protein is crucial for motor neurons to maintain homeostasis. A healthy individual has two sets of gene copies that encode SMN protein, one being the telomeric *SMN1* and the other being the centromeric copy *SMN2*. Although *SMN1* and *SMN2* are more than 99% identical, *SMN2* has a C to T transition in exon 7. Thus, the great majority of functional SMN protein is produced by *SMN1*-derived mRNA7 [1].

SMA is mainly caused by homozygous deletion or mutations in the *SMN1* gene on chromosome 5q13.2 resulting in insufficient and dysfunctional synthesis of SMN protein. Approximately 95% of typical SMA cases carry homozygous deletion on exon 7, and it results in the *SMN2* gene taking the lead in the production of SMN protein. This new protein, SMNΔ7, is highly unstable and dysfunctional. However, 10–15% of mRNAs from *SMN2* have exon 7 and so they may have the ability to synthesise a small amount of functional SMN protein (Fig. 19.1). Thus, the lack of a functional *SMN1* gene can be only partially compensated by *SMN2*. Disease severity and the time of onset of symptoms usually correlate inversely with the amount of *SMN2* copies [1].

Up until 2017, the main treatment options for patients with SMA consisted of multidisciplinary supportive strategies, nutritional care, and orthopaedic interventions. However, the introduction of breakthrough disease-modifying agents has changed the clinical approach. These agents will be discussed below:

Nusinersen, an antisense oligonucleotide (ASO), modifies the splicing of *SMN2* pre-mRNA and results in the inclusion of exon 7 in *SMN2*-derived mRNA. Thus, the drug leads

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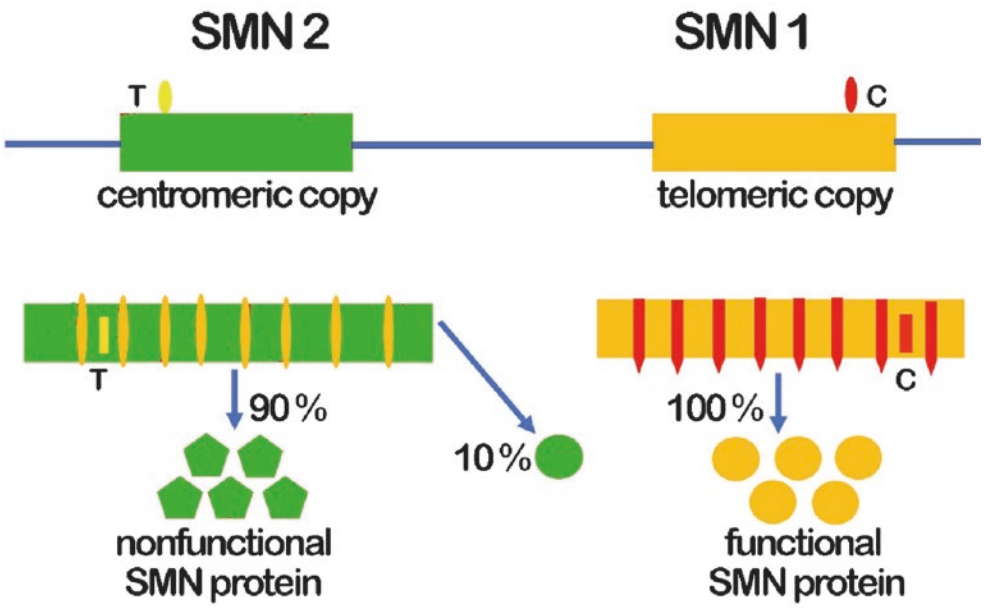

Fig. 19.1 The SMN1 and SMN1 genes are responsible for the production of the SMN protein. The C > T transition in exon 7 of the SMN2 gene causes the production of non-functional SMN protein

to the increased transcription of full-length, functional SMN protein. It was the first disease-modifying agent to be implemented in the clinical management of SMA following its licensing by the FDA in December 2016 and the European Medicines Agency (EMA) in May 2017. This ASO cannot cross the blood–brain barrier, and preclinical studies revealed that when administered via intravenous injection, the agent causes renal tubular degeneration by accumulating in the kidney. Thus, the preferred route is via intrathecal injection initiated with loading doses on day 0, 14, 28, and 63, followed by a maintenance dose once every 4 months [2].

The ENDEAR trial, a phase 3 study that paved the way for the ASO's approval, was conducted on infants diagnosed with type 1 SMA, aged 7 months or younger. The study revealed that the agent was significantly superior to the sham procedure regarding the improvement of motor function (measured by The Hammersmith Infant Neurological Examination) and event-free survival. As the interim analyses indicated that the sham group showed signs of evident deterioration, the study was terminated early, and Nusinersen was granted regulatory approval for clinical use in SMA [3]. Similarly, the CHERISH study that was conducted on children diagnosed with type 2 SMA, aged 2–12, found that the children receiving Nusinersen had significantly better motor scores compared to the sham group at the end of the 15-month treatment course [4]. A single-arm, open-label study, called NURTURE, evaluating the efficacy of intrathecal Nusinersen treatment in pre-symptomatic infants who have a genetic diagnosis of SMA is still ongoing (Clinicaltrials.gov ID: NCT02386553). The interim analysis of the study reports that 15 infants with two *SMN2* copies and 10 infants with three *SMN2* copies were enrolled, and all of the participants were alive with none of them requiring tracheostomy. Eighty-eight percent of them had achieved independent walking [5]. The most common Nusinersen-related adverse events in clinical trials are constipation, lumbar-puncture-related problems, proteinuria, and coagulation abnormalities. Moreover, the clinical data gathered from 346 patients receiving Nusinersen revealed that 4% of the patients developed anti-drug antibodies, of which five had a persistent immunogenic response. However, the influence of immunogenicity on the efficacy and safety of the drug is not yet understood [2].

SMN1 gene replacement therapy: Onasemnogene abeparvovec-xioi is a recombinant adeno-associated viral vector that carries complementary DNA encoding functional human SMN protein. The vector received its first approval from the FDA in 2019 to be used in infants younger than 2 years of age, carrying bi-allelic *SMN1* mutations. As of May 2020, onasemnogene abeparvovec-xioi has conditional marketing authorisation in Europe. The data from a Phase I study, which evaluated the safety and efficacy of the vector in 15 SMA type 1 infants younger than 9 months old, indicated that all infants were alive during the 20-month follow-up and had significantly better motor milestone achievement compared to the historical control group [6]. A Phase III study conducted using a similar population revealed that the therapy was superior to the control in terms of the ability to sit without support for longer than 30 s and survival free from ventilation. Although the therapy is given as a one-time intravenous infusion, the clinical studies investigating the long-term efficacy and safety of the agent are still ongoing. Furthermore, the need for immunosuppression before vector transfer, hepatotoxicity, cardiac toxicity, and thrombocytopenia are the main concerns regarding the safety of the therapy. Although inflammation of dorsal root ganglia (DRG) in cynomolgus monkeys was reported in preclinical studies, no

patient was reported to have findings of DRG inflammation in clinical settings [7]. Another limitation regarding the use of this therapy is that the influence of pre-existing immunity to *wild-type* viruses from which the vector is derived on the efficacy of the treatment is not fully known [8].

Risdiplam modifies the splicing of *SMN2* and binds to *SMN2* pre-messenger RNA, eventually leading to increased production of full-length SMN protein. The drug is used daily via the oral route and was licensed in August 2020 by the FDA for use in patients aged 2 months or older, with a clinical diagnosis of SMA. In March 2021, Risdiplam obtained marketing authorisation throughout the European Union. Phase II/III studies conducted with type 1, 2, and 3 SMA patients indicated a significant improvement in motor functions compared to the control groups. The adverse events that have been reported so far include fever, rash, and diarrhoea. The potential inhibition of hepatic and intestinal cytochrome 3A enzymes by Risdiplam raises concerns regarding drug-drug interactions [9].

Monoclonal anti-myostatin antibodies: Another therapeutic approach used for SMA are interventions that aim to reduce the loss of skeletal muscle mass and strength, in turn improving the quality of life. Preclinical studies revealed that specific inhibition of myostatin, a negative regulator of skeletal muscle mass, might be a potential therapeutic target to prevent muscle atrophy [10]. A phase III trial investigating the efficacy and safety of Risdiplam combined with a novel monoclonal anti-myostatin antibody, *GYM329*, is currently ongoing (Clinicaltrials.gov ID: NCT05115110). Another anti-promyostatin monoclonal antibody called *Apitegromab* is also being investigated with a phase III clinical trial, where it is being used as an adjunct therapy to Risdiplam and Nusinersen in children with SMA type 2 or 3 (Clinicaltrials.gov ID: NCT05156320).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease that affects upper and/or lower motor neurons. Although it is acknowledged that various aetiological factors such as inherited, environmental, and age-related risks contribute to the pathogenesis of the disease, no specific therapeutic target has been identified so far.

Riluzole, the first agent to be licensed for clinical use in ALS, is known to work by decreasing the hyperexcitability of cells. Another drug used in the management of ALS, that has been used in clinical practice since 2017, Edaravone, lowers intracellular oxidative stress. Even though both drugs can slow down disease progression in the early phases, they are far from being curative [11]. Another therapeutic approach used ALS functions to target mitochondrial dysfunction. *Dextromethorphan*, an opioid-derivative licensed for use in Parkinson's disease, is reported to increase ATP synthesis. It does this by inhibiting the leaky conductance of mitochondria, ultimately preventing neuronal cell death. There are promising results from clinical studies indicating that the combination of Dextromethorphan and Quinidine has ameliorating effects on pseudobulbar symptoms of ALS [12–14]. However, other agents, such as Olesoxime and Cutamesine, which regulate mitochondrial function, did not prove efficacious in clinical trials [15].

Arimoclomol, an agent that amplifies the response of heat shock proteins and has the potential to reduce the aggregation of misfolded proteins, has shown promising results in early-phase clinical trials [15]. Yet, the results from a Phase III trial investigating the drug have not been published so far. The phase II study of sodium phenylbutyrate–taurursodiol, called AMX0035, revealed promising results, with the intervention slowing down the loss of functionality and providing a longer median survival period compared to placebo [16]. The Phase III trial for this agent is expected to be completed in 2024 (Clinicaltrials.gov ID: NCT05021536).

Masitinib, a tyrosine kinase inhibitor, has been shown to slow functional decline and prolong survival by over 2 years compared to placebo in a Phase III clinical trial [17]. However, marketing authorisation for this agent was rejected by the EMA in 2018 due to the lack of a risk management plan for safety concerns.

Reldesemtiv, ION363, TPN-101, Triumeq, FAB122, Cannabinoids, Lenzumestrocel, Tauroursodeoxycholic acid, Huolingshengji granules, Deferiprone, Tofersen, Memantine, Trazadone, Ibudilast, Zilucoplan, Verdiperstat, CNM-Au8, Pridopidine, and SLS-005 Trehalose are other agents that are currently under Phase III trials for use in ALS.

Drugs that inhibit glutamate transport and excitotoxicity include *Topiramate*, *Talampanel*, and *various beta-lactam antibiotics*, and axonal transport regulators, such as *Noscapine*, *Lithium*, and *Valproic acid*, are other drugs that have been studied regarding their use in the management of ALS [15]. Despite all efforts to build novel therapeutic strategies that are aimed at various therapeutic targets, the studies fail to discover a new agent that might positively affect disease progression in the long term.

Neuromuscular Junction Diseases

Myasthenia Gravis

Myasthenia gravis (MG) is the commonest autoimmune disease that affects the neuromuscular junction. While most patients develop antibodies against acetylcholine (ACh) receptors, a smaller group of patients have autoantibodies

against muscle-specific kinase, low-density lipoprotein receptor-related protein 4, agrin, titin, ryanodine receptors, or the alpha subunit of voltage-gated K^+ channels. Currently, the mainstay treatment of MG consists of immunosuppression and cholinesterase enzyme inhibitors. Some of the conventional treatments that are used in immunosuppression are corticosteroids that reduce T-cell activation. The agents that interfere with T- and B-cell proliferation include azathioprine, mycophenolate, and methotrexate. T-cell activation inhibitors include tacrolimus and cyclosporine. Additionally, intravenous immunoglobulin and plasma exchange therapy are further immunomodulatory strategies that inhibit the interaction between antigen-presenting cells and the Fc receptor on the T-cell surface. A better understanding of MG pathophysiology has made it possible to focus on target-specific novel immunotherapeutics. These new therapies are mentioned below:

Complement inhibitors: Novel anti-C5 antibodies designed to inhibit the common complement pathway have not been implemented in clinical use due to the increased risk of opportunistic infections. On the other hand, preclinical studies investigating the use of small interfering RNAs that selectively inhibit classical complement pathways are still ongoing [18].

Eculizumab is the first recombinant humanised monoclonal antibody to prevent cleavage by binding to the C5 complement. The drug was licensed for clinical use in nocturnal haemoglobinuria by the FDA in 2007. Within the scope of drug repurposing studies, the efficacy and safety of the drug have been investigated in patients with anti-ACh receptor-positive refractory generalised MG. A Phase III study, REGAIN, and its open-label extension study demonstrated that intravenous Eculizumab treatment, added to standard immunosuppressive therapy, provided a significant improvement in disease symptoms compared to placebo [19]. The main concern regarding the safety of the drug is the risk of infection. Up to one-fifth of the patients were reported to have invasive infections caused by *Pseudomonas, Cytomegalovirus,* or *Aspergillus* species. It is recommended that patients have the *Neisseria meningitides* vaccine or prophylaxis with antibiotics before Eculizumab administration. However, due to the apparent efficacy in the management of disease symptoms, the drug has been approved for clinical use in patients with anti-ACh receptor-positive refractory generalised MG [18]. Following these promising results, studies investigating novel complement inhibitors are underway. A Phase III study of *Zilucoplan* estimated it would finish in 2022 whilst its open-label extension study continues. However, the results are yet to be published (Clinicaltrials.gov ID: NCT04115293). Another agent, *Ravulizumab*, has shown promising results and is reported to have a higher affinity to complement C5 and a longer half-life. Yet, the results of the phase III study investigating this drug in patients with MG have not yet been published (Clinicaltrials.gov ID: NCT03920293).

Inhibitors of Fc receptors: Preclinical studies demonstrated that the inhibition of the Fc receptor significantly reduces the severity of MG symptoms and lowers the level of anti-ACh receptor IgG. A novel human IgG1 antibody Fc fragment that has been developed to lower the pathogenic autoantibody levels of IgG, called Efgartigimod, showed promising results in a Phase III trial. The results indicate that Efgartigimod provides a significant improvement in the clinical manifestations of MG in both anti-ACh receptor antibody positive and negative patients with refractory generalised MG [20]. Following these studies, the drug was approved by the FDA in December 2021 to manage generalised MG in adult patients who are anti-ACh receptor antibody positive. Nipocalimab and Rozanolixizumab are other novel Fc receptor inhibitor candidates, of which Phase III studies investigating them are soon to be completed (Clinicaltrials.gov ID: NCT04951622, NCT04124965). It should be noted that the data regarding the long-term efficacy and the interaction between the other therapeutic monoclonal antibodies is sparse.

Chimeric Antigen Receptor-T (CAR-T) cell therapy relies on the concept of harvesting T-cells from patients and manipulating them ex-vivo so that they can detect and attack targeted cells [18]. Although it is mainly used in haematological malignancies, there is an ongoing Phase I/II study investigating the safety, tolerability, and efficacy of CD8-positive CAR-T therapy directed against plasma cells secreting autoantibodies in generalised MG (Clinicaltrials.gov ID: NCT04146051).

Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton Myasthenic Syndrome (LEMS) is a disease that occurs as a part of a paraneoplastic syndrome or a primary autoimmune disorder that affects neuromuscular junctions. The antibodies against voltage-gated P/Q-type calcium channels (VGCC) on presynaptic nerve terminals, which lead to a decreased release of ACh, are the main pathology behind the disease. The conventional management approach relies on the treatment of the underlying malignancy or treatment options that affect the immune system. Amifampridine, an orphan drug, increases the action potential duration and ACh release in nerve terminals by blocking the potassium influx and lengthening the open time of Ca^{2+} channels. The drug has been classified as an orphan designation by the EMA since 2002 and was finally

approved by the FDA in November 2018 for treating patients with primary LEMS [21].

Pompe Disease

Pompe is a glycogen storage disease that occurs due to the decreased activity of a key enzyme in intralysosomal glycogen degradation, called acid alpha-glucosidase (GAA). The effect of the disease on muscles is usually characterised by cellular degeneration within muscle fibers. The time of onset and the severity of symptoms vary depending on residual GAA activity. The mainstay treatment strategy is the replacement of the missing enzyme. *Alglucosidase alfa*, the recombinant GAA enzyme, was the first and only agent that has been used in clinical practice since 2006. A second-generation recombinant GAA enzyme, called *Avalglucosidase alfa*, was granted approval by the FDA for use in late-onset Pompe disease in 2021 [22]. Another novel recombinant GAA enzyme, *Cipaglucosidase alpha,* combined with *Miglustat* did not achieve superiority over conventional recombinant GAA enzyme in Phase III clinical trials [23].

Adeno-associated virus-mediated alpha-glucosidase gene therapy is another therapeutic strategy that has been investigated in Pompe disease. Although some of the gene transfer therapies hold an orphan designation status from the EMA, clinical trials aiming to determine the efficacy and safety of these novel therapeutics are still ongoing [24]. So far, none of them have been granted marketing authorisation.

Mitochondrial Myopathies

Mitochondrial diseases are mostly multisystemic disorders that are characterised by disruption in aerobic metabolism. The tissues that are highly dependent on aerobic metabolism, especially muscle tissue, are the ones that are most affected. Conventional therapy mainly consists of symptomatic supportive care. Modulation of oxidative stress, augmentation of mitochondrial function, nitric oxide restoration, modulation of the mitochondrial genome, restoration of nucleotide pool to improve mitochondrial DNA maintenance, and stem cell-based therapies are the current experimental therapeutic approaches available for mitochondrial diseases [25]. So far, none of the therapeutics are considered curative or gold standard in managing mitochondrial myopathies. Pharmacologic treatment mainly consists of replacing cofactors, antioxidants, amino acids, coenzymes, or their synthetic analogues involved in the respiratory chain. The efficacy of some of these supplements is still under investigation.

Elamipretide is a tetrapeptide that targets cardiolipin on the inner mitochondrial membrane. Preclinical studies demonstrated that the drug boosts ATP synthesis and reduces the production of reactive oxygen species irrespective of the type of mitochondrial abnormality. A multicentre phase I/II study evaluating the safety, tolerability, and efficacy of Elamipretide has shown that the drug increased exercise performance in a dose-dependent manner after 5 days of treatment in patients with mitochondrial myopathies compared to placebo [26]. As much as the results were promising in preclinical and early-phase clinical studies, the Phase III trial investigating the drug is reported terminating early due to lack of efficacy (Clinicaltrials.gov ID: NCT03323749). The candidate drug *Omaveloxolone*, a potent activator of the nuclear factor erythroid 2–related factor 2, was reported to have antioxidant and anti-inflammatory effects in preclinical trials. However, the results from a Phase II study conducted using adult patients who had mitochondrial myopathies revealed that the drug did not cause a significant improvement in terms of peak exercise workload compared to placebo [27]. Another small molecule called *REN001*, a peroxisome proliferator-activated receptor delta (PPAR-δ) agonist, is currently under investigation. A phase II study evaluating the efficacy of the drug in adult patients with primary mitochondrial myopathies is still ongoing (Clinicaltrials.gov ID: NCT04535609).

Myogenic stem cell-based therapies, a newly emerged treatment strategy for muscular dystrophies, are currently under investigation for use in heteroplasmic m.3243A > G point mutation carriers providing the mutation is in mitochondrial DNA (Clinicaltrials.gov ID: NCT05063721). Therefore, this treatment strategy is not applicable to patients with nuclear DNA mutations or patients who do not have mtDNA-mutation-free stem cells [28]. *KL1333* (Clinicaltrials.gov ID: NCT03888716), an antioxidant called *KH176* (Clinicaltrials.gov ID: NCT04165239), *Deoxynucleosides Pyrimidines* (Clinicaltrials.gov ID: NCT04802707), a central nervous system penetrant soluble guanylyl cyclase stimulator called *IW-6463* (Clinicaltrials.gov ID: NCT04475549), *L-citrulline*, and a combination of Deoxynucleosides called *MT1621* (Clinicaltrials.gov ID: NCT04581733) are other agents currently being investigated for use in mitochondrial disorders.

Genetic Muscular Disorders

No precise or specific treatments for genetic muscle diseases have yet been discovered. Current treatments and approaches are limited to palliative treatment for symptoms related to complications of these diseases and physical therapy/rehabilitation that can help maintain muscle strength and function. Gene therapy may be a promising option for these diseases. Genetic muscle diseases are a group of diseases that can be examined under different classifications according to their characteristics. These include inheritance pattern,

affected body region, or time of onset. Here, we only present diseases treated with pharmacologic agents (drugs or biologic products) whose clinical trials are ongoing and are approved for marketing. For detailed information about the diseases, please see the relevant sections. All searches have been performed in June 2022.

Muscular Dystrophies

Duchenne and Becker Muscular Dystrophies (DMD and BMD): Both represent a range of phenotypes caused by mutations in the DMD gene located on the X chromosome. The disorder varies according to the type and severity of the mutation. Dystrophin is an essential protein for muscle fiber integrity. Due to various mutations, including nonsense mutations and exon deletions in the DMD gene, dystrophin cannot be produced, or is insufficiently or abnormally synthesised. DMD is characterised by the absence of functional dystrophin. Lack of functional dystrophin leads to the sarcolemma becoming vulnerable to mechanical stress, increased production of reactive oxygen metabolites that cause chronic inflammation, decreased regenerative capacity of muscle fibers, and the development of fibrosis. Therefore, muscle weakness develops. Muscle weakness may occur in voluntary muscles and involuntary muscles such as the myocardium and diaphragm. Continuous stress in the left ventricle (LV) causes thinning of the ventricle wall and eventually dilatation. Cardiac fibrosis that develops over time may contribute to a decrease in LV contractility and cardiac output causing fatal arrhythmias. It is predicted that all DMD patients by the age of 18 at the latest will experience some degree of cardiac involvement, mostly dilated cardiomyopathy (CMP). DMD-associated CMP is one of the most important reasons for death in these patients. Therefore, the treatment and prevention of DMD-associated CMP is of vital importance [29]. BMD maintains a reading frame that allows for partially functional dystrophin expression. Therefore, it is a milder disorder compared to DMD; symptoms usually begin later, and progression is slower. However, cardiac involvement may also occur in patients with BMD. Because DMD has high mortality and the highest incidence among inherited muscular diseases, most drugs/biological products being developed are directed toward the treatment of DMD.

Current Options and New Approaches in the Treatment of DMD

Current therapeutic strategies in DMD include treatment or prevention of DMD-associated complications, improving muscle function and quality by targeting the pathological changes triggered by an absence of dystrophin, and restoration of dystrophin expression and/or function.

Treatment or Prevention of DMD-Associated Complications

DMD can cause complications in almost all body systems. DMD-associated complications and their severity may vary from patient to patient; therefore, treatment should be individualised using additions to standard care, including surgical procedures for scoliosis, diet management to prevent weight gain or obesity, bronchodilator therapy for respiratory dysfunction, etc.

CMP is the most important cause of mortality in patients with DMD. We present here only the pharmacological agents used in treating DMD-associated CMP. In treating DMD-associated CMP, standard care and interventional procedures such as implantable cardioverter defibrillators have been involved as non-pharmacological therapies. The classes of medications that have been shown to have beneficial effects on DMD-associated CMP are as followsAngiotensin-converting-enzyme (ACE) inhibitors

- Angiotensin II receptor blockers (ARBs).
- Beta-adrenergic receptor blockers.
- Mineralocorticoid receptor antagonists (MRAs).
- Glucocorticoids (GCs).

The anti-fibrotic effects of ACE inhibitors are well known. Angiotensin II (AT-II) is an endogenous substance that leads to fibrosis by inducing hypertrophy and hyperplasia in myocardial and vascular smooth muscle. ACE inhibitors such as enalapril and lisinopril prevent the formation of AT-II by inhibiting the ACE, which converts angiotensin I to Angiotensin II. They are currently the first-line drugs for treating heart failure unless there is any contraindication. They reduce the rate of mortality and the need for hospitalisation by reducing vascular resistance and cardiac remodelling. The use of ACE inhibitors is strongly recommended in all DMD patients from the age of 10 with or without LV dysfunction (it can be prescribed earlier) [30]. ARBs are preferred in patients who cannot tolerate ACE inhibitors, as alternative drugs. MRAs such as eplerenone and beta-adrenergic receptor blockers such as carvedilol have beneficial effects on DMD-associated CMP. Both drug classes are often combined with ACE inhibitors to improve cardiac function [31]. GCs are currently in clinical use for standard treatment of DMD. They are mainly used to prevent disease progression by slowing muscle breakdown. In addition, they also can delay the development of CMP. There are available few ongoing clinical trials investigating the treatment or prevention of DMD-associated CMP (Table 19.1).

Table 19.1 Ongoing clinical trials regarding the treatment or prevention of DMD-associated CMP (adapted from: Clinicaltrials.gov, Search was performed in June 2022)

Agents	Effect mechanisms	Ongoing clinical trials: Clinicaltrials.gov ID
Metoprolol	Beta-adrenergic receptor blocker	Phase III: NCT05066633
Bisoprolol	Beta-adrenergic receptor blocker	Phase II/III: NCT03779646
Ifetroban	Potent and selective thromboxane A_2/prostanoid receptor (TPr) antagonist	Phase II: NCT03340675
CAP-1002 (allogeneic cardiosphere-derived cells)	HOPE-Duchenne clinic trials (see, "cell-based therapies")	Phase II: NCT04428476 Phase III: NCT05126758

Improving Muscle Function and Quality by Targeting the Pathological Changes Triggered by an Absence of Dystrophin

Therapeutic Strategies Targeting Inflammation

Glucocorticoids (GCs) are the most effective anti-inflammatory agents. The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), is the major activator of the genes coding for immunoreceptors, proinflammatory cytokines, and chemokines. The major effect of GCs in DMD is believed to be the inhibition of NF-κB [32]. Prednisone and deflazacort, which are GC drugs, are currently available for standard treatment of DMD. GCs can improve muscle strength and pulmonary function, reduce the need for scoliosis surgery, and delay the onset of cardiomyopathy. Additionally, it has been reported that deflazacort may increase the survival by 5–15 years of treatment and delay time to loss of ability to walk (ambulation). On the other hand, long-term and high-dose use of GCs in childhood is of concern because of the many adverse effects associated with GCs, including growth retardation, loss in bone density, increased risk of fractures and systemic infections, weight gain, obesity, increased propensity for diabetes mellitus type 2, and osteoporosis. The American Academy of Neurology recommends the short-term use of GCs in treating DMD due to their beneficial effects on muscle strength and function since data are insufficient on their long-term efficacy [33]. This situation highlights the importance of developing new treatment options. *Vamorolone* is a modified GC developed to reduce the adverse effects of GCs. It exerts anti-inflammatory effects by inhibiting NF-kB pathways like GCs.

Givinostat is a histone deacetylase (HDAC) inhibitor. Inhibiting HDAC up-regulates the expression of follistatin protein in dystrophic muscle. Follistatin inhibits the production of myostatin protein, which prevents muscle growth and regeneration by inducing fibrosis. In conclusion, HDAC inhibitors may help activate muscle repair mechanisms indirectly by reducing inflammation and fibrosis and increasing muscle fiber regeneration [32]. It has been designated as an orphan drug for both DMD and BMD.

TAS-205 is a haematopoietic prostaglandin D synthase (HPGDS) inhibitor. This enzyme can promote the production of PGD2, which is an inflammatory mediator. Hence, it is thought that TAS-205 may have a potential anti-inflammatory effect and prevent muscle tissue necrosis.

Canakinumab is a monoclonal antibody developed against human IL-1 beta proinflammatory cytokines. It is thought that Canakinumab may reduce muscle inflammation in DMD due to this effect.

Therapeutic Strategies Targeting Fibrosis

Pamrevlumab is a monoclonal antibody designed to target connective tissue growth factor (CTGF). Increased expression of CTGF may trigger the formation of scar tissue and fibrosis; therefore, Pamrevlumab may improve symptoms of DMD.

Tamoxifen is a selective oestrogen receptor regulator (SERM). In mice lacking dystrophin, tamoxifen reduced fibrosis in the heart and diaphragm and increased the amount of contractile tissue available for respiratory function by increasing the number and diameter of myofibers in the diaphragm [34]. Therefore, it is thought that it may be beneficial in patients with DMD.

Therapeutic Strategies Targeting Muscle Damage

Rimeporide is sodium (Na^+)/proton (H^+) exchanger type 1 inhibitor (NHE-1 inhibitor). Under normal conditions, approximately 70–90% of intracellular Ca^{2+} is moved from the cytoplasm to the sarcoplasmic reticulum (SR) by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and via sarcolemmal Ca^{2+} transport proteins. In dystrophin-free cardiac and skeletal muscles, Ca^{2+} uptake into SR is significantly reduced due to decreased SERCA function. Secondary to the increase in intracellular Ca^{2+} concentration, intracellular Na^+ concentration and pH levels also increase. These changes damage muscle cells by triggering inflammation and fibrosis and contribute to the progression of DMD. Rimeporide may partially compensate for this by preventing the increase of Na + ions and pH (Fig. 19.2) and is also thought to have a cardioprotective effect, which may prolong survival in patients with DMD-associated CMP. A phase Ib study on the safety of Rimeporide in young boys with DMD was completed in 2018. A larger and longer placebo-controlled clinical trial is being prepared to evaluate whether Rimeporide has beneficial effects in decreasing or preventing muscle damage in patients with DMD, as demonstrated in preclinical studies [35].

Phosphodiesterase 5 (PDE-5) inhibitors: Protective functional sympatholysis is a mechanism by which muscle blood flow is increased with exercise to provide oxygen and nutrients to the muscles. The nitric oxide (NO)-cyclic guanosine

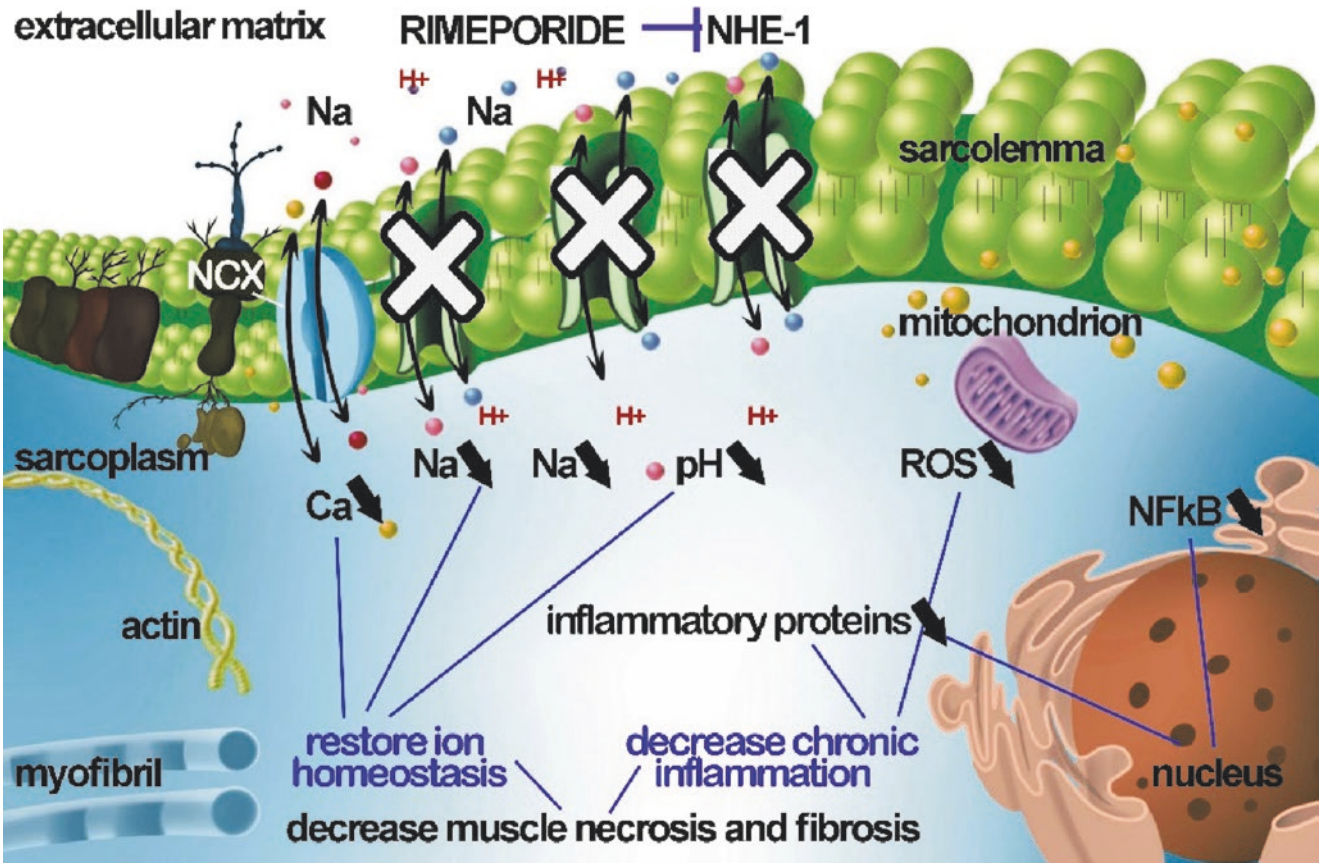

Fig. 19.2 The mechanism of action of Rimeporide at the cellular level [35]

monophosphate (cGMP) pathway participates in this mechanism. NO stimulates cGMP, which mediates vasodilation and increases muscle blood flow. In a healthy skeletal muscle, dystrophin contains a domain to which neuronal NO synthase (nNOS), one of the NO synthesising enzymes, binds. Conversely, nNOS levels are reduced in dystrophin-free muscles. This is important because the decreased production of NO, as a result of nNOS deficiency, can cause irregular blood flow, impaired muscle growth, inflammation, and fibrosis [36]. NO also has a potential regenerative effect in muscles because it regulates myoblast fusion and myotube growth by inducing cGMP and follistatin production. Cyclic GMP, the second messenger of NO, is broken down by phosphodiesterase (PDE) enzymes. It has been reported that sildenafil, a PDE-5 inhibitor, increases the regenerative capacity of muscles in rats (Fig. 19.3), and chronic sildenafil treatment improves the cardiac performance of aged mdx mice [37, 38]. Therefore, it was previously thought that sildenafil may be beneficial in patients with DMD due to its potential cardioprotective effects. However, a placebo-controlled phase II clinical trial was terminated due to the worsening of cardiomyopathy in some patients, including patients with BMD treated with sildenafil [39]. The worsening of cardiomyopathy is thought to be due to the increase in nitrosative stress [40]. It is common for the success of therapeutic innovations assessed in preclinical animal studies not to be achieved in clinical trials. Despite these unforeseen cir-

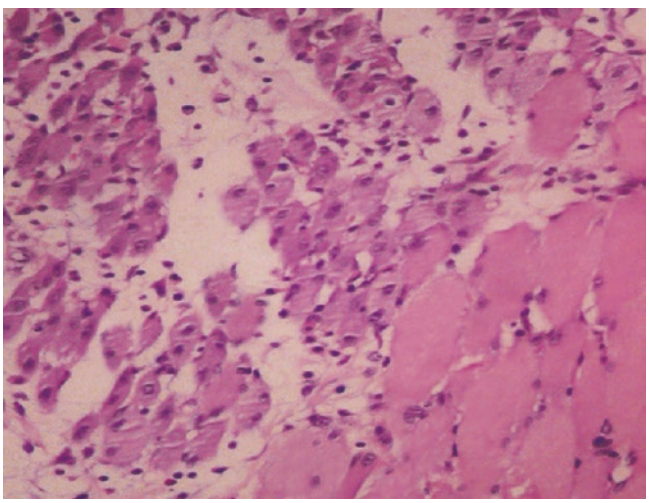

Fig. 19.3 The rat group treated with Sildenafil for wound healing showed marked regeneration in abdominal muscle cells without inflammation (HE ×200) [37]

Table 19.2 Current treatments are targeting secondary pathology triggered by an absence of dystrophin (adapted from: Clinicaltrials.gov, ema.europa.eu, fda.gov; Search was performed in June 2022)

Therapeutic strategies	Effect mechanisms	Current status	Ongoing clinical trials: Clinicaltrials.gov ID
Anti-inflammatory agents			
Corticosteroids – Prednisone – Deflazacort	NF-κB inhibition	Approved for marketing (FDA)	None
Vamorolone	NF-κB inhibition	Designated as OD (EMA, FDA)	Phase II: NCT05185622
Givinostat	Histone deacetylase inhibitor	Designated as OD (EMA, FDA)[a]	Phase II/III: NCT03373968
TAS-205	HPGDS inhibitor	None	Phase III: NCT04587908
Canakinumab	Anti-human IL-1β antibody	None	Phase I/II: NCT03936894
Anti-fibrotic agents			
Pamrevlumab (FG-3019)	Monoclonal anti-CTGF antibody	Designated as OD (EMA, FDA)	Phase II: NCT02606136 Phase III: NCT04632940, NCT04371666
Tamoxifen	SERM	None	Phase III: NCT03354039
Reduction of muscle damage			
Rimeporide	NHE-1 inhibitor	Designated as OD (EMA, FDA)	None
Tadalafil	PDE-5 inhibitor	Designated as OD (FDA)	Phase II/III: NCT05195775
L-arginin+metformin	NO precursors	Designated as OD (EMA)	None
Energy production			
Bocidelpar (also known as ASP0367 or MA-0211)	PPAR-δ activator	None	Phase I: NCT04184882 Phase II/III: NCT04641962[b]

BMD becker muscular dystrophy, *CTGF* connective tissue growth factor, *DMD* duchenne muscular dystrophy, *EMA* European medicines agency, *FDA* U.S. food and drug administration, *HPGDS* haematopoietic prostaglandin D synthase, *NF-κB* transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells, *NHE-1* sodium/proton exchanger type 1 inhibitor, *NO* nitric oxide, *OD* orphan drug, *PDE* phosphodiesterase, *PPAR-δ* peroxisome proliferator-activated receptor delta, *SERM* selective oestrogen receptor modulator

[a] For both DMD and BMD

[b] A clinical trial of bocidelpar is ongoing in participants with primary mitochondrial myopathy

cumstances, the importance of animal studies in the development of a novel agent cannot be disputed. PDE-5 inhibitors are still thought to be able to provide a therapeutic benefit in DM patients without cardiomyopathy. Tadalafil, another PDE-5 inhibitor, is currently being trailed in an ongoing phase II/III study (Table 19.2).

Combination of L-arginine and Metformin: L-arginine is an amino acid and metformin is an antidiabetic agent (a biguanide). Both are NO precursors. Furthermore, it has been reported that L-citrulline has a direct protective effect on skeletal muscle cells in cell culture [41], and metformin increases muscle function and protects muscle in mdx mice by stimulating adenosine monophosphate-activated protein kinase (AMPK) [42]. In phase III clinical trial, it has been concluded that the combined use of these drugs may slow the reduction of muscle function in a certain subgroup of patients with DMD [43].

Bocidelpar is a PPAR-δ activator. PPARs are nuclear hormone receptors that affect biological processes by altering gene expression. PPARs have three subtypes: α, γ, and δ. The predominant form of PPARs in skeletal muscle is PPAR-δs. Activation of PPAR-δ induces fatty acid oxidation and provides energy for oxidative phosphorylation by activating energy uncoupling proteins and mitochondrial biogenesis. It also increases the expression of genes related to mitochondrial respiration and oxidative metabolism and other proteins in skeletal muscle. Additionally, it inhibits macrophage-derived inflammation. Mitochondrial oxidative phosphorylation disorders may impair the regeneration of muscle fibers. Therefore, it is thought that the agonism of PPAR-δ could benefit mitochondrial metabolism disorders characterised by exercise intolerance, fatigue, and muscle wasting, as well as DMD. Clinical trials of Bocidelpar are ongoing in participants with DMD and primary mitochondrial myopathy (Table 19.2).

Restoration of Dystrophin Expression and/or Function

Gene-Based Therapies

Exon Skipping: One or more exons may be missing from the gene associated with dystrophin production in some patients with DMD. However, this loss prevents the remaining exons from being able to fit together. Therefore, a functional dystrophin protein cannot be produced.

Table 19.3 Antisense oligonucleotides developed for the treatment of DMD (adapted from: Clinicaltrials.gov, ema.europa.eu, fda.gov; Search was performed in June 2022)

Agent	Effect mechanisms	Current status	Ongoing Clinical trials: Clinicaltrials.gov ID
Viltolarsen (NS-065/ NCNP-01)	Exon 53 skipping	Approved for marketing (FDA); designated as OD (EMA)	Phase III: NCT04060199
Golodirsen (SRP-4053)	Exon 53 skipping	Approved for marketing (FDA)	Phase III: NCT02500381
WVE-N531	Exon 53 skipping	None	Phase I/II: NCT04906460
Eteplirsen (AVI-4658)	Exon 51 skipping	Approved for marketing (FDA)	Phase III: NCT03992430
Vesleteplirsen (SRP-5051)	Exon 51 skipping	None	Phase II: NCT04004065
Casimersen (SRP-4045)	Exon 45 skipping	Approved for marketing (FDA)	Phase III: NCT02500381
Renadirsen (DS-5141b)	Exon 45 skipping	None	Phase II: NCT04433234
NS-089/ NCNP-02	Exon 44 skipping	None	Phase I/II: NCT05135663

EMA European medicines agency, *FDA* U.S. food and drug administration, *OD* orphan drug

Antisense oligonucleotides (AONs) help the exons on both sides of the deleted exon to reach each other during RNA splicing. Thus, an in-frame mRNA is produced, which functions to produce semi-functional dystrophin. As not all DMD patients carry the same mutation, this approach is mutation specific. AONs developed for the treatment of DMD are described in Table 19.3.

Stop Codon Readthrough: Some nonsense mutations can produce a "premature stop signal" during dystrophin protein synthesis. In this case, a functional dystrophin protein cannot be produced. Thirteen to fifteen percent of patients with DMD have nonsense mutations [44]. *Ataluren,* accepted as a translational read-through–inducing drug, has been approved by the EMA for clinical use in treating DMD. Although the effect mechanism of Ataluren is not fully understood, it has been suggested that it targets ribosomes, and it produces full-length protein by promoting the insertion of near-cognate tRNAs at the site of the nonsense codon without apparent effects on the transcription and translation process [45]. Its phase II and III clinical trials are still ongoing (Clinicaltrials.gov ID: NCT04336826 and NCT03179631, respectively). Ataluren also has been authorised as an orphan drug by the EMA and the FDA for treating BMD.

Gene Transfer: It is based on the approach of delivering micro-dystrophin-encoding genes or GALGT2 genes into the body via adeno-associated viruses (AAVs) used as delivery vectors. Overexpression of GALGT2 increases the expression of dystrophin surrogate proteins such as utrophin which preserve muscle cell integrity by preventing damage during contraction. In other words, rAAVrh74.MCK. GALGT2 therapy aims to compensate for the absence of dystrophin by enhancing the production of other proteins which mediate muscle regeneration and muscle preservation. AAV-mediated gene therapies developed for the treatment of DMD are described in Table 19.4.

Cell-based therapies: Cell-based therapies aim to insert healthy genomes into dystrophic muscles by transplanting normal muscle precursor cells. Clinical trials are ongoing: 1) Myoblast transplantation (Phase I/II; Clinicaltrials.gov ID: NCT02196467) 2) HOPE-Duchenne (Halt cardiomyOPathy progrEssion in Duchenne) clinical trials: It is aimed to increase and protect cardiac functions by infusing cells derived from the allogeneic cardiosphere (CAP-1002) to patients with DMD. (Phase II and III; Clinicaltrials.gov IDs: NCT04428476 and NCT05126758, respectively) EN001 (Infusion of allogeneic umbilical cord-derived mesenchymal stem cells (Phase I; Clinicaltrials.gov ID: NCT05338099. A genome-editing technique, called *CRISPR/Cas9*, is a promising approach in treating DMD. However, it has not yet reached the stage of clinical use. With the CRISPR/Cas9 system, changes can be made to certain limits in the DNA sequence. It was previously proven effective in exon-skipping strategies with long-lasting effects. However, when using AAV vectors to deliver and express the Cas9 system, there is a risk of off-target mutagenesis and decreased effectiveness due to the immune response. Recently the use of lipid nanoparticles (LNPs) was introduced as a tool to deliver Cas9 mRNA and sgRNA into skeletal muscle by repeated intramuscular injections. Although the effects were transient, the LNPs system provided stable exon skipping and restored the dystrophin protein. New Approaches in Treatment of Becker Muscular Dystrophy: Except for exon-skipping, most pharmacological agents developed or under development for the treatment of DMD and DMD-associated complications can also be used in patients with BMD if necessary. Examples of these are "GCs and givinostat" aimed at reducing inflammation and fibrosis, and "ataluren" as gene-based therapy. The following are the agents that have been tried or are being tried only in patients with BMD so far. (−)-Epicatechin increases the synthesis of follistatin, a protein that induces muscle growth, and diminishes production of myostatin, which blocks muscle growth. It has been showed that epicatechin has positive effects on mitochondrial biogenesis and muscle regeneration and, improves the graded exercise testing parameters in patients with BMD [46]. It has been assigned by EMA as orphan drug in treatment of BMD. EDG-5506: It has been suggested that a very recently discovered by Russel et al., a small molecule, EDG-

Table 19.4 AAV-mediated gene therapies developed for the treatment of DMD (adapted from: Clinicaltrials.gov, ema.Europa.eu, fda.gov; Search was performed in June 2022)

Targeted gene	Current status	Ongoing clinical trials: Clinicaltrials.gov ID
– *SRP-9001* also known as:AVV-serotype rh74, containing the human micro-dystrophin gene – rAAVrh74.MHCK7.Microdystrophin, – Delandistrogene moxeparvovec	Designated as OD (EMA)	Phase I: NCT04626674 Phase II: NCT03769116 Phase I/II: NCT03375164 Phase III: NCT05096221
SGT-001 also known as: – Recombinant AVV vector encoding a human micro-dystrophin gene under the control of a muscle-specific promoter	Designated as OD (EMA)	Phase I/II: NCT03368742
PF-06939926 known as: – Adeno-associated viral vector serotype 9, containing the human mini-dystrophin gene – Fordadistrogene movaparvovec	Designated as OD (EMA)	Phase I: NCT03362502 Phase III: NCT04281485
scAAV9.U7.ACCA Also known as: – AAV viral vector, containing a modified U7 snRNA gene	Designated as OD (EMA)	Phase I/II: NCT04240314
rAAVrh74.MCK.GALGT2 Also known as: – AAV serotype rh74, carrying the GALGT2 gene under the control of an MCK promoter	None	Phase I/II: NCT03333590

AAV adeno-associated virus, *EMA* European medicines agency, *OD* orphan drug

5506 stabilizes skeletal muscle fibers and prevents the extreme stress response resulting from muscle contraction. Russel et al. reported that the elevation of creatine kinase decreased after exercise in mdx mice dosed with EGD-5506 both a single dose and for 6 weeks and fibrosis accumulation in the diaphragm decreased when EDG-5506 administered for long-term and similar effects also obtained in different animal species. It is thought that EGD-5506 may have potential to prevent muscle fiber damage and improve muscle function in muscular dystrophies including DMD, BMD and [47]. Phase 1a (in adult healthy volunteers) and Phase 1b in adults with BMD) were completed (ClinicalTrials.gov ID: NCT04585464). A phase 1 study is ongoing to determine the long-term safety and pharmacokinetic and pharmacodynamic profiles of EGD-5506 in adults with BMD (ClinicalTrials.gov ID: NCT05160415). Future studies will show whether the successes from preclinical studies can also be achieved in humans.

Limb-Girdle muscular dystrophies (LGMDs) are a group of diseases, and each type is differentiated by the protein missing in the muscle and genetic transition. They are characterised by progressive muscle weakness, especially in the hip and shoulders. The severity of symptoms varies depending on the mutation type. Two AAV-mediated gene therapy products and a molecule (Ribitol) have been developed, which EMA has assigned as an orphan drug:

SRP-9003 (also known as AVV-serotype rh74, containing the human sarcoglycan beta gene) has been developed for the treatment of LGMD type 2E, which is an autosomal recessive disorder with gene mutations in beta-sarcoglycan protein. Phase I/II clinical trials are ongoing (Clinicaltrials.gov ID: NCT03652259).

AVV-serotype 9, which expresses the human fukutin-related protein and target sequence of the miR-208a, has been developed for the treatment of LGMDs that have a pattern of autosomal recessive inheritance, and gene mutations of fukutin-related protein (FKRP). FKRP mediates the attachment of muscle fibers to the extracellular matrix (ECM). *Ribitol,* acyclic sugar alcohol ($C_5H_{12}O_5$), is a component of the O-mannose glycan located in mammalian α-dystroglycan. Glycosylated α-dystroglycan protects the cytoskeleton and prevents the deterioration of muscle cells. Ribitol is essential for the glycosylation of α-dystroglycan protein. FKRP contributes to the glycosylation of α-dystroglycan protein using the ribitol 5-phosphate molecule. The function of FKRPs is insufficient in some subgroups of LGMDs. The purpose of using Ribitol in such cases is to compensate for this by providing more Ribitol for use by FKRPs. Ribitol (BBP-418) has been assigned by the EMA and FDA as an orphan drug in the treatment of LGMDs.

Facioscapulohumeral muscular dystrophy (FSHMD) is a muscle disease inherited in an autosomal dominant pattern that begins at puberty and is characterised by weakness, especially in the face and shoulders. It is believed that abnormal synthesis of the double homeobox protein 4 (DUX4) transcription factor in skeletal muscle is responsible for the pathogenesis of FSHMD [48], and oxidative stress also triggers muscle weakness. *Combination of α-tocopherol + Ascorbic acid + L-selenomethionine + Zinc gluconate:* α-tocopherol (a form of vitamin E), ascorbic acid (also known as vitamin C), L-selenomethionine, and zinc gluconate has an antioxidative effect. This combination has been assigned by the EMA as an orphan drug in the treatment of FSHMD. *Losmapimod* has the potential to prevent muscle cell injury since it blocks the abnormal activity of the DUX4 protein. It has been assigned

Table 19.5 Ongoing clinical trials for the treatment of myotonic dystrophies (adapted from: Clinicaltrials.gov, Search was performed in June 2022)

Agent	Effect mechanism	Indication	Ongoing clinical trials: Clinicaltrials.gov ID
Mexiletine	Sodium channel blocker	DMs	Phase III: NCT04624750
Tideglusib	GSK3β inhibitor	Congenital DM1	Phase II/III: NCT03692312; NCT05004129
AOC 1001	Oligonucleotide-based therapy	Mild DM1	Phase I/II: NCT05027269
Pitolisant	H_3 receptor antagonist/inverse agonist	Excessive daytime sleepiness in DM1	Phase II: NCT04886518

DM myotonic dystrophy, *GSK3β* glycogen synthase kinase 3 beta

by the EMA and FDA as an orphan drug in the treatment of FSHMD. Ongoing clinical studies on this product are as follows: Phase II, Clinicaltrials.gov ID: NCT04004000 and Clinicaltrials.gov ID: NCT04264442; Phase III, Clinicaltrials.gov ID: NCT05397470.

Myotonic dystrophies are also known as *dystrophic myotonias (DMs)*. They are autosomal dominant muscle diseases characterised by episodes of myotonia and progressive muscle weakness. Episodes of myotonia are characterised by rigidity and pain due to the excessively slow relaxation of the muscle. DMs have two major subtypes: DM1 and DM2. Although it may vary according to different geographic or ethnic populations, the most common type of DM is DM1. DM1 has different phenotypic subtypes with variable onset. Mild DM1 is the most common and adult-onset form. Congenital DM1 is often apparent at birth. Abnormal tri-(CTG) nucleotide repeats in the DM protein kinase (DMPK) gene, located on chromosome 19, are responsible for DM1. In DM2, there is tetra-(CCTG) nucleotide expansion in intron 1 of the ZNF9 (zinc finger 9) gene. Therefore, following DNA transcription, mRNAs are formed with abnormal repeats and longer than they should be. These mRNAs accumulate in the nucleus and cause the development of toxic events which suppress the synthesis of many proteins and disrupt the physiological functions of skeletal, smooth, and cardiac muscle cells. Cataracts, diabetes mellitus, and infertility (especially in men) may accompany muscle involvement. Currently, there are no specific treatments for DMs. The agents developed for the treatment of DM-related symptoms/complications are as follows (Table 19.5):

Mexiletine is a sodium channel blocker, also known as a Type I antiarrhythmic drug. Sodium channel blockage prevents the repetitive firing of action potentials that result in the generation of myotonic muscle rigidity. Mexiletine reduces rigidity caused by prolonged contractions by blocking the sodium channels in the muscle cells. Mexiletine received marketing approval from the EMA in 2018 to treat myotonic disorders. It also has been assigned by the EMA and FDA as an orphan drug in the treatment of non-dystrophic myotonic disorders (non-dystrophic myotonia results from ion channelopathies). A post-marketing observational clinical trial is ongoing (Clinicaltrials.gov ID: NCT04616807).

Tideglusib is a glycogen synthase kinase 3 beta (GSK3β) inhibitor. Activation of GSK3β is one of the signalling pathways in DM1. Tideglusib may inhibit GSK3β activity in DM1 and promote cellular maturation.

AOC 1001 is a conjugate of small interfering (si) RNA and a monoclonal antibody targeting the transferrin receptor 1 on the cell surface. A monoclonal antibody helps siRNA enter the cell. It is thought that siRNA may decrease the DMPK mRNA in a dose-dependent manner by degrading DMPK.

Pitolisant is an antagonist/inverse agonist of the histamine-3 receptor. It has received marketing approval for treating excessive daytime sleepiness or cataplexy in adult patients with narcolepsy, both by the FDA in 2019 and by the EMA in 2021. Excessive daytime sleepiness is an important non-muscular symptom thwarting the daily life of patients with DM. It is thought that Pitolisant may also be beneficial in patients with DM.

Congenital Muscular Dystrophies (CMDs) are a heterogeneous group of neuromuscular disorders with early onset. They are characterised by muscle weakness, hypotonia, and early mortality. The main pathology is the failure to establish a healthy connection between the ECM and the sarcolemma membrane. Muscle weakness usually occurs immediately after birth; if not, it can occur up to the age of 2 at the latest.

LAMA2-related CMD: Laminins synthesised in the basement membrane support the connection between muscle cells and the ECM. This connection is required for muscle cell survival. LAMA2-related CMD is the most common form of CMD. Alpha 2 subunit of laminin-111 (also known as merosin) cannot be produced due to LAMA2 gene mutation. *Recombinant human laminin-111* has been assigned by the EMA as an orphan drug in the treatment of LAMA2-Related CMD. It is considered replacement therapy. *Omigapil* is a glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-Siah1-mediated apoptosis inhibitor. It is thought that apoptotic events play a role in the pathogenesis of *LAMA2-Related CMD*. It has been shown that, Omigapil, which blocks GAPDH-Siah1-CBP/p300-p53 signalling (an apoptotic pathway), ameliorates some pathologic signs in mice with LAMA2-Related CMD. Hence, it has been suggested that Omigapil may reduce early mortality by slowing muscle wasting [49]. Omigapil has been assigned by the EMA and

FDA as an orphan drug for both treatment of LAMA2-related and Collagen VI–related muscular dystrophy.

Congenital Myopathies

Congenital myopathies are early onset, like CMDs. However, muscle weakness becomes evident more slowly compared with CMDs. The main pathology is usually defects in supporting structures such as T tubules and the SR, which are necessary for excitation–contraction coupling.

Centronuclear Myopathies

Centronuclear myopathy related to dynamin 2 (DNM2) gene mutations: DNM2 gene mutations, inherited in an autosomal dominant manner, are one cause of centronuclear myopathies. Dynamin 2 protein, also known as a guanosine triphosphatase (GTPase), is encoded by the DNM2 gene, is widely expressed in the body, and has an important role in membrane traffic. DNM2 gene mutations lead to a change in the structure of dynamin 2 protein. Increased dynamin 2 activity can cause disorganisation of T tubule structures.

DYN101 (Dynamin 101) is an antisense oligonucleotide that is thought to reduce the production of Dynamin 2 by binding to the DNM2 gene. It has been assigned by EMA as an orphan drug in the treatment of centronuclear myopathies caused by mutations in the DNM2 gene. DYN101 is currently being trialled in an ongoing phase I/II study (ClinicalTrials.gov ID: NCT04033159).

X-linked myotubular myopathy (MTM) is a kind of centronuclear myopathy in which myotubule production is impaired due to mutations in the myotubularin gene (MTM1 gene) located on the X chromosome.

Adeno-associated viral vector serotype 8 containing the human MTM1 gene (also known as resamirigene bilparvovec or AT132) has been assigned by EMA as an orphan drug in the treatment of X-linked myotubular myopathy. It is currently being trialed in an ongoing phase I/II study (ClinicalTrials.gov ID: NCT03199469).

Tamoxifen is a selective estrogen receptor regulator (SERM). It has been shown that tamoxifen prolonged survival, enhanced motor function, and improved muscle architecture histopathologically in MTM1 gene knockout mice. A phase I/II study investigating the efficacy and safety of tamoxifen in improving motor and respiratory functions is ongoing in patients with X-linked myotubular myopathy (Clinicaltrials.gov ID: NCT04915846).

GNE Myopathy

Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) myopathy, also known as *Nonaka myopathy, Hereditary inclusion body myopathy,* or *Rimmed vacuole myopathy*, is a rare and progressive distal myopathy. It is an autosomal recessive disorder. It is often seen between 10–30 years old and primarily affects skeletal muscles related to movement, especially extensor calf muscles. It is thought that GNE myopathy is caused by a mutation in the gene that encodes an enzyme used for the synthesis of aceneuramic acid (typical sialic acid) (Fig. 19.4). Sialic acid has a variety of roles in cellular functions, such as cell migration, cell adhesion, and intercellular signal transmission.

N-acetyl-D-mannosamine monohydrate (*ManNAc*) is a sialic acid precursor and a substrate of MAnNAc kinase encoded by the GNE gene. Although ManNAc kinase activity is reduced in GNE myopathy, it has been shown that the oral administration of ManNAc increased sialic acid levels in patients with GNE myopathy in a phase I study [50]. ManNAc could be beneficial for metabolic supplementation in patients with GNE myopathy. It has been assigned by EMA and FDA as an orphan drug and a phase II clinical trial is ongoing (Clinicaltrials.gov ID: NCT04231266).

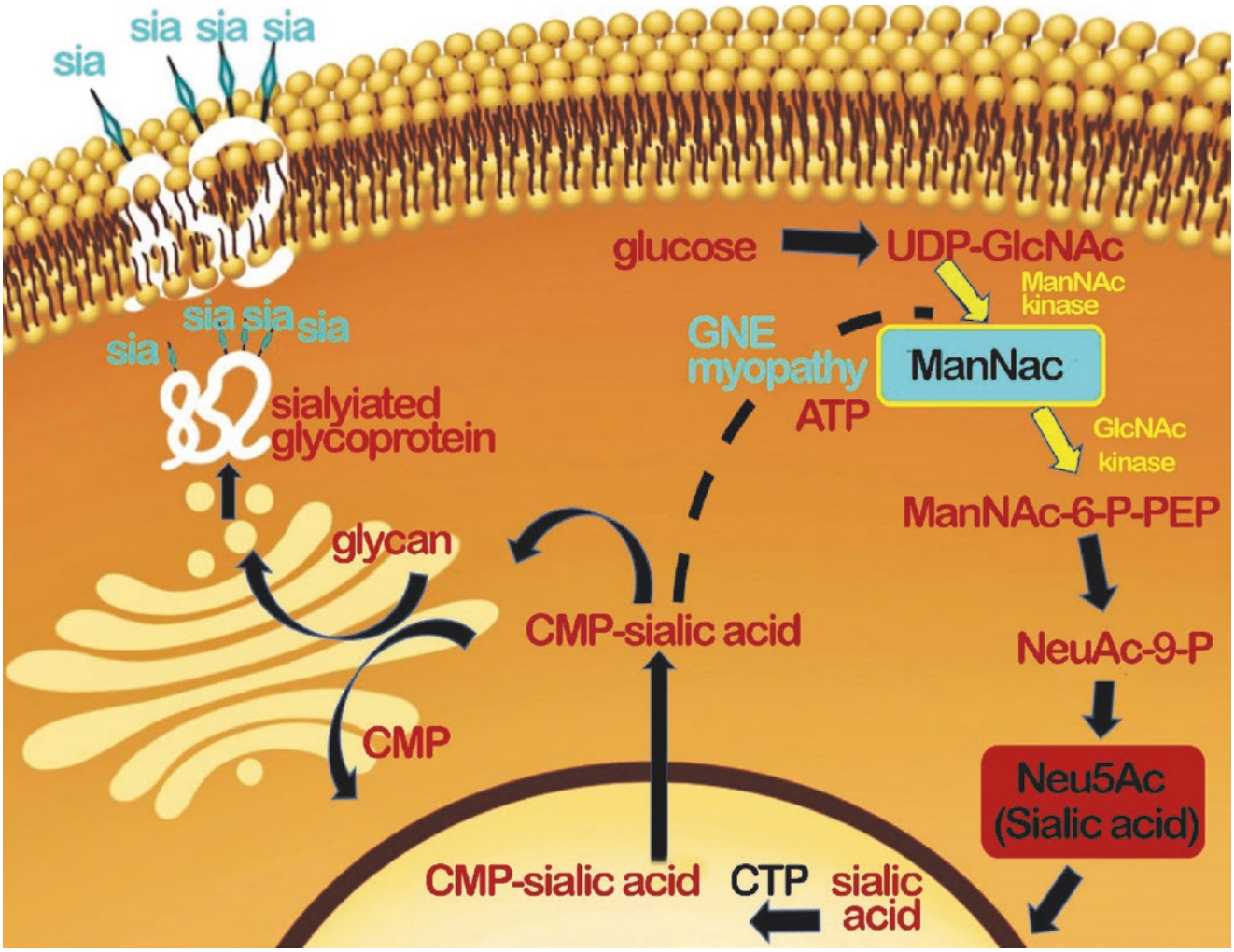

Fig. 19.4 Synthesis of sialic acid (Neu5Ac) and the pathogenesis of GNE myopathy. GNE myopathy is the result of enzyme deficiency, which converts UDP-GlcNAc to ManNAc and/or subsequently, mediates the phosphorylation of ManNAc [50]

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