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RESEARCH

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Relevance of muscle biopsies in the neonatal and early infantile period: a 52 years retrospective study in the gene-sequencing era

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Abstract

Neuromuscular disorders (NMD) with neonatal or early infantile onset are usually severe and differ in symptoms, complications, and treatment options. The establishment of a diagnosis relies on the combination of clinical examination, morphological analyses of muscle biopsies, and genetic investigations. Here, we re-evaluated and classified a unique collection of 535 muscle biopsies from NMD infants aged 0–6 months examined over a period of 52 years. We aimed to assess the importance and contribution of morphological muscle biopsy analyses for the establishment of a precise and accurate molecular diagnosis. Altogether, 82% of the biopsies showed typical structural myofiber anomalies highly suggestive of specific NMD classes (congenital myopathies, metabolic myopathies, lower motor neuron (LMN) and neuromuscular junction (NMJ) disorders, muscular dystrophies, inflammatory myopathies), while the remaining 18% showed no or only non-specific histological abnormalities. The diagnostic success rate differed among the NMD classes and was particularly high for congenital myopathies as illustrated by the identification of causative genes in 61% of cases. This is essentially due to the presence of characteristic histopathological hallmarks on biopsies visible by light or electron microscopy often pointing to specific genes. In contrast, metabolic myopathies commonly displayed non-specific features on muscle sections, led to the identification of causative genes in only 19% of the patients, and typically required additional enzymatic tests to establish a more precise diagnosis. The evolution of sequencing technologies fundamentally improved molecular diagnosis and also shifted the relevance of muscle biopsies within the diagnostic process. Depending on the clinical presentation of the patients, direct gene or panel sequencing may be the preferred method nowadays. However, histological and ultrastructural examinations of muscle sections are still frequently useful and can constitute an elemental step in the diagnostic process—either by directing purposeful gene sequencing or pointing to genes and pathogenic variants identified by next-generation sequencing (NGS), or by complementing clinical findings and biochemical analysis methods.

Keywords Neuromuscular disorder, NMD, Muscle biopsy, Congenital myopathy, Metabolic myopathy, Electron microscopy

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Introduction

The diagnosis of neuromuscular diseases with neonatal or perinatal onset requires rigorous and expeditious studies to determine the cause of the disease, establish the vital prognosis, improve disease management, and provide adequate genetic counselling for the family. Muscle biopsies took and still take a central place in the diagnostic process, and the indication to perform muscle biopsies on newborns and infants is based on the severity of the clinical phenotype and the presence of life-threatening signs including marked hypotonia, muscle weakness, akinesia, and difficulties in breathing and swallowing. Other common signs suggestive of neuromuscular disorders such as hydramnios, joint contractures, arthrogryposis, fetal hypomobility, and pulmonary hypoplasia can be present before birth [26, 30].

However, in the light of major advancements in molecular genetics and the routine use of next-generation sequencing (NGS) techniques in research institutes and accredited public and private diagnostic laboratories, the value and usefulness of muscle biopsies for the precise diagnosis of neuromuscular disorders has been subject to debates.

Here, we present a retrospective study on muscle biopsies performed on infants aged 0-6 months at the neuromuscular reference center of the GHU Pitié-Salpêtrière in Paris and examined at the French National Institute of Health and Medical Research (Inserm)/Neuromuscular Morphology Unit of the Institute of Myology over a period of 52 years from 1970 to 2021. For all patients, the diagnostic flow implied clinical examinations followed by muscle morphology analyses and genetic investigations. The objectives were to (1) provide a representative cross-section of the different groups of neuromuscular diseases in neonates and infants requiring a muscle biopsy, (2) evaluate the importance of morphological muscle biopsy analyses for the establishment of an accurate diagnosis, and (3) determine the value of electron microscopy in directing molecular diagnosis.

Materials and methods

Patients and muscle biopsies

From 15300 muscle biopsies referred to our laboratory between 1970 and 2021, a series of more than 600 specimens were from infants aged 0–6 months, and 535 were selected for this study. The remaining biopsies were not included due to lack of additional clinical information and/or poor muscle sampling conditions not allowing thorough analysis. For all 535 selected cases, the available muscle section slides were reviewed by light and—if appropriate—electron microcopy. Numerous samples underwent accessory histological or immunofluorescence experiments in the light of novel scientific findings over the past decades, but no additional experiment was specifically performed for the present study.

Most of the samples were taken from quadriceps and deltoid muscles, and less frequently from biceps and tibialis anterior. Biopsies were usually divided into four samples for histoenzymological, electron microscopy, enzymatic, and molecular studies. The region containing NMJs was determined by inducing small twitches, and the presence of NMJs on muscle biopsies was confirmed using the classic Koelle method revealing cholinesterase activity. Clinical, biological, morphological, and molecular data were retrospectively and separately analyzed for patients examined between 1970 and 1999, and for patients examined from 2000 to 2021. This is justified by the similar number of cases (248 versus 287) and the possibility to compare the contribution of muscle biopsies to the establishment of a definite diagnosis in two fundamentally distinct periods. Indeed, the routine use of Sanger sequencing in diagnostic laboratories and the recent evolution of NGS technologies significantly increased the number of known myopathy genes and improved molecular diagnosis since the turn of the millennium.

The patients were classified based on (1) clinical criteria and the predominant features observed at clinical examination of the patients, and (2) morphological criteria resting upon histoenzymological studies of muscle samples.

Clinical and muscle morphology criteria

All patients included into the survey had a minimum gestational age of 21 weeks and presented with generalized hypotonia as the principal sign, most often associated with muscular atrophy. Based on the severity and the presence of additional signs, the patients were divided into six groups:

(1) Muscle weakness (with or without respiratory assistance)
(2) Muscle weakness with arthrogryposis and/or dysmorphia/malformations (with or without respiratory assistance)
(3) Muscle weakness with multi-systemic involvement of multiple organs and tissues including brain, liver, bone marrow, intestine, or others (detectable e.g. by elevated lactate levels or hepatic cytolysis)
(4) Muscle weakness with cardiomyopathy (separated from group 3 due to structural and physiological similarities between skeletal muscle and heart, and the implication of paralogue genes in myopathies and cardiomyopathies [27])
(5) Muscle weakness with elevated creatine phosphokinase (CK)

(6) Muscle weakness with central nervous system (CNS) involvement

The morphological classification was exclusively based on muscle biopsy findings. The muscle samples were studied in a standardized way with systematic application of a range of histological and histoenzymological techniques [31]. Ultrastructural investigations by electron microscopy were considered whenever a sample was available and fixed under appropriate conditions [16]. Depending on the muscle morphology, the biopsies were included into one of the following groups:

- Congenital myopathies, characterized by the presence of cores, nemaline bodies, cytoplasmic bodies, caps, central and internalized nuclei, nuclear envelope abnormalities, type 1 fiber predominance
- Metabolic myopathies involving abundance of lipid droplets, subsarcolemmal mitochondrial aggregates, ragged red fibers (RRF), glycogen accumulations, type 1 fiber predominance
- Lower motor neuron (LMN) and neuromuscular junction (NMJ) disorders showing numerous isolated or grouped atrophic fibers, grouping of muscle fibers of the same histochemical type, type 2 fiber atrophy in NMJ disorders
- Muscular dystrophies, characterized by myofiber diameter variability, rounded fibers, internalized nuclei, necrotic/regenerating fibers, increased connective tissue
- Inflammatory myopathies showing foci of inflammatory cells (mainly lymphocytes and eosinophils)
- Non-significant abnormalities (e.g. minor variation in fiber size as the only detectable anomaly)
- Normal muscle morphology

Importantly, all of these neuromuscular disorder categories are rare diseases, which are essentially monogenic. Multigenic disorders were not addressed in this study.

Muscle ultrastructure

Electron microscopy findings were classified as decisive, contributory or confirmatory according to their relevance and contribution to molecular diagnosis. "Decisive" refers to the identification of significant elements that were undetectable by histoenzymology such as caps, cytoplasmic bodies, nemaline rods, neuromuscular junction defects, nuclei abnormalities, or other rarer lesions (swollen reticular cisterns, autophagy, other inclusions). EM was considered "contributory" when it confirmed the abnormalities observed by histoenzymology and detected additional elements not seen by optical microscopy. Furthermore, "confirmatory" describes the observation of the same type of anomalies by light and electron microscopy without providing additional elements to direct molecular diagnosis or to refine the initial diagnostic hypothesis.

Results

Description of the cohort

The cohort of 535 infants was composed of 54% boys and 46% girls. Patients were assigned to one of the six clinical groups according to the phenotypic presentation at the time of muscle biopsy, and—after biopsy analysis—to one of the muscle morphology groups based on the histopathological and ultrastructural features in myofibers. An overview of the individual groups with distribution of patients is shown in Figs. 1 and 2, and examples of typical histopathological and ultrastructural findings on muscle biopsies are illustrated in Figs. 3, 4, 5 and 6.

In total, 40% of the clinically examined neonates presented with profound hypotonia and muscle weakness, and 13% manifested additional arthrogryposis with or without dysmorphia and other malformations (Fig. 1A). Muscle weakness and hypotonia were also seen with multi-systemic signs including elevated lactate levels or hepatic cytolysis (19% of the patients), with CNS involvement (16%), or in combination with cardiomyopathy (9%) or elevated serum creatine levels (CK, 3%).

As illustrated in Fig. 1B, around 43% of the analyzed muscle biopsies featured metabolic abnormalities suggesting an inborn error of metabolism (IEM). These anomalies encompassed abundant lipid droplets, subsarcolemmal mitochondrial aggregations and ragged red fibers (RRFs), COX-deficient muscle fibers, or glycogen accumulations. Other common groups were congenital myopathies (20% of all patients), followed by lower motor neuron (LMN) and neuromuscular junction (NMJ) disorders (10%), muscular dystrophies (8%), and inflammatory myopathies (1%). In 10% of the patients, muscle biopsies showed nonspecific anomalies and were not suggestive of distinct disease entities, and 8% of the biopsies were without detectable lesions and were considered as normal. Among the congenital myopathies, the vast majority of patients exhibited structural anomalies such as cores, nemaline rods, or centralized nuclei, while the remaining cases corresponded to rare congenital myopathies without classical morphological hallmarks on the biopsy, but showing a high frequency of immature muscle fibers or increased expression of embryonic myosin indicating developmental anomalies.

Distribution of morphological findings through different periods

We then compared the distribution of the muscle morphology groups between the biopsies analyzed from 1970 to 1999 (248 cases) and from 2000 to 2021 (287 cases),

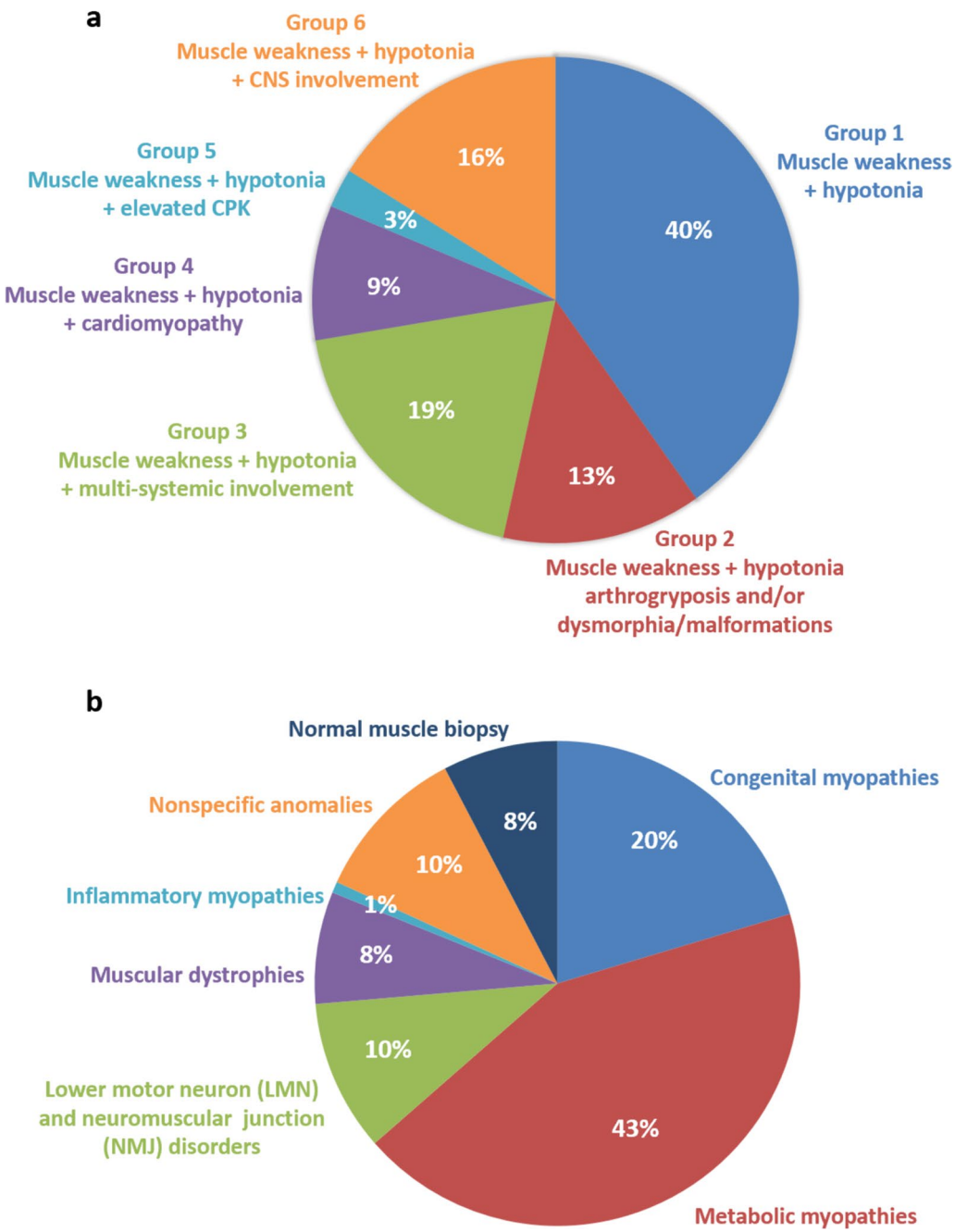
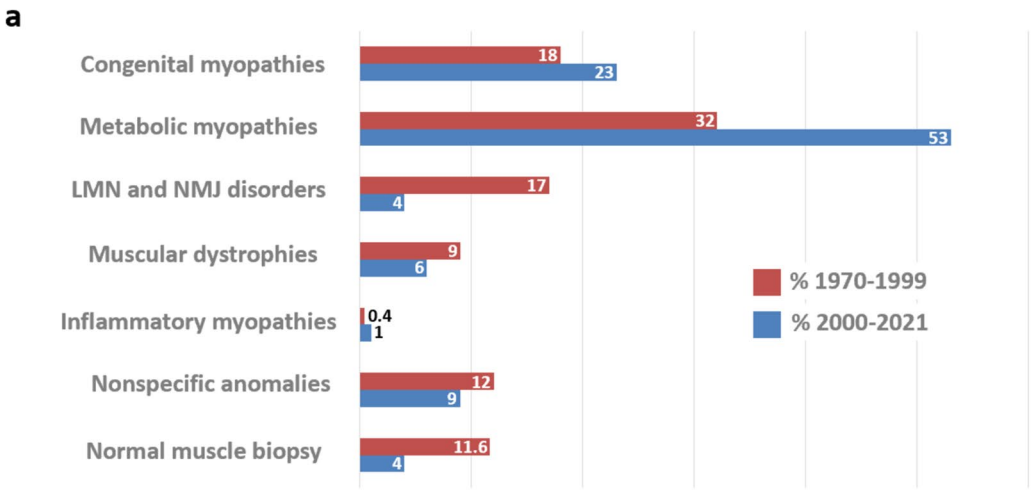


Fig. 1 Distribution (in %) of 535 analyzed cases. (**a**) Classification of patients by clinical criteria. (**b**) Classification of patients by muscle morphology

reflecting two periods with a comparable number of cases but profoundly differing by the available molecular diagnostic methods.

As shown in Fig. 2A, 32% of the biopsies analyzed from 1970 to 1999 displayed metabolic anomalies, while 18% were suggestive of congenital myopathies and 17% were compatible with lower motor neuron (LMN) or neuromuscular junction (NMJ) disorders. In the period from 2000 to 2021, we observed an increased ratio of metabolic myopathies (53%), which is possibly due to a recruitment bias linked to the close collaboration of our laboratory with teams working on IEM. In parallel to enzymatic and molecular studies carried out in hospitals, we were regularly commissioned with histological analyses of muscle biopsies from affected individuals.



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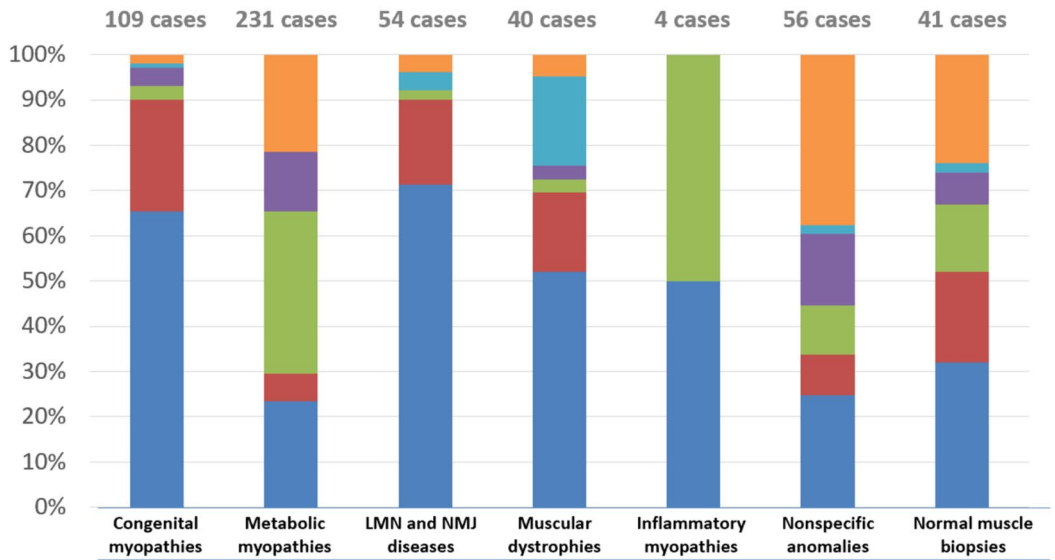

	Congenital myopathies	Metabolic myopathies	LMN and NMJ diseases	Muscular dystrophies	Inflammatory myopathies	Nonspecific anomalies	Normal muscle biopsies
Group 1	66 %	23 %	72 %	53 %	50 %	25 %	32 %
Group 2	25 %	6 %	19 %	18 %	0 %	9 %	20 %
Group 3	3 %	35 %	2 %	3 %	50 %	11 %	15 %
Group 4	4 %	13 %	0 %	3 %	0 %	16 %	7 %
Group 5	1 %	0 %	4 %	20 %	0 %	2 %	2 %
Group 6	2 %	21 %	4 %	5 %	0 %	38 %	24 %

Fig. 2 Distribution (in %) of muscle morphology groups. (**a**) Ratio of cases with congenital myopathies, metabolic myopathies, lower motor neuron (LMN) and neuromuscular junction (NMJ) disorders, inflammatory myopathies, nonspecific muscle anomalies, or normal muscle biopsy in the period from 1970 to 1999 (red bars) and 2000–2021 (blue bars). (**b**) Correlation of muscle morphology with the clinical presentation of the patients

Biopsies in favor of LMN and NMJ disorders were more frequently seen in the period from 1970 to 1999, with around 17% compared to 4% between 2000 and 2021. This is at least partially related to the discovery of the *SMN1* gene in 1995 [18] and the shift towards direct gene sequencing for the diagnosis of infantile spinal muscular atrophy (SMA). Within a few years, Sanger sequencing / multiplex PCR of *SMN1* (and *SMN2*) was

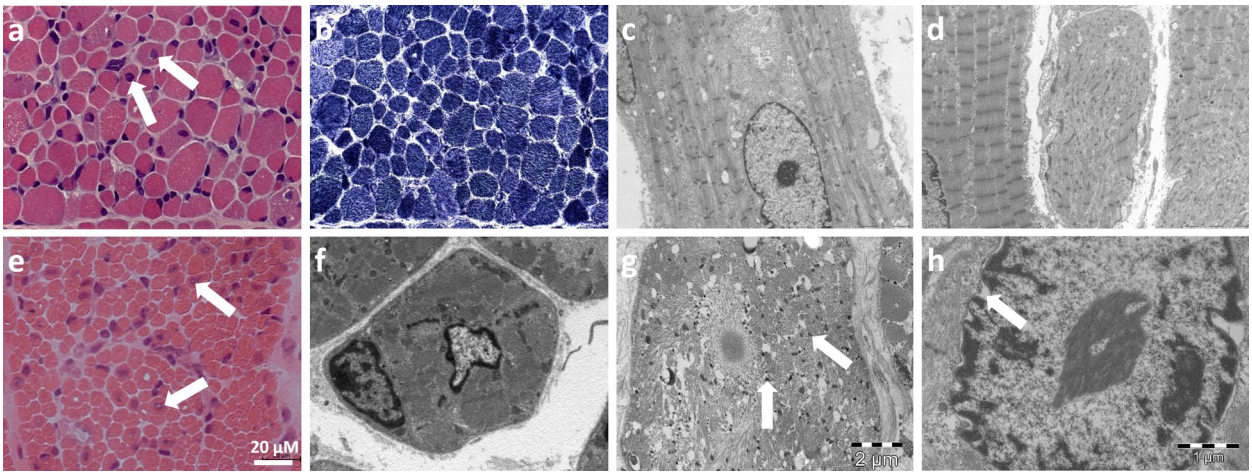

Fig. 3 Congenital myopathies—histopathological hallmarks on muscle biopsies. (**a**–**d**) Serial sections of a muscle biopsy from a 45-day-old infant with *RYR1*-related autosomal recessive congenital myopathy. (**a**) HE and (**b**) NADH staining showed significant fiber size variability with numerous centralized/internalized nuclei (arrows) and disorganization of the internal structure of the muscle fibers. (**c**, **d**) Electron microscopy sections revealed the presence of internalized nuclei and large areas of disorganization devoid of mitochondria indicative of unstructured cores. (**e**, **f**) Muscle biopsy from a 1-month-old infant (gestational age 29 GA) with *DNM2*-related centronuclear myopathy CNM) showing numerous muscle fibers with centralized nuclei resembling myotubes (arrows, **e**). (**f**) On electron microscopy, satellite cells appear in normal numbers in DNM2-related CNM—contrasting MTM1-related CNM [29]. (**g**, **h**) In a 40-day-old newborn with *ACTA1*-related nemaline myopathy, electron microscopy sections revealed cytoplasmic body inclusions, intranuclear rods (arrows) and nuclei with enlargement of the perinuclear space of up to 1200 nm (arrow, normal distance 30–50 nm between the inner and outer nuclear membrane of the nuclear envelope).

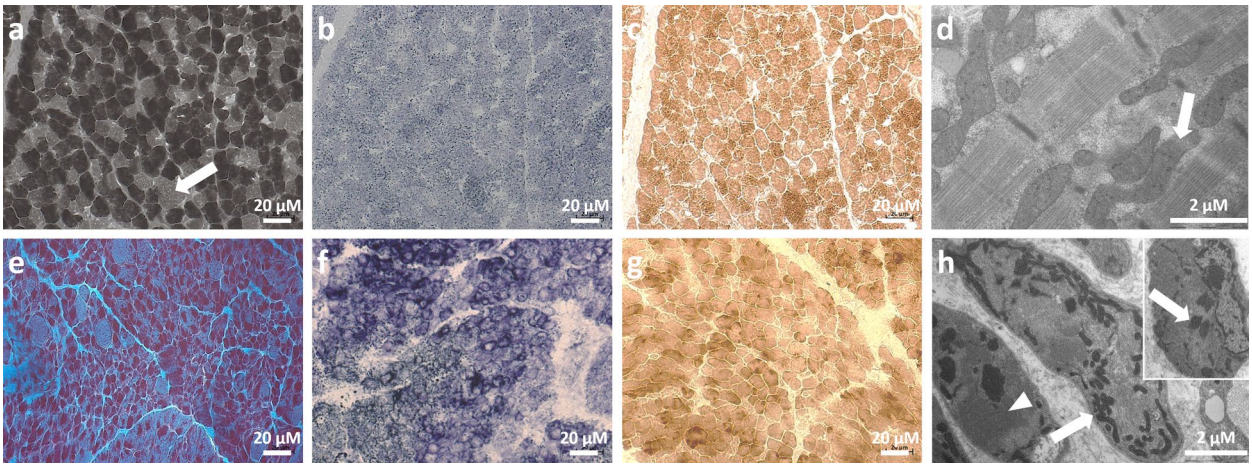

Fig. 4 Muscle biopsies in metabolic myopathies. (**a**–**d**) Muscle biopsy from a 5-day-old patient with pyridox(am)ine 5′-phosphate oxidase (PNPO) deficiency. (**a**) ATPase pH 9.4 reaction evidenced a microvacuoles appearance of type I myofibers probably related to abundant lipid droplets (arrow). (**b**, **c**) Enzymatic SDH and COX reactions disclosed intense signals in type 1 muscle fibers in the absence of red-ragged fibers. (**d**) Electron microscopy revealed numerous mitochondrial aggregates among the myofibrils (arrow). (**e**–**h**) Muscle sections from an infant with *PNPT1* mutation showed marked fiber size variability, small red deposit/inclusions on Gomori trichrome staining (**e**), increased signal intensities on SDH (**f**) and few COX-pale fibers (**g**). (**h**) Electron microscopy revealed diverse lesions such as areas with dense filament material (arrowhead), mitochondrial aggregates at the periphery of the muscle fiber (arrow), and rods (inset, arrow).

routinely applied in most diagnosis laboratories, and muscle biopsies were rarely requested since. Similarly, following the identification of the first causative mutations in *DMD* [22] and the advancement of efficient DNA sequencing techniques, the number of muscle biopsies from patients with muscular dystrophies declined with time. There was also a significant difference in the relative number of cases with apparently normal muscle morphology. Indeed, in the time from 1970 to 1999, 11.6% of all biopsies were considered as normal compared to only

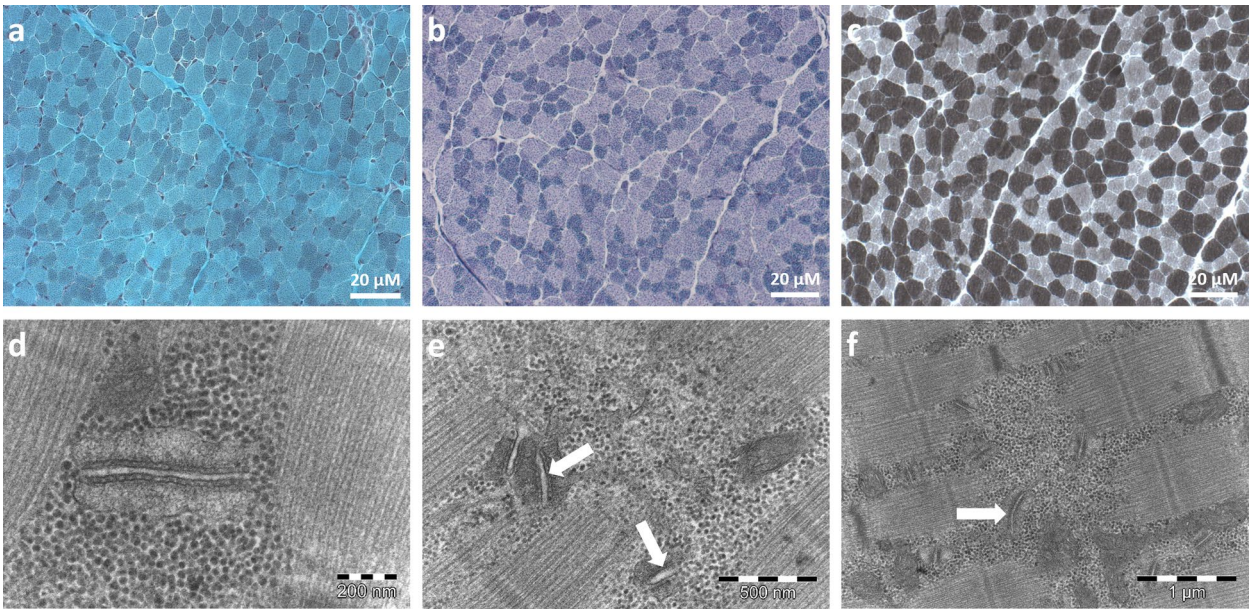

Fig. 5 Muscle biopsy from a 2-months-old infant with *STAC3*-related congenital myopathy. Muscle sections stained with Gomori trichrome (**a**), NADH-TR (**b**), and ATPase pH 9.4 (**c**) show fiber size disproportion with slight fiber type I atrophy and predominance. (**d**–**f**) Electron microscopy revealed numerous dilated cisternae (arrows), presumably reflecting swollen T-tubules and sarcoplasmic reticulum.

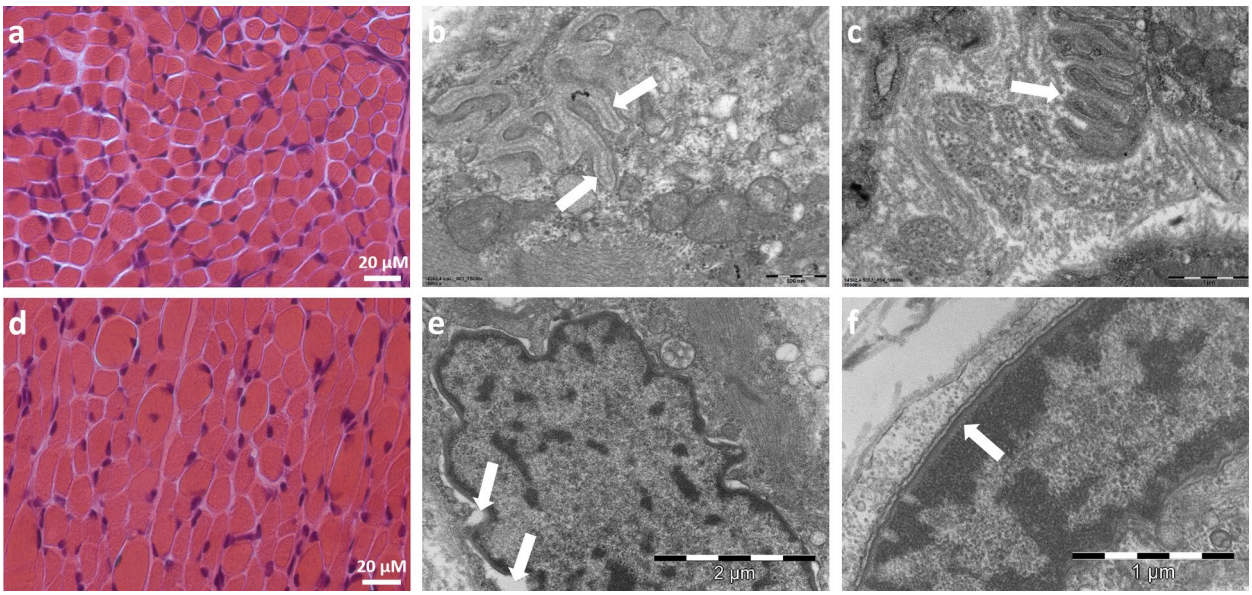

Fig. 6 Muscle biopsies in LMN and NMJ disorders. (**a**–**c**) Muscle biopsy from a 14-day-old infant (gestational age 34 GA) with *CNTNAP1*-related myopathy. (**a**) HE sections showed fiber size variability but no specific abnormality suggestive of a particular myopathy. (**b**, **c**) In contrast, EM sections evidenced numerous abnormal neuromuscular junctions with ridge depletion (arrows). (**d**–**f**) Muscle biopsy from a 1-month-old congenital myopathy patient with aberrant nuclear architecture. (**d**) Non-specific fiber size variability is the only visible anomaly on HE. Electron microscopy revealed significant abnormalities of the nuclei, in particular an enlargement of the perinuclear space (**e**) (arrows) and abnormal thickening of the lamina (**f**) (arrow). The patient still awaits molecular diagnosis

4% from 2000 to 2021. This is possibly due to the technical improvements of microscopes, the availability of specific antibodies, and the development of sophisticated imaging software, all allowing the detection of smaller lesions at first examination of biopsies collected since 2000. Moreover, the constantly increasing knowledge

on rare diseases significantly improved the recognition and classification of neuromuscular disorders at clinical examination, and decreased the likelihood that muscle biopsies were requested for disorders without primary muscle involvement.

Correlation between clinical picture and muscle morphology

Next, we compared the clinical groups with the muscle biopsy classifications, and several correlations could be drawn (Fig. 2B). As an example, groups 1 and 2, comprising patients with muscle weakness associated or not with arthrogryposis, formed the vast majority of cases diagnosed with congenital myopathies and LMN and NMJ disorders. Muscle weakness with multi-systemic signs (group 3) was mostly accompanied by metabolic muscle lesions, possibly because aberrant metabolic pathways also affect other tissues than muscle such as liver and heart in different glycogen storage disorders and mitochondrial myopathies. Muscular dystrophies are characterized by increased myofiber degeneration and the release of creatine kinase into blood circulation and were congruously associated with muscle weakness and elevated CK levels (group 5). Lastly, patients with CNS involvement (group 6) are more likely to have a normal or non-specific biopsy compared with other clinical groups, supposedly because a subset of CNS disorders do not directly impact on myofiber morphology and may cause hypotonia as a secondary consequence of neurological impairments.

Muscle biopsies studied by electron microscopy

From the 535 muscle biopsies, 120 samples were analyzed by electron microscopy (EM), reflecting a ratio of 22%. In 20% of the cases, no EM-compatible muscle specimen was received, and in the remaining cases, ultrastructural investigations were not considered necessary.

From the 120 muscle biopsies analyzed by EM, the majority of 62% were from patients with congenital myopathies. The other biopsies were from patients with metabolic myopathies (22%), LMN and NMJ disorders (6%) and muscular dystrophies (6%). In patients with nonspecific or absent histopathological features, EM was rarely carried out. The contribution of ultrastructural analyses was classified as "decisive", "contributory", or "confirmatory" depending on their relevance for diagnosis. An overview is provided in Online Resource 1.

The significance of complementary ultrastructural explorations of muscle sections strongly varied among the disease entities. Indeed, EM was contributory and even decisive for the establishment of a diagnosis in most patients with congenital myopathies, but had a rather confirmatory role in patients with metabolic myopathies and muscular dystrophies. Both metabolic myopathies and muscular dystrophies are usually diagnosed through the combination of medical examination of the patient and biochemical tests on blood samples. Histological and especially electron microscopic analyses of muscle biopsies are often not necessary anymore, but may nevertheless help to validate the initial hypothesis in individual cases. In contrast, biochemical blood parameters are commonly unremarkable in patients with congenital myopathies, shifting the focus on the analysis of muscle biopsies. Structural anomalies typically observed in congenital myopathies such as cores, nemaline rods, caps, and cytoplasmic bodies can be undetectable in atrophic fibers and fibers from neonates by histological methods and often require electron microscopy to be identified.

Genetic studies

Molecular studies were carried out in all disease groups where possible. Online Resource 2 summarizes the genes found in patients of each muscle morphology group.

Among the 109 cases classified as congenital myopathies, genetic studies led to the identification of the causative genes in 57 of the 94 patients with classical structural congenital myopathies (61%). Of note, 46 of the 57 cases were molecularly diagnosed in the period from 2000 to 2021 with the majority diagnosed through exome or panel sequencing since 2010. This emphasizes the impact of NGS technologies on the diagnostic process of rare muscle disorders, and highlights the markedly shortened time span between clinical examination, muscle biopsy analysis, and the validation of genetic investigations.

For the remaining cases, molecular analyses are either ongoing through exome or genome sequencing projects, or are on hold due to the lack of sufficient DNA samples from the index patient and unaffected family members. This particularly concerns patients examined and biopsied in the period from 1970 to 1999, when DNA was not systematically extracted. All DNA samples collected before the year 2000 nevertheless underwent Sanger sequencing of known and newly identified genes at different time points, and led to a molecular diagnosis of individual cases up to 21 years after the muscle biopsy. In total, mutations in 13 different genes were found in the molecularly diagnosed congenital myopathy patients with a particular recurrence of mutations in *MTM1, ACTA1, RYR1* and *NEB* (Online Resource 3) [4, 7, 8, 14, 16, 19, 20, 29]. The comparison of the age at the time of muscle biopsy revealed a higher diagnosis success rate in neonates (< 2 months) compared with early infants (2-6 months). This is possibly due to the fact that neonates with life-threatening clinical manifestations undergo biopsy within the first

days or weeks of life and that these severe forms of congenital myopathies frequently arise from mutations in well-known genes like *MTM1* or *ACTA1*.

Contrasting with congenital myopathies, metabolic myopathies were genetically highly heterogeneous in our cohort. In the 43 molecularly diagnosed patients, mutations in 34 different genes were identified, including a large number of nuclear-encoded mitochondrial genes. In addition, mutations in the mitochondrial DNA and in glycogenosis and fatty acid oxidation disorder (FAOD) genes were found [13] (Online Resource 4). Importantly, a large number of patients with metabolic myopathies still await molecular diagnosis (81%) because genetic investigations were inconclusive. This is partially due to the absence of parental DNA samples for segregation analysis in several families, but also suggests that additional causative genes remain to be identified, emphasizing the wide genetic heterogeneity of metabolic myopathies. Another distinctive feature of metabolic myopathies is the higher diagnostic success rate in infants (2–6 months) over neonates (< 2 months), which can be explained by the idiosyncratic disease course. Indeed, metabolic alterations typically lead to the accumulation of metabolites, intermediate molecules, and by-products, which often reach a pathogenic threshold after weeks or months and induce the occurrence of the first clinical signs.

Molecular analyses for patients with rather non-specific histoenzymological abnormalities as COX-negative fibers or isolated overload of lipid droplets were sometimes inconclusive, indicating that the current imaging techniques may be insufficient to uncover specific structural hallmarks at early disease stages and direct genetic investigations, or that yet unknown exonic, intronic, or intergenic mutations account for these muscle disorders. It should also be noted that myofiber abnormalities may not always be of genetic origin and can result from viral infections or toxic injuries of specific drugs.

Discussion

In this retrospective study, we reviewed 535 muscle biopsies from patients with different muscle disorders and complete clinical data, and we evaluated the contribution of histological and ultrastructural analyses of the muscle samples to the establishment of a definite diagnosis. The biopsies were taken and examined in a single reference center over a period of 52 years and constitute a unique collection of neonatal muscle samples. We provide an overview of the frequency of specific muscle disorders and the diagnostic success rate in light of the major progress of DNA sequencing technologies and the increasing use of genetic tests.

Contribution of muscle biopsies to diagnosis

From the 535 reviewed biopsies, a minority of 18% displayed no or nonspecific structural anomalies, precluding a histopathological classification of the patients and a purposeful sequencing of myopathy genes. Conversely, 82% of all biopsies were informative and suggestive of a specific myopathy or a group of myopathies, and significantly reduced the diagnostic odyssey of the affected families. The identification of the causative gene improves disease management, permits precise genetic counselling and—in some cases—also prenatal and preimplantation diagnosis.

From the 439 informative muscle biopsies, 77% were from patients with metabolic myopathies or congenital myopathies. Although muscular dystrophies—including Duchenne (DMD) and Becker (BMD) muscular dystrophy—are more common in the population, they are often suspected by highly elevated serum CK levels and/or a family history of neuromuscular disorders, and do not necessarily require a muscle biopsy. Anyhow, myofibers in muscular dystrophy patients undergo necrosis and enhanced degeneration and regeneration cycles, resulting in a typical histopathological picture of the muscle biopsy without pointing to a specific gene. Immunohistochemical analyses on muscle sections using specific antibodies can nonetheless be useful and conclusive for the diagnosis as for *LAMA2*-related muscular dystrophy, and may provide relevant information complementing genetic investigations [11]. However, antibodies are costly and nowadays considered less efficient than panel or exome sequencing. As another example, lower motor neuron (LMN) and neuromuscular junction (NMJ) disorders involve an abnormal nerve-to-muscle signal transmission and can be efficiently detected and diagnosed through electroneuromyography (ENMG) and the disease-typical decremental response upon repetitive nerve stimulations, rendering muscle biopsies unnecessary in many cases.

Noteworthy, the value of muscle biopsies in the diagnosis process was dissimilar among the main myopathy groups. They were generally decisive or contributory for congenital myopathies and played a confirmatory role for metabolic myopathies. This is essentially due to the disparate type and specificity of the histopathological hallmarks. Indeed, the main congenital myopathy entities are often identifiable by characteristic structural anomalies such as rods in nemaline myopathy (NM), cores in *RYR1*-related myopathy, or central nuclei in centronuclear myopathy (CNM) (Fig. 3). Histological and ultrastructural examinations of muscle biopsies can even point to single genes among the genetically heterogeneous congenital myopathy subgroups. As an example, CNM can be caused by mutations in *BIN1*, *DNM2*, *MTM1*, *RYR1*,

SPEG, or *TTN* [1, 5, 6, 9, 17, 23, 32], and the different disease forms clinically overlap, ranging from severe neonatal hypotonia with poor prognosis to milder adult-onset forms. All CNM patients display abnormal nuclear centralization on muscle biopsies, but can be distinguished by additional histopathological features as necklace fibers in *MTM1* patients [2], radial arrangements of sarcoplasmic strands in *DNM2* patients [5], or the occurrence of unstructured cores in *RYR1* patients [3, 32]. Rarely, muscle biopsies from CNM patients are not indicative of a specific gene and may even be misleading at first glance. The muscle histology illustrated in Fig. 3E was suggestive of *MTM1*, but additional electron microscopy uncovered a normal number of satellite cells, which is typical for *DNM2*-related CNM. Subsequent gene analysis confirmed the causality of *DNM2*.

Importantly, the wide clinical and genetic heterogeneity of congenital myopathies was at least partially discovered by the systematic and unbiased use of NGS for diagnostic purposes. Indeed, *RYR1* mutations were previously associated with autosomal dominant central core disease (CCD) until exome sequencing detected pathogenic *RYR1* variants in recessive muscle disorders with unreported clinical phenotype and atypical morphological myofiber anomalies such as centronuclear myopathy (CNM) or dusty core disease (DuCD) [12, 32].

In contrast to congenital myopathies, metabolic myopathy biopsies often display rather non-specific features including abnormal mitochondrial activity or lipid or glycogen accumulations (Fig. 4). The detection of these anomalies is useful to confirm the diagnosis of metabolic myopathies, but is often insufficient to indicate a distinct causative gene. In general, additional enzymatic tests on blood and muscle samples are necessary to establish a more precise diagnosis.

Relevance of electron microscopy

In total, 120 muscle biopsies were analyzed by electron microscopy, including 74 biopsies from patients with congenital myopathies. Other muscle morphology groups were less represented, either because histological examinations in combination with clinical and enzymatic data were sufficient to confirm metabolic myopathies or muscular dystrophies, or because the absence of histopathological signs did not justify ultrastructural investigations.

From the 74 congenital myopathy biopsies, EM studies were considered decisive or contributory in the vast majority. This is primarily due to the severity of most congenital myopathy subgroups and the necessity to perform muscle biopsies in the early neonatal period. However, the restricted myofiber diameter in newborns can complicate the detection and classification of structural anomalies by histoenzymological studies and commonly require complementary EM analyses. Small nemaline rods, caps, inclusions, or cytoplasmic bodies are often solely detectable by electron microscopy, just as the misalignment of Z-lines or the disorganization of the intermyofibrillar network. As for histoenzymology, electron microscopy can also point to specific disease genes. By way of example, nemaline myopathies are genetically and clinically heterogeneous muscle disorders characterized by early-onset hypotonia, respiratory distress, delayed motor milestones, and skeletal deformities with 15 causative genes identified to date (*ACTA1*, *ADSSL1*, *CFL2*, *KBTBD13*, *KLHL40*, *KLHL41*, *LMOD3*, *MYO18B*, *MYPN*, *NEB*, *RYR3*, *TNNT1*, *TNNT3*, *TPM2*, *TPM3*) [16, 21, 24, 28]. Muscle biopsies from affected individuals typically show abnormal accumulations of sarcomeric structures known as nemaline rods, which are visible by Gomori trichrome stain on light microscopy. However, intranuclear rods are exclusively observed in *ACTA1*-related NM and are typically detected by electron microscopy (Fig. 3G) [16, 25]. In the same line, patients with severe *ACTA1*-related NM occasionally display anomalies of the nuclei as thickening of the nuclear lamina or the enlargement of the perinuclear space, and both are solely discernible by electron microscopy (Fig. 3H) [16]. As another example of a decisive contribution of EM pointing to specific muscle disorders, patients with *STAC3* mutations associated with neonatal hypotonia, muscle weakness, short stature, susceptibility to malignant hyperthermia (MH), and high childhood mortality rates [15] typically display type I fiber atrophy on muscle biopsies under light microscopy, while electron microscopy can additionally disclose dilated cisternae indicating swollen T-tubules or sarcoplasmic reticulum (Fig. 5). And in a neonate with severe muscle weakness and swallowing difficulties, histochemical analyses of the muscle biopsy failed to identify the disease group, while electron microscopy revealed an abnormal neuromuscular junction with ridge depletion (Fig. 6). Ultrastructural investigations robustly disclose anomalies of the neuromuscular junction and play an important role in the diagnosis of myopathies with neuromuscular transmission defects—especially if ENMG was inconclusive or not conducted. This shows that the higher resolution of EM is not only a feature complementing light microscopy, but also a separate and powerful diagnostic method.

Concluding remarks

In the era of routine panel, exome, and genome sequencing in accredited diagnostic laboratories, the morphological analysis of muscle biopsies may not always constitute a critical step in the diagnosis process of NMD anymore—especially if NGS detects a pathogenic variant in

a known gene matching the clinical presentation of the patient [10].

However, muscle disorders are clinically and genetically heterogeneous, and NGS uncovers a great number of variants of uncertain significance (VUS). In specialized histopathological laboratories, results and conclusions of muscle biopsies are generally obtained within one week and can confirm a diagnosis by complementing clinical and biochemical lines of evidence, or can decisively orient molecular diagnosis and purposeful gene sequencing in patients with non-specific clinical features and normal metabolic parameters. Morphological analyses of muscle sections can also help to distinguish between different muscle disorders induced by mutations in same gene and giving rise to clinically and histologically heterogeneous disorders such as *RYR1* or *TTN*-related myopathies, and enable the establishment of genotype/phenotype correlations. Finally, further investigations on the causes and consequences of specific structural anomalies might identify novel therapeutic targets and pave the way for the development of efficient treatments.

From our experience, histological and ultrastructural examinations of muscle sections were frequently accurate and either pointed to group of myopathies or to single genes and mutations causing characteristic lesions in myofibers. Even in the absence of a final diagnosis, biopsies can indicate the disease subgroup and direct molecular analyses to rapidly take measures for an appropriate care of the patient. They can also be used for functional studies to confirm or discard the pathogenicity of potential mutations and VUS identified by NGS, and may serve as a resource of DNA allowing genetic or expression studies on DNA, RNA, or mitochondrial DNA when no other biological material is available.

Taken together, the increasing use of NGS as a routine tool significantly improved molecular diagnosis and changed the overall diagnostic process. While the morphological muscle biopsy analysis and genetic tests were previously considered as distinct, consecutive, and interdependent steps, they are nowadays rather performed in parallel and provide complementary information.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40478-024-01882-0.

Additional file 1.

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Author contributions
NBR designed and coordinated the project and obtained funding; MTB, GFE, TE, EL, GB, CL, AM, AC, MB, FBL, VB, GB, PDL, JL, JB, NBR performed the experiments and analyzed the data; MTB, GFE, TE, PDL provided clinical data and biological samples; NBR, JB drafted the manuscript with inputs from the other authors.

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Data availability
All data generated or analyzed during this study and concerning clinical and histological characteristics are included in this article or were presented in previous publications listed in the references. Other DNA variants identified by panel or exome sequencing are not publicly accessible.

Declarations

Ethics approval and consent to participate
All patients and legal guardians consented to the publication of the clinical, histological, and genetic data. Molecular diagnosis was carried out with written informed consent from the patients or legal guardians. Muscle biopsy storage and usage were IRB-approved.

Competing interests
The authors declare no competing interests.

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References

1. Agrawal PB, Pierson CR, Joshi M, Liu X, Ravenscroft G, Moghadaszadeh B, Talabere T, Viola M, Swanson LC, Haliloglu G et al (2014) SPEG interacts with myotubularin, and its deficiency causes centronuclear myopathy with dilated cardiomyopathy. Am J Hum Genet 95:218–226. https://doi.org/10.1016/j.ajhg.2014.07.004
2. Bevilacqua JA, Bitoun M, Biancalana V, Oldfors A, Stoltenburg G, Claeys KG, Lacene E, Brochier G, Manere L, Laforet P et al (2009) "Necklace" fibers, a new histological marker of late-onset MTM1-related centronuclear

myopathy. Acta Neuropathol 117:283–291. https://doi.org/10.1007/s00401-008-0472-1
3. Bevilacqua JA, Monnier N, Bitoun M, Eymard B, Ferreiro A, Monges S, Lubieniecki F, Taratuto AL, Laquerriere A, Claeys KG et al (2011) Recessive RYR1 mutations cause unusual congenital myopathy with prominent nuclear internalization and large areas of myofibrillar disorganization. Neuropathol Appl Neurobiol 37:271–284. https://doi.org/10.1111/j.1365-2990.2010.01149.x
4. Biancalana V, Rendu J, Chaussenot A, Mecili H, Bieth E, Fradin M, Mercier S, Michaud M, Nougues MC, Pasquier L et al (2021) A recurrent RYR1 mutation associated with early-onset hypotonia and benign disease course. Acta Neuropathol Commun 9:155. https://doi.org/10.1186/s40478-021-01254-y
5. Bitoun M, Maugenre S, Jeannet PY, Lacene E, Ferrer X, Laforet P, Martin JJ, Laporte J, Lochmuller H, Beggs AH et al (2005) Mutations in dynamin 2 cause dominant centronuclear myopathy. Nat Genet 37:1207–1209. https://doi.org/10.1038/ng1657
6. Bohm J, Biancalana V, Malfatti E, Dondaine N, Koch C, Vasli N, Kress W, Strittmatter M, Taratuto AL, Gonorazky H et al (2014) Adult-onset autosomal dominant centronuclear myopathy due to BIN1 mutations. Brain 137:3160–3170. https://doi.org/10.1093/brain/awu272
7. Bohm J, Malfatti E, Oates E, Jones K, Brochier G, Boland A, Deleuze JF, Romero NB, Laporte J (2019) Novel ASCC1 mutations causing prenatal-onset muscle weakness with arthrogryposis and congenital bone fractures. J Med Genet 56:617–621. https://doi.org/10.1136/jmedgenet-2018-105390
8. Bohm J, Vasli N, Malfatti E, Le Gras S, Feger C, Jost B, Monnier N, Brocard J, Karasoy H, Gerard M et al (2013) An integrated diagnosis strategy for congenital myopathies. PLoS ONE 8:e67527. https://doi.org/10.1371/journal.pone.0067527PONE-D-12-35890[pii]
9. Ceyhan-Birsoy O, Agrawal PB, Hidalgo C, Schmitz-Abe K, DeChene ET, Swanson LC, Soemedi R, Vasli N, Iannaccone ST, Shieh PB et al (2013) Recessive truncating titin gene, TTN, mutations presenting as centronuclear myopathy. Neurology 81:1205–1214. https://doi.org/10.1212/WNL.0b013e3182a6ca62
10. de Feraudy Y, Vandroux M, Romero NB, Schneider R, Saker S, Boland A, Deleuze JF, Biancalana V, Bohm J, Laporte J (2024) Exome sequencing in undiagnosed congenital myopathy reveals new genes and refines genes-phenotypes correlations. Genome Med 16:87. https://doi.org/10.1186/s13073-024-01353-0
11. Fardeau M, Tome FM, Helbling-Leclerc A, Evangelista T, Ottolini A, Chevallay M, Barois A, Estournet B, Harpey JP, Faure S et al (1996) Congenital muscular dystrophy with merosin deficiency: clinical, histopathological, immunocytochemical and genetic analysis. Rev Neurol (Paris) 152:11–19
12. Garibaldi M, Rendu J, Brocard J, Lacene E, Faure J, Brochier G, Beuvin M, Labasse C, Madelaine A, Malfatti E et al (2019) "Dusty core disease" (DuCD): expanding morphological spectrum of RYR1 recessive myopathies. Acta Neuropathol Commun 7:3. https://doi.org/10.1186/s40478-018-0655-5
13. Gorman GS, Chinnery PF, DiMauro S, Hirano M, Koga Y, McFarland R, Suomalainen A, Thorburn DR, Zeviani M, Turnbull DM (2016) Mitochondrial diseases. Nat Rev Dis Primers 2:16080. https://doi.org/10.1038/nrdp.2016.80
14. Hernandez-Lain A, Husson I, Monnier N, Farnoux C, Brochier G, Lacene E, Beuvin M, Viou M, Manere L, Claeys KG et al (2011) De novo RYR1 heterozygous mutation (I4898T) causing lethal core-rod myopathy in twins. Eur J Med Genet 54:29–33. https://doi.org/10.1016/j.ejmg.2010.09.009
15. Horstick EJ, Linsley JW, Dowling JJ, Hauser MA, McDonald KK, Ashley-Koch A, Saint-Amant L, Satish A, Cui WW, Zhou W et al (2013) Stac3 is a component of the excitation-contraction coupling machinery and mutated in Native American myopathy. Nat Commun 4:1952. https://doi.org/10.1038/ncomms2952
16. Labasse C, Brochier G, Taratuto AL, Cadot B, Rendu J, Monges S, Biancalana V, Quijano-Roy S, Bui MT, Chanut A et al (2022) Severe ACTA1-related nemaline myopathy: intranuclear rods, cytoplasmic bodies, and enlarged perinuclear space as characteristic pathological features on muscle biopsies. Acta Neuropathol Commun 10:101. https://doi.org/10.1186/s40478-022-01400-0
17. Laporte J, Hu LJ, Kretz C, Mandel JL, Kioschis P, Coy JF, Klauck SM, Poustka A, Dahl N (1996) A gene mutated in X-linked myotubular myopathy defines a new putative tyrosine phosphatase family conserved in yeast. Nat Genet 13:175–182. https://doi.org/10.1038/ng0696-175
18. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M et al (1995) Identification and characterization of a spinal muscular atrophy-determining gene. Cell 80:155–165. https://doi.org/10.1016/0092-8674(95)90460-3
19. Lornage X, Quijano-Roy S, Amthor H, Carlier RY, Monnier N, Deleuze JF, Romero NB, Laporte J, Bohm J (2020) Asymmetric muscle weakness due to ACTA1 mosaic mutations. Neurology 95:e3406–e3411. https://doi.org/10.1212/WNL.0000000000010947
20. Malfatti E, Bohm J, Lacene E, Beuvin M, Romero NB, Laporte J (2015) A Premature stop codon in MYO18B is associated with severe nemaline myopathy with cardiomyopathy. J Neuromuscul Dis 2:219–227. https://doi.org/10.3233/JND-150085
21. Malfatti E, Romero NB (2016) Nemaline myopathies: state of the art. Rev Neurol (Paris) 172:614–619. https://doi.org/10.1016/j.neurol.2016.08.004
22. Monaco AP, Bertelson CJ, Middlesworth W, Colletti CA, Aldridge J, Fischbeck KH, Bartlett R, Pericak-Vance MA, Roses AD, Kunkel LM (1985) Detection of deletions spanning the Duchenne muscular dystrophy locus using a tightly linked DNA segment. Nature 316:842–845. https://doi.org/10.1038/316842a0
23. Nicot AS, Toussaint A, Tosch V, Kretz C, Wallgren-Pettersson C, Iwarsson E, Kingston H, Garnier JM, Biancalana V, Oldfors A et al (2007) Mutations in amphiphysin 2 (BIN1) disrupt interaction with dynamin 2 and cause autosomal recessive centronuclear myopathy. Nat Genet 39:1134–1139. https://doi.org/10.1038/ng2086
24. Nowak KJ, Ravenscroft G, Laing NG (2013) Skeletal muscle alpha-actin diseases (actinopathies): pathology and mechanisms. Acta Neuropathol 125:19–32. https://doi.org/10.1007/s00401-012-1019-z
25. Nowak KJ, Wattanasirichaigoon D, Goebel HH, Wilce M, Pelin K, Donner K, Jacob RL, Hubner C, Oexle K, Anderson JR et al (1999) Mutations in the skeletal muscle alpha-actin gene in patients with actin myopathy and nemaline myopathy. Nat Genet 23:208–212. https://doi.org/10.1038/13837
26. Romero NB, Clarke NF (2013) Congenital myopathies. Handb Clin Neurol 113:1321–1336. https://doi.org/10.1016/B978-0-444-59565-2.00004-6B978-0-444-59565-2.00004-6[pii]
27. Schartner V, Laporte J, Bohm J (2019) Abnormal excitation-contraction coupling and calcium homeostasis in myopathies and cardiomyopathies. J Neuromuscul Dis 6:289–305. https://doi.org/10.3233/JND-180314
28. Sewry CA, Laitila JM, Wallgren-Pettersson C (2019) Nemaline myopathies: a current view. J Muscle Res Cell Motil 40:111–126. https://doi.org/10.1007/s10974-019-09519-9
29. Shichiji M, Biancalana V, Fardeau M, Hogrel JY, Osawa M, Laporte J, Romero NB (2013) Extensive morphological and immunohistochemical characterization in myotubular myopathy. Brain Behav 3:476–486. https://doi.org/10.1002/brb3.147
30. Tulinius M, Oldfors A (2011) Neonatal muscular manifestations in mitochondrial disorders. Semin Fetal Neonatal Med 16:229–235. https://doi.org/10.1016/j.siny.2011.04.001
31. Udd B, Stenzel W, Oldfors A, Olive M, Romero N, Lammens M, Kusters B, Sewry C, Goebel HH, Evangelista T (2019) 1st ENMC European meeting: The EURO-NMD pathology working group recommended standards for muscle pathology Amsterdam, The Netherlands, 7 December 2018. Neuromuscul Disord 29:483–485. https://doi.org/10.1016/j.nmd.2019.03.002
32. Wilmshurst JM, Lillis S, Zhou H, Pillay K, Henderson H, Kress W, Muller CR, Ndondo A, Cloke V, Cullup T et al (2010) RYR1 mutations are a common cause of congenital myopathies with central nuclei. Ann Neurol 68:717–726. https://doi.org/10.1002/ana.22119

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