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SANNA PUUSEPP

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Comparison of molecular genetics and morphological findings of childhood-onset neuromuscular disorders

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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II. **Puusepp S***, Kovacs-Nagy R*, Alhaddad B, Braunisch M, Hoffmann GF, Kotzaeridou U, Lichvarova L, Liiv M, Makowski C, Mandel M, Meitinger T, Pajusalu S, Rodenburg RJ, Safiulina D, Strom TM, Talvik I, Vaarmann A, Wilson C, Kaasik A, Haack TB, Õunap K. Compound heterozygous *SPATA5* variants in four families and functional studies of SPATA5 deficiency. *Eur J Hum Genet*. 2018;26(3):407–419.

III. **Puusepp S**, Reinson K, Pajusalu S, van Kuilenburg ABP, Dobritzsch D, Roelofsen J, Stenzel W, Õunap K. Atypical presentation of Arts syndrome due to a novel hemizygous loss-of-function variant in the *PRPS1* gene. *Mol Genet Metab Rep*. 2020;25:100677.

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My contributions to the preparation of the original publications:

Publication I: Analyzing clinical data; analyzing and interpreting muscle biopsy samples; preparing the tables; writing the manuscript. *These authors contributed equally to this work.

Publication II: Analyzing clinical data; preparing the tables and figure 4; writing the manuscript. *These authors contributed equally to this work.

Publication III: Collecting and analyzing clinical data; analyzing and interpreting muscle histology; preparing figures 1 and 3; writing the manuscript.

Publication IV: Collecting and analyzing clinical data and interpreting muscle histology of the Estonian patient.

ABBREVIATIONS

AAA	ATPase Associated with diverse cellular Activities
AD	Autosomal dominant
AR	Autosomal recessive
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AVM	Autophagic vacuolar myopathy
AVSF	Autophagic vacuoles with sarcolemmal features
BAG3	BCL2-associated athanogene 3
BM	Basement membrane
BMD	Becker muscular dystrophy
BMI	Body mass index
bp	Basepair
BWA	Burrows-Wheeler Aligner
cDNA	Complementary DNA
CASA	Chaperone-assisted selective autophagy
COX	Cytochrome C oxidase
CK	Creatine kinase
CMA	Chromosomal microarray
CMD	Congenital muscular dystrophy
CMS	Congenital myasthenic syndrome
CMT	Charcot-Marie-Tooth disease
CMTX5	X-linked Charcot-Marie-Tooth disease type 5
CNS	Central nervous system
CNV	Copy number variation
CPT	Carnitine palmitoyltransferase
Crea	Creatinine
CSF	Cerebrospinal fluid
CVI	Cortical visual impairment
DD	Developmental delay
DEE	Developmental and epileptic encephalopathy
DFN2	Nonsyndromic X-linked deafness
DG	Dystroglycan
DHPR	Voltage-sensing dihydropyridine-sensitive calcium channel
Dll	Delta-like
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
DTR	Deep tendon reflex
EC	Excitation-contraction
ECG	Electrocardiography
ECM	Extracellular matrix
EDMD	Emery-Dreifuss muscular dystrophy
EEG	Electroencephalography

EM	Electron microscopy
ENMG	Electroneuromyography
FSHD	Facioscapulohumeral muscular dystrophy
FTD	Fiber-type disproportion
FTT	Failure to thrive
FVC	Forced vital capacity
GATK	Genome Analysis Toolkit
GERD	Gastroesophageal reflux disease
GSD	Glycogen storage disease
GW	Gestation week
HE	Hematoxylin and eosin
het	Heterozygous
hNMD	Hereditary NMD
hom	Homozygous
HSM	Hepatosplenomegaly
HSP70	Heat-shock protein 70
ID	Intellectual disability
IHC	Immunohistochemistry
indel	Insertion/deletion
IUGR	Intrauterine growth retardation
kb	Kilobase
LAMP2	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein 1 light chain 3
LGMD	Limb-girdle muscular dystrophy
LHON	Leber hereditary optic neuropathy
Mb	Megabase
MD	Mitochondrial disorder
MDC	MD diagnostic criteria
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonic epilepsy associated with ragged-red fibers
mGT	Modified Gömöri trichrome
MHC I	Major histocompatibility complex class I
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnet resonance imaging
mtDNA	Mitochondrial DNA
MyHCd	Myosin heavy chain developmental/embryonic
MyHCf	Myosin heavy chain fast
MyHCn	Myosin heavy chain neonatal/fetal
MyHCs	Myosin heavy chain slow/beta cardiac
NA	Not applicable
NADH-TR	Reduced nicotinamide adenine dinucleotide-tetrazolium reductase
NCAM	Neural cell adhesion molecule
nDNA	Nuclear DNA

NGS	Next generation sequencing
NMD	Neuromuscular disorder
nNOS	Neuronal nitric oxide synthase
OFC	Occipitofrontal circumference
ORO	Oil Red O
OXPHOS	Oxidative phosphorylation
PAS	Periodic Acid-Schiff
PNS	Peripheral nervous system
PP	Purine and pyrimidine
PRPP	Phosphoribosylpyrophosphate
PRPS	Phosphoribosylpyrophosphate synthetase
RCC	Respiratory chain complex
RIM-BP1	RIM-binding protein 1
RNA	Ribonucleic acid
RRF	Ragged-red fiber
rRNA	Ribosomal RNA
S	Serum
SARC	Sarcoglycan
SD	Standard deviation
SDH	Succinate dehydrogenase
SERCA	Sarco(endo)plasmic reticulum calcium ATPase
shRNA	Short hairpin RNA
SMA	Spinal muscular atrophy
SMA-LED	SMA, lower extremity predominant
SNHL	Sensorineural hearing loss
SNV	Single nucleotide variant
SPAF	Spermatogenesis associated factor
SPATA5	Spermatogenesis-associated protein 5
SPECT	Single-photon emission computerized tomography
SR	Sarcoplasmic reticulum
TIEF	Transferrin isoelectric focusing
tRNA	Transfer RNA
U	Urine
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing
WISC	Wechsler Intelligence Scale for Children
WPPSI	Wechsler Preschool and Primary Scale of Intelligence
WWS	Walker-Warburg syndrome
XMEA	X-linked myopathy with excessive autophagy
ZASP	Z-band alternatively spliced PDZ motif-containing protein

1. INTRODUCTION

In 1836, Italian physicians G. Conte and L. Gioja described two brothers with muscle hypertrophy and progressive weakness and were the first to speculate that the cause of this disease was a change in the „nutritional process“ of the muscle itself [Tyler 2003]. The English physician Edward Meryon (1809–1880) further elaborated this notion in pathological descriptions of a series of patients with muscle hypertrophy and paralysis in 1851. Meryon microscopically investigated the autopsy material and stated that „the nervous tissues had preserved their integrity; and the only structural change observed was that which presented itself in the muscular fibers, which were broken down, and converted into granular and fatty matter [*sic*]“ [Emery and Emery 2011; Meryon 1852; Tyler 2003].

The French neurologist Guillaume Benjamin Amand Duchenne (1806–1875) made significant contributions to the neuromuscular disorders (NMDs) field. In 1842, he began to study clinical electrophysiology using a portable device of his invention for the electrical stimulation of single muscles and nerves. He also developed an instrument known as Duchenne's trocar, which enabled percutaneous sampling of muscle tissue from living patients, and he was one of the first physicians to collect photographs of patients and histological slides. Duchenne gathered an extensive collection of patients with pseudohypertrophic muscular paralysis (now known as Duchenne muscular dystrophy or DMD) with detailed clinical, electrophysiological, and neuropathological descriptions at different stages of the disease [Parent 2005; Tyler 2003; Waclawik and Lanska 2019].

When the English neurologists John Walton and Frederick Nattrass published the first classification of muscular dystrophies in 1954, it consisted of seven subtypes along with DMD [Walton and Nattrass 1954]. After that, many descriptions of new muscle disease subgroups emerged based on characteristic histopathological findings in the muscle (e.g., glycogen accumulation, rods, and cores) [Kakulas 2008]. The first description of clinical, biochemical, and morphological features of a patient with "a defect in the mitochondrial enzyme organization" was that of Luft *et al.* [Luft *et al.* 1962]. This case report included the first photographs of pathological mitochondria with lipid and paracrystalline inclusions and concentric cristae. Furthermore, the discovery of the dystrophin protein [Kunkel *et al.* 1985] and many other muscle proteins paved the way for muscle immunohistochemistry (IHC).

The rapid evolution of genetics began with Mendel's hybridization laws, the chromosomal theory, the notion of deoxyribonucleic acid (DNA) as the substance of genes, and the invention of DNA sequencing methods (e.g., Sanger sequencing) and developed into the modern molecular diagnostics with next generation sequencing (NGS) and multi-omics [Gayon 2016; Giani *et al.* 2020; Hasin *et al.* 2017]. The triumph of genetics shifted the classification system of NMDs towards a more gene-based one, with over 600 genes associated with different neuromuscular phenotypes to date (http://www.musclegenetable.fr/).

In Estonia, Prof. Andres Piirsoo initiated the light and electron microscopic investigations of muscle biopsy samples in 1993 in collaboration with the late Prof. Tiina Talvik, a pediatrician known for leading an epidemiological study of DMD in childhood in Estonia. The study compiled the main characteristics of Estonian DMD patients [Talkop *et al.* 1999]. The point prevalence of definite DMD in the male population of Estonia on January 1, 1998, was 1:7,837 and the incidence of DMD during 1986–1990 was 1:5,652 [Talkop *et al.* 2003]. Prof. Katrin Õunap led extensive studies of patients suspected of mitochondrial disorders (MDs). The first study concluded that serum lactic acid measurement is a suitable screening method in children for early-onset MDs. The live-birth prevalence of MDs in Estonia during 2003–2009 was 1:20,764 [Joost *et al.* 2012]. The second project assessed the effectiveness of whole exome sequencing (WES) in patients with a clinical suspicion of an MD, which was 57%. Of note, 14% of the patients had a confirmed diagnosis of MD [Puusepp *et al.* 2018b].

The current dissertation analyzes and discusses the myopathological features of patients from the last mentioned project and an additional muscle biopsy cohort. Moreover, this study compares the pathohistological findings with the genetic background of these patients, presents new disease-associated genes and variants, and analyzes the value of muscle biopsy in the study groups. In addition, the present work is illustrated by three case reports with insights into the pathomechanism of the diseases.

2. REVIEW OF THE LITERATURE

2.1 Classification and etiology of neuromuscular disorders

NMDs comprise highly heterogeneous diseases of the peripheral nervous system (PNS). The anatomical location of a disorder is the primary basis for classifying NMDs: lower motor neuron, peripheral nerve, neuromuscular junction, and muscle diseases. Further subdivisions are based on disease onset, the presenting symptoms, the distribution of muscle weakness, the histopathological features, the inheritance pattern, the defective structural components or physiological pathways, and the underlying gene defect [Goebel *et al.* 2013]. Figure 1 illustrates the gross anatomy of the PNS, the microscopic anatomy of muscle tissue, and the molecular components of the muscle fiber.

In adults, the majority of NMDs are acquired. However, in children, most NMDs arise due to genetic alterations. The main disease entities and etiology of hereditary NMDs (hNMDs), grouped by anatomical location, are outlined below.

The most common hNMD affecting the lower motor neurons is spinal muscular atrophy (SMA), caused by biallelic variants in the *SMN1* gene. Other forms of lower motor neuron diseases are sporadic with over 60 gene associations, and many of them present with additional extramuscular symptoms. The proteins associated with lower motor neuron disorders function in autophagy, protein quality control, ribonucleic acid (RNA) processing, cytoskeletal dynamics, cation channeling, or vitamin uptake into the motor neurons [Cohen *et al.* 2021; Teoh *et al.* 2017].

The numerous subtypes of Charcot-Marie-Tooth disease (CMT) make up most of the genetic neuropathies. Although variants in more than 90 genes can cause CMT, the *PMP22* gene duplication comprises 60% of all genetically confirmed cases of CMT. The following most frequent causes of CMT are variants in the *MPZ*, *GJB1*, or *MFN2* genes accounting for 30%. The pathomechanisms of CMT involve defects in protein synthesis, posttranslational processing, intracellular trafficking, and the function of ion channels and mitochondria [Morena *et al.* 2019].

Hereditary diseases of the neuromuscular junctions are termed congenital myasthenic syndromes (CMSs). The 32 disease-associated genes encode presynaptic, synaptic, or postsynaptic proteins that either form the subunits of the acetylcholine receptor or function in axonal transport, exocytosis of synaptic vesicles, glycosylation of synaptic molecules, or synthesis or recycling of acetylcholine. Of note, defects in the acetylcholine receptor account for about 75% of CMS cases [Finsterer 2019].

Muscle diseases constitute the most complex subgroup of hNMDs, of which dystrophinopathy, resulting in DMD, Becker muscular dystrophy (BMD), or symptomatic female carriers, is the most frequent entity. Table 1 lists a clinicopathological classification of hereditary muscle diseases and their genetic etiology [Cohen *et al.* 2021]. The pathogenesis of muscle diseases involves defects

in extracellular matrix, sarcolemma, and nuclear membrane-associated proteins, ion channels, cytosolic enzymes, sarcomeric assembly and function, excitation-contraction coupling, intracellular calcium homeostasis, autophagy, respiratory chain function, and glycogen and lipid metabolism, and altered transcription and splicing [Claeys 2020; Desaphy *et al.* 2020; Himeda and Jones 2019; LoRusso *et al.* 2018; Margeta 2020; Mercuri *et al.* 2019; Toscano *et al.* 2017].

The use of NGS technologies has considerably blurred the boundaries between different muscle disease entities making the classification complicated. Variants in one gene can cause diverse phenotypes/myopathology, and conversely, one clinicopathological entity can result from variants in different genes. For example, several genes implicated in congenital muscular dystrophies (CMDs) can also cause a later-onset limb-girdle type muscle weakness. In addition, the 229th European Neuromuscular Centre international workshop recently revised the definition and nomenclature of limb-girdle muscular dystrophies (LGMDs) [Straub *et al.* 2018]. As a result, some entities were removed, and some were added to LGMDs. While the old nomenclature classified autosomal dominant forms as LGMD1 and autosomal recessive forms as LGMD2, followed by an alphabetical suffix assigned for each locus, the new classification follows the formula "LGMD, inheritance (D or R), order of discovery (number), affected protein" [Straub *et al.* 2018].

Moreover, the genetic etiology of the subtypes of congenital myopathies is particularly complex. For example, nemaline myopathies are associated with 15 genes (Table 1). In addition, large copy number variations (CNVs) and rearrangements, which are not easily detectable by sequencing, might be responsible for a significant number of congenital myopathy cases [Pelin and Wallgren-Pettersson 2019]. On the other hand, many congenital myopathy-related genes are implicated in more than one subtype. In particular, defects in the *RYR1* gene can cause five distinct congenital myopathy entities (central core disease, core-rod myopathy, centronuclear myopathy, congenital fiber-type disproportion, and multi-minicore disease) without clear genotype-phenotype correlations [Lawal *et al.* 2020]. Hence, muscle biopsy is necessary to diagnose a congenital myopathy subtype correctly.

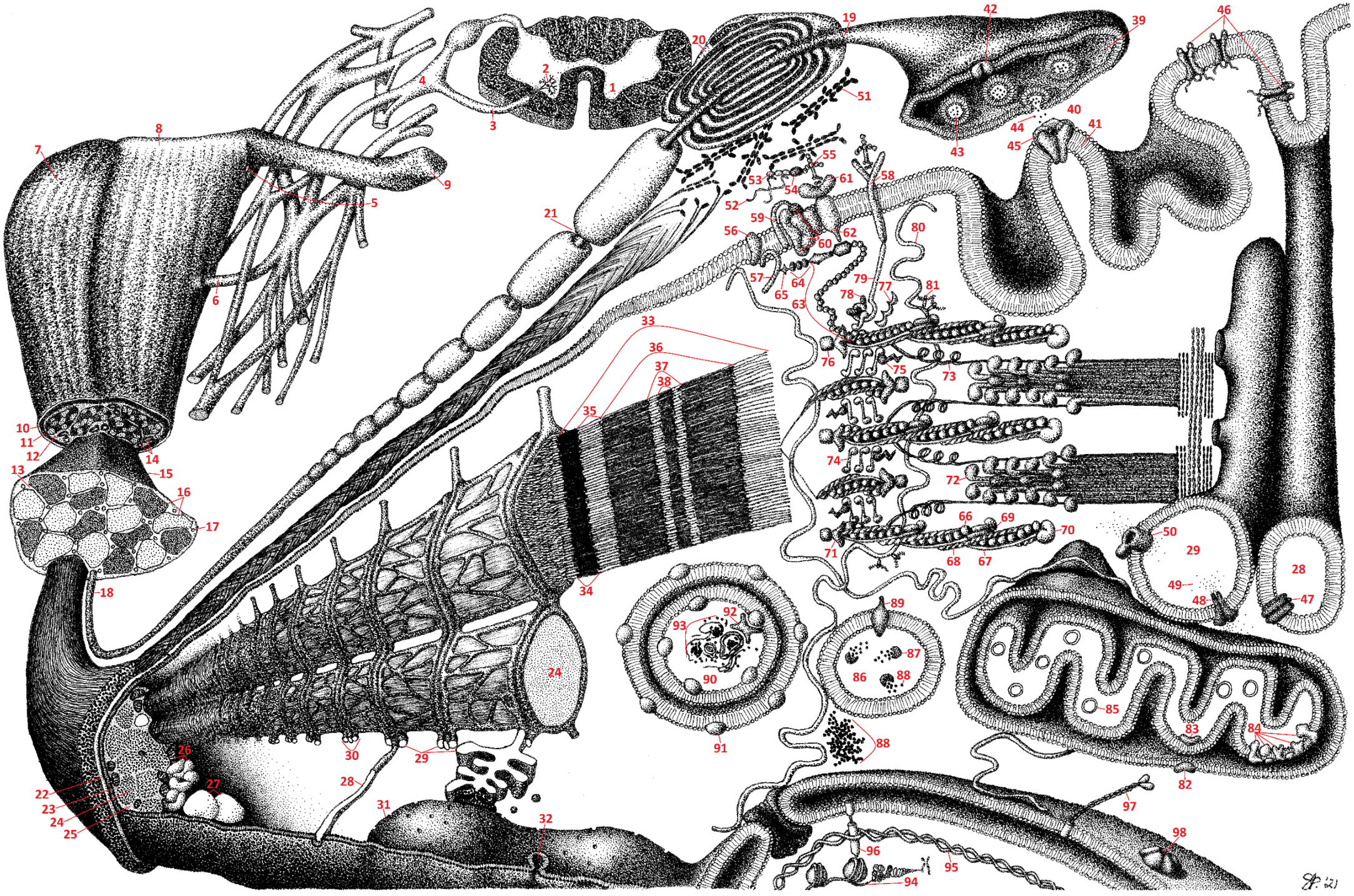


Figure 1. An integrative illustration of the anatomy of the peripheral nervous system, the structure of the muscle fiber, and the location of the main structural and membrane proteins in the muscle. The illustration is made by the author of the dissertation, S. Puusepp, based on information obtained from Goebel *et al.* [Goebel *et al.* 2013].

Figure 1. An integrative illustration of the anatomy of the peripheral nervous system, the structure of the muscle fiber, and the location of the main structural and membrane proteins in the muscle. The illustration is made by the author of the dissertation, S. Puusepp, based on information obtained from Goebel *et al.* [Goebel *et al.* 2013].

The upper-left part of the illustration represents the anatomical structures that constitute the PNS. It depicts the innervation of the deltoid muscle: (1) anterior horn of the C7 segment of the spinal cord, (2) lower motor neuron, (3) ventral root, (4) spinal nerve C7, (5) brachial plexus, (6) *nervus axillaris,* (7) *musculus deltoideus,* (8) tendon, and (9) clavicle. Microscopically, the muscle consists of (10) fascia, (11) epimysium, (12) perimysium, (13) endomysium, (14) arteries and veins, (15) muscle fascicles, (16) type 1 and type 2 muscle fibers, (17) capillaries, and (18) intramuscular nerve branches. The upper-middle part of the figure depicts an (19) axon with (20) Schwann cells forming the myelin sheath and (21) nodes of Ranvier′. The lower-left part illustrates the ultrastructure of the muscle fiber with (22) basement membrane, (23) sarcolemma, (24) myofibrils, (25) sarcoplasm, (26) mitochondria, (27) lipid droplets, (28) T-tubules, (29) sarcoplasmic reticulum, (30) triad, (31) nucleus, and (32) caveola. In the center of the illustration is a (33) sarcomere with (34) Z line, (35) I bands, (36) A band, (37) H zone, and (38) M line.

The right-hand side of the illustration depicts the molecular level of the muscle fiber. In the upper-right part is a neuromuscular junction composed of (39) presynaptic terminal, (40) synaptic cleft, and (41) postsynaptic membrane. An action potential opens (42) voltage-gated calcium channels, and the (43) synaptic vesicles release (44) acetylcholine, which binds to (45) acetylcholine receptors (*CHRNA1*, *CHRNB1*, *CHRND*, *CHRNE*, and *CHRNG* genes). The (46) voltage-gated sodium (*SCN4A* gene), chloride (*CLCN1* gene), and potassium (*KCNE3* and *KCNJ18* genes) ion channels, (47) voltage-sensing dihydropyridine-sensitive calcium channel or DHPR (*CACNA1S* gene), and the (48) ryanodine receptor (*RYR1* and *RYR3* genes) are involved in excitation-contraction coupling resulting in (49) calcium release into the sarcoplasm. The (50) sarco(endo)plasmic reticulum calcium ATPase or SERCA protein (*ATP2A1* gene) transports calcium back into the sarcoplasmic reticulum. The endomysium contains (51) collagen VI fibrils (*COL6A1*, *COL6A2*, and *COL6A3* genes). Basement membrane proteins include (52) collagen IV, (53) perlecan (*HSPG2* gene), (54) nidogen, and (55) laminin 211 or merosin (*LAMA2* gene encoding the α2 subunit). The sarcolemmal proteins depicted are (56) dysferlin (*DYSF* gene), (57) caveolin (*CAV3* gene), (58) integrin α7 (*ITGA7* gene), and the dystrophin-glycoprotein complex composed of (59) sarcospan, (60) α-, β-, γ-, and δ-sarcoglycans (*SGCA*, *SGCB*, *SGCG*, and *SGCD* genes), (61) α- and (62) β-dystroglycans (*DAG1* gene), (63) dystrophin (*DMD* gene), (64) syntrophins, and (65) neuronal nitric oxide synthase (nNOS). The sarcomeres contain thin filaments composed of (66) actin (*ACTA1* gene), (67) nebulin (*NEB* gene), (68) tropomyosins (*TPM2* and *TPM3* genes), (69) troponin T, I, and C (*TNNT1* gene) (70) tropomodulin, and (71) CapZ and thick filaments composed of (72) myosins (*MYH2* and *MYH7* genes). The (73) titin protein (*TTN* gene) extends from the Z line to the M line. The Z line includes (74) α-actinin (*ACTN2* gene), (75) Z-band alternatively spliced PDZ motif-containing protein or ZASP (*LDB3* gene), (76) telethonin (*TCAP* gene), (77) BCL2-associated athanogene 3 or BAG3 (*BAG3* gene), (78) myotilin (*MYOT* gene), and (79) filamin C (*FLNC* gene) proteins. The (80) desmin proteins (*DES* gene) interlink the Z lines and connect the myofibrils to the sarcolemma, mitochondria, and nuclei. The (81) αB-crystallin (*CRYAB* gene) colocalizes with desmin at the Z lines. The mitochondrial proteins depicted are (82) carnitine palmitoyltransferase (CPT) I on the outer and (83) CPT II (*CPT2* gene) on the inner membrane, and the (84) respiratory chain with complex I (*MT-ND1–6* genes), complex II, coenzyme Q10 (*COQ2*, *COQ4*, *COQ6*, *COQ7*, and *COQ9* genes), complex III (*MT-CYB* gene), cytochrome C, complex IV (*MT-CO1–3*, and *COX6A2* genes), and adenosine triphosphate (ATP) synthase or complex V on the inner membrane. The mitochondrial matrix contains several copies of (85) mitochondrial DNA (mtDNA). The (86) lysosome contains various acid hydrolases, e.g. (87) acid alpha-glucosidase (*GAA* gene) that degrades (88) glycogen, and (89) lysosome-associated membrane protein 2 (*LAMP2* gene). The (90) autophagosome contains (91) microtubule-associated protein 1 light chain 3 (LC3) proteins, (92) autophagy receptor p62 (*SQSTM1* gene), and (93) cytoplasmic cargo. The nucleus contains (94) nuclear DNA, (95) lamin A/C (*LMNA* gene), (96) emerin (*EMD* gene), (97) nesprin 1 and 2 (*SYNE1* and *SYNE2* genes) proteins, and (98) nuclear pores.

Table 1. Clinicopathological classification of hereditary muscle diseases and the disease-associated genes, based on Cohen *et al.* [Cohen *et al.* 2021].

Muscle disease	Disease-associated genes
Muscular dystrophies	
DMD/BMD	Gene encoding dystrophin: *DMD*
EDMDs and similar syndromes (nuclear envelopathies)	Genes encoding nuclear envelope-associated proteins: *EMD*, *LMNA*, *SYNE1*, *SYNE2*, *TMEM43*, *FHL1, TOR1AIP1*
LGMDs	Genes encoding • collagen VI: *COL6A1*, *COL6A2*, *COL6A3* • BM/sarcolemmal proteins: *LAMA2*, *SGCA*, *SGCB*, *SGCG*, *SGCD, DYSF*, *DAG1* • proteins involved in the O-glycosylation of α-DG: *POMT1*, *POMT2*, *POMGNT1*, *POMGNT2*, *POGLUT1*, *FKTN*, *FKRP*, *ISPD*, *GMPPB*, *TRAPPC11* • sarcomeric proteins: *TCAP*, *TTN* • other proteins: *DNAJB6, CAPN3, ANO5, PLEC, HNRNPDL, TNPO3, TRIM32*, *BVES*, *POPDC3, JAG2*
FSHD type 1 and type 2	Inappropriate reactivation of a transcription factor *DUX4* caused by a D4Z4 unit repeat contraction on chromosome 4q35 or an *SMCHD1* or *LRIF1* gene defect with a permissive 4qA haplotype
Myotonic dystrophy type 1 and type 2	Repeat expansions in *DMPK* or *CNBP* genes cause the accumulation of ribonuclear inclusions that alter the splicing of different genes
Other muscular dystrophies	*CAVIN1*, *LIMS2*, *DPM3, POMK, PYROXD1, PABPN1, GGPS1*
Congenital muscular dystrophies (CMDs)	
CMD with merosin deficiency	Gene encoding the α2 subunit of merosin: *LAMA2*
Ullrich CMD / Bethlem myopathy	Genes encoding ECM proteins: *COL6A1*, *COL6A2*, *COL6A3*, *COL12A1*
CMD-dystroglycanopathies (Fukuyama CMD, WWS, muscle-eye-brain disease, and others)	Genes encoding proteins involved in the O-glycosylation of α-DG: *DAG1*, *POMT1*, *POMT2*, *POMGNT1*, *POMGNT2*, *FKTN*, *FKRP*, *LARGE1*, *B4GAT1*, *B3GALNT2*, *POMK*, *RXYLT1*, *ISPD*, *GMPPB*, *DPM1*, *DPM2, MPDU1*, *TRAPPC11*, *GOSR2*

Muscle disease	Disease-associated genes
Other CMDs	Genes encoding • structural proteins: *ITGA7*, *ACTA1*, *TCAP, LMNA* • SR proteins: *SELENON, RYR1* • other proteins: *GOLGA2*, *DNM2, CHKB, INPP5K*, *TRIP4*, *MSTO1*
Congenital myopathies	
Nemaline myopathies	Genes encoding • proteins involved in sarcomeric assembly and function: *ACTA1*, *NEB*, *TPM2*, *TPM3*, *TNNT1*, *TNNT3, CFL2*, *LMOD3*, *MYPD*, *KLHL40*, *KLHL41* • SR proteins: *RYR1, RYR3* • other proteins: *MYO18B*, *KBTBD13*
Centronuclear myopathies	Genes encoding • proteins involved in triad formation and EC coupling: *MTM1*, *DNM2*, *BIN1*, *RYR1*, *SPEG*; • other proteins: *TTN*, *CCDC78, MYF6, ZAK*
Core myopathies	Genes encoding • sarcomeric proteins: *TTN*, *ACTA1*, *ACTN2, MYH7* • SR proteins: *RYR1*, *SELENON*; • other proteins: *MEGF10*, *FXR1, CCDC78*
Congenital fiber-type disproportion	Genes encoding • sarcomeric proteins: *ACTA1*, *TPM2*, *TPM3*, *MYH7*, *MYL2*, *TTN* • SR proteins: *RYR1*, *SELENON* • other proteins: *HACD1*, *SCN4A*
Myosin storage myopathy	Gene encoding beta heavy chain of cardiac myosin: *MYH7*
Other congenital myopathies	*MYH2, HNRNPA1, MYBPC3, CNTN1, TRIM32, CACNA1S, SPTBN4, HRAS, MYMK, STAC3, MYL1, CACNA1H, PAX7, PYROXD1, TNPO3, UNC45B, MCOLN1, TNNC2, MYOD1*
Distal myopathies	Genes encoding • sarcolemmal proteins: *DYSF*, *CAV3* • sarcomeric proteins: *TTN, NEB*, *MYH7*, *MYOT*, *LDB3*, *FLNC*, *ACTN2, DES, CRYAB* • proteins involved in autophagy: *VCP*, *SQSTM1*, *DNAJB6*, *HSPB8*, *GNE* • other proteins: *TIA1*, *MATR3*, *ANO5*, *DNM2*, *KLHL9*, *ADSSL1*, *HNRNPA2B1, FHL1, SMPX, PLIN4*

Muscle disease	Disease-associated genes
Myofibrillar myopathies	Genes encoding • Z-line-associated proteins: *DES*, *CRYAB*, *MYOT*, *LDB3*, *FLNC*, *BAG3*, *TTN*, *KY* • other proteins: *PYROXD1*, *SVIL*, *SELENON*, *TRIM63+TRIM54*
AVMs	Genes encoding proteins involved in autophagic flux: *LAMP2, VMA21, CLN3*
Metabolic myopathies	
Disorders of glycogen metabolism	*GAA, AGL, GBE1, PYGM, PFKM, PHKA1, PGM1, GYG1, GYS1, GYG1, PGK1, PGAM2, LDHA, ENO3, SLC16A1, PRKAG2, RBCK1*
Disorders of lipid metabolism	*CPT2, SLC22A5, SLC25A20, ETFA, ETFB, ETFDH, ACADVL, ACAD9, ABHD5, PNPLA2, LPIN1, PNPLA8, FLAD1*
Mitochondrial myopathies	Nuclear genes encoding • proteins causing mtDNA deletions or depletion: *POLG, POLG2, SLC25A4, OPA1, TYMP, RRM2B, TK2, DGUOK, SUCLA2, SUCLG1, TWNK, DNA2, RNASEH1, TOP3A, MGME1, FBXL4, LIG3* • respiratory chain proteins: *COX6A2, FASTKD2, COQ2, COQ9, COQ6, COQ4, COQ7, ISCU* • other mitochondrial proteins: *PUS1, YARS2, SLC25A42, CHCHD10, AIFM1, MRPS25, MSTO1, TIMM22, APOO, MICU1, TMEM126B, GFER, NSUN3, COX16* 37 mtDNA genes
Other myopathies	*LRP12, GIPC1, NOTCH2NLC, STIM1, ORAI1, CASQ1, MB*
Non-dystrophic myotonic syndromes	Genes encoding • ion channels: *CLCN1*, *SCN4A* • other proteins: *CAV3*, *HSPG2*, *ATP2A1*
Periodic paralyses	Genes encoding ion channels: *SCN4A*, *ATP1A2*, *CACNA1S*, *KCNE3*, *KCNJ18*

AVM – autophagic vacuolar myopathy; BM – basement membrane; CMD – congenital muscular dystrophy; DG – dystroglycan; DMD/BMD – Duchenne/Becker muscular dystrophy; EC – excitation-contraction; ECM – extracellular matrix; EDMD – Emery-Dreifuss muscular dystrophy; FSHD – facioscapulohumeral muscular dystrophy; LGMD – limb-girdle muscular dystrophy; mtDNA – mitochondrial DNA; SR – sarcoplasmic reticulum; WWS – Walker-Warburg syndrome

2.2 Muscle pathology in neuromuscular disorders

2.2.1 The role of muscle biopsy in neuromuscular disorders diagnostics

In addition to the clinical evaluation of the patients and their signs and symptoms, the diagnosis of a specific type of NMD is reached using different investigative techniques: electroneuromyography (ENMG), magnet resonance imaging (MRI), enzyme analysis, muscle and nerve biopsy, and genetic testing [Goebel *et al.* 2013]. Muscle biopsy plays an evolving but significant role in the diagnostics of NMDs. Many histopathological features have defined and helped to diagnose and identify a disorder. During the early 2000s, when one could perform molecular diagnostics only on a gene-by-gene basis, muscle histopathology gave valuable information to direct genetic testing. The role of muscle biopsy has slightly changed after the application of NGS, but it is not redundant. Investigating the affected tissue becomes extremely important in cases with variants of unknown significance (VUSs), variants in a new gene, or when no genetic alteration is found [Dubowitz *et al.* 2021].

The diagnostic value of muscle biopsy depends on several aspects. Firstly, some muscle diseases may not show any histopathology or may present with only non-specific myopathological changes. Furthermore, distinct muscle disorders affect different muscle groups, and some muscles may characteristically be spared. Therefore, choosing the correct muscle to biopsy is very important. In general, the biopsied muscle should be moderately affected. Muscle MRI is of great assistance in locating the muscle biopsy site. The muscle can be biopsied using an open or semi-open method with a needle or conchotome, both of which have advantages and disadvantages. It is essential not to inject the local anesthetic into the muscle tissue, as this will cause fluid retention between the muscle fibers. The muscle tissue for histological studies needs to be snap-frozen in isopentane pre-cooled in liquid nitrogen with a maximal delay of two hours after surgery. One small specimen should be fixed in glutaraldehyde for electron microscopy. Additional samples frozen in liquid nitrogen may be needed for specific methods such as Western blotting, enzyme biochemistry, and extraction of DNA and RNA [Dubowitz *et al.* 2021; Meola *et al.* 2012; Nix and Moore 2020].

The microscopical investigations of frozen muscle tissue consist of histology, enzyme histochemistry, and IHC. Histological and enzyme histochemistry stains show the overall structure of the muscle tissue and different chemical and enzymatic properties of the muscle fibers. IHC enables visualization of the presence/absence of various proteins and is based on the specific affinity of a primary antibody for its antigen. Finally, electron microscopy highlights the ultrastructure of the muscle fibers.

In 2018, the EURO-NMD pathology working group agreed upon “Recommended Standards for Muscle Pathology” [Udd *et al.* 2019]. The following routine stains should be performed for all new muscle biopsy specimens:

1. Conventional histology:
    - Hematoxylin and eosin (HE);
    - Modified Gömöri trichrome (mGT);
    - Oil red O (ORO) / Sudan black staining lipids;
    - Periodic acid Schiff (PAS) staining glycogen.
2. Enzyme histochemistry:
    - Reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), an enzyme located in mitochondria and the sarcoplasmic reticulum;
    - Succinate dehydrogenase (SDH) and cytochrome C oxidase (COX), which are mitochondrial respiratory chain complex II and IV enzymes, respectively;
    - Acid phosphatase, a lysosomal enzyme;
    - Adenosine triphosphatase (ATPase) reactions at different pH levels highlighting the fiber types:
        - at pH 4.3, type 1 fibers are dark brown, type 2C (immature) fibers lighter brown, and type 2A and 2B fibers do not stain;
        - at pH 4.6, type 1, type 2C, 2B, and 2A fibers show staining gradation from dark to light brown in the respective order;
        - at pH 9.4 or 10.2, all type 2 fibers are dark brown and type 1 fibers light brown.
3. IHC:
    - Myosin heavy chain neonatal/fetal (MyHCn) and myosin heavy chain developmental/embryonic (MyHCd) showing expression in regenerating fibers;
    - Myosin heavy chain slow/beta cardiac (MyHCs) and myosin heavy chain fast (MyHCf) staining type 1 and type 2 fibers, respectively;
    - Major histocompatibility complex class I (MHC I), used for detecting its sarcolemmal and sarcoplasmic over-expression in muscle fibers;
    - p62 showing expression in autophagic vacuoles and protein aggregates.

The recommended extended methods are context-dependent.

2.2.2 Basic histopathological features in the skeletal muscle tissue

The first aspects of a biopsied muscle sample to recognize and account for are the quality and integrity of the muscle sections, as handling of the specimen can cause different artifacts. Firstly, intramuscular infiltration of local anesthetic or hemorrhage can induce rounding up and hypercontraction of muscle fibers (Figure 2A). However, muscle fiber hypercontraction is also a feature of muscular dystrophies. Secondly, mixed muscle fiber orientation (Figure 2B) complicates the comparison of fiber sizes, and errors in freezing the sample create ice crystal holes in the muscle fibers, which, in extreme cases, make fiber structure

assessment impossible (Figure 2C). However, thawing and refreezing the sample may restore the fiber morphology. Furthermore, incorrect tissue cutting can result in scratched, wrinkled, or compressed sections. Lastly, unevenness of stain or dirt can appear due to staining artifacts [Dubowitz *et al.* 2021; Nix and Moore 2020; Roth *et al.* 2000].

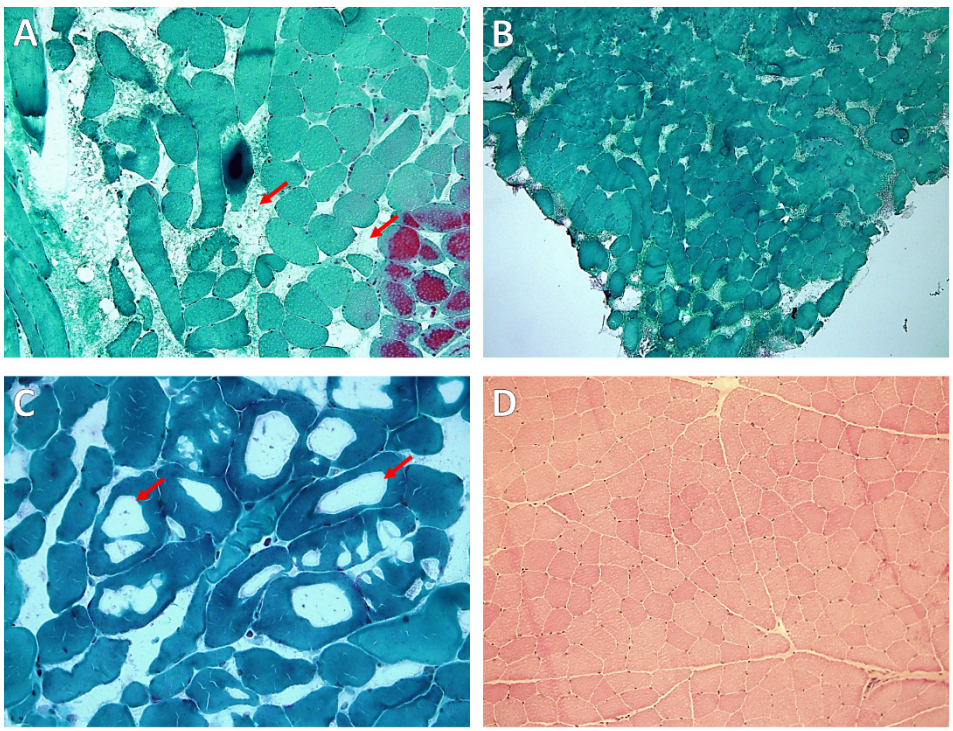


Figure 2. Muscle sections demonstrating (A) fluid infiltration between muscle fibers (arrows; mGT), (B) varying orientation of the muscle fibers (mGT), (C) severe ice crystal damage inside muscle fibers (arrows; mGT), and (D) normal skeletal muscle histology (HE).

In a healthy muscle, the muscle fibers are polygonal and tightly arranged next to each other (Figure 2D). The myonuclei reside beneath the sarcolemma, and the myofiber structure is homogenous. The normal diameter of the fibers in an infant is 12–15 µm, at 10 years of age, 40–55 µm, and in adults, 45–70 µm in females and 65–90 µm in males [Esbjornsson *et al.* 2021]. However, when the muscle fibers have not attained their regular size, they are hypotrophic, and when the fibers are shrunken or enlarged, they are atrophic or hypertrophic, respectively. Thickening of endo- and perimysium can result from inflammatory cell infiltration, fat cell proliferation, and/or fibrosis (increased amount of connective tissue).

HE is a standard histological technique that stains the myofibrils and cytoplasm of all cells light to dark pink and nuclei dark blue, making the presence

of increased cellularity easily detectable (Figure 3A). However, in myopathology, the mGT stain is of greater value than HE. mGT stains the myofibrils green/ blue, nuclei red/purple, and connective tissue bright green, enabling the visualization of fibrosis much better than on HE (Figure 3B). In addition, mGT nicely highlights the localization and abundance of mitochondria, which appear red. Any aggregates, vacuoles, rods, or defects of and in the sarcoplasm can be identified with excellent precision by mGT. Necrotic fibers appear pale, and regenerating fibers bluish with large vesicular nuclei and prominent nucleoli on both mGT and HE (Figure 3C and D) [Dubowitz *et al.* 2021; Sewry and Goebel 2013].

Muscle fiber necrosis and regeneration reflect an acute or active disease process, and fibrosis, fatty tissue infiltration, and internal nuclei reflect the disorder's chronicity and long duration, irrespective of the underlying etiology. While some histopathological features (e.g., absence of myophosphorylase or dystrophin) are independently pathognomonic for a specific disease, typically, combinations of several histological changes define a particular disorder or NMD group. The following sections describe the different myopathological patterns of changes, emphasizing childhood-onset hNMDs.

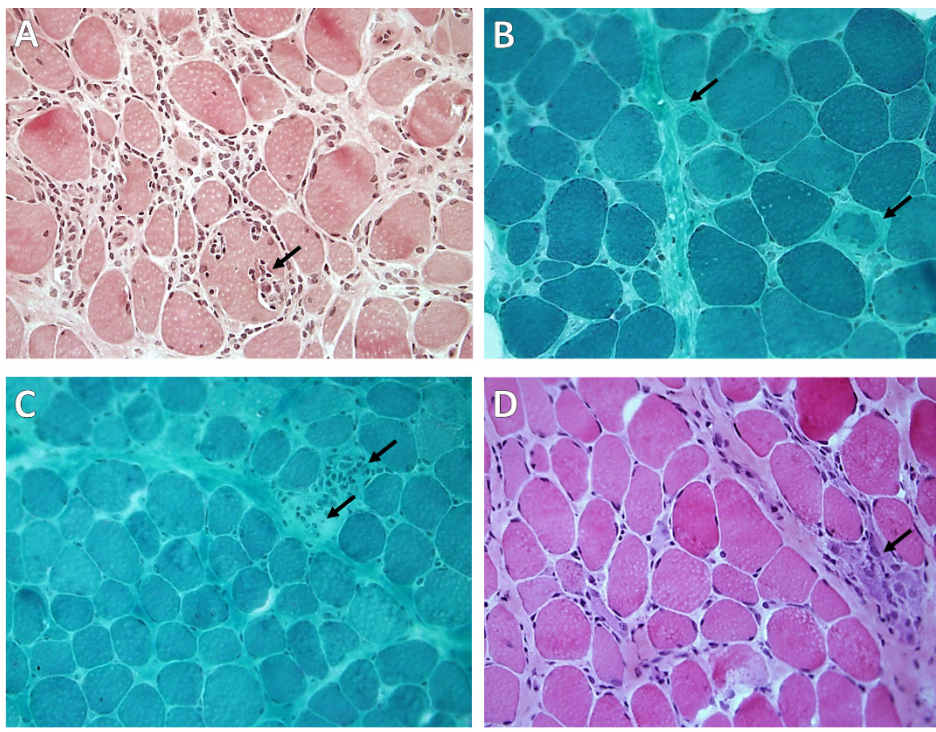


Figure 3. Muscle sections of (A) an adult patient with inclusion body myositis showing inflammatory cells surrounding the muscle fibers and invading one non-necrotic muscle fiber (arrow; HE) and a boy with DMD showing (B) endomysial fibrosis (arrows; mGT), (C) pale necrotic fibers in the phagocytosis stage (arrows; mGT), and (D) a small group of basophilic regenerating fibers (arrow; HE).

2.2.3 Neurogenic pattern of myopathological changes

When muscle fibers lose their supply of innervation, they become atrophic, show intense non-specific esterase and oxidative enzyme staining and neural cell adhesion molecule (NCAM) expression, and lose immunoreactivity to neuronal nitric oxide synthase (nNOS). While the denervated fibers typically become angular in shape, in SMA types I and II, the atrophic fibers are round. Furthermore, some fibers become so atrophic that only pyknotic nuclear clumps are seen. This whole process is called denervation or neurogenic atrophy (Figure 4A). Interestingly, some denervated fibers may express MyHCn, a marker of immaturity and regeneration [Sewry *et al.* 2021]. As one motor neuron innervates many muscle fibers and the neurogenic diseases of humans involve neurons supplying both type 1 and type 2 fibers, the atrophic muscle fibers form small or large groups of both fiber types. The other muscle fibers with intact motor supply may show compensatory hypertrophy. In the early stages of SMA type I and II, hypertrophy primarily involves type 1 fibers (Figure 4B). In addition, the surviving motor nerves undergo collateral sprouting and reinnervate the denervated fibers, which results in groups of both fiber types. A group consists of fibers of one type surrounding at least one fiber of the same type. This is called fiber type grouping (Figure 4C), and its extent reflects the reinnervation potential [Doppler *et al.* 2008; Gosztonyi *et al.* 2001; Kugelberg *et al.* 1970].

A histopathological feature of an active neurogenic process is the presence of target or targetoid fibers. Targets are areas of three distinctive circular zones with alternate oxidative enzyme activity (Figure 4D), and targetoids appear as areas of absent or reduced oxidative enzyme activity. Various proteins may accumulate in the center of targets/targetoids, and cytoplasmic bodies may be associated with them (Figure 4E and F). Furthermore, if the process is very active, muscle fiber necrosis may also occur. In chronic, long-lasting neuropathies, myopathological features of chronicity (nuclear internalization and fibrosis) may accompany the neurogenic changes. In some cases, moth-eaten fibers, whorled fibers, and autophagic vacuoles can also be seen. However, in SMA type III and other lower motor neuron diseases, the muscle pathology may be very mild with only small group atrophy or even show only myopathic changes. On the other hand, some myopathies may be accompanied by secondary neurogenic changes, e.g., in mitochondrial myopathies, facioscapulohumeral muscular dystrophy (FSHD), and myofibrillar myopathies, especially in *BAG3*-related cases [Dubowitz *et al.* 2021; Lu *et al.* 2019; Vogel 2013].

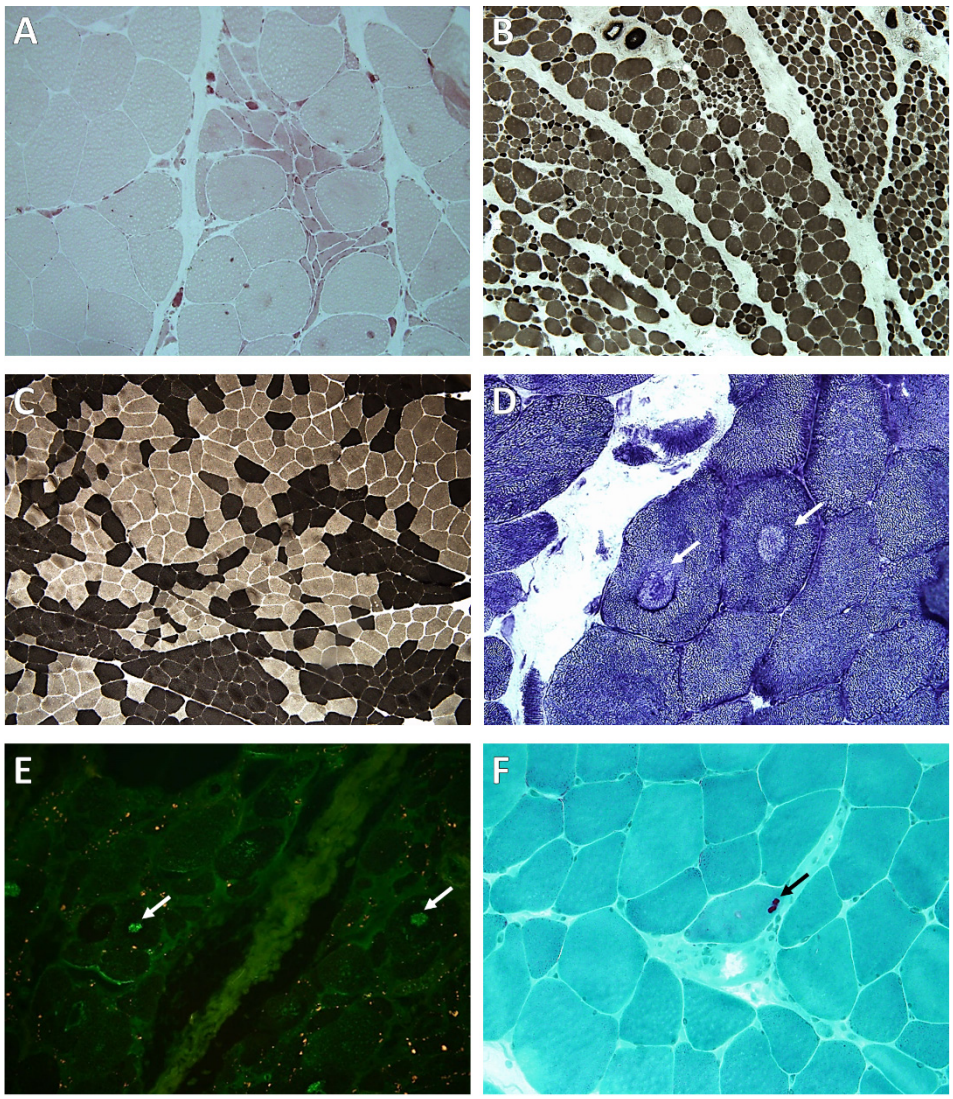


Figure 4. Muscle sections demonstrating neurogenic myopathological changes: (A) a small group of intensely stained denervated fibers (non-specific esterase), (B) hypertrophy of type 1 fibers (light) and atrophy of both type 1 and type 2 (dark) fibers in a girl with SMA type I (ATPase at pH 10.2), (C) fiber type grouping (ATPase at pH 10.2), (D) target fibers (arrows; NADH-TR), (E) myotilin aggregation in the center of targets (arrows), and (F) a cytoplasmic body, which is a non-specific finding (arrow; mGT).

2.2.4 Dystrophic pattern of myopathological changes

In severe progressive muscle diseases, primarily muscular dystrophies, the muscle biopsy specimen displays dystrophic features comprising both active and chronic histopathological changes. The muscle fibers become rounded, and the variation in fiber size increases considerably due to diffuse atrophy and hypertrophy of the fibers of both types. However, in myotonic dystrophy type 1, predominant atrophy of type 1 and hypertrophy of type 2 fibers, and in myotonic dystrophy type 2, predominant atrophy of type 2 fibers are seen [Schoser *et al.* 2004; Vihola *et al.* 2003]. In addition, hypertrophic fibers may split, and multiple splitting can seem like a group of small fibers. While pyknotic nuclear clumps are infrequent in most muscular dystrophies, they are a particular feature of myotonic dystrophy type 2 early in the disease course (Figure 5A). An increased number of internal nuclei per age is a non-specific sign of muscle pathology; however, numerous internalized myonuclei are a characteristic feature in myotonic dystrophies type 1 and especially type 2 [Naukkarinen 2011; Schoser *et al.* 2004].

The activation of apoptosis pathways results in muscle fiber necrosis, which is often segmental. The necrotic part of a cell initially coagulates and becomes pale on HE, mGT, and other stains. After that, macrophages appear to phagocytose the necrotic material and eventually fill the whole fiber (Figure 3C). Simultaneously, inside the basement membrane of the necrotic fiber, satellite cells become activated, differentiate into myoblasts, and bridge the gap of the muscle cell or fuse into regenerating fibers [Nix and Moore 2020]. Hence, groups of regenerating fibers may be seen (Figure 3D), which must be differentiated from the grouped atrophic fibers in neurogenic muscle. In some muscular dystrophies, primarily in DMD, many hypercontracted fibers staining eosinophilic on HE and dark green/blue on mGT are a particular feature (Figure 5B) [Lotz and Engel 1987]. The hypercontracted fibers are considered a form of degeneration [Dubowitz *et al.* 2021]. Varying degrees of endo- and perimysial fibrosis and adipose tissue infiltration may occur because the muscle cannot regenerate as much as it is degenerating [Cai *et al.* 2019].

Furthermore, the internal architecture may be altered, resulting in whorled (Figure 5C), moth-eaten, or ring fibers (Figure 5D) or core-like areas. Autophagic vacuoles may also be present, especially in some muscular dystrophies (e.g., LGMD D1 *DNAJB6*-related). In addition, the differential diagnosis between muscular dystrophies and inflammatory myopathies may be challenging. For example, significant infiltration of inflammatory cells, sarcolemmal expression of MHC I, and complement deposition can be present in FSHD and LGMD R2 dysferlin-related [Benveniste and Romero 2011]. However, not all muscle diseases termed muscular dystrophy show prominent dystrophic features (e.g., Emery-Dreifuss muscular dystrophy), and some disorders classified as myopathies may conversely show apparent dystrophic changes (e.g., distal myopathies).

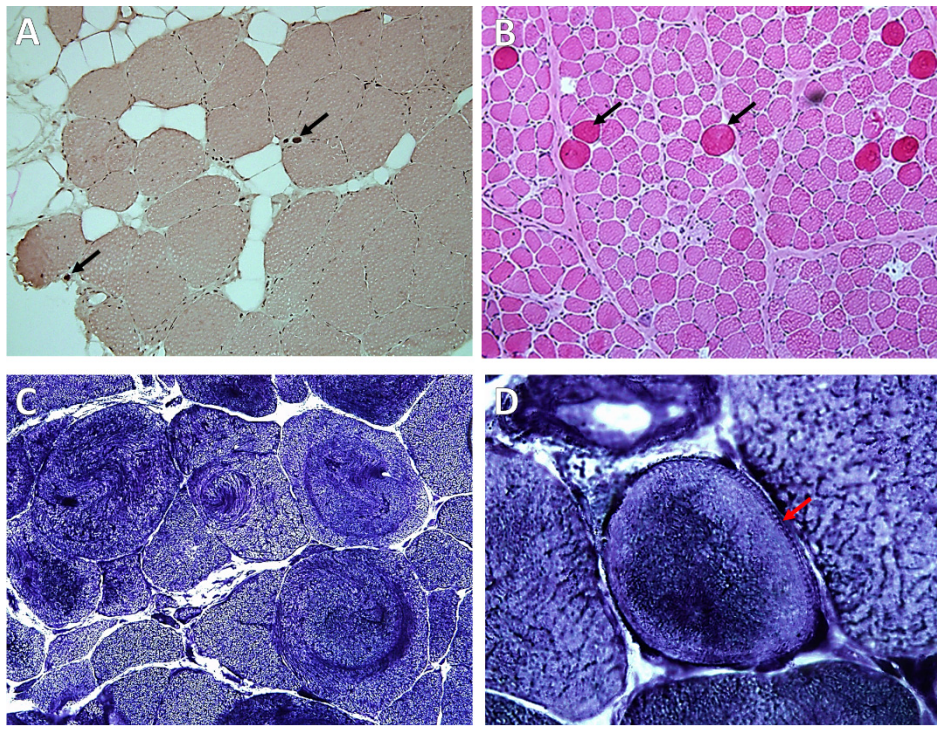


Figure 5. Muscle sections of (A) a patient with myotonic dystrophy type 2 showing infiltration of fatty tissue, variation in fiber size, several internal nuclei, and pyknotic nuclear clumps (arrows; HE) and (B) a boy with DMD demonstrating fibrosis, rounded fibers, variation in fiber size, necrosis, regeneration, and hypercontracted fibers (arrows; HE). Muscle sections stained with NADH-TR highlighting (C) whorled fibers and (D) a ring fiber (arrow).

2.2.5 Myopathological immunohistochemistry patterns of muscular dystrophies

While autosomal dominant LGMDs, some CMDs, autosomal dominant Emery-Dreifuss muscular dystrophy, and FSHD show general non-specific dystrophic/myopathic changes in the muscle, many subgroups of muscular dystrophies can be histopathologically diagnosed based on IHC. In DMD, all muscle fibers (except revertant fibers) show the absence of dystrophin (Figure 6B) and nNOS and upregulation of utrophin. However, antibodies directed against different dystrophin epitopes should always be used as one antibody reflects the presence of only that particular epitope. In some cases of BMD, only one dystrophin epitope might be absent or reduced (in most cases, the N-terminus). Furthermore, the reduction may be so mild that it cannot be visually detected on IHC. Western blot analysis of dystrophin and secondary IHC changes provide additional information in these cases. A mosaic pattern of dystrophin absence is present in DMD/BMD carrier females with skewed X-inactivation (Figure 6C).

Lastly, the expression of other dystrophin-glycoprotein complex proteins is commonly secondarily reduced [Barresi 2011; Nix and Moore 2020].

Antibodies directed against several proteins associated with autosomal recessive LGMDs are commercially available for IHC and Western blotting. In LGMD R1, a reduction of calpain-3 can be detected on Western blot. LGMD R2 presents an absence or severe decrease of dysferlin and secondary reduction of caveolin-3. A defect of one of the sarcoglycans responsible for LGMD R3–R6 typically but not always leads to decreased immunoreactivity of all four. In sarcoglycanopathies, dystrophin and β-dystroglycan can be secondarily reduced, and utrophin upregulated [Schroder *et al.* 2014]. The absence of telethonin refers to LGMD R7. In LGMD R23 and *LAMA2*-related CMD, laminin α2 sarcolemmal labeling is absent or variably decreased, and secondarily, laminin α5 is upregulated, and α-dystroglycan may be reduced. The absence or reduction of collagen VI is characteristic of Ullrich CMD. However, collagen VI labeling in Bethlem myopathy is usually normal, whereas laminin β1 expression is frequently decreased. Antibodies targeted against carbohydrate moieties of α-dystroglycan show loss or reduction of sarcolemmal labeling in cases of defective O-glycosylation of α-dystroglycan, causing several types of LGMDs and CMD-dystroglycanopathies. In these cases, a secondary decrease of laminin α2 and β-dystroglycan and upregulation of laminin α5 can occur. Lastly, X-linked Emery-Dreifuss muscular dystrophy shows the absence of emerin from all nuclei. In addition, it is essential to recognize that regenerating fibers show a reduction of nNOS and co-express laminin α5, utrophin, MyHCn, MyHCd, and MHC I [Barresi 2011; Sewry *et al.* 2021].

Figure 6. Immunofluorescence with an antibody directed against the rod domain of the dystrophin protein. Muscle sections of (A) a control, (B) a boy with DMD showing complete absence of dystrophin, and (C) a symptomatic girl with a heterozygous variant in the *DMD* gene highlighting mosaic loss of dystrophin.

2.2.6 Myopathological patterns of congenital myopathies

Congenital myopathies are historically defined by distinctive myopathological features. Typically, congenital myopathies feature type 1 fiber predominance and atrophy (Figure 7A). However, type 1 fiber uniformity may also occur, e.g., in *RYR1*-related myopathy. A phenomenon of type 1 fibers being at least 35–40% smaller than type 2 fibers is called fiber-type disproportion (Figure 7B), which is considered a separate subgroup of congenital myopathies. Centronuclear myopathies feature prominent single central nuclei with a dark central stain and a pale peripheral halo on NADH-TR. However, central nuclei are also characteristic of congenital myotonic dystrophy, an important differential diagnosis in infants. Core myopathies may present with central or peripheral, single or multiple cores, or multi-minicores. Typical cores are demarcated areas devoid of oxidative enzyme stain extending down the whole length of the fiber (Figure 7C). Ultrastructurally, cores can show only mild contraction or misalignment of myofibrils, Z line irregularities, Z-streaming, or marked myofibrillar disruption (Figure 7D). Minicores are small focal areas of Z-streaming that lack mitochondria. Notably, cores and minicores are indistinguishable from targetoids seen in neurogenic diseases. In addition, uneven oxidative enzyme stain and core-like areas may be seen in many different muscle diseases [Claeys 2020; Phadke 2019].

The hallmark of nemaline myopathy is nemaline bodies or rods (Figure 7E), which are Z-line derivatives. Nemaline bodies appear red-bluish on mGT and have a distinctive rod-like shape. Ultrastructurally, the rods often appear parallel and connected with the Z line with a similar lattice structure (Figure 7F). On the other hand, rods can also occur as a minor feature in various other myopathies, in atrophic fibers in neurogenic disorders, in normal extraocular muscles, near myotendinous junctions, and in aging muscles. Pathological variants of nemaline myopathies include actin thin filament aggregates, caps, and zebra bodies. Caps are peripheral areas of disorientated myofilaments that are sharply demarcated on HE, mGT, and NADH-TR but lack ATPase activity. Zebra bodies consist of dark filamentous lines alternating with lighter ones, seen only ultrastructurally. Finally, myosin storage myopathy is categorized separately and shows hyaline bodies in type 1 fibers, which stain with ATPase but not with NADH-TR and ultrastructurally appear granular [Claeys 2020; Phadke 2019].

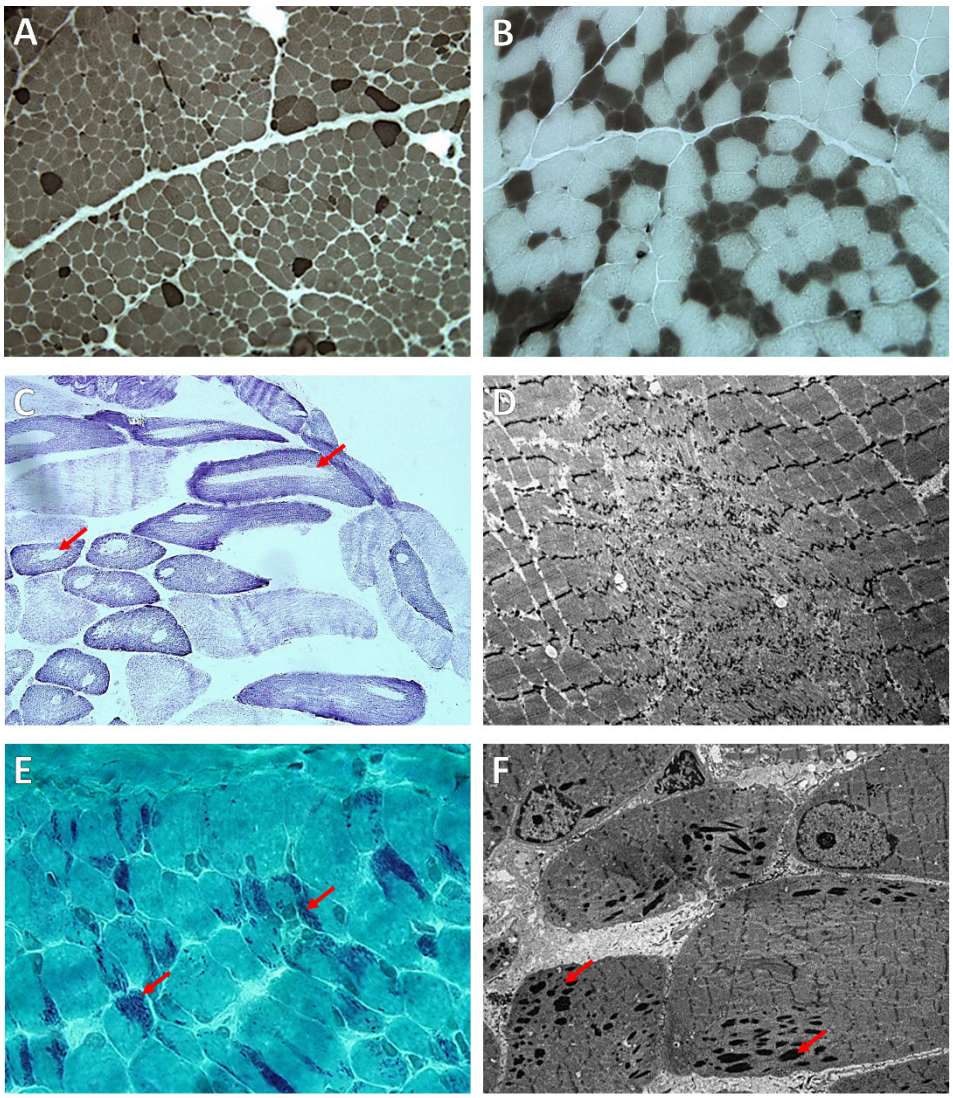


Figure 7. Muscle sections demonstrating histopathological changes of congenital myopathies: (A) predominance and atrophy of type 1 fibers (light; ATPase at pH 10.2), (B) fiber-type disproportion in a patient with a variant in the *MYH7* gene (ATPase at pH 4.3), (C) typical cores extending longitudinally along the fiber (arrows) in a patient with a variant in the *RYR1* gene (SDH), (D) ultrastructure of a core with myofibrillar disarray and lack of mitochondria, (E) numerous nemaline rods (arrows) in a patient with variants in the *NEB* gene (mGT), and (F) ultrastructure of nemaline rods (arrows).

2.2.7 Myopathological patterns of defects in autophagy

As the muscle fibers and especially Z line proteins are under significant mechanical stress, the tension-induced chaperone-assisted selective autophagy (CASA) pathway constantly targets damaged Z line proteins to maintain muscle homeostasis. Therefore, defects in either the Z line proteins (desmin, myotilin, ZASP, and filamin C) or the CASA proteins (e.g., chaperones αB-crystallin and BAG3) result in the accumulation of autophago(lyso)somes and cargo targeted for degradation and cause myofibrillar myopathies. The hallmark features of myofibrillar myopathies are rimmed vacuoles (Figure 8A), which are membrane-bound areas of a muscle fiber surrounded by basophilic material, and protein aggregates, which are immunoreactive to various proteins, including desmin, myotilin, BAG3, ubiquitin, heat-shock protein 70 (HSP70), and the autophagy receptor p62. The protein aggregates appear dark green/blue on mGT (Figure 8B) and lack mitochondria producing a wiped-out appearance on oxidative enzyme histochemistry. However, similar protein accumulation can also be seen in the center of targets, targetoids, and cores. The ultrastructural features of myofibrillar myopathies include myofibrillar disruption ranging from mild Z line irregularities to vast disarray of myofibrils, 15–18 nm tubulofilamentous and granulofilamentous inclusions (Figure 8C), cytoplasmic and spheroid bodies, and accumulation of cellular debris (Figure 8D). A very similar pathology with rimmed vacuoles, protein aggregation, and tubulofilamentous inclusions is seen in *GNE*- and *VCP*-related distal myopathies. In addition, autophagic vacuoles are a feature of LGMD D1 caused by variants in *DNAJB6*, a gene encoding a CASA chaperone [Margeta 2020].

Defective autophagosome-lysosome fusion and lysosomal homeostasis, the causes of Danon disease and X-linked myopathy with excessive autophagy (XMEA), respectively, result in the presence of numerous vacuoles lined by sarcolemmal and basement membrane proteins (e.g., dystrophin and laminin α2) called autophagic vacuoles with sarcolemmal features (AVSFs). AVSFs are immunoreactive for LC3 located on autophagosome membranes and p62 and stain with the lysosomal enzyme acid phosphatase. In Danon disease, the deficiency of LAMP2 can be visualized on IHC. In XMEA, muscle fibers show upregulation of MHC I, complement deposition, duplication of the basement membrane, and debris between basement membrane layers in addition to vacuolization [Margeta 2020].

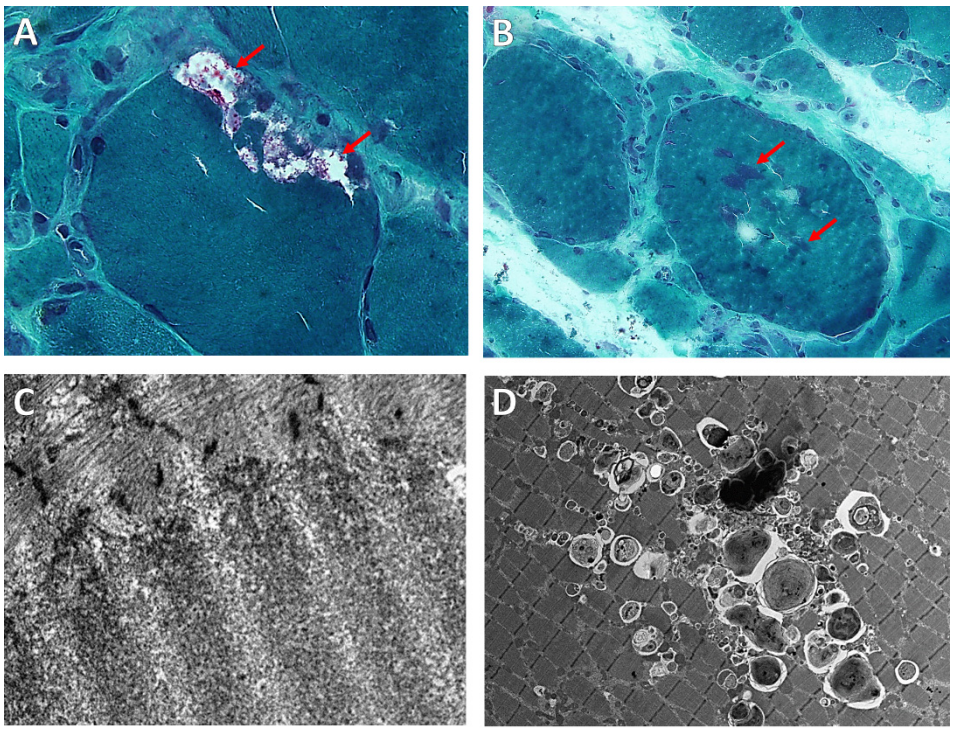


Figure 8. Muscle sections showing histopathological changes of myofibrillar myopathies: (A) rimmed vacuoles with red granular material (arrows; mGT), (B) protein aggregation (arrows; mGT), and ultrastructure of (C) a granulofilamentous inclusion and (F) cellular debris.

2.2.8 Myopathological features of metabolic defects

The muscle histology of patients with glycogen storage diseases (GSDs) and lipid metabolism disorders may be normal or show mild myopathic changes. In Pompe disease (GSD type II), caused by acid α-glucosidase deficiency, glycogen accumulates inside lysosomes, which can be detected on PAS and acid phosphatase stains (Figure 9A–C). Autophagic vacuoles and AVSFs are also frequently present. The extent of glycogen deposition varies in milder Pompe cases, but increased areas of acid phosphatase activity are always evident. Pronounced glycogen deposits are also seen in GSD type III. However, other GSDs may or may not show glycogen accumulation. Two GSDs have pathognomonic features in the muscle: absence of myophosphorylase in McArdle disease (GSD type V) and lack of phosphofructokinase in GSD type VII. Polyglucosan bodies can be seen in a few GSDs, primarily GSD type IV. Primary carnitine deficiency and neutral lipid storage disease present massive lipid accumulation in the muscle fibers. However, CPT II deficiency, short-, medium-, and very long-chain, and multiple acyl-CoA dehydrogenase deficiency, trifunctional protein deficiency, and lipin-1 deficiency may or may not show lipid accumulation. On

the other hand, an increased amount of lipid droplets can also be a prominent feature in some mitochondrial myopathies [Dubowitz *et al.* 2021; Liang and Nishino 2013; Vissing 2013].

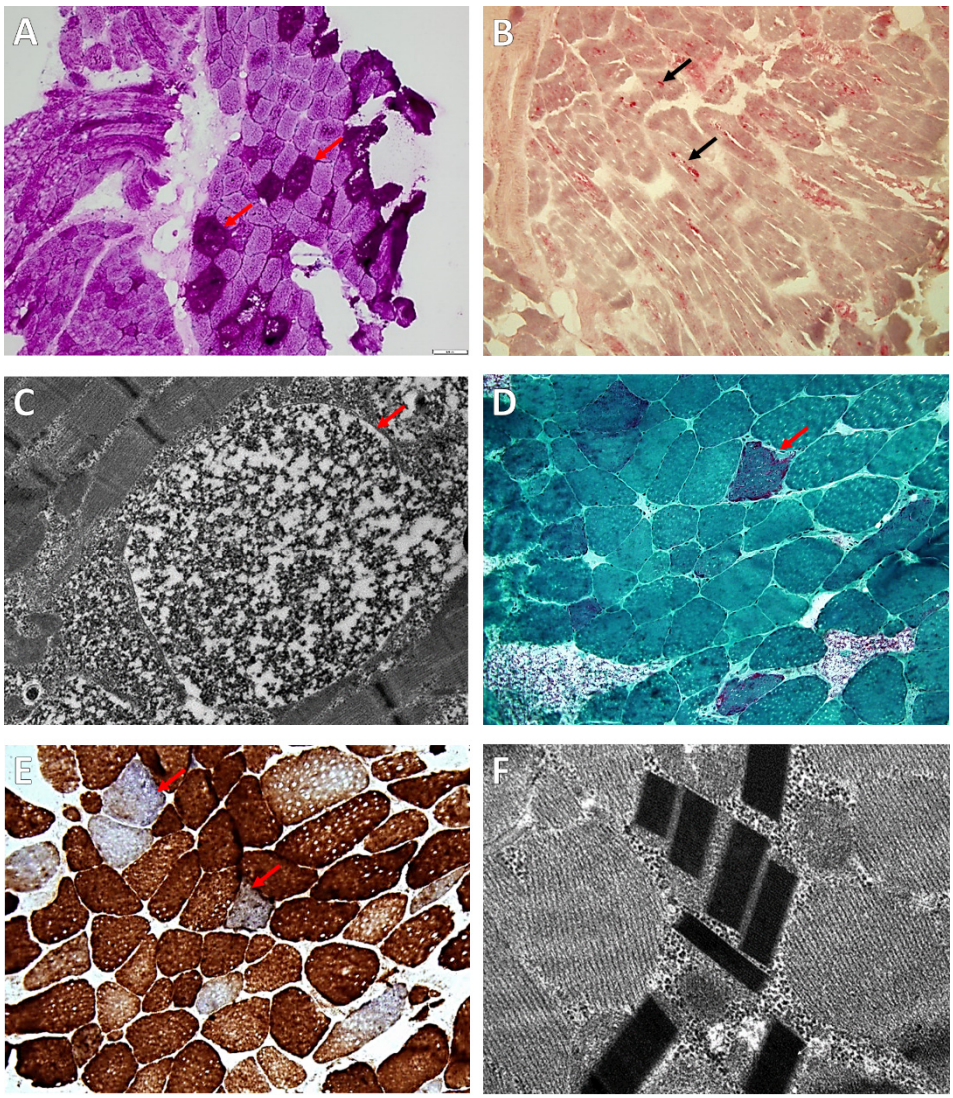


Figure 9. Muscle sections of a patient with Pompe disease highlighting (A) accumulation of glycogen (arrows; PAS), (B) increased lysosomal activity (arrows; acid phosphatase), and (C) ultrastructure of membrane-bound glycogen (arrow), and a patient with an mtDNA deletion revealing (D) RRFs (arrow; mGT), (E) COX-negative fibers (arrows; COX-SDH), and (F) ultrastructure of pathological mitochondria with paracrystalline inclusions.

Mitochondriopathy is characterized by segmental subsarcolemmal and intermyofibrillar mitochondrial proliferation, structural abnormalities of mitochondria, and fibers lacking COX enzyme activity. The terms ragged-red fibers (RRFs) and ragged blue/brown fibers refer to the extreme proliferation of mitochondria in muscle fibers revealed as a red, blue, or brown stain on mGT, SDH, or COX, respectively (Figure 9D). These fibers usually display mitochondria with ultrastructural abnormalities. The mitochondria may show glycogen, lipid, paracrystalline, or other electron-dense inclusions, enlargement, cristae linearization, concentric cristae, compartmentalization, nanotunneling, or hyperbranching. The paracrystalline inclusions are rigid rectangular crystals consisting of stacked sheets (Figure 9F) [Vincent *et al.* 2016]. Fibers lacking COX enzyme activity (COX-negative fibers) can be best seen on the combined COX-SDH stain, where they appear blue (Figure 9E). Strongly SDH-positive blood vessels can be present in MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) syndrome. Antibodies targeted against the mitochondrial respiratory chain enzyme complexes are also available [Rocha *et al.* 2015].

Primary and secondary mitochondriopathy cannot be differentiated based on these features. However, a mitochondrial myopathy can be defined as a myopathy with abnormal mitochondrial proliferation and/or COX-negative fibers without evidence of another primary muscle disease. The general histology may be normal or show various degrees of myopathic or neurogenic changes. Secondary mitochondriopathy can be present in several NMDs, including SMA, DMD, LGMDs, and myofibrillar myopathies. In addition, the presence of COX-negative fibers appears in normal aging from approximately 40 years of age [Haas *et al.* 2008; Katsetos *et al.* 2013; Ripolone *et al.* 2015].

2.2.9 Myopathology in muscle ion channelopathies

The general histology of non-dystrophic myotonic syndromes and periodic paralyses is typically normal or shows various degrees of myopathic changes. However, in potassium-aggravated myotonia, subsarcolemmal vacuolation related to the dilation of the T-tubules may be seen. In addition, in some cases of *paramyotonia congenita* and periodic paralysis, proliferation and dilation of the T-tubular and sarcoplasmic reticulum systems resulting in vacuoles and tubular aggregates can be detected. Tubular aggregates are also a particular feature of tubular aggregate myopathies caused by variants in the *STIM1*, *ORAI1*, and *CASQ1* genes, which affect calcium homeostasis. However, single tubular aggregates can be present in healthy individuals (Figure 10) [Dubowitz *et al.* 2021; Jurkat-Rott and Lehmann-Horn 2013].

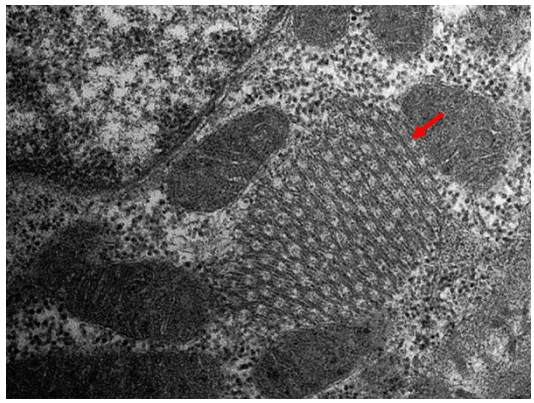


Figure 10. A single small tubular aggregate (arrow) seen with electron microscopy in a muscle with normal histology.

2.2.10 Myopathology in congenital myasthenic syndromes

Similarly to muscle ion channelopathies, the general histology of CMSs is typically normal or shows various degrees of myopathic changes. Most, but not all, CMS subtypes show neuromuscular junction abnormalities, including small synaptic vesicles, small endplates, wide synaptic spaces, and degeneration of the junctional folds. However, synapses are not commonly seen on regular muscle biopsy specimens. Nevertheless, some CMSs, primarily *GFPT1*-related cases, frequently feature tubular aggregates. In addition, cases of CMS with type 1 fiber predominance and atrophy resembling fiber-type disproportion, core-like areas, minicores, occasional nemaline rods, fibrosis, necrosis, and autophagic vacuoles have also been reported [Chaouch and Lochmüller 2013; Dubowitz *et al.* 2021].

2.3 Diagnostics of mitochondrial disorders

2.3.1 Mitochondrial physiology and genetics

Mitochondria form a dynamic network within all human cells, except erythrocytes, and undergo constant fusion and fission processes. They are surrounded by outer and inner mitochondrial membranes, of which the latter forms cristae. Between the cristae is the matrix, which contains enzymes and other proteins executing the various functions of mitochondria: oxidative phosphorylation (OXPHOS), carbohydrate, lipid, and amino acid metabolism, calcium homeostasis, mitochondrial DNA (mtDNA) maintenance, transcription, and translation, and many others. On the inner mitochondrial membrane lies the electron transport chain or respiratory chain where OXPHOS takes place by shuttling electrons from complex I (NADH: ubiquinone oxidoreductase) to complex II (SDH), complex III (ubiquinol-cytochrome c oxidoreductase), and complex IV (COX), which creates a proton gradient enabling complex V (ATP synthase) to generate ATP [Dard *et al.* 2020; Saneto 2020].

Each nucleated human cell contains a widely variable number of mitochondria and 100–10,000 copies of mtDNA, depending on the energy demand of the tissue. The mtDNA is a circular double-stranded molecule residing in the mitochondrial matrix, which encodes 13 OXPHOS proteins, 22 transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs). However, the sequence of each mtDNA copy is not identical. This phenomenon gives rise to heteroplasmy, meaning that only a certain percentage of mtDNA molecules in a cell harbor a specific variant. Inversely, homoplasmy is a state where all mtDNA molecules have an identical sequence regarding the locus in question [Dard *et al.* 2020; Saneto 2020]. Nevertheless, the vast majority of the proteins needed for mitochondrial functions are encoded by almost 1,100 nuclear genes [Rath *et al.* 2021].

2.3.2 Clinical, biochemical, and pathological features of mitochondrial disorders

While mitochondria are involved in many biological processes, MDs are usually defined as defects in the OXPHOS system. However, no consensus exists for a clear, standardized definition of MDs [Schlieben and Prokisch 2020]. Initially, during the premolecular era, MDs were diagnosed and grouped based on clinical, neuroimaging, biochemical, and pathological findings. However, after the identification of the first disease-causing variants in mtDNA, knowledge regarding mitochondrial genetics and biology rapidly expanded, blurring the classification of MDs [DiMauro and Garone 2010].

The MDs present extensive phenotypic and genotypic heterogeneity because mitochondria reside in almost all human cells and possess unique features. Therefore, any organ system can be involved. However, as MDs are energy production disorders, the most frequently affected organs are the ones consuming the most ATP (brain, cardiac and skeletal muscles, and liver). Due to the dual genetic control of mitochondrial proteins, the inheritance of MDs can be autosomal dominant or recessive or X-linked when nuclear DNA (nDNA) is altered or follow the maternal inheritance of mtDNA variants. In addition, the level of heteroplasmy of an mtDNA variant can significantly vary between different tissues, and a variant-specific threshold level of heteroplasmy needs to be exceeded for the dysfunction to occur. Furthermore, the degree of heteroplasmy can change during subsequent mitoses, called replicative segregation, altering the clinical expression even more [DiMauro and Hirano 2005]. Therefore, MDs can present with either isolated symptoms or multi-systemic involvement. Secondly, the disease-onset varies from prenatal to adulthood, and many clinical features of MDs are non-specific and overlap with other neurometabolic and neurodegenerative diseases and NMDs. In addition, one disease entity can be caused by variants in different genes, and a specific variant in one gene can cause various phenotypes [Saneto 2020; Schlieben and Prokisch 2020].

A deficient OXPHOS system and decreased ATP production result in a plethora of cellular responses affecting many metabolites [Esterhuizen *et al.* 2017]. Increased plasma lactate levels represent a relevant clue for MD, and it was initially used as a first-tier screening method in children [Haas *et al.* 2007; Joost *et al.* 2012]. However, lactic acid elevation may occur in various conditions and, therefore, it is a non-specific finding. Conversely, in many MDs, the lactate concentration in blood and cerebrospinal fluid (CSF) can be completely normal [Haas *et al.* 2007]. More detailed metabolic testing for MDs includes measurement of plasma and CSF amino acids, urine organic acids, and plasma acylcarnitines. Elevated lactate, pyruvate, lactate/pyruvate ratio, and alanine in blood and increased excretion of tricarboxylic acid cycle intermediates, ethylmalonic acid, and 3-methyl glutaconic acid are all indicators of MD. Secondary fatty acid oxidation defects and carnitine deficiency can also occur. However, these biomarkers have suboptimal specificity and sensitivity [Haas *et al.* 2008]. Therefore, new biomarkers for MDs have been suggested. For example, neurofilament light-chain was significantly increased in patients with multisystemic MD with central nervous system (CNS) involvement, especially in patients with epilepsy. In addition, fibroblast growth factor 21 and growth and differentiation factor 15 were significantly elevated in patients with a skeletal muscle dominant MD [Suomalainen *et al.* 2011; Varhaug *et al.* 2021].

OXPHOS enzyme activity measurement from muscle using different methods is a more sensitive analysis. However, it is relevant to note that the results vary depending on the technique and if the muscle sample is fresh or frozen. Cultured fibroblasts can be used as an alternative to muscle, but a high false-negative rate has to be accounted for [Haas *et al.* 2008]. The histopathological hallmarks of MDs in the muscle tissue are mitochondrial proliferation, RRFs, COX-negative fibers, and ultrastructural pathology of mitochondria in myofibers. While a mosaic pattern of COX-positive and COX-negative SDH-positive fibers indicates an mtDNA defect, a generalized deficiency of COX activity refers to nDNA alterations, and a complex II defect might result in a lack of SDH stain. However, normal biochemical or histological analyses do not exclude an MD, and mitochondrial dysfunction can be a secondary finding in many genetic diseases [Haas *et al.* 2008; Katsetos *et al.* 2013].

Two different MD diagnostic criteria (MDC) have been developed to assess the probability of an MD and guide genetic analysis [Bernier *et al.* 2002; Morava *et al.* 2006; Wolf and Smeitink 2002]. For example, the Nijmegen MDC scoring system [Morava *et al.* 2006; Wolf and Smeitink 2002] incorporates the clinical, biochemical, neuroimaging, and muscle histopathological features and has been of great help before the widespread clinical use of NGS techniques. However, with the application of WES, it has been shown that many patients with a clinical suspicion of MD harbor disease-causing variants in genes not associated with mitochondria, highlighting the extensive phenotypic and biochemical overlap between MDs and other neurological and multi-system diseases [Schlieben and Prokisch 2020].

2.3.3 Molecular genetics of mitochondrial disorders

As nuclear gene variants are uniformly present in all nucleated cells, except in the case of mosaicism, the nDNA can reliably be investigated from blood. However, the variable level of heteroplasmy of mtDNA in different tissues and cells makes mtDNA variant detection especially challenging. An easily accessible alternative for blood is urinary epithelial cells [de Laat *et al.* 2019], and cultured fibroblasts from a skin biopsy are another relatively non-invasive option. However, in some instances, especially in patients with isolated muscle symptoms, the mtDNA should be extracted from muscle tissue [Haas *et al.* 2008].

The mutation rate of mtDNA is significantly higher than nDNA [Dard *et al.* 2020]. Almost all mtDNA genes harbor disease-associated variants, including point mutations, single large-scale deletions, insertions, duplications, multiple deletions, and mtDNA depletions, the latter two being caused by nDNA variants. However, only a minority of patients with MDs harbor variants in mtDNA, especially children. Notably, around 400 nuclear genes have been related to MDs, with an expected continuous increase [Schlieben and Prokisch 2020]. Growing knowledge about mitochondria significantly contributes to diagnostics. For example, it has been shown that mitochondrial dynamics play an essential role in neuronal development, and its dysfunction has been related to several rare and common neurological diseases and MDs [Navaratnarajah *et al.* 2021]. We have recently described the role of SPATA5 (spermatogenesis-associated protein 5) in maintaining the balance of mitochondrial fusion-fission rate and axonal growth, which will be discussed in more detail in sections 2.3 and 5.2 [Puusepp *et al.* 2018a].

Sequencing of single genes chosen based on the clinical, biochemical, and pathological features has largely been replaced by NGS-based gene panel analyses and WES. The diagnostic yield of WES in patients with a clinical suspicion of an MD varies between 40–60% [Pronicka *et al.* 2016; Puusepp *et al.* 2018b; Taylor *et al.* 2014; Theunissen *et al.* 2018; Wortmann *et al.* 2015]. However, a major challenge in interpreting WES data is the presence of VUSs. Therefore, a comprehensive clinical investigation of the patient is needed together with radiological imaging, metabolic analyses, and tissue analysis, including skin and muscle biopsies, to confirm or exclude the pathogenicity of VUSs. Hence, the clinical utility of the abovementioned investigations is still highly relevant [Alston *et al.* 2021].

However, many patients remain without a molecular diagnosis after WES analysis in clinical settings. These patients could harbor variants in genes not associated with a disease before warranting scientific research with additional functional studies primarily from the affected tissue. Poor quality of the reads can be another reason for a negative result. For example, WES revealed no potentially pathogenic variants in a family with biochemically diagnosed mitochondrial short-chain enoyl-CoA hydratase deficiency, indicating a defect in the *ECHS1* gene. However, due to low read depth, Sanger sequencing of *ECHS1* was performed, and a homozygous pathogenic variant was found.

The latter case again highlights the importance of clinical and biochemical analyses guiding genetics. In addition, some disease-causing variants are undetectable by WES or not assigned as pathogenic by the investigator. In these cases, the current research focuses on the combined transcriptomic, proteomic, and metabolomic analyses performed preferably from the affected tissue [Alston *et al.* 2021].

2.4 The *SPATA5* gene and epilepsy, hearing loss, and intellectual disability syndrome

A complementary DNA (cDNA) fragment initially termed spermatogenesis associated factor (*SPAF*) was identified from a differential gene expression study of a squamous cell carcinoma model [Liu and Kulesz-Martin 1998]. Cloning of the full-length mouse *SPAF* cDNA revealed that it encodes a polypeptide of 892 amino acids, which belongs to the ATPase Associated with diverse cellular Activities (AAA) protein family. In addition to two highly conserved ATPase modules, *SPAF* contains a putative mitochondrial matrix-targeting sequence [Liu *et al.* 2000].

While the *SPAF* cDNA, now termed *SPATA5* (MIM# 613940), was predominantly expressed in the testis in mice [Liu *et al.* 2000], the *SPATA5* expression is ubiquitous in humans, including neuronal cells of the cerebral cortex (https://www.proteinatlas.org/ENSG00000145375-SPATA5/tissue, accessed on 20.02.2021). IHC analysis of mouse testis sections located SPAF protein in the cytoplasm, and an ultrastructural immunoperoxidase pre-embedding technique revealed SPAF immunoreactivity in mitochondria. As most other members of the AAA-protein subfamily with two ATPase modules are involved in the membrane fusion of different organelles, it was hypothesized that SPAF could play a role in mitochondrial membrane fusion events [Liu *et al.* 2000].

Tanaka *et al.* were the first to associate *SPATA5* variants with a Mendelian disease. [Tanaka *et al.* 2015]. Shortly after, additional case reports were published [Buchert *et al.* 2016; Kurata *et al.* 2016]. All of the patients harbored either homozygous or compound heterozygous *SPATA5* variants and presented with global developmental delay with more severe involvement of cognition and speech than motor abilities and abnormal electroencephalogram (EEG). Most patients were also diagnosed with symptomatic epilepsy and sensorineural hearing loss. Hence, this phenotype was named epilepsy, hearing loss, and intellectual disability syndrome (MIM# 616577). Interestingly, two of these patients showed enlarged, abnormally shaped mitochondria and mild mitochondrial proliferation on muscle biopsy specimens. It was suggested that *SPATA5* might be involved in mitochondrial morphogenesis during neuronal development [Tanaka *et al.* 2015].

We have described five new patients and summarized the phenotype and genotype of all reported cases with *SPATA5* biallelic variants. In addition, we

have studied SPATA5 deficiency in rat cortical neurons and revealed a significant imbalance in the mitochondrial fusion-fission rate [Puusepp *et al.* 2018a].

2.5 Molecular diagnostics in neuromuscular disorders

2.5.1 DNA sequencing

Classically, the genetic analyses of NMD patients have been based on single gene testing chosen by the clinical features and complementary investigations, including brain and muscle MRI, ENMG, and muscle tissue analyses. However, NGS-based gene panel sequencing has been widely adopted in the clinic as first-tier genetic testing for most hereditary disease groups, including NMDs [Thompson *et al.* 2020; Volk and Kubisch 2017]. The diagnostic yield of WES for patients with an LGMD phenotype who already had extensive single gene testing has been reported to be 37–45% [Ghaoui *et al.* 2015; Harris *et al.* 2017; Reddy *et al.* 2017]. In another study of pediatric patients with heterogeneous phenotypes (congenital myopathy, muscular dystrophy, peripheral neuropathy, and complex conditions with neuromuscular involvement), the diagnostic yield of WES was 26%. However, these patients were also previously well studied [Tsang *et al.* 2020]. In a cohort of patients with severe muscle weakness presenting at birth or during the first year of life with or without additional findings, the combined diagnostic yield of targeted single gene sequencing (based on either characteristic myopathological findings or specific clinical features) and NGS-based gene panel or WES was 64%. However, WES was not performed in all patients with a non-diagnostic single gene or panel analysis [Vill *et al.* 2017].

A great study by Schofield *et al.* highlighted the effectiveness of WES over a traditional approach of candidate gene testing [Schofield *et al.* 2017]. They reviewed 56 pediatric patients with a clinical and histopathological diagnosis of CMD or nemaline myopathy. All patients were initially investigated with the traditional diagnostic algorithm (complementary studies, histological muscle analysis, candidate gene sequencing, and chromosomal microarray), which yielded a diagnosis in 46% (26/56) of patients. Afterward, the 30 undiagnosed patients received NGS-based NMD gene panel sequencing (diagnosis in 4/7 patients) or WES (diagnosis in 16/26). Considering that the variants of the 28 patients diagnosed using candidate gene or gene panel sequencing would also have been detected using WES, the diagnostic yield of WES was estimated to be 79% (44/56).

NGS-based genetic testing has considerably expanded the phenotypic spectrum of many known NMD genes. For example, Evangelista *et al.* [Evangelista *et al.* 2020] described a mother and a son with adult-onset proximal lower limb and distal upper limb muscle weakness and a pathogenic heterozygous variant in the *FLNC* gene associated with myofibrillar myopathy. However, in the male proband, the muscle histology revealed changes characteristic of congenital

myopathy with type 1 fiber predominance, nemaline bodies in approximately 20% of atrophic fibers, and many ring fibers without abnormal protein aggregation. On the other hand, we have described a boy who was initially suspected of having a congenital myopathy; however, his muscle histology showed only non-specific changes, and we identified a hemizygous variant in the *PRPS1* gene causing a purine/pyrimidine metabolism defect [Puusepp *et al.* 2020]. This case is discussed in more detail in sections 2.5 and 5.4.

WES and whole genome sequencing (WGS) approaches have significantly contributed to new disease-causing gene discovery. As a result, the number of NMD-associated genes has increased more than twice in 11 years, from 290 genes known in 2010 to 608 genes in 2021 (http://www.musclegenetable.fr/). For example, a new muscle disease-associated gene has recently been described in 21 patients from four families with autosomal dominant distal muscular dystrophy [Savarese *et al.* 2019] and two patients with early-onset progressive myopathy with unique structured cores in the muscle [Lornage *et al.* 2019], who were found to harbor pathogenic heterozygous variants in the *ACTN2* gene. Furthermore, in collaboration with international research teams, we have confirmed the disease causality of biallelic variants in the *JAG2* gene in patients with congenital or childhood-onset proximal muscular dystrophy [Coppens *et al.* 2021]. The new gene-disease association of *JAG2* is further discussed in sections 2.6 and 5.5.

2.5.2 Targeted genetic testing

As the currently available NGS techniques and bioinformatics tools have limited ability to call intragenic CNVs and cannot detect repeat expansions and contractions, other targeted gene analyses must be considered before NGS in some NMDs [Thompson *et al.* 2020]. Considering that 65–80% of pathogenic variants in the *DMD* gene and 95–98% of variants in *SMN1* are intragenic CNVs, targeted multiplex ligation-dependent probe amplification (MLPA) or array comparative genomic hybridization methods should be used in patients with a clinical suspicion of DMD/BMD or SMA as first-tier [Darras *et al.* 2020; Prior *et al.* 2020]. CNVs have also been reported in other NMD genes, although in a lower proportion than *DMD* and *SMN1*. For example, large pathogenic CNVs in the *NEB* gene contribute to approximately 10–15% of nemaline myopathy cases [Pelin and Wallgren-Pettersson 2019].

In addition, myotonic dystrophies type 1 and 2 are caused by unstable CTG repeat expansion in the *DMPK* gene and a CCTG repeat expansion in the *CNBP* gene, respectively. Patients with the classical phenotype of myotonic dystrophy type 1 have 50–1,000 CTG repeats, but congenital cases show 1,000–4,000 repeats. However, in myotonic dystrophy type 2, the CCTG repeat size does not correlate with disease severity, and the size varies considerably from 75 to 11,000 [LoRusso *et al.* 2018]. Lastly, FSHD type 1 is caused by a contraction of D4Z4 repeat units at chromosome 4q35, which is in *cis* with a permissive 4qA subtelomere. In healthy people, the 4q35 region harbors 11–100 D4Z4 repeats,

while FSHD type 1 patients have only 1–10 D4Z4 repeats. FSHD type 2 is clinically identical to type 1, but it is caused by defects in proteins associated with epigenetic silencing of the 4q35 locus with the permissive 4qA subtelomere. The genomic defects of both FSHD types reactivate the *DUX4* gene, a highly cytotoxic transcription factor [Himeda and Jones 2019].

2.5.3 RNA sequencing

Although WES and WGS have significantly increased the diagnostic capabilities for Mendelian disorders, many challenges remain. The main difficulty is interpreting the clinical significance of different DNA variants, many of which fall into the category of VUS due to the lack of knowledge of their functional consequences. To resolve this issue, one can use RNA sequencing methods to analyze the transcriptome of different tissues. After extraction from tissue cells, the RNA fragments are converted into cDNA and sequenced using NGS technology. After that, the data is compared to a control data set and analyzed for gene expression outliers, abnormal splicing isoforms, and allele-specific expression in which only one allele is expressed and the other is silenced [Murdock 2020].

2.6 Alterations in the enzyme activity of phosphoribosylpyrophosphate synthetase

The enzyme phosphoribosylpyrophosphate synthetase (PRPS, EC 2.7.6.1) catalyzes phosphoribosylpyrophosphate (PRPP) synthesis, the first step of *de novo* purine synthesis. PRPP is also used in the *de novo* synthesis of pyrimidines. Purine and pyrimidine bases form nucleosides and nucleotides, which are involved in various essential biological functions, including conservation and transfer of energy, cell signaling, and formation of coenzymes, DNA, and RNA [Balasubramaniam *et al.* 2014a; Balasubramaniam *et al.* 2014b].

Alterations of the PRPS enzyme activity were first studied and reported in the 1970s in patients with both hyper- and hypouricemia [Sperling *et al.* 1972; Wada *et al.* 1974]. Many families with PRPS superactivity (MIM# 300661) were described, and an X-linked transmission was noticed [Yen *et al.* 1978]. Most of these patients presented with early adult-onset gout, but some individuals also had a history of early-onset sensorineural hearing loss, intellectual disability, muscle hypotonia, or ataxia [Becker *et al.* 1988; Simmonds *et al.* 1985]. Afterward, Becker *et al*. identified two PRPS cDNA isoforms and the respective genes, *PRPS1* (MIM# 311850) and *PRPS2* (MIM# 311860) [Becker *et al.* 1990]. In addition, two *PRPS1* variants, Asn113Ser and Asp182His, were detected in two patients with purine nucleotide feedback-resistant PRPS superactivity [Roessler *et al.* 1993].

Concurrently, Arts *et al.* described an X-linked recessive disease in a five-generation family with 12 affected boys [Arts *et al.* 1993]. The patients pre-

sented intellectual disability, developmental delay, early-onset muscular hypotonia, ataxia, deafness, nystagmus, loss of vision, flaccid tetraplegia, areflexia, and frequent infections causing early death in all but one. Some carrier females also developed hearing loss, ataxic diplegia, or muscle hypotonia with early adulthood-onset. Over a decade later, this phenotype, referred to as Arts syndrome (MIM# 301835), was associated with *PRPS1* missense loss-of-function variants, resulting in decreased PRPS activity in erythrocytes and fibroblasts [de Brouwer *et al.* 2007]. Loss-of-function *PRPS1* variants were also found in patients with X-linked Charcot-Marie-Tooth disease type 5 (CMTX5, MIM# 311070) presenting with a triad of hearing loss, visual impairment, and peripheral neuropathy and nonsyndromic X-linked deafness (DFN2, MIM# 304500) [Kim *et al.* 2007; Liu *et al.* 2010].

After that, several cases with variants in the *PRPS1* gene have been reported, including patients with intermediate phenotypes and intrafamilial variation of clinical features. Therefore, it was suggested that *PRPS1*-related diseases represent a disease continuum rather than four separate clinical entities [Al-Maawali *et al.* 2015; Gandia *et al.* 2015; Kim *et al.* 2016; Maruyama *et al.* 2016; Moran *et al.* 2012; Nishikura *et al.* 2019; Park *et al.* 2013; Porrmann *et al.* 2017; Robusto *et al.* 2015; Synofzik *et al.* 2014].

We have described a boy with a novel *PRPS1* loss-of-function variant and an atypical phenotype. Our patient presented with some features characteristic of Arts syndrome (e.g., global developmental delay and frequent respiratory infections) but had not developed hearing loss, the main clinical symptom of Arts syndrome, CMTX5, and DFN2 [Puusepp *et al.* 2020].

2.7 The *JAG2* gene encodes a Notch ligand

While pursuing human Notch ligands, the *JAG2* gene, encoding the Jagged2 protein, was assembled from cDNA clones similar to Serrate, a ligand of *Drosophila melanogaster* Notch [Luo *et al.* 1997; Valsecchi *et al.* 1997]. The Notch signaling pathway mediates developmental patterning cues in multicellular organisms. In humans, this pathway comprises four Notch receptor paralogs (Notch1–4) and five Notch ligands, Delta-like (Dll) 1, Dll3, Dll4, Jagged1, and Jagged2, which are all transmembrane proteins. A ligand on one cell binds to a receptor on another contacting cell resulting in the liberation of the Notch intracellular domain, which translocates to the nucleus and activates transcriptional regulation [Sjoqvist and Andersson 2019].

Notch receptors and ligands, including Jagged2, are ubiquitously expressed (https://www.proteinatlas.org/ENSG00000184916-JAG2/tissue, accessed on 20.02. 2021). However, of importance in the context of NMDs, is the significance of Notch signaling in myogenesis and maintenance of muscle stem cells / satellite cells. During development, Notch maintains the proliferative state of the satellite cells, whereas, in the adult, the satellite cells are kept quiescent [Mourikis and Tajbakhsh 2014]. Notch loss-of-function in mouse embryos resulted in

premature and excessive myogenic differentiation, which led to a loss of progenitor cells and muscle hypotrophy [Schuster-Gossler *et al.* 2007; Vasyutina *et al.* 2007]. Another study of immobilized chick fetuses demonstrated that flaccid paralysis decreases Notch activity. Of note, a reduced expression of Jagged2 was detected in the muscle fibers of the immobilized fetuses. The authors also revealed that the loss of Notch ligand in the muscle fibers is sufficient to decrease the number of fetal muscle progenitors [Esteves de Lima *et al.* 2016]. We report the first patients with disease-causing variants in the *JAG2* gene who present with a muscular dystrophy phenotype [Coppens *et al.* 2021].

2.8 Summary of the literature

Significant progress has been made in diagnosing NMDs and MDs during the last century, especially in the previous two decades. Powerful advancements in technology and bioinformatics have made essential contributions to molecular genetics, enabling the analysis and study of massive amounts of data. In addition, gene panel analyses and WES have become affordable and cost-effective, and the relatively high diagnostic yield makes these tests suitable as first-tier analyses for many inherited disease groups, including NMDs and MDs.

However, the clinical, imaging, biochemical, and pathological investigations have not lost their relevance. On the contrary, detailed phenotypic information is ever more important. With more and more genetic data, many unanswered questions and problems arise. To solve the emerging “unknowns”, we need to gather patients with similar phenotypes and genotypes. Therefore, data about the patients’ disease history, imaging studies, ENMG, muscle histology, enzyme activities, and metabolite concentrations are crucial. In addition, muscle, as the affected tissue in most NMDs and many MDs, is an invaluable resource for further confirmation of gene variants by IHC, Western blotting, enzyme analyses, transcriptomics, and proteomics.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To analyze the pathomorphological aspect of the muscle in patients with a clinical suspicion of a mitochondrial disorder (Publication I);
2. To characterize the association of *SPATA5* gene variants with mitochondrial dysfunction (Publication II);
3. To discover new disease-associated pathogenic variants in Estonian patients with a clinically suspected muscle disease of childhood-onset;
4. To investigate the clinical and functional effect of a novel hemizygous variant in the *PRPS1* gene (Publication III);
5. To delineate the phenotype, muscle histopathology, and the transcriptome profile of biallelic variants in a novel muscle disease-associated gene *JAG2* (Publication IV).

4. MATERIALS AND METHODS

4.1 Study subjects

4.1.1 Study group of patients with a suspicion of a mitochondrial disorder, including a patient characterized in detail (Publications I and II)

From January 2014 to March 2016, we gathered retro- and prospective study groups of patients with a clinical suspicion of an MD but without a precise genetic diagnosis to analyze the effectiveness of WES in these patients and to investigate the myopathological features in those patients of the study group who had a muscle biopsy. While the results of the effectiveness of WES in our study group are presented and discussed in the dissertation of Dr. Karit Reinson [Reinson 2018], the histopathological changes in the muscle are described and analyzed in the current dissertation.

We selected the retrospective study group from 181 patients consulted by a clinical geneticist from January 2003 to December 2013 with fibroblast cell cultures stored in the Department of Clinical Genetics, Tartu University Hospital, Estonia. We included 17 patients who had a strong suspicion of an MD, but genetic investigations at the time (targeted mtDNA and single nuclear gene analyses) resulted in no abnormal findings. The prospective study group consisted of eleven patients referred to a clinical geneticist in the Department of Clinical Genetics, Tartu University Hospital, from January 2014 to March 2016, with a childhood-onset disease, whose clinical geneticist had a suspicion of an MD.

Of the 28 study group patients, a muscle biopsy had been performed on 18 patients. However, we were able to reanalyze the frozen muscle biopsy specimens of 16 patients. One patient's muscle biopsy was performed at Helsinki University Clinic, and the muscle of another patient was unfortunately fixed in formalin and was therefore not usable for reanalysis.

The patient described in detail in section 5.2 was included in the retrospective study group; however, she did not have a muscle biopsy.

4.1.2 Study group of patients with a suspicion of a childhood-onset hereditary neuromuscular disorder, including two patients characterized in detail (Publications III and IV)

We gathered a retrospective study group of patients suspected of a childhood-onset hNMD to discover new disease-associated pathogenic variants and to analyze and correlate the histopathology of their muscle biopsy specimens.

We selected the study group from 201 patients who had a histopathological analysis of a frozen muscle biopsy specimen from January 2013 to December 2016 at the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. After revising the request forms of the muscle biopsy and

acquiring additional information from the referring physician, we included 74 patients with disease-onset during childhood (<18 years). Further stratification of the patients was based on reviewing the patients' muscle histology reports and medical records acquired from the referring physician or the electronic medical record software eHL. Figure 11 depicts the patient selection process.

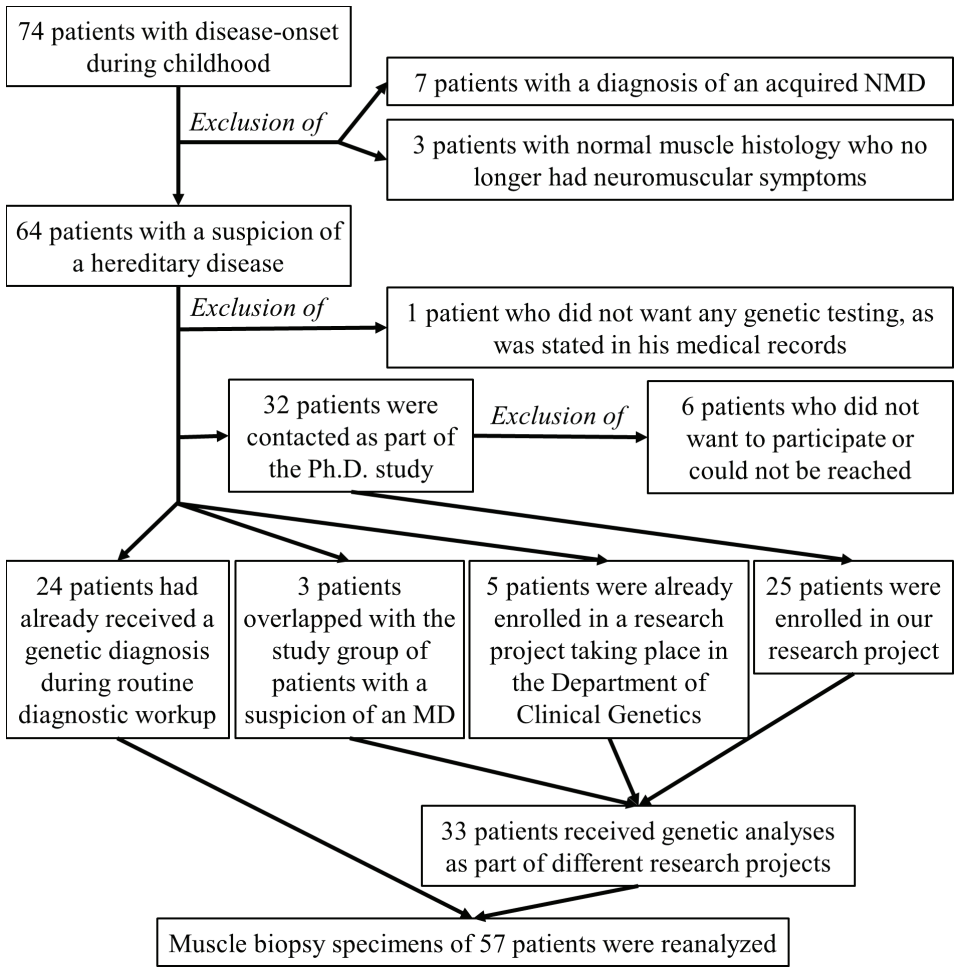


Figure 11. Scheme of the selection process of the study group of patients with a suspected childhood-onset hNMD.

4.2 Methods

4.2.1 Muscle biopsy specimen investigations (Publications I, III, and IV)

We investigated all available light microscopy slides, electron microscopy grids, and digitally archived photographs of the preparations of the muscle samples of the patients in both study groups. The light microscopy slides of all patients included HE, mGT, PAS, ORO, non-specific esterase, NADH-TR, and ATPase at pH 4.3 and 10.2 stains. SDH and COX stains had also been done on some muscle specimens. IHC had been performed by the immunofluorescence method, so the slides were no longer available for reanalysis. However, digitally archived photographs of the IHC stains were available for most muscle samples. IHC with primary antibodies targeted against the following proteins had been carried out on the majority of the muscles: laminin α2, α-, β-, and γ-sarcoglycans, dystrophin C-terminal epitope, laminin α4, and a marker for macrophages (an in-house antibody 1m2d7). In some cases, antibodies targeting δ-sarcoglycan, β-dystroglycan, dystrophin N-terminal and rod-domain epitopes, dysferlin, and laminin β1 had also been used. In addition, separate muscle biopsy pieces had been fixed in 2.5% glutaraldehyde-cacodylate solution and 1% osmium tetroxide and embedded in Epon resin for electron microscopy for all patients. Semi-thin sections of 2 μm stained with methylene blue and alkaline fuchsin and ultrathin sections of 70 nm stained with 2% uranyl acetate and lead citrate on 3 mm metal grids were available for most muscle samples.

We performed new stains on all available muscle biopsy specimens frozen in isopentane pre-cooled in liquid nitrogen stored at -80°C in the Department of Clinical Genetics, Tartu University Hospital. Additional 8 μm thick muscle sections were cut with a Leica CM1860 cryostat. We performed acid phosphatase, SDH, and combined COX-SDH enzyme reactions according to standard protocols [Dubowitz *et al.* 2021]. The primary antibodies used for IHC are listed in Table 2.

Table 2. List of primary antibodies used for IHC on the available muscle biopsy specimens.

Primary antibody	Supplier	Clone	Dilution
Anti-α-dystroglycan	Millipore	IIH6C4	1:200
Anti-β-dystroglycan	Novocastra	43DAG1/8D5	1:100
Anti-laminin β1	Millipore	4E10	1:200
Anti-collagen type VI	Millipore	VI-26	1:100
Anti-dysferlin	Abcam	ab124684	1:20
Anti-utrophin	Leica Biosystems	DRP3/20C5	1:10
Anti-myotilin	Leica Biosystems	RSO34	1:20
Anti-SERCA1	GeneTex	VE121G9	1:200
Anti-SERCA2	GeneTex	IID8	1:200
Anti-MyHCf	Leica Biosystems	WB-MHCF	1:25

Primary antibody	Supplier	Clone	Dilution
Anti-MyHCd	Leica Biosystems	RNMy2/9D2	1:20
Anti-MyHCn	Leica Biosystems	WB-MHCN	1:10
Anti-MHC I	Dako	W6/32	1:500
Anti-C5b-9	Dako	aE11	1:50
Anti-SQSTM1/p62	Abcam	ab56416	1:500
Anti-LC3	Nanotools Art	LC3-2G6	1:50
Anti-HSP70	Abcam	ab6535	1:100
Anti-BAG3	Abcam	polyclonal	1:500
Anti-PAX7	Covalab	polyclonal	1:50
Anti-myogenin	Quartett	polyclonal	1:50

4.2.2 Whole exome sequencing performed on the patient with variants in the *SPATA5* gene and functional studies (Publication II)

The DNA was extracted from blood lymphocytes. ServiceXS (Leiden, The Netherlands) prepared the library using SureSelect XT Human All Exon v5 enrichment kit (Agilent Technologies, Santa Clara, CA) and sequenced the run with an Illumina HiSeq sequencer. We aligned the raw sequencing reads from fastq files to the hg19 reference genome using Burrows-Wheeler Aligner (BWA) [Li and Durbin 2010] and performed the bioinformatic processing, variant calling, and annotation following Genome Analysis Toolkit (GATK) best practice guidelines [Van der Auwera *et al.* 2013] using Picard, GATK [DePristo *et al.* 2011; McKenna *et al.* 2010], Annovar [Wang *et al.* 2010] and SnpSift [Cingolani *et al.* 2012] software.

Prof. Allen Kaasik and his group performed functional studies on rat cortical neurons to analyze the effect of SPATA5 deficiency on the CNS. Detailed descriptions of the methods used are available in Puusepp *et al.* [Puusepp *et al.* 2018a]. In short, primary cultures of rat cortical cells were prepared from neonatal Wistar rats. The *SPATA5* gene was silenced by transfecting the neurons with plasmids expressing short hairpin RNA (shRNA) targeted against rat *SPATA5*. Transfection with scrambled shRNA plasmids was used as control. The subcellular localization of *SPATA5* was detected with IHC using rabbit anti-myc primary antibody (Abcam, clone ab9106, dilution 1:300). Mouse anti-TOMM20 antibody (Abcam, clone ab56783, dilution 1:200) was used as a mitochondrial outer membrane marker. The cortical cell cultures were transfected with mito-KikGR1 plasmid and examined by a laser scanning confocal microscope to analyze the mitochondrial fusion and fission events. ATP/ADP ratio and axonal growth were measured using plasmids expressing GW1-PercevalHR and neuron-specific pAAV-hSyn-DsRedExpress, respectively.

4.2.3 Molecular investigations performed on the patients with a suspicion of a hereditary neuromuscular disorder (Publications III and IV)

The DNA was extracted from blood lymphocytes in all cases. In five patients, including the patient with a variant in the *PRPS1* gene, we performed a panel sequencing covering >4,800 genes associated with monogenic disorders. The panel was sequenced and analyzed as described by Pajusalu *et al.* [Pajusalu *et al.* 2018]. In short, libraries were generated using TruSight One kits (Illumina Inc., San Diego, CA, USA) and sequenced on MiSeq or HiSeq platforms (Illumina) to mean sequencing depth of at least ×70 and ×150, respectively. In-house bioinformatics and variant annotation pipelines were used.

WES was carried out at different institutions. For 24 patients, including two patients investigated with the expanded gene panel sequencing with no abnormal findings, the DNA was sent to GenomeScan (Leiden, The Netherlands). Libraries were prepared using SureSelect XT Human All Exon v7 enrichment kit (Agilent Technologies, Santa Clara, CA). Sequencing was carried out on NovaSeq 6000 (Illumina) platform. The GATK v4 best practice guidelines using wdl scripts and Cromwell workflow management system were used for fastq file processing and variant calling [Van der Auwera *et al.* 2013]. Shortly, raw sequencing reads were mapped to the hg19 reference genome using the BWA MEM algorithm (v 0.7.17) [Li and Durbin 2010], followed by the processing using GATK (v4.1.4.0), including flagging duplicated reads and recalibrating base quality scores. Variants were first called for individual samples using GATK HaplotypeCaller, before multi-sample joint aggregation and re-annotation using GATK GenotypeGVCFs. The variant call set was annotated using hail 0.1 (Hail Team. Hail 0.1. https://github.com/hail-is/hail) and then uploaded to the Seqr platform (https://github.com/broadinstitute/seqr) for collaborative variant analysis.

In the patient with variants in the *JAG2* gene and three other patients, WES was performed as part of routine clinical investigations. The sequencing was performed at the Estonian Genome Centre of the University of Tartu (Tartu, Estonia) using SureSelect XT Human All Exon v5 (Agilent Technologies, Santa Clara, CA) WES enrichment kit and the HiSeq 4000 (Illumina) platform, which resulted in no abnormal findings. As part of a collaboration project, the WES data of these four patients were reanalyzed at the Broad Institute of MIT (Massachusetts Institute of Technology) and Harvard (Cambridge, MA, USA). Aligned WES data were reverted to fastq files ensuring the original base quality was restored. A temporary unaligned sam file was created to generate independent alignment metrics and identify sample QC outliers. The data was processed through a pipeline based on Picard, using base quality score recalibration and local realignment at known insertions/deletions (indels). We used the BWA aligner for mapping reads to the human genome build 37 (hg19). Single nucleotide variants (SNVs) and indels were jointly called across all samples using GATK HaplotypeCaller package version 3.4. Default filters were applied

to SNV and indel calls using the GATK Variant Quality Score Recalibration approach. Lastly, the variants were annotated using Variant Effect Predictor. The variant call set was uploaded to the Seqr platform, and analysis was performed using the various inheritance patterns.

Sanger sequencing was used to confirm all candidate variants found on the gene panel or WES and to perform familial segregation analysis.

One patient's DNA was sent to Leiden University Medical Center (The Netherlands) for FSHD type 1 genetic analysis. The length of the polymorphic D4Z4 subtelomeric repeat region at 4q35 was analyzed by Southern blotting. The D4Z4 region was delineated by EcoRI sites and detected with the probe P13E-11. Subsequent restriction analysis with BlnI enzyme was used to digest a 10q26 fragment to which the probe also hybridizes due to similarity.

4.2.4 Purine and pyrimidine metabolite and phosphoribosylpyrophosphate synthetase analyses of the patient with a variant in the *PRPS1* gene (Publication III)

Urinary purine and pyrimidine metabolites were measured with ultra-performance liquid chromatography-tandem mass spectrometry (Waters, Milford, MA, USA), modified from Hartmann *et al.* [Hartmann *et al.* 2006]. PRPS activity was determined in a reaction mixture containing an aliquot of erythrocytes, 3 mM or 32 mM sodium phosphate, 1 mM dithiothreitol, 4.5 mM $MgCl^2$, 1.0 mM ATP, 1 mM ribose-5-phosphate, and 50 mM Tris HCl (pH 7.4). The purines were removed by dilution of 100 µL frozen-packed erythrocytes with 400 µL 0.9% (w/v) NaCl and concentrated on an Amicon Ultra Ultracel 10 K Membrane filter (Millipore) by centrifugation (14,000 g at 4°C for 60 minutes), essentially as described before [Al-Maawali *et al.* 2015]. Separation of AMP, ADP, and ATP was performed using a gradient from 100% to 70% 0.75 mM sodium phosphate (pH 4.55) in 20 minutes at a flow rate of 1.0 mL/min by HPLC on an ion exchange column (Whatman PartiSphere SAX 125 mM × 4.6 mM, 5 µm particle size; VWR International, Amsterdam, the Netherlands) and a guard column (Whatman PartiSphere AX 10 mM × 2.5 mM, 5 µm particle size; VWR International) with online UV detection at 254 nm. The PRPS activity was calculated as follows: ([AMP] + ½[ADP])/(mg protein × incubation time).

4.2.5 RNA sequencing of the patient with variants in the *JAG2* gene (Publication IV)

RNA was extracted from a muscle sample and sequenced by the Genomics Platform at the Broad Institute of MIT and Harvard. The transcriptome product combines poly(A)-selection of mRNA transcripts with strand-specific cDNA library preparation with a mean insert size of 550 basepairs (bp). Libraries were sequenced on the HiSeq 2500 platform to a minimum depth of 50 million STAR-aligned reads. External RNA Controls Consortium RNA spike-in control

mixes were included for all samples, allowing additional control of variability between samples. STAR (v.2.5.3) aligner [Dobin *et al.* 2013] was used to map sequencing reads to the hg38 reference genome, and GENCODE v.26 [Frankish *et al.* 2019] was used to define genes and transcripts. We detected expression outliers with R package OUTRIDER [Brechtmann *et al.* 2018], comparing the patient's skeletal muscle RNA sequencing results with skeletal muscle RNA sequencing data from 12 individuals with other myopathies and 100 control individuals from GTEx (dbGaP:phs000424.v8.p2). We calculated gene expression alterations (log2 fold changes) by comparing the normalized read counts for each sample and gene to normalized mean read counts from all affected and control individuals. The *p* values were calculated based on the fitted negative binomial model and then adjusted via the Benjamini-Yekutieli false-discovery rate method. Statistical significance was determined with adjusted *p* values with a cutoff of 0.05.

4.3 Ethics

The Research Ethics Committee of the University of Tartu approved the study of patients with a suspicion of an MD (Publications I and II) [approval date 17.11.2014, number 242/M-10; renewal date 20.11.2017, number 242/M-11(2)] and the study of patients with a suspicion of an hNMD (Publications III and IV) [approval date 19.12.2016, number 265/T-12; renewal date 12.16.2019, number 299/M-13] in agreement with the Declaration of Helsinki. Written informed consent was obtained from all patients, their parents, or legal guardians in cases where we performed genetic analyses as part of either of the two studies, except when the proband was deceased.

The muscle biopsy specimens, which were analyzed for the two studies, had already previously been taken as requested by the patients' treating physicians. Therefore, no new muscle biopsies were performed as part of the studies. In addition, all patients who received genetic analyses as part of the studies presented different symptoms, and they or their parents showed interest in finding the cause of their or their child's disease. Lastly, we only reported those variants to the patients and their families, which we could confirm to be associated with the patient's phenotype. The disease association was affirmed based on previous publications on the same variant, additional biochemical analyses showing, e.g., decreased enzymatic activity as was the case in the patient with a variant in the *PRPS1* gene, or extensive international collaboration to establish the disease-association of a new gene as was the case in the patient with variants in the *JAG2* gene.

5. RESULTS AND DISCUSSION

5.1 The comparison of myopathology and genotype in patients with a clinical suspicion of a mitochondrial disorder (Publication I)

A detailed histological analysis of frozen and glutaraldehyde-fixed skeletal muscle tissue specimens was performed in all study group patients suspected of an MD who had a muscle biopsy. Table 3 summarizes these patients' genotype, phenotype, and myopathology (the patient identification numbers correlate with our publication) [Puusepp *et al.* 2018b]. In addition, a detailed discussion of the patients' genotype and myopathology is presented below.

The WES analysis revealed pathogenic/likely pathogenic variants in known genes in eight patients, matching known phenotypes (Table 3A). Patient 2 had compound heterozygous variants, c.1377G>A, p.(Met459Ile) and c.3154G>C, p.(Gly1052Arg), in the nuclear *POLG* gene. As the *POLG* gene encodes DNA polymerase γ, which is responsible for mtDNA replication, its defects are associated with mtDNA depletion or multiple deletions. Clinically, patients present with a spectrum of overlapping phenotypes. Muscle histology may be normal or show features of mitochondrial damage [Rahman and Copeland 2019]. Our patient presented with several features characteristic of a *POLG*-related disorder. While the mtDNA deletion-duplication analysis from muscle did not reveal any aberrations, the mtDNA depletion study was not performed. Muscle histopathology revealed typical features of mitochondrial myopathy (Figure 12A) and signs of a neurogenic process correlating well with the genetic diagnosis. In addition, this patient had a heterozygous c.1655G>A, p.(Arg552Gln) variant in the *RYR1* gene inherited from his mother. This particular variant has previously been reported in the literature in two patients with hereditary neuropathy and two individuals with malignant hyperthermia [Klingler *et al.* 2014; Schabhuttl *et al.* 2014]. The muscle histology of our patient did not show any cores, minicores, central nuclei, or fiber-type disproportion, which would be characteristic of an autosomal dominant *RYR1*-related pathology. The patient's mother reported mild limb pain and fatigue, but her neurological examination was normal. ENMG showed signs of myopathy in proximal upper limbs; however, her muscle revealed normal histology. Nevertheless, *RYR1*-related disorders present a broad range of phenotypes, from severe congenital myopathy to malignant hyperthermia susceptibility with no muscle involvement. Similarly, muscle microscopy may show typical structural changes or normal histology [Lawal *et al.* 2020].

Patient 3 harbored the m.9176T>C variant in the mitochondrial *MT-ATP6* gene (Table 3A), which causes an approximate 30% deficit in mitochondrial ATP production in yeast, as revealed by previously performed functional studies [Kucharczyk *et al.* 2010]. The two muscle biopsy samples of our patient showed features of a mitochondriopathy (Figure 12B and C) correlating well

with an MD diagnosis. Interestingly, no neurogenic changes were seen in the biopsied muscle, although the patient's ENMG revealed the presence of axonal neuropathy. The heteroplasmy level of the m.9176T>C variant in our patient was measured from three tissues showing essentially equal levels in all. A high correlation of degrees of heteroplasmy of *MT-ATP6* variants in different tissues was also reported in a large cohort of patients [Stendel *et al.* 2020]. Therefore, a diagnosis of *MT-ATP6*-associated disease can be reached by mtDNA analysis from blood.

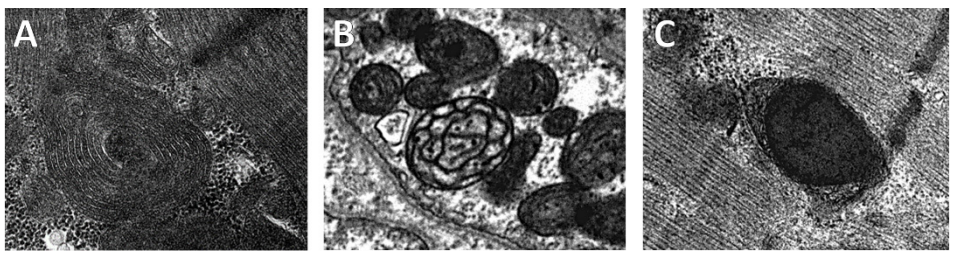


Figure 12. Ultrastructure of skeletal muscle tissue revealing (A) mitochondria with concentric cristae in patient 2 with *POLG* variants and mitochondria with (B) loss of cristae and (C) a lipid inclusion in patient 3 with an *MT-ATP6* variant.

Patient 4 presented with a phenotype characteristic for SMA type I (Table 3A). However, the deletion/duplication analysis of the *SMN1* gene revealed only a heterozygous deletion of exons 7 and 8. Therefore, a muscle biopsy was performed, which showed histopathological changes specific for SMA type I consisting of grouped atrophic type II fibers while many type I fibers were hypertrophic. However, a nearly absent COX reaction was also found (Figure 13), which prompted the possibility of a concomitant MD. WES revealed a novel frameshift c.410dup, p.(Asn137LysfsTer11) variant on the other allele of *SMN1,* confirming the diagnosis of SMA [Sarv *et al.* 2021]. However, disease-causing variants in MD-associated genes were not found. Nevertheless, growing evidence shows that mitochondrial dysfunction plays an important role in the pathogenesis of SMA. Several studies on the muscle tissue of the patients and animal and cell culture models have revealed defects in OXPHOS activity [James *et al.* 2021]. A systematic review highlighted that all muscle samples from 24 molecularly confirmed SMA type I, II, and III patients showed an overall decrease in COX activity with many COX-negative fibers. The most severe COX deficiency was evident in SMA type I patients [Ripolone *et al.* 2015]. The COX stain of our patient was even more strikingly reduced than that reported by Ripolone *et al.* [Ripolone *et al.* 2015], but the staining pattern correlates well with the published photos.

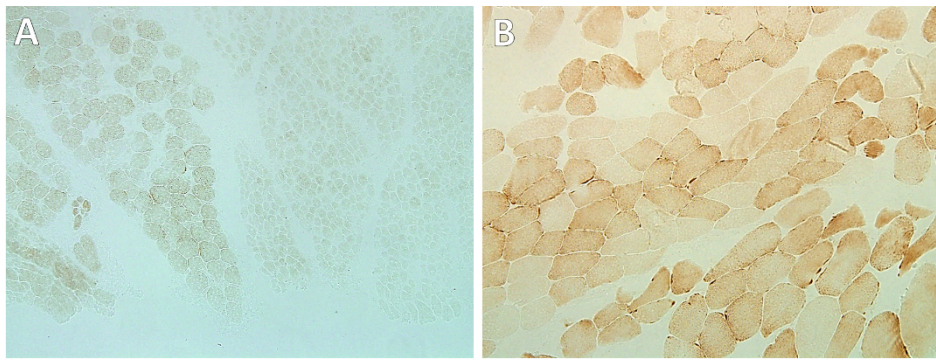


Figure 13. Muscle sections stained with COX showing (A) a strong reduction of the enzyme in patient 4 with SMA type I compared to (B) the normal stain of a control.

Patient 8 had a heterozygous c.5609 T > C, p.(Leu1870Pro) variant in the *MYH2* gene (Table 3A). The same variant in the heterozygous state has previously been reported in a patient with a similar phenotype to ours with severe dysphagia requiring gastrostomy after birth. However, the reported child also had congenital ptosis and external ophthalmoparesis, which was not noted in our patient [D'Amico *et al.* 2013]. While both autosomal dominant and recessive modes of inheritance have been described, clear phenotypic distinctions cannot be made between the two entities [Cabrera-Serrano *et al.* 2015; D'Amico *et al.* 2013; Findlay *et al.* 2018; Hernandez-Lain *et al.* 2017; Lossos *et al.* 2013; Madigan *et al.* 2021; Martinsson *et al.* 2000; Tajsharghi *et al.* 2014; Tajsharghi *et al.* 2010; Telese *et al.* 2020; Tsabari *et al.* 2017; Willis *et al.* 2016]. The phenotypes include congenital joint contractures, feeding problems in infancy, dysphagia, ptosis, external ophthalmoparesis, and general, limb-girdle, distal, or facial weakness. Hand tremor, a feature present in our patient, was also reported in 13 patients from a large pedigree with autosomal dominant myopathy, the first report of *MYH2* disease association [Darin *et al.* 1998; Martinsson *et al.* 2000]. Weakness of the extraocular muscles is a consistent finding in *MYH2*-related myopathy. However, the onset age of ophthalmoparesis is variable. Of note, one patient presented with weak suck in infancy but developed ophthalmoparesis after 40 years of age [Tajsharghi *et al.* 2014]. Therefore, it is most probable that our patient will develop ophthalmoparesis at some point in the disease course. As *MYH2* encodes the myosin heavy chain type IIa, it is not surprising that muscle histology revealed atrophy/hypotrophy or absence of type 2A muscle fibers in all the published cases. The type 2B fibers were of normal size. Most of the patients also presented with type 1 fiber predominance or uniformity. Some muscle samples from adult patients showed myopathic or dystrophic changes with rimmed vacuoles [Findlay *et al.* 2018; Madigan *et al.* 2021; Martinsson *et al.* 2000; Telese *et al.* 2020]. In addition, core-like structural changes [Cabrera-Serrano *et al.* 2015; Willis *et al.* 2016], filamentous tangles with nemaline rods [Madigan *et al.* 2021], and hybrid fibers [Willis *et al.* 2016] were reported in some cases. Our patient's skeletal muscle showed

type 1 fiber predominance, many small type 2 fibers co-expressing MyHCn and/or MyHCd, and cytoplasmic and rod-like bodies (Figure 14). Our case shows further evidence of hybrid fibers and abnormal inclusions in *MYH2*-related pathology.

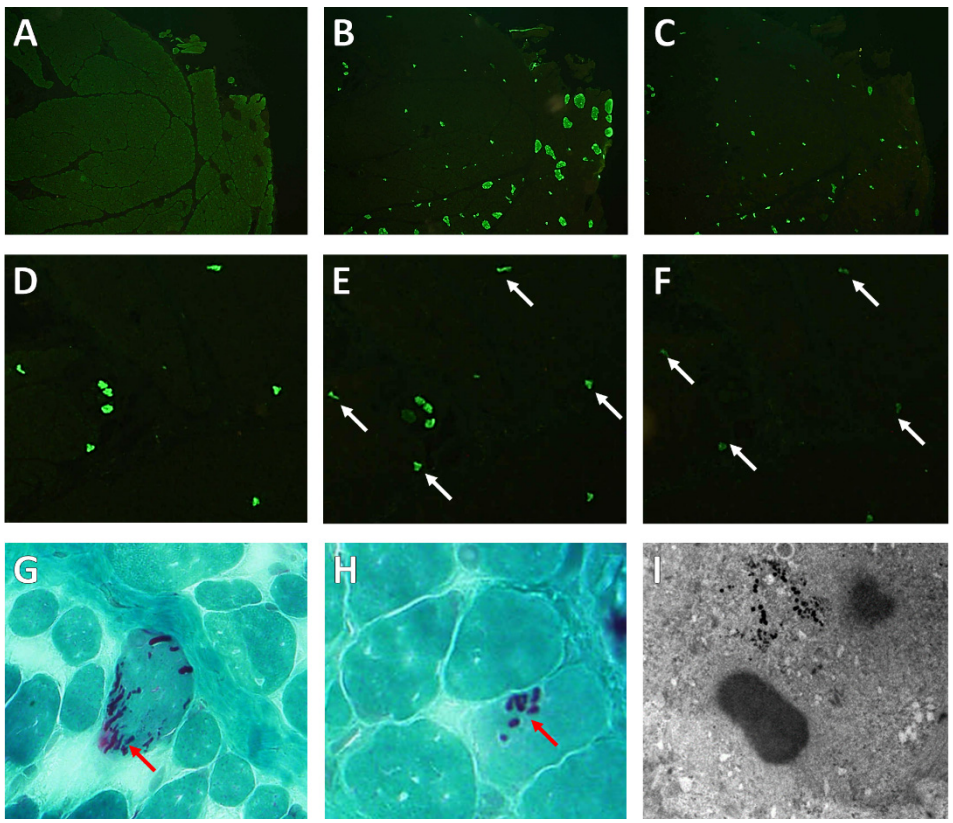


Figure 14. Muscle sections of patient 8 with an *MYH2* variant showing (A) type 1 fiber predominance (anti-MLC2E8), (B) some normal-sized and many tiny type 2 fibers (anti-MyHCf), (C) MyHCn co-expression in small type 2 fibers, (D) higher magnification of the small type 2 fibers (anti-MyHCf) co-expressing (E) MyHCn and (F) MyHCd (arrows), (G) rod-like bodies (arrow; mGT), (H) cytoplasmic bodies (arrow; mGT), and (I) ultrastructure of the cytoplasmic bodies.

Patient 9 was diagnosed with Vici syndrome (MIM# 242840) caused by a novel homozygous frameshift variant, c.6690delT, p.(Asn2230LysfsTer6), in the *EPG5* gene (Table 3A). Since the original description of the syndrome [Dionisi Vici *et al.* 1988] and the association with variants in the *EPG5* gene [Cullup *et al.* 2013], more than 50 molecularly confirmed cases of Vici syndrome have been published. The principal diagnostic features are absence of the corpus

callosum, profound developmental delay, failure to thrive, hypopigmentation of skin and hair, immune problems, progressive microcephaly, cardiomyopathy, and cataracts [Byrne *et al.* 2016]. Vici syndrome is caused by defective autophagosome-lysosome fusion [Hori *et al.* 2017]. Hence, the muscle pathology was consistent with a vacuolar myopathy in most studied cases. Glycogen accumulation and mitochondrial pathology were also frequently reported [Balasubramaniam *et al.* 2018; Hedberg-Oldfors *et al.* 2017]. Our patient suffers from genetically confirmed Vici syndrome with a typical phenotype and myopathology consisting of myopathic changes, autophagic vacuoles, abundant intracellular debris, which is often membrane-bound and also deposited within basal lamina layers with evidence of ongoing exocytosis, and poorly formed myofibrils admixed with the debris (Figure 15).

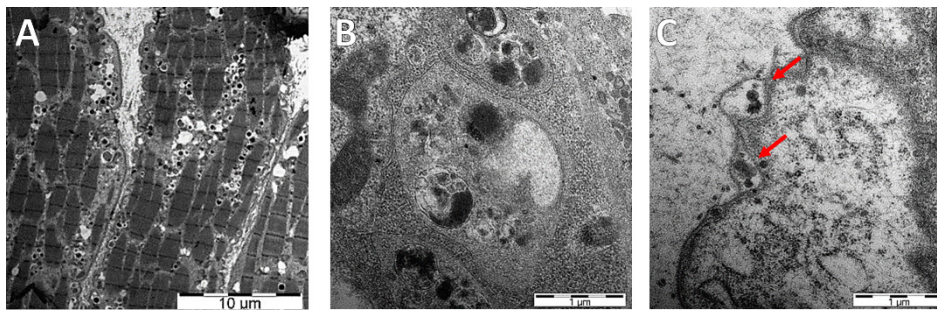


Figure 15. Ultrastructure of the tibial muscle of patient 9 with Vici syndrome revealing (A) intracellular autophagic debris consisting of (B) electron-dense granular and membranous material and (C) debris located between the layers of the basal lamina (arrows).

Patient 16 was found to have a *de novo* c.207_209delGGT, p.(Val70del) variant in the *LMNA* gene (Table 3A). *LMNA* encodes intermediate filaments lamin A and C (by differential splicing), which form the nucleoskeleton beneath the inner nuclear membrane. Defects in these proteins mainly affect the skeletal and cardiac myocytes, epithelial cells, and adipose tissue [Maggi *et al.* 2021]. The phenotype of our patient matched with *LMNA*-related CMD (MIM# 613205). In a large cohort of *LMNA*-related CMD patients, muscle histology disclosed dystrophic changes (76%, 96/127), non-specific myopathic changes (22%, 28/127), inconclusive findings (two patients), or normal histology (one patient) [Ben Yaou *et al.* 2021]. Nuclear anomalies have also been detected [Sabatelli *et al.* 2001]. Our patient's muscle biopsy specimen demonstrated myopathic/dystrophic changes and abnormal nuclei (Figure 16A–D). It has been hypothesized that in addition to impaired gene expression and mechanosignaling, altered desmin localization resulting in mitochondrial dysfunction and impairment of autophagy may also play a role in the pathogenesis of *LMNA*-related muscle disease [Maggi *et al.* 2021]. In this regard, we found small aggregations

of swollen mitochondria with electron microscopy (Figure 16E and F), although this finding could also be an artifact. Nevertheless, we did not see mitochondrial inclusions, COX-negative fibers, or RRFs.

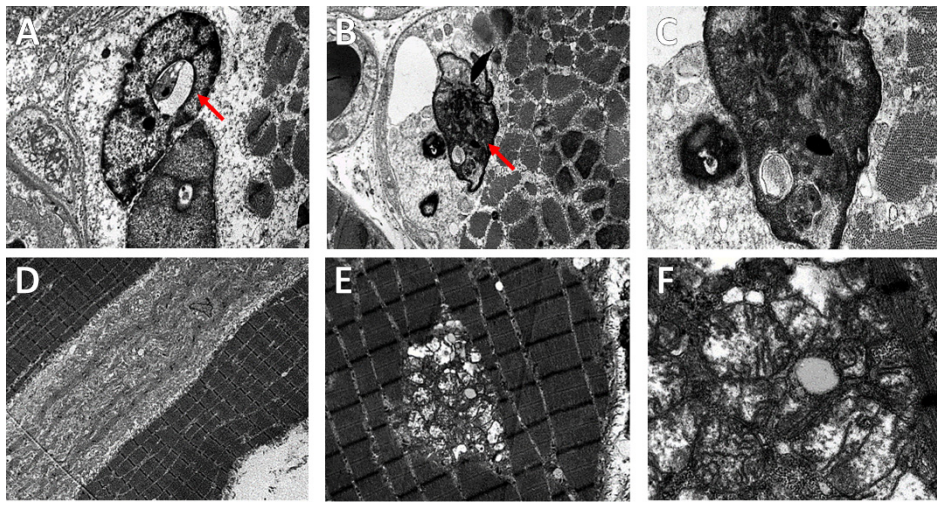


Figure 16. Ultrastructure of the deltoid muscle of patient 16 with an *LMNA* variant showing (A) nuclei with invaginations (arrow), (B and C) a nucleus with strange inclusions (arrow), (D) severe myofibrillar degeneration in the fiber between two fibers with normal myofibrils, and (E and F) small aggregations of swollen mitochondria.

Patients 19 and 24 were diagnosed with the brain-lung-thyroid syndrome (MIM# 610978) and developmental and epileptic encephalopathy (MIM# 613721) due to a *de novo* variant in the *NKX2-1* gene, c.578A > C, p.(His193Pro), and the *SCN2A* gene, c.4405A>T, p.(Ile1469Phe), respectively (Table 3A). Primary myopathy is not part of these diseases' clinical spectrum; hence, no information regarding muscle pathology can be found in the literature. Our patients' muscle biopsy specimens revealed only non-specific changes.

In two cases (patients 1 and 17), we found variants in known disease genes but with features not previously described (Table 3B). In patient 1, a *de novo* c.239G>A, p.(Arg80His) variant in the *SLC25A4* gene was found, which encodes a mitochondrial ADP/ATP carrier isoform 1 protein. Analyses of the patient's muscle tissue confirmed the presence of mitochondrial dysfunction with characteristic histopathological changes, reduced respiratory chain complex I and IV enzyme activities, and decreased mtDNA copy number (approximately 34%). However, the phenotype of our patient was much more severe than previously described in the literature. Nevertheless, six other patients with similar severe phenotypes and *de novo SLC25A4* variants were found through international collaboration, and further functional studies confirmed a new disease phenotype for *SLC25A4* [Thompson *et al.* 2016].

In patient 17, compound heterozygous variants, c.4315T>A, p.(Trp1439Arg) and c.472_478delGCCTTCC, p.(Ala158ThrfsTer6), in the *CACNA1A* gene were found (Table 3B). Segregation analysis of the variants revealed that the parents and three siblings with mild symptoms harbored one of the two variants, and one deceased sibling with a severe phenotype similar to the proband was also compound heterozygous. Our family represents the first reported case of an autosomal recessive inheritance pattern for *CACNA1A*-related disorders [Reinson *et al.* 2016]. The patient's muscle specimen showed only non-specific changes.

In one case (patient 18, Table 3C), we established a novel gene-disease association with a homozygous c.2449_2450delinsTG, p.(Gln817Ter) variant in the *TSPOAP1* gene causing an autosomal recessive dystonia syndrome [Mencacci *et al.* 2021]. *TSPOAP1*, also known as *BZRAP1* was first identified as a peripheral benzodiazepine receptor-associated protein localizing in the cytoplasm and partially in the mitochondria [Galiegue *et al.* 1999]. Subsequently, its protein product was associated with RIMs (presynaptic active zone proteins) and hence, was named RIM-binding protein 1 (RIM-BP1). RIM-BP1 is a scaffolding protein between RIMs and voltage-gated calcium channels in the presynaptic axon terminals, ensuring fast neurotransmitter release [Acuna *et al.* 2015]. It was also found that RIM-BP1 was specifically expressed throughout rat brains [Mittelstaedt and Schoch 2007; Wang *et al.* 2000]. However, Hibino *et al.* [Hibino *et al.* 2002] demonstrated that RIM-BPs were expressed not only in axons but also in dendrites and neuronal cell bodies, indicating that RIM-BPs could be implicated in other cellular processes as well. Of note, a recent computational study analyzing canonical pathways of *TSPOAP1* indicated mitochondrial dysfunction as one of the top pathways [Suthar *et al.* 2021]. In addition, according to the Human Protein Atlas database (https://www.proteinatlas.org/ENSG00000005379-TSPOAP1/tissue, accessed on 07.05.2022), *TSPOAP1* mRNA is ubiquitously expressed across tissues. Furthermore, our patient's muscle histology revealed scattered denervated fibers, which could indicate a neuromuscular junction dysfunction, but also myopathic changes with lobulation and one COX-negative fiber (Figure 17). Hence, our findings further hint at the possible association of *TSPOAP1* with mitochondrial pathology / morphological alterations.

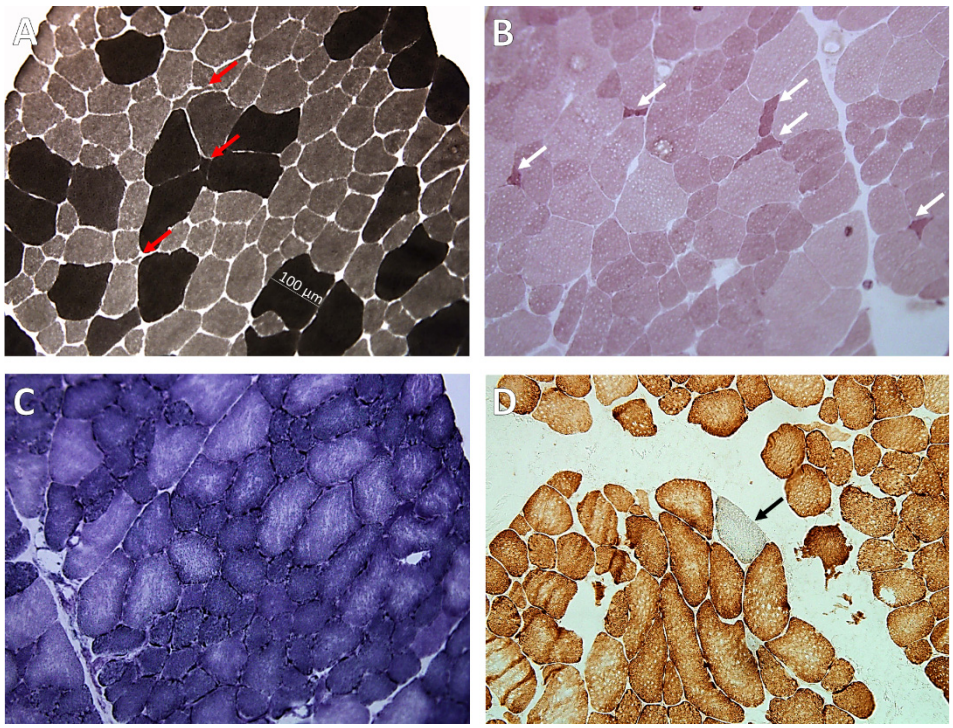


Figure 17. Muscle sections of patient 18 with a homozygous *TSPOAP1* variant showing (A) fiber size variation with fiber diameter 10–100 µm and scattered angular atrophic fibers of both fiber types (arrows; ATPase at pH 10.2) staining intensely on (B) non-specific esterase (arrows), (C) an uneven internal structure and subsarcolemmal accumulations of mitochondria (NADH-TR), and (D) one COX-negative fiber (arrow; COX-SDH).

We found variants in novel candidate genes in two cases (patients 6 and 20, Table 3D). In patient 6 with congenital developmental delay, sensorineural hearing loss, spasticity, and dystonia, we found a *de novo* splice donor variant, c.578+1_578+2del, in the *KIAA0408* gene. RNA sequencing from the patient's muscle tissue showed that this variant causes exon skipping. Very little is known about the *KIAA0408* gene. According to the Human Protein Atlas database (https://www.proteinatlas.org/ENSG00000189367-KIAA0408, accessed on 07.05.2022), the gene has a high expression in brain and skeletal muscles. Its tissue expression is clustered with genes involved in action potential transmission in striated muscle, and cell line expression is clustered with genes involved in nervous system development. Therefore, *KIAA0408* is a promising candidate for our patient.

In patient 20 (Table 3D), we identified a *de novo* 90 kilobase (kb) deletion involving the last two exons of the *RBFOX1* gene which encodes an RNA-binding protein and regulates tissue-specific splicing [Jin *et al.* 2003]. It is

highly expressed in the brain and skeletal muscles. Several animal models have been engineered to study the function of *RBFOX1*. For example, knockout of cytoplasmic or nuclear isoforms of *RBFOX1* in ventricular zone progenitor cells of mouse embryos resulted in neuronal migration defects and impaired axonal growth, mice with CNS knockout of *RBFOX1* developed spontaneous seizures, and adult mice deficient in skeletal muscle *RBFOX1* demonstrated progressive weakness, tubular aggregate formation, and impaired calcium handling [Conboy 2017]. Moreover, *RBFOX1* gene haploinsufficiency caused by a *de novo* translocation was reported in three patients with epilepsy, intellectual disability, and autism spectrum disorder [Bhalla *et al.* 2004; Martin *et al.* 2007]. In addition, several other patients with intragenic deletions or duplications in *RBFOX1* and autism spectrum disorder, developmental delay, intellectual disability, or epilepsy have been reported [Bill *et al.* 2013]. Therefore, *RBFOX1* is a promising candidate gene as our patient presents both CNS and muscle involvement with developmental delay, intellectual disability, epilepsy, muscle weakness and atrophy, and myopathic histopathological changes.

Table 3. Summary of the genotype, phenotype, and myopathology of patients suspected of an MD with variants in known, novel, or candidate genes.

№	Genotype[a]	Phenotype	Myopathology
A. Patients with variants in known disease genes with matching phenotypes			
2	*POLG* (NM_002693.2): 1) c.1377G>A, p.(Met459Ile) – het, maternal, novel, LP 2) c.3154G>C, p.(Gly1052Arg) – het, paternal, novel, LP *RYR1* (NM_001042723.2): c.1655G>A, p.(Arg552Gln) – het, maternal, recurrent, LP	Male, disease onset at 6y, moderate progression, died at 26y. Features: severe spastic tetraparesis, scoliosis, distal limb muscle atrophy, hypomimic face, severe dysarthria and dysphagia, ataxia and tremor in upper limbs, and severe dementia. Brain MRI: progressive diffuse cerebral and mild cerebellar atrophy. SPECT: presynaptic dopaminergic hypofunction in the striatum. ENMG: signs of lower motor neuron damage. CK normal.	At 12y, muscle unknown: three SDH+ COX– fibers. At 14y, *m. tibialis anterior*: mitochondrial myopathy (fiber size variation, internal nuclei, three RRFs, EM: mitochondria with concentric wheels and loss of cristae) and mild neurogenic changes. No COX– fibers.
3	*MT-ATP6* (NC_012920.1): m.9176T>C, p.ATP6:(Leu217Pro) – recurrent, pathogenic, heteroplasmy 96% in muscle, 95% in blood, 96% in urinary epithelial cells	Female, disease onset at 2y, slow progression, currently 29y. Features: speech delay, high-arched feet and hammer toes, mild scoliosis, spastic tetraparesis, dysarthria, dysphagia, hyperopia, astigmatism, and bilateral SNHL. Brain MRI: mild enlargement of lateral ventricles and cisterna magna. ENMG: sensorimotor axonal neuropathy. CK normal/slightly elevated. CSF lactate elevation (2.9 mmol/L).	At 10y, *m. tibialis anterior*: mitochondriopathy (mild mitochondrial proliferation, EM: mitochondria with concentric wheels, paucity of cristae, lipid and small electron dense inclusions, and Z-streaming). No COX– fibers. At 14y, muscle unknown: same findings as previously. No COX– fibers.
4	*SMN1* (NM_000344.3): 1) deletion of exons 7 and 8 – het, recurrent, pathogenic 2) c.410dup, p.(Asn137LysfsTer11) – het, novel, pathogenic	Female, disease onset *in utero*, rapid progression, died at 3m. Features: decreased fetal movements, severe muscular hypotonia after birth, muscle weakness requiring tube feeding, breathing difficulties, absent DTR, tongue fasciculations, contractures of hips and knees, GERD, and congenital hypothyroidism. Brain MRI: normal. ENMG: lower motor neuron damage. CK normal.	At 2m, *m. tibialis anterior*: specific pattern for SMA type I (large and small group atrophy of both fiber types and hypertrophic type 1 fibers), strongly reduced/nearly absent COX.

№	Genotype[a]	Phenotype	Myopathology
8	*MYH2* (NM_017534.6): c.5609 T > C, p.(Leu1870Pro) – het, *de novo*, recurrent, pathogenic	Male, disease onset at 3 weeks of life, minimal progression, currently 14y. Features: inertness in infancy, motor DD, speech delay, severe dysphagia (gastrostomized), dysarthria, proximal muscle weakness, muscular hypotonia and atrophy, gait ataxia, and tremor in hands. Brain MRI normal. ENMG normal. CK 1,486–3,000 U/L at onset, later normal.	At 2m, muscle unknown: myopathic (fiber size variation, internal/central nuclei, regenerating fibers), cytoplasmic and rod-like bodies. COX stain not performed. At 1y, muscle unknown: myopathic (type 1 fiber predominance, fiber size variation), type 2 fiber atrophy. No COX– fibers.
9	*EPG5* (NM_020964.2): c.6690delT, p.(Asn2230LysfsTer6) – hom, *in trans*, novel, pathogenic	Male, disease onset at birth, died at 1y. Features: lethargy and poor sucking since birth, severe DD, failure to thrive, spastic tetraparesis, dysphagia requiring tube feeding, hypotonic trunk, hypomimic face, focal epilepsy, horizontal nystagmus, bilateral optic atrophy, cataracts, mild hepatosplenomegaly, and hypertrophic cardiomyopathy. Brain MRI: agenesis of the corpus callosum. ENMG not done. CK normal.	At 9m, *m. tibialis anterior*: vacuolar myopathy (fiber size variation, atrophy of both fiber types, internal nuclei, few vacuoles, EM: lots of autophagic debris in the sarcoplasm and between the layers of the basal lamina, Z-streaming, and swelling of some mitochondria). No COX– fibers.
16	*LMNA* (NM_170707.2): c.207_209delGGT, p.(Val70del) – het, *de novo*, novel, pathogenic	Female, disease onset at 2m, rapid progression, died at 5y9m. Features: muscular hypotonia, motor DD (later regression), dropped head, axial, limb, and respiratory muscle weakness, partial ptosis dex>sin, myopathic face, dysarthria, muscular atrophy, absent DTR, lumbar hyperlordosis, scoliosis, and elbow and ankle contractures. ECG: partial blockade of the right bundle of His. Brain MRI normal. ENMG: myopathic changes. CK 1,200–2,200 U/L.	At 8m, *m. deltoideus*: myopathic (fiber size variability, internal nuclei), two abnormal nuclei on EM. No COX– fibers. At 4y, muscle unknown: myopathic/dystrophic (fiber size variability, internal nuclei, single necrotic fibers, several MyHCd and many MyHCn fibers, EM: marked myofibrillar degeneration in several fibers). No COX– fibers.

№	Genotype[a]	Phenotype	Myopathology
19	*NKX2-1* (NM_003317.3): c.578A > C, p.(His193Pro) – het, *de novo*, novel, pathogenic	Male, onset at birth, moderate progression, currently 13y. Features: neonatal hypotonia, mild motor DD, abnormal gait, hypothyroidism, frequent viral infections, allergic asthma, and choreoathetosis in arms and trunk. CK and ENMG normal.	At 4y, *m. tibialis anterior*: non-specific (increased amount of lipid droplets, three immature fibers, EM: many small lipid droplet clusters and Z-streaming). No COX– fibers.
24	*SCN2A* (NM_021007.2): c.4405A>T, p.(Ile1469Phe) – het, *de novo*, novel, pathogenic	Female, disease onset at birth, died at 1y3m. Features: born in severe asphyxia, birth weight -2 SD, severe muscular hypotonia, arthrogryposis, bilateral club foot, tracheostomized and gastrostomized, no motor development, bilateral cataract, and one possible seizure episode. Brain MRI: mild cerebral atrophy. ENMG: no clear findings. CK normal.	At 1.5m, *m. quadriceps femoris*: non-specific myopathic (mild fiber size variation, one central nucleus, and EM: Z-streaming). No COX– fibers.
B. Patients with variants in known disease genes but presenting with new features			
1	*SLC25A4* (NM_001151.3): c.239G>A, p.(Arg80His) – het, *de novo*, novel, pathogenic	Female, disease onset at birth, fast progression, died at 7y. Features: severe neonatal muscular hypotonia with hyporeflexia, respiratory muscle weakness requiring tracheostomy, proximal muscle weakness with loss of ambulation, severe speech delay, mild ID, generalized seizures, and mild hypertrophic cardiomyopathy. Brain MRI: progressive white matter atrophy. Serum: progressive lactate increase after birth. CK normal.	At 15 days of life, muscle unknown: mitochondrial myopathy (fiber size variability, RRFs, large lipid depositions in type 1 fibers, abundant accumulation of glycogen, EM: mitochondria with loss of cristae, concentric cristae, and lipid inclusions). COX stain not performed and muscle tissue not available.
17	*CACNA1A* (NM_023035.2): 1) c.4315T>A, p.(Trp1439Arg) – het, maternal, novel, LP 2) c.472_478delGCCTTCC, p.(Ala158ThrfsTer6) – het, paternal, novel, pathogenic	Male, disease onset at 4m, fast progression, died at 9y. Features: marked hypotonia, absent DTR, seizures, horizontal nystagmus, optic atrophy, severe DD, macrocephaly +3 SD, marked muscular atrophy, and dysphagia. Brain MRI: hypoplastic corpus callosum, diffuse hypomyelination, cerebral and cerebellar atrophy. ENMG and CK normal.	At 10m, *m. tibialis anterior*: non-specific (several MyHCn fibers, EM: some small lipid droplet clusters). No COX– fibers.

№	Genotype[a]	Phenotype	Myopathology
C. Patient with a variant in a novel disease-gene			
18	*TSPOAP1* (NM_004758.3): c.2449_2450delinsTG, p.(Gln817Ter) – hom, LP	Male, disease onset at 7y, fast progression, currently 25y. Features: learning difficulties → mild ID → moderate ID, severe progressive dystonia, dysarthria, dysphagia, spastic right-side hemiparesis → tetraparesis. Brain MRI: progressive cerebellar atrophy. ENMG normal. Mild CK elevation (581 U/L). Decreased 5-methyltetrahydrofolate in CSF.	At 19y, *m. tibialis anterior*: signs of mild mitochondrial myopathy (fiber size variability, few internal nuclei, lobulated fibers, oxidative stain unevenness, one COX– fiber) and scattered denervated fibers.
D. Patients with variants in candidate genes			
6	*KIAA0408* (ENST00000483725): c.578+1_578+2del – het, *de novo*	Male, disease onset at birth, moderate progression, currently 9y. Features: born prematurely 31+2 GW → sepsis, meningitis with lactate increase, severe DD, bilateral SNHL, spasticity, dystonia, and moderate splenomegaly. Brain MRI: bilateral periventricular white matter lesions. ENMG and CK normal.	At 1y7m, *m. tibialis anterior*: non-specific (EM: Z-streaming and lipid accumulation in several fibers).
20	*RBFOX1* 90,051 bp deletion at position chr16:7,683,019–7,773,070 (hg19) – het, *de novo*	Male, onset in early infancy, slow progression, currently 23y. Features: DD, epilepsy since 5y, learning difficulties → mild ID, exercise intolerance, mild proximal muscle weakness, scapular winging, muscle atrophy, and flat feet. Brain MRI: mild brain atrophy → normal. Muscle MRI of the thighs and calves normal. ENMG: mild myopathic changes. CK normal.	At 13y, muscle unknown: mild myopathic (fiber size variation, single internal nuclei, fat cells in perimysium, EM: Z-streaming, misalignment, abnormally shaped nuclei, lipid droplet clusters in some fibers).

[a] The pathogenicity of the variants was predicted using VarSome [Kopanos *et al.* 2019]

№ – patient identification number; bp – basepair; CK – creatine kinase; COX – cytochrome C oxidase; CSF – cerebrospinal fluid; DD – developmental delay; DTR – deep tendon reflex; ECG – electrocardiography; ENMG – electroneuromyography; EM – electron microscopy; GERD – gastroesophageal reflux disease; GW – gestation weeks; het – heterozygous; hom – homozygous; ID – intellectual disability; LP – likely pathogenic; m – months; MRI – magnet resonance imaging; MyHCd – myosin heavy chain developmental/embryonic; MyHCn – myosin heavy chain neonatal/fetal; RRF – ragged red fiber; SD – standard deviation; SDH – succinate dehydrogenase; SMA – spinal muscular atrophy; SNHL – sensorineural hearing loss; SPECT – single-photon emission computerized tomography; y – years

The remaining patients are still unsolved. Patient 11 presented with Reye syndrome at 2 months of age and subsequently developed progressive spastic tetraparesis, dysphagia, dysarthria, scoliosis, joint contractures, moderate intellectual disability, microcephaly, and mild cerebral and cerebellar atrophy. His muscle histology revealed fiber size variation, some subsarcolemmal vacuoles with slight acid phosphatase activity, abundant lobulated fibers, several ring fibers, a few fibers expressing MyHCn, disoriented myofibrils, single dense tubules, and mitochondria with paracrystalline inclusions in one fiber. In addition, a decreased mtDNA/nDNA ratio was found. However, respiratory chain complex enzyme activities in muscle were normal, making the diagnosis of mtDNA depletion syndrome unlikely. Comprehensive genetic analyses (WES, WGS, RNA sequencing, and metabolome analysis) have not revealed suitable candidate variants. However, the patient's phenotype and myopathology correlate with an MD diagnosis.

WES did not reveal any findings in patients 10 and 12. However, further genetic analyses were not pursued as they only had a single disease episode in infancy resolving after treatment with several vitamins, their muscle specimens showed normal histology, and they currently have diagnoses of selective mutism and behavioral disorder, respectively.

In conclusion, muscle histology revealed characteristic changes in all three patients diagnosed with MD and those with SMA type I, *MYH2*-related myopathy, and Vici syndrome. Typical, although relatively non-specific, changes were also seen in the patient with *LMNA*-related CMD. Interestingly, an association with mitochondrial dysfunction, either as part of the pathogenesis of the disease or as a secondary pathological finding, was found in some disorders not classified as MD. Indeed, secondary mitochondrial dysfunction has been described in many NMDs, neurodegenerative diseases, and organic acidurias [Katsetos *et al.* 2013; McAvoy and Kawamata 2019; Wajner *et al.* 2020]. The muscle specimens of four patients with primary CNS involvement and upper motor neuron signs showed only non-specific changes. On the other hand, all patients whose clinical symptoms indicated muscle involvement presented with some degree of myopathic changes in the muscle. Interestingly, signs of a mild mitochondrial myopathy were detected in the muscle specimen of the patient with *TSPOAP1*-related dystonia. The muscle biopsy played an essential role in confirming the genetic diagnosis in several patients by revealing typical histopathological changes or biochemical abnormalities supporting the pathogenicity of novel variants in known genes and enabling the analysis of tissue-specific mtDNA and transcriptome alterations.

5.2 The phenotype of patients with biallelic variants in the *SPATA5* gene and association with mitochondrial dynamics (Publication II)

The index patient of our family was an Estonian girl who died at 4 years 11 months of age. Her parents were non-consanguineous, of mixed Russian, Ukrainian, and Finnish origin. She was born at term with an average birth weight (3,150 g) and length (50 cm). She achieved head control at 4 months and rolling over at 5 months of age. However, since 7 months of life, she presented with remarkable developmental regression (all motor abilities, including head control, were lost) and tonic epileptic seizures. At 1 year of age, her weight was 7,350 g (-3 SD), height 73 cm (-1.5 SD), and occipitofrontal circumference (OFC) 43 cm (-2 SD). At 2 years 3 months, she had developed microcephaly (OFC 44 cm, -3.5 SD) and presented with no head control, elevated deep tendon reflexes, bilateral Babinski sign, spasticity, dystonic movements, strabismus, no eye contact, and mild microanomalies (relatively big ears, up-slanted palpebral fissures, high narrow palate, and retrognathia). Pyramidal symptoms were progressive in time. She was also diagnosed with sensorineural hearing loss.

Ictal EEG showed hypokinetic and tonic-clonic seizures, and repeated interictal EEG revealed progressive slowing of the background and multifocal epileptiform discharges (*dexter>sinister*). Antiepileptic treatment was started with valproate and later changed to clonazepam. MRI scans revealed progressive brain atrophy, a slightly elevated signal intensity on T2-weighted images in white matter, and an atrophic caudate nucleus suggesting a degenerative neuronal disorder. Complete blood count, electrolytes, and liver enzymes were normal. Serum lactate was 3.6 mmol/L (reference <2.2 mmol/L) and homocysteine 14 µmol/L (reference <7.6 µmol/L). However, CSF lactate was in the reference range (2.1 mmol/L, reference 1.1–2.8 mmol/L), and all other metabolic analyses from serum, urine, and CSF did not reveal alterations. Enzyme analyses ruled out lysosomal disorders, and the respiratory chain enzyme, complex V, and citrate synthase activities from cultured skin fibroblasts were also normal. In addition, chromosomal microarray (CMA) and sequencing of the *POLG* gene resulted in no abnormal findings.

The elder brother of the index patient had a similar progressive disease. He died at 3 years of age. He was also born at term with an average birth weight (3,230 g) and length (55 cm). He presented delayed development during his first months of life and regression at 10 months. He developed epileptic seizures at 13 months of age. At 2 years 5 months, his length was 88 cm (-1 SD), weight 11.5 kg (-2.5 SD), and he presented with microcephaly (OCF was 47 cm, -2.5 SD). He showed no head control or eye contact, elevated deep tendon reflexes, bilateral Babinski sign, marked spasticity, and dystonia. He was also diagnosed with sensorineural hearing loss.

Interictal EEG showed multifocal sharp waves, and ictal EEG revealed generalized paroxysmal bursts of polyspikes with deprivation of the baseline.

Due to myoclonic epilepsy, valproate treatment was initiated. Abdominal ultrasound showed mild hepatosplenomegaly, and MRI revealed brain atrophy and delayed myelinization. The values of complete blood count, electrolytes, liver enzymes, serum lactate, amino acids, acylcarnitines, and urinary amino and organic acids were in the reference range.

The index patient was included in our study group of patients with a suspected MD, and a singleton WES analysis revealed two rare protein-altering heterozygous variants in *SPATA5* (NM_145207.2): c.250C>T, p.(Arg84Ter) and c.989_991del, p.(Thr330del). The c.250C>T is a nonsense variant presumably causing loss-of-function with an allele frequency of <0.0001 (i.e., one heterozygous carrier) in the gnomAD database [Karczewski *et al.* 2020]. The c.989_991del in-frame deletion has previously been reported in multiple patients with *SPATA5*-related disorders [Buchert *et al.* 2016; Kurata *et al.* 2016; Tanaka *et al.* 2015]. Sanger sequencing in the index patient and her affected brother confirmed both variants. Their mother carried only the c.250C>T variant; however, DNA from the father was not available for testing.

In addition, our group described three other families with single affected cases detected at the Institute of Human Genetics, Technical University Munich, Germany, whom all carried compound heterozygous variants in the *SPATA5* gene. Two of these patients harbored the same c.989_991del in-frame deletion as our patients in addition to the c.554G>A, p.(Gly185Glu) and c.394C>T, p.(Gln132Ter) variants. The third case carried a nonsense c.700C>T, p.(Gln234Ter) and a missense c.2384C>G, p.(Pro795Arg) variant.

Together with our five subjects, 30 patients with homozygous or compound heterozygous variants in the *SPATA5* gene have been reported in the literature [Buchert *et al.* 2016; Kurata *et al.* 2016; Tanaka *et al.* 2015]. We described the characteristic phenotype of this syndrome as all patients share similar features (Table 4). Disease-onset is in early infancy. All patients present global developmental delay, severely limited speech or no words, hearing impairment (most frequently sensorineural hearing loss), and a pathological EEG with symptomatic epilepsy in 73% of patients. Other prominent features are microcephaly, gastrointestinal problems, abnormal brain MRI (predominantly brain atrophy and delayed myelination), visual impairment, most frequently cortical visual impairment, and no or reduced eye contact. In addition, the patients tend to have a combination of axial hypotonia and peripheral hypertonia with spasticity.

The 25 variants described in the *SPATA5* gene in the reported patients include 14 missense, five nonsense, three frameshift, two in-frame deletions, and one intronic splice-site variant. The in-frame deletion c.989_991del is the most common variant occurring in 6/18 families (33%) and was detected in a homozygous state in one girl with a severe phenotype [Tanaka *et al.* 2015]. Strangely, a homozygous c.989_991del variant in one individual from Sweden is also present in the gnomAD database [Karczewski *et al.* 2020]. Three other variants, c.251G>A, p.(Arg84Gln), c.556C>T, p.(Arg186Ter), and c.1883A>G, p.(Asp628Gly), have occurred in two different families each. There are no variant hot spots in the gene, and clear genotype-phenotype associations cannot

be made. Intrafamilial variability in disease severity can also be seen [Tanaka *et al.* 2015].

Our index patient was clinically suspected of having an MD because of the various CNS symptoms and elevated serum lactate. However, mitochondrial respiratory chain enzyme analyses from cultured skin fibroblasts showed normal activities. Nevertheless, our experiments on cultured rat cortical neurons showed that SPATA5 deficient neurons demonstrate a twofold statistically significant decrease in mitochondrial fusion-fission ratio, a 20% decrease in mitochondrial length, and a 12% decrease in ATP levels compared to controls. Our results confirm the role of SPATA5 in mitochondrial morphogenesis and function, as was previously suggested [Liu *et al.* 2000]. In addition, we have demonstrated that the *in vitro* axonal growth of SPATA5 deficient primary cortical neurons is significantly impaired compared to controls.

Table 4. Phenotype overview of 30 patients with *SPATA5* variants reported in the literature.

Phenotype	**Our study**	**Tanaka *et al.*, 2015**	**Kurata *et al.*, 2016**	**Buchert *et al.*, 2016**	**All 30 patients**	**% of a feature**
	5 patients in 4 families	**14 patients in 10 families**	**3 patients in 2 families**	**8 patients in 2 families**	**30 patients in 18 families**	
Sex	Female (3/5)	Female (8/14)	Female (1/3)	Female (3/8)	Female (15/30)	50%
	Male (2/5)	Male (6/14)	Male (2/3)	Male (5/8)	Male (15/30)	50%
Family history	Familial case (2/5)	Familial case (8/14)	Familial case (2/3)	Familial case (7/8)	Familial case (19/30)	63%
	Single case (3/5)	Single case (6/14)	Single case (1/3)	Single case (1/8)	Single case (11/30)	37%
Consanguinity	-	-	-	Yes (7/8)	Yes (7/30)	23%
Pregnancy/delivery	Uneventful (4/5)	Not mentioned, but probably uneventful (14/14)	Uneventful (2/3)	Uneventful (4/8)	Uneventful (10/30); not mentioned, but probably uneventful (14/30)	80%
	Emergency cesarean section (1/5)	-	IUGR (1/3)	Complicated (maternal diabetes, neonatal hypotonia, born with cyanosis) (4/8)	Complicated (6/30)	20%
Age of onset	Early infancy (5/5)	Not mentioned	Early infancy (3/3)	Early infancy (8/8)	Early infancy (16/16)	100%
Microcephaly	Yes (5/5)	Yes (12/13)	Yes (2/3)	Yes (7/8)	Yes (26/29)	90%
Global DD	Yes (5/5)	Yes (14/14)	Yes (3/3)	Yes (8/8)	Yes (30/30)	100%
Developmental regression	Yes (3/5)	Yes (1/14)	-	-	Yes (4/30)	13%

Phenotype	Our study	Tanaka *et al.*, 2015	Kurata *et al.*, 2016	Buchert *et al.*, 2016	All 30 patients	% of a feature
Speech	No words (5/5)	No words / one word (13/13)	No words (3/3)	Severely limited (8/8)	No words / severely limited (29/29)	100%
Hearing	-	-	Hearing impairment (3/3)	Hearing impairment (3/4)	Hearing impairment including SNHL (26/26)	100%
	SNHL (5/5)	SNHL (14/14)	-	SNHL (1/4)	SNHL (20/26)	77%
Ocular symptoms	Visual impairment (2/3)	Visual impairment (2/13)	-	Visual impairment (2/8)	Visual impairment including CVI (17/27)	63%
	CVI (1/3)	CVI (9/13)	-	Cortical blindness (1/8)	CVI (11/27)	41%
	Strabismus (2/5)	Strabismus (2/13)	-	Strabismus (1/8)	Strabismus (5/29)	17%
	-	Astigmatism (1/13)	Astigmatism (1/3)	-	Astigmatism (2/29)	7%
	Pendelnystagmus (1/5)	Nystagmus (3/13)	-	-	Nystagmus (4/29)	14%
	No eye contact (3/5)	Visual disinterest (1/13)	No/reduced eye contact (2/3)	Reduced eye contact (8/8)	No/reduced eye contact (14/29)	48%
Muscle tonicity	Hypotonia (axial) (2/5)	Hypotonia (primarily axial) (11/14)	Hypotonia (1/3)	Hypotonia (4/8)	Hypotonia (18/30)	60%
	Hypertonia (5/5)	Hypertonia (primarily peripheral) (8/14)	Hypertonia (2/3)	-	Hypertonia (15/30)	50%
	Spasticity (5/5)	Spasticity (7/14)	Spasticity (2/3)	-	Spasticity (14/30)	47%
	Dystonia (5/5)	Dystonia (2/14)	Dystonia (1/3)	Dystonia (1/8)	Dystonia (9/30)	30%

Phenotype	Our study	Tanaka *et al.*, 2015	Kurata *et al.*, 2016	Buchert *et al.*, 2016	All 30 patients	% of a feature
	Dyskinesia (1/5)	-	Dyskinesia (1/3)	-	Dyskinesia (2/30)	7%
Hyperreflexia	Yes (5/5)	Not mentioned	Yes (1/3)	Not mentioned	Yes (6/8)	NA
Epilepsy	Yes (5/5)	Yes (13/14)	Yes (3/3)	Yes (1/8)	Yes (22/30)	73%
	-	Infantile spasms (5)	-	Infantile spasms (1)	Infantile spasms (6)	-
	Myoclonic (3)	Myoclonic (2)	-	-	Myoclonic (5)	-
	Tonic (3)	Tonic (4)	Tonic (2)	-	Tonic (9)	-
EEG	Abnormal (5/5)	Abnormal (14/14)	Abnormal (3/3)	Abnormal (2/2)	Abnormal (24/24)	100%
Brain MRI	Abnormal (5/5)	Abnormal (7/12)	Abnormal (3/3)	Abnormal (1/4)	Abnormal (16/24)	67%
	Brain atrophy (4)	Brain atrophy (4)	Brain atrophy (3)	Brain atrophy (1)	Brain atrophy (12)	-
	Hypoplasia of corpus callosum (1)	Hypoplasia of corpus callosum (2)	Thin corpus callosum (3)	-	Hypoplasia of corpus callosum (6)	-
	Delayed myelination (3)	Delayed myelination / hypomyelination (3)	Delayed myelination (3)	-	Delayed myelination / hypomyelination (9)	-
Gastrointestinal problems	Yes (puree feeding, GERD, HSM, gastroenteritis) (5/5)	Yes (puree feeding, constipation, vomiting, G-tube, FTT) (13/14)	Yes (vomiting, GERD) (1/3)	Yes (constipation, FTT) (3/8)	Yes (22/30)	73%
Skeletal findings	-	Scoliosis (4/14)	-	Scoliosis (1/8)	Scoliosis (5/30)	17%
	Hip dysplasia/dislocation (2/5)	Hip dysplasia/dislocation (4/14)	-	-	Hip dysplasia/dislocation (6/30)	20%
Cardiac problems	-	-	Yes (1/3)	Yes (1/8)	Yes (2/30)	7%
Immunodeficiency	-	Yes (4/14)	-	-	Yes (4/30)	13%
Thrombocytopenia	-	Yes (3/14)	-	-	Yes (3/30)	10%
Blood copper level	Not tested	Not mentioned	Elevated (2/3)	Not mentioned	Elevated (2/3)	NA

Phenotype	Our study	Tanaka *et al.*, 2015	Kurata *et al.*, 2016	Buchert *et al.*, 2016	All 30 patients	% of a feature
Muscle biopsy	Normal (2/2)	Abnormal mitochondria (2/2)	Not done	Not done	Abnormal mitochondria (2/4)	NA
Respiratory chain enzyme analysis	Complex I and IV defects (1/3)	Reduced activity (1/2)	Not tested	Not tested	Abnormal (2/5)	NA
Microanomalies	Retrognathia (3/5)	-	Retrognathia (3/3)	-	Retrognathia (6/30)	20%
	Large ears (2/5)	-	Large ears (1/3)	-	Large ears (3/30)	10%
	-	-	Low-set ears (2/3)	-	Low-set ears (2/30)	7%
	-	-	-	Long nose (7/8)	Long nose (7/30)	23%
	-	-	Depressed nasal ridge/bridge (3/3)	Low nasal bridge (1/8)	Depressed nasal ridge/bridge (4/30)	13%
	-	-	Broad eyebrows (3/3)	Broad eyebrows (1/8)	Broad eyebrows (4/30)	13%

CVI – cortical visual impairment; DD – developmental delay; EEG – electroencephalography; FTT – failure to thrive; GERD – gastroesophageal reflux disease; HSM – hepatosplenomegaly; IUGR – intrauterine growth retardation; MRI – magnet resonance imaging; NA – not applicable; SNHL – sensorineural hearing loss

Although we observed that SPATA5 was dominantly cytosolic and not co-localized with mitochondria, there are many examples where non-mitochondrial proteins play an active role in mitochondrial function. For example, the *KIF5A* gene encodes a cytosolic protein that interacts with mitochondrial adaptor proteins and is responsible for mitochondrial transport in axons. Variants in this gene cause hereditary spastic paraplegia [Reid *et al.* 2002].

In conclusion, our results provide insight into the pathomechanism of the development of the CNS symptoms (developmental delay, intellectual disability, epilepsy, sensorineural hearing loss, cortical visual impairment, and pyramidal signs) and brain atrophy in patients with a defect in the *SPATA5* gene.

5.3 Genetic and myopathological analysis of a muscle biopsy cohort of patients with a childhood-onset disease

5.3.1 Results of the genetic analyses

The initial study group included 64 patients suspected of a childhood-onset hNMD or MD (Figure 11). Of these patients, 56 had had some genetic testing (e.g., single gene analyses, CMA, gene panel sequencing, karyotyping) as part of the clinical diagnostic workup, which yielded a diagnosis in 24/56 (43%) patients (Table 5A). Eleven of the 24 patients were diagnosed with gene panel sequencing using the Illumina TruSight One kit (TSO). Six patients received the diagnosis with a trio WES analysis, of which two patients had a prior TSO gene panel sequencing. One of those patients was found to harbor a 19p13.3 microdeletion, which was detected using WES read depth data to predict CNVs and later confirmed by CMA. The other patient's WES revealed compound heterozygous variants in the *CAPN3* gene, one of which was a 15 bp deletion probably not called on the TSO sequencing due to bioinformatic reasons. In three patients, the diagnosis was confirmed by a single gene or targeted gene panel testing based on the muscle histology results. Three other patients were diagnosed by mtDNA sequencing from muscle tissue. Lastly, one patient was confirmed to have FSHD type 1.

Of the remaining patients without a genetic diagnosis, 33 agreed to participate in one of our research projects (Figure 11). We reached a definite genetic diagnosis for 11/33 patients (Table 5B). Patient II-28, diagnosed with FSHD type 1, had a TSO gene panel sequencing during routine diagnostics. However, she presented with a typical FSHD phenotype. The genetic alteration causing FSHD type 1 cannot be detected by sequencing; instead, complicated analyses on agarose embedded very high-molecular-weight genomic DNA need to be undertaken [Himeda and Jones 2019]. Patient II-25 was diagnosed by TSO gene panel sequencing. He was previously tested only with CMA and for single gene variants. Interestingly, while we eventually diagnosed an inborn error of purine and pyrimidine metabolism related to the *PRPS1* gene, he was initially suspected

of having a congenital myopathy or an MD [Puusepp *et al.* 2020]. A detailed description and discussion regarding this case are given in section 5.4.

We performed a singleton, duo, or trio WES on 25 patients, of whom six received a definite genetic diagnosis. Patient II-26, diagnosed with Bethlem myopathy, had no genetic testing before our research project. Patient II-27 had previously been analyzed with the TSO gene panel sequencing in 2016, and the c.1712A>C, p.(Lys571Thr) variant in *COL6A1* was reported as a VUS. Although the patient had proximal muscle weakness, this variant was not considered disease-causing at that time because she did not have obvious joint contractures or dermatological features. However, the WES analysis did not reveal other potentially disease-associated variants besides the one in *COL6A1*. Nevertheless, at re-examination in 2022, the patient presented the characteristic Bethlem sign (inability to extend the fingers on wrist dorsiflexion completely) [Bonnemann 2011] and recalled having rough skin with small papules (*keratosis pilaris*) as a child. As these features are typical of Bethlem myopathy, the *COL6A1* variant was confirmed as disease-causing.

Patient II-29, who was clinically diagnosed with Leber hereditary optic neuropathy (LHON), had received testing for the three most frequent LHON variants from blood and sequencing of the entire mtDNA from muscle. As LHON was, until recently, associated only with mtDNA variants, further genetic analyses were not performed. However, in 2021, an association was made between LHON and the first nuclear gene, *DNAJC30*, manifesting as an autosomal recessive LHON [Stenton *et al.* 2021]. Our patient harbored a recurrent homozygous Eastern European founder variant c.152A>G, p.(Tyr51Cys) in the *DNAJC30* gene. This variant was estimated to have arisen 85 generations ago [Stenton *et al.* 2021]. The carrier frequency of this variant in Estonia is 1:60. This finding enabled us to molecularly confirm the diagnosis of LHON and offer idebenone treatment to the patient. For patient II-30, several genetic analyses, including the TSO gene panel sequencing, were clinically performed without abnormal findings. We found a heterozygous recurrent c.283T>G, p.Trp95Gly variant in *SET*, a known disease gene. This finding was not reported with the TSO gene panel sequencing because the analysis was done before the *SET* gene was associated with an intellectual disability disorder [Richardson *et al.* 2018; Stevens *et al.* 2018]. Two patients overlapping with the study group suspected of an MD were confirmed to have *TSPOAP1*-related dystonia (patient 18, Table 3C) and *NKX2-1*-related brain-lung-thyroid syndrome (patient 19, Table 3A), discussed in section 5.1.

Five patients with a diagnostic trio WES without any abnormal findings were recruited for a collaboration project with the Broad Institute, and their WES data were reanalyzed. One of those patients was found to harbor variants in a novel *JAG2* gene confirmed to be disease-associated through international collaboration [Coppens *et al.* 2021]. A detailed case report of our patient II-32 and a summary of the clinical features characteristic of *JAG2*-related muscular dystrophy, which has already been classified as LGMD R27 (MIM# 619566), is given in section 5.5. Patient II-33 was found to have compound heterozygous

variants, c.1210G>A, p.(Glu404Lys) and c.4415C>G, p.(Thr1472Ser), in a novel *CELSR1* gene, classified as a tier 1 candidate gene. Five additional patients with a matching phenotype and biallelic variants in the *CELSR1* gene have been found through the GeneMatcher platform [Philippakis *et al.* 2015]. *CELSR1* encodes a protein, which has an essential role in ectoderm patterning, neural tube closure, cilia development and organization, neuronal migration, and cerebral cortex development [Boucherie *et al.* 2018; Goffinet and Tissir 2017].

Lastly, patient II-31, whose WES showed no clear abnormal findings, was eventually diagnosed with SMA type III by MLPA analysis of the *SMN1* gene, which revealed the classic homozygous deletion of exons 7 and 8. Patients with unclear or partially phenotype-associated genetic findings (Table 5C) are discussed in sections 5.3.2 and 5.3.3.

The previously described instances highlight the importance of being aware that some hNMDs are caused by genetic changes, which cannot be detected by sequencing. These include FSHD, DMD, SMA, myotonic dystrophies, and OPMD. Secondly, reanalysis of the sequencing data may improve the diagnostic yield owing to new gene-disease associations, phenotype expansion of a known gene, updated reference databases, or reannotation of sequencing data using updated bioinformatics pipelines. For example, a recent review reported that the median new diagnosis rate of sequencing data reanalysis was 15% [Tan *et al.* 2020]. Thirdly, sharing data and collaborating with international research groups significantly enhances the discovery of new gene-disease associations.

Table 5. The genetic findings of the study group patients with corresponding disease entities matching the patients' phenotypes.

№	Genotype[a]	Disease association
A. Genetic diagnoses reached during routine diagnostic workup		
II-1	19p13.3 ~1 Mb deletion at position chr19:1,132,017–2,121,918 (hg19) – het, *de novo*, pathogenic	19p13.3 microdeletion syndrome [Tenorio *et al.* 2020]
II-2	*WAC* (NM_100264.3): c.1120_1121del, p.(Leu374AspfsTer15) – het, *de novo*, novel, pathogenic	DeSanto-Shinawi syndrome (MIM# 616708)
II-3	*MVK* (NM_000431.3): c.610G>C, p.(Asp204His) – het, maternal, novel, pathogenic; c.1000G>A, p.(Ala334Thr) – het, paternal, recurrent, pathogenic	Mevalonic aciduria (MIM# 610377)
II-4	*DYNC1H1* (NM_001376.4): c.1869C>G, p.(Phe623Leu) – het, maternal, novel, likely pathogenic	SMA-LED (MIM# 158600)
II-5	*DMD* (NM_004006.2): c.1177C>T, p.(Gln393Ter) – hemizygous, maternal, novel, pathogenic	DMD (MIM# 310200)
II-6	*DMD* (NM_004006.2) exon 54 deletion – het, *de novo*, recurrent, pathogenic. Skewed X-inactivation 76% versus 24%.	Symptomatic *DMD* carrier

№	Genotype[a]	Disease association
II-7	*COL6A3* (NM_004369.3): c.6199G>A, p.(Glu2067Lys) – het, recurrent, pathogenic	Bethlem myopathy, LGMD D5 (MIM# 158810)
II-8	*COL6A1* (NM_001848.2): c.877G>A, p.(Gly293Arg) – het, recurrent, pathogenic	Bethlem myopathy, LGMD D5 (MIM# 158810)
II-9	*COL6A1* (NM_001848.2): c.868G>A, p.(Gly290Arg) – het, mosaic 23.5%, recurrent, pathogenic	Bethlem myopathy, LGMD D5 (MIM# 158810)
II-10	*CAPN3* (NM_000070.2): c.550del, p.(Thr184ArgfsTer36) – het, paternal, recurrent, pathogenic; c.598_612del, p.(Phe200_Leu204del) – het, maternal, recurrent, pathogenic	LGMD R1 (MIM# 253600)
II-11	*CAPN3* (NM_000070.2): c.550del, p.(Thr184ArgfsTer36) – het, maternal, recurrent, pathogenic; c.598_612del, p.(Phe200_Leu204del) – het, paternal, recurrent, pathogenic	LGMD R1 (MIM# 253600)
II-12	*SGCA* (NM_000023.4): c.229C>T, p.(Arg77Cys) – het, paternal, recurrent, pathogenic; c.274T>C, p.(Ser92Pro) – het, maternal, novel, likely pathogenic	LGMD R3 (MIM# 608099)
II-13	*LMNA* (NM_170707.4): c.94A>G, p.(Lys32Glu) – het, *de novo*, recurrent, pathogenic	EDMD (MIM# 181350)
II-14	7±1 D4Z4 repeat units on chromosome 4q35	FSHD type 1 (MIM# 158900)
II-15	*RYR1* (NM_001042723.2): c.325C>T, p.(Arg109Trp) – het, paternal, recurrent, pathogenic; c.6721C>T, p.(Arg2241Ter) – het, maternal, recurrent, pathogenic	Central core disease (MIM# 117000)
II-16	*NEB* (ENST00000397345.8): c.6937C>T, p.(Arg2313Ter) – het, *de novo*, recurrent, pathogenic; c.25070G>A, p.(Trp8357Ter) – het, maternal, novel, pathogenic	Nemaline myopathy (MIM# 256030)
II-17	*MYH7* (NM_000257.4): c.5655G>A, p.(Ala1885=) leading to 38. exon skipping – het, *de novo*, novel, pathogenic	*MYH7*-related congenital myopathy [Pajusalu *et al.* 2016]
II-18	*DYSF* (ENST00000410020.8): c.4941del, p.(Lys1648ArgfsTer4) – het, in trans, recurrent, pathogenic; c.4989del, p.(Glu1663AspfsTer10) – het, in trans, recurrent, pathogenic	Miyoshi muscular dystrophy (MIM# 254130)
II-19	*MT-TE*: m.14674T>C (tRNA Glu) – homoplasmic (muscle), recurrent, pathogenic	Reversible infantile RCC deficiency [Roos *et al.* 2021]
II-20	*MT-TL1*: m.3243A>G (tRNA Leu) – heteroplasmic (59% muscle, 38% urinary epithelial cells, 0% blood leukocytes), recurrent, pathogenic	m.3243A>G associated multisystemic MD [Chin *et al.* 2014]
II-21	*MT-TK*: m.8344A>G (tRNA Lys) – heteroplasmic (90% muscle), recurrent, pathogenic	MERRF [Altmann *et al.* 2016]

№	Genotype[a]	Disease association
II-22	*BCS1L*: c.232A>G, p.(Ser78Gly) – het, maternal, recurrent, pathogenic; c.245C>T, p.(Ser82Leu) – het, paternal, novel, likely pathogenic	RCC III deficiency, nuclear type 1 (MIM# 124000)
II-23	*CLCN1* (ENST00000343257.7): c.503del, p.(Pro168HisfsTer2) – het, recurrent, pathogenic; c.2680C>T, p.(Arg894Ter) – het, recurrent, VUS	Becker myotonia (MIM# 255700)
II-24	*SCN1A* (ENST00000674923.1): c.1252A>C, p.(Ile418Leu) – het, maternal, novel, pathogenic	DEE (MIM# 619317)
B. Genetic diagnoses found through our research projects		
II-25	*PRPS1* (NM_002764.4): c.130A>G, p.(Ile44Val) – hemizygous, maternal, novel, pathogenic	Arts syndrome (MIM# 301835)
II-26	*COL6A1* (NM_001848.3): c.1056+1G>A – het, recurrent, pathogenic	Bethlem myopathy, LGMD D5 (MIM# 158810)
II-27	*COL6A1* (NM_001848.3): c.1712A>C, p.(Lys571Thr) – het, recurrent, likely pathogenic	Bethlem myopathy, LGMD D5 (MIM# 158810)
II-28	3±1 D4Z4 repeat units on chromosome 4q35	FSHD type 1 (MIM# 158900)
II-29	*DNAJC30* (NM_032317.3): c.152A>G, p.(Tyr51Cys) – hom, paternal and maternal, recurrent, pathogenic	LHON (MIM# 619382)
II-30	*SET* (NM_001374326.1): c.283T>G, p.(Trp95Gly) – het, not maternal, recurrent, pathogenic	Intellectual developmental disorder (MIM# 618106)
II-31	*SMN1* (NM_000344.3): deletion of exons 7 and 8 – hom, recurrent, pathogenic	SMA type III (MIM# 253400)
II-32	*JAG2* (NM_002226.5): c.283A>G, p.(Thr95Ala) – het, maternal, novel, VUS; c.2473C>T, p.(Arg825Cys) – het, *de novo*, novel, VUS	LGMD R27 (MIM# 619566)
II-33	*CELSR1* (ENST00000674500.2): c.1210G>A, p.(Glu404Lys) – het, benign; c.4415C>G, p.(Thr1472Ser) – het, VUS	Novel disease association, not yet published
C. Other genetic findings		
II-34	*DMD* (NM_004006.3): c.4978T>C, p.(Trp1660Arg) – hemizygous, maternal, novel, VUS	Probable very mild BMD (MIM# 300376)
II-35	*ARFGEF1* (NM_006421.5): c.986T>A, p.(Val329Glu) – het, not maternal, novel, VUS	Possible disease association
II-36	*RYR2* (NM_001035.3): c.836C>T, p.(Thr279Met) – het, novel, VUS	*RYR2*-related epilepsy [Yap and Smyth 2019]
II-37, 38	*UROD* (ENST00000246337.9): c.400_401delGTinsCA, p.(Val134Gln) – het, inherited, recurrent, pathogenic	*Porphyria cutanea tarda* (MIM# 176100)
II-39	*ATP2A1* (NM_004320.6): c.2480C>T, p.(Pro827Leu) – het, novel, VUS	Not confirmed
II-40	*SELENON* (NM_020451.3): c.997_1000del, p.(Val333ProfsTer6) – het, maternal, recurrent, pathogenic	Not confirmed

№	Genotype[a]	Disease association
II-41	9p21.3 ~200 kb deletion at position chr9:21,260,106–21,468,754 (hg19) – het, *de novo*	Candidate CNV
II-42	*TNRC6A* (NM_014494.4): c.4405C>T, p.(Gln1469Ter) – het, *de novo*, novel, likely pathogenic	Candidate gene

[a] The pathogenicity of the variants was predicted using VarSome [Kopanos *et al.* 2019]
№ – patient identification number; het – heterozygous; hom – homozygous; CNV – copy number variant; DEE – developmental and epileptic encephalopathy; DMD/BMD – Duchenne/Becker muscular dystrophy; EDMD – Emery-Dreifuss muscular dystrophy; FSHD – facioscapulohumeral muscular dystrophy; kb – kilobase; LGMD – limb-girdle muscular dystrophy; LHON – Leber hereditary optic neuropathy; Mb – megabase; MD – mitochondrial disorder; MERRF – myoclonic epilepsy associated with ragged-red fibers; RCC – respiratory chain complex; SMA – spinal muscular atrophy; SMA-LED – SMA, lower extremity predominant; VUS – variant of unknown significance

5.3.2 Myopathological findings in patients with predominant peripheral nervous system involvement

The phenotype of the 57 study group patients, whose muscle biopsy specimens were reanalyzed (Figure 11), was heterogeneous. Table 6 summarizes their clinical and myopathological features. Peripheral nervous system (PNS) involvement was a predominant feature in 35 patients (Table 6A), of whom 25 (71%) received a genetic diagnosis. Most patients presented with muscle weakness, but three had only myotonia, muscle cramps, or hyperCKemia. In addition, six patients had concomitant neurological symptoms (cognitive deficiency, migraine, epilepsy, sensorineural hearing loss, and pontocerebellar hypoplasia). One patient was diagnosed with cutaneous porphyria and hemochromatosis in addition to skeletomuscular problems. Two patients with an MD had multisystemic involvement.

Of the 35 patients with PNS involvement, specific myopathological changes matching the genotype were present in nine (26%) biopsy specimens (Table 6A). Of note, during 2013–2016, muscle histopathology aided the genetic testing of four patients. Patient II-5 presented with a typical DMD phenotype; however, the *DMD* deletion/duplication analysis revealed no alterations. Nevertheless, muscle histological investigations confirmed the diagnosis with totally absent dystrophin expression. After that, a nonsense variant, c.1177C>T, p.(Gln393Ter), was found in the *DMD* gene. A mosaic absence of dystrophin in the muscle specimen of patient II-6 prompted the deletion/duplication analysis of the *DMD* gene, revealing a heterozygous deletion of exon 54. Similarly, a reduced expression of α-sarcoglycan suggested a defect in the *SGCA* gene in patient II-12, which was confirmed by targeted sequencing revealing compound heterozygous variants, c.229C>T, p.(Arg77Cys) and c.274T>C, p.(Ser92Pro). The muscle histology of patient II-16 showed abundant nemaline rods; therefore, a nemaline myopathy gene panel was ordered, revealing two nonsense

variants, c.6937C>T, p.(Arg2313Ter) and c.25070G>A, p.(Trp8357Ter), in the *NEB* gene. In addition, three patients with mitochondrial pathology were confirmed to have mtDNA variants. Notably, the m.3243A>G variant of patient II-20 could not be detected in blood, showing the relevance of muscle biopsy. For patient II-15 with compound heterozygous variants, c.325C>T, p.(Arg109Trp) and c.6721C>T, p.(Arg2241Ter), in the *RYR1* gene, the muscle biopsy was performed after the genetic finding to confirm disease causality. Indeed, his muscle histology showed typical changes seen in autosomal recessive *RYR1*-related core myopathy with numerous cores and very uneven structure (Figure 18). Lastly, while dysferlin IHC was initially not performed on the muscle specimen of patient II-18 diagnosed with Miyoshi myopathy, we later confirmed the absence of dysferlin.

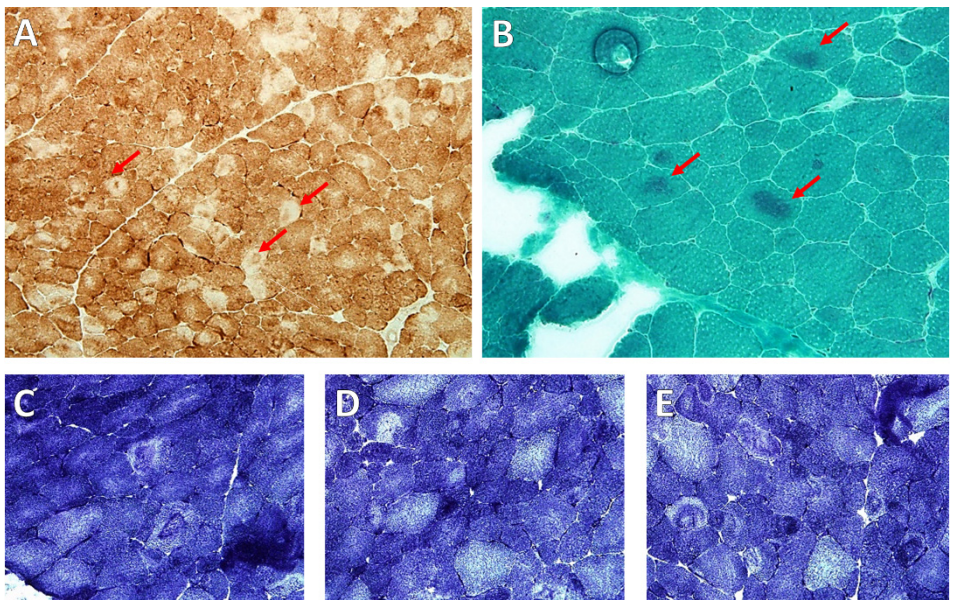


Figure 18. Muscle sections of patient II-15 with *RYR1* variants showing (A) numerous large cores (arrows; COX-SDH) with (B) protein aggregation (arrows; mGT) and (C–E) higher magnification demonstrating the uneven structure of many fibers (NADH-TR).

Neurogenic changes were evident in patient II-4, matching his diagnosis of SMA-LED (Table 6A) caused by a maternally inherited c.1869C>G, p.(Phe623Leu) variant in the *DYNC1H1* gene. Dystrophic or myopathic changes without specific IHC or structural anomalies were present in 14 (40%) cases. Interestingly, patient II-31 with muscle histopathological features corresponding to chronic dystrophic/myopathic changes (endomysial fibrosis, randomly distributed atrophic and hypertrophic fibers, increased amount of internal nuclei, single necrotic fibers, and no fiber type grouping) was found to harbor the typical homozygous deletion of exons 7 and 8 of the *SMN1* gene causing SMA.

Of note, the patient's clinical features, CK value, and ENMG results (Table 6A) also referred to a muscle rather than a motor neuron disease. However, there are reports of SMA type III patients with calf pseudohypertrophy, high CK, and myopathic/dystrophic changes in the muscle evoking diagnostic confusion with muscular dystrophies [Alsaman and Alshaikh 2013; Dubowitz *et al.* 2021; Muqit *et al.* 2004].

Five patients (II-7, II-8, II-9, II-26, and II-27, Table 6A) with autosomal dominant Bethlem myopathy showed myopathic changes but normal collagen type VI expression on the sarcolemma, which was expected. While a secondary reduction of sarcolemmal laminin β1 can be seen in Bethlem myopathy, it is a non-specific finding [Dubowitz *et al.* 2021]. In addition, decreased laminin β1 expression is not always present, as illustrated by our patients, of whom two had a reduction, and three did not.

Patient II-17 (Table 6A) harbored a *de novo* synonymous c.5655G>A, p.(Ala1885=) variant in the *MYH7* gene on trio WES. While most synonymous variants are benign, this variant was predicted to disrupt splicing by MutationTaster [Schwarz *et al.* 2014] and MutPred Splice [Mort *et al.* 2014]. Indeed, by performing additional studies with RNA extracted from the patient's muscle tissue, we could confirm that this variant causes in-frame skipping of the 38th exon of the *MYH7* gene [Pajusalu *et al.* 2016]. The *MYH7* gene is associated with cardiomyopathy, Laing distal myopathy, myosin storage myopathy, and congenital fiber-type disproportion [Tajsharghi and Oldfors 2013]. Our patient's muscle histology revealed the presence of hypotrophic type 1 fibers, while apparent myosin aggregation could not be detected by light microscopy or ultrastructurally. However, small strange fibers with a circular lack of NADH-TR and a dark rim on ATPase at pH 10.2 without myosin accumulation were evident (Figure 19).

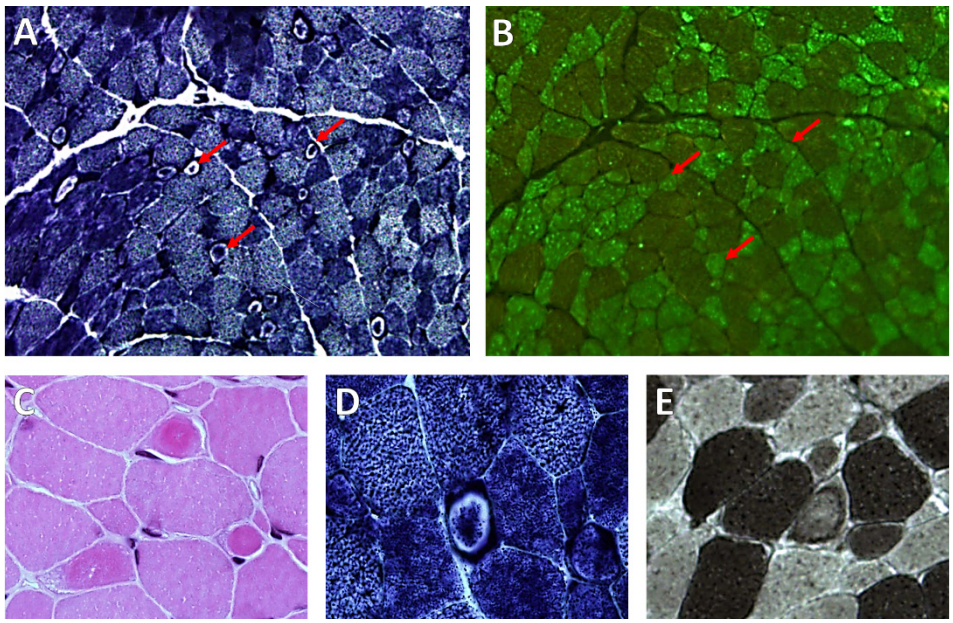


Figure 19. Muscle sections of patient II-17 with an *MYH7* variant showing strange fibers with (A) an uneven NADH-TR stain (arrows) but (B) no accumulation of myosin (arrows; anti-MLC2E8); higher magnification of the structure of these fibers on (C) HE, (D) NADH-TR, and (E) ATPase at pH 10.2.

Interestingly, two non-related patients (II-10 and II-11) diagnosed with LGMD R1 had the same compound heterozygous variants in the *CAPN3* gene (Table 5A). According to the gnomAD database [Karczewski *et al.* 2020], the allele frequency of the c.550del variant in Estonia is high (0.002068), resulting in a carrier frequency of 1:242. However, the c.598_612del variant is globally rare (total allele count is 6). Notably, the phenotype and myopathological changes were very similar in the two boys (Table 6A). Both had disease-onset at 5 years of age after a febrile illness with very high CK and developed slowly progressive proximal muscle weakness. Their muscle biopsy samples showed focal dystrophic changes with necrosis and regeneration limited to single fascicles, while the other fascicles appeared nearly normal (Figure 20). One publication has previously reported the same two variants in the compound state in a patient with proximal lower limb muscle weakness since 5 years of age. However, detailed clinical data were not provided, and a muscle biopsy was not performed [Stehlikova *et al.* 2014].

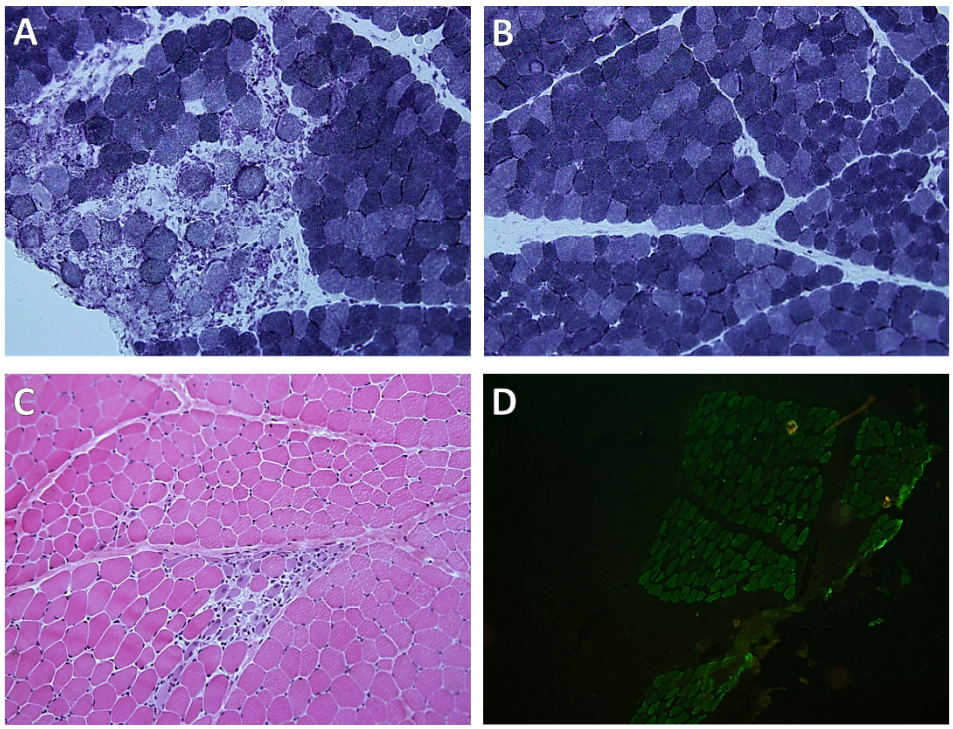


Figure 20. Muscle sections of patients II-10 (A and B) and II-11 (C and D) with variants in the *CAPN3* gene showing (A) necrosis of a whole fascicle (NADH-TR) while (B) the other fascicles appear normal (NADH-TR), (C) a group of basophilic regenerating fibers (HE), and (D) whole fascicles expressing MyHCd.

Patient II-13 (Table 6A) with an *LMNA* c.94A>G, p.(Lys32Glu) variant did have abnormally shaped nuclei, a non-specific finding, but no chromatin anomalies were detected, which have been described in some Emery-Dreifuss muscular dystrophy patients [Sabatelli *et al.* 2001]. Patient II-34 had a novel VUS, c.4978T>C, p.(Trp1660Arg), in the *DMD* gene inherited from his mother. His phenotype did correlate with a very mild BMD with elevated CK and calf pseudohypertrophy (Table 6A). However, the muscle histology did not reveal signs of dystrophinopathy. Furthermore, IHC for all three dystrophin epitopes, nNOS, utrophin, and other sarcolemmal proteins, did not show any alterations. Nevertheless, the IHC can be normal in very mild BMD cases, and only WB for dystrophin may reveal slight changes [Dubowitz *et al.* 2021].

Patient II-40 was found to harbor a heterozygous recurrent pathogenic c.997_1000del, p.(Val333ProfsTer6) variant in the *SELENON* gene, inherited from her unaffected mother. Our patient's clinical features (Table 6A) match the *SELENON*-related myopathy phenotype described in the literature [Villar-Quiles *et al.* 2020]. However, it is an autosomal recessive disease, and other rare variants were not found in the *SELENON* gene in our patient. Interestingly, her muscle specimens revealed autophagic vacuoles, pathological mitochondria,

myofibrillar disorganization, and a large osmiophilic granular-filamentous plaque (Figure 21), which is somewhat similar to the Mallory body-like inclusions described by Ferreiro *et al.* [Ferreiro *et al.* 2004]. Unfortunately, we could not perform additional stains to evaluate the presence of protein aggregation, as the muscle samples were no longer available.

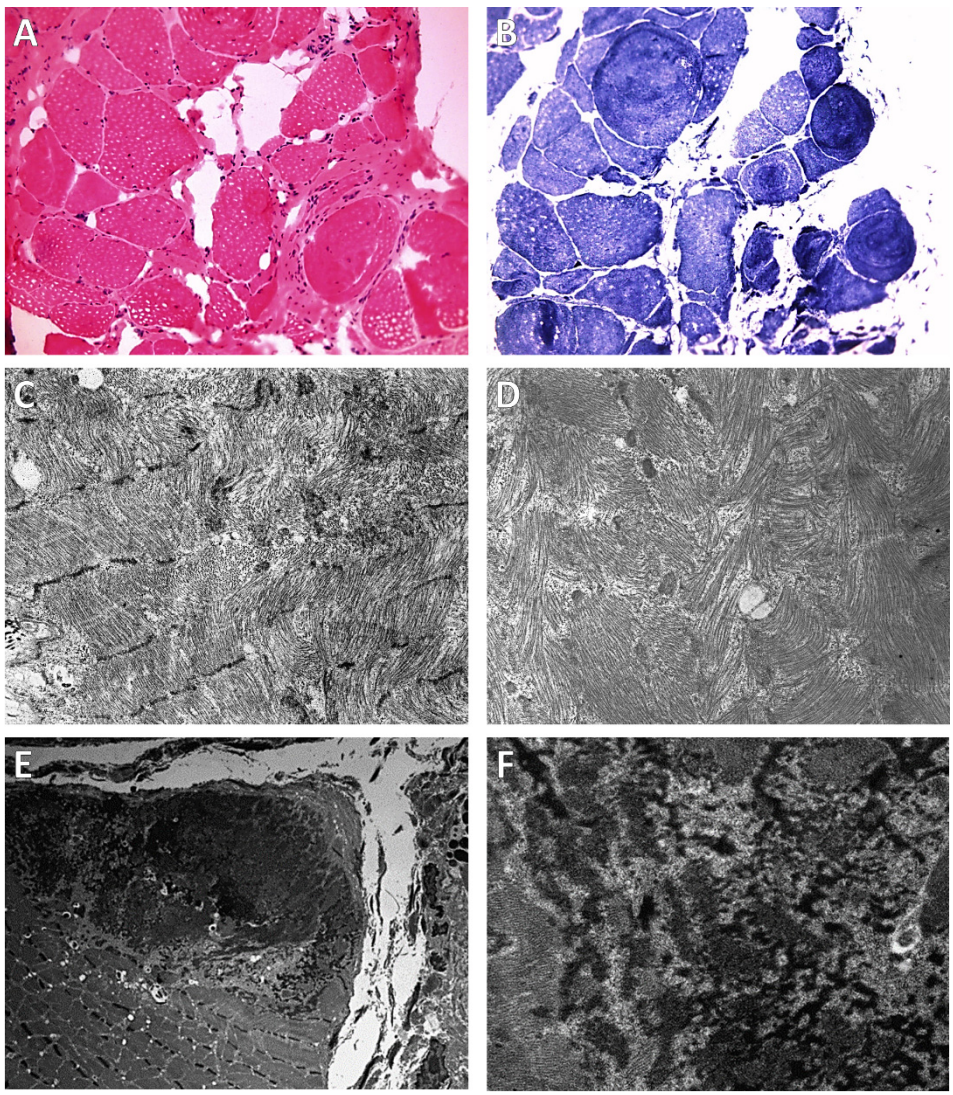


Figure 21. Muscle sections of patient II-40 demonstrating (A) fibrosis, fat cells, marked fiber size variation, and internalized nuclei on HE, (B) whorled fibers (NADH-TR), and ultrastructure of (C) myofibrillar disorganization with (D) complete lack of sarcomeric structure, and (E) an osmiophilic granular-filamentous plaque with (F) showing higher magnification.

The muscle histology of patient II-48 revealed mild fiber-type disproportion (Table 6A); however, genetically, the case remains unsolved for the time being. The muscle samples of eight patients showed only single non-specific findings or normal histology. Of these patients, only one (II-23, Table 6A) received a genetic diagnosis. He was diagnosed with Becker myotonia (Table 5A), which typically does not cause any myopathological alterations [Dubowitz *et al.* 2021]. Two brothers (II-37 and II-38, Table 6A) had a c.400_401delGTinsCA, p.(Val134Gln) variant in the *UROD* gene, which is the cause of cutaneous porphyria in one brother, but this variant cannot explain the skeletomuscular symptoms. Another patient (II-39, Table 6A) harbored a heterozygous novel VUS, c.2480C>T, p.(Pro827Leu), in the *ATP2A1* gene, which is associated with Brody myopathy characterized by exercise-induced muscle stiffness and cramps, matching our patient's symptoms [Molenaar *et al.* 2020]. However, the disease is autosomal recessive, and other rare variants in the *ATP2A1* gene were not found. In addition, our patient presents with bilateral ptosis and some fibers with decreased COX activity in the biopsied muscle, referring to an MD instead.

5.3.3 Myopathological findings in patients with predominant central nervous system involvement

CNS involvement was a predominant feature in 18 patients, with seven (39%) receiving a precise genetic diagnosis (Table 6B). Eight patients also presented a definite or possible PNS involvement with arthrogryposis, Gowers' sign, myopathic face, CK elevation, or myopathic changes on ENMG. Five patients had a CNS or a multisystemic disease with or without muscular hypotonia with no apparent muscle weakness. Eight patients presented signs of upper motor neuron damage (Babinski sign, spasticity, or clonus).

Mild myopathic changes were present in the muscle samples of five patients, all of whom showed some clinical signs of PNS involvement. One of those patients (II-3, Table 6B) was diagnosed with mevalonic aciduria (Table 5A), a defect in cholesterol biosynthesis. While myopathy is not a frequent finding, it has been described in patients with mevalonic aciduria [Haas and Hoffmann 2006], and one patient showing fiber hypotrophy on histological muscle analysis has also been reported [Brennenstuhl *et al.* 2021]. Another patient with myopathic histopathological changes (II-24, Table 6B) had an *SCN1A* gene variant, c.1252A>C, p.(Ile418Leu), which was also detected in her affected sister, while their mother was 5% mosaic. Another sibling of patient II-24 most probably also carried the *SCN1A* variant, but as he died at 7 years of age, he could not be tested. All three children had a similar phenotype: congenital skeletal problems, arthrogryposis, seizures, and psychomotor developmental delay. *SCN1A* gene defects were initially associated with Dravet syndrome and other epileptic encephalopathies [Ding *et al.* 2021]. However, the phenotypic spectrum of *SCN1A*-related diseases has expanded recently, including

arthrogryposis [Marco-Hernandez *et al.* 2022]. Therefore, we can conclude that the *SCN1A* variant in this family is the cause of the entire phenotype.

Patient II-36 (Table 6B) harbored a heterozygous novel VUS, c.836C>T, p.(Thr279Met), in the *RYR2* gene. *RYR2* is a known disease gene causing cardiac arrhythmias. However, it has recently been shown that *RYR2* can manifest as primary generalized epilepsy without cardiac involvement [Yap and Smyth 2019]. Therefore, we can assume that the *RYR2* variant is the cause of our patient's epilepsy. However, the elevated CK and myopathic changes on ENMG and the biopsied muscle cannot be clearly associated with *RYR2*, which has a high expression in cardiac but not skeletal muscles (https://www.proteinatlas.org/ENSG00000198626-RYR2/tissue, accessed on 29.05.2022).

Patient II-46 (Table 6B), born from a monozygotic twin pregnancy at 28+2 gestation weeks, developed sepsis-induced apnea episodes on the 5th day of life. She was diagnosed with bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, spastic tetraparesis, arthrogryposis, and bilateral hip luxation. At 6 years of age, her development was severely delayed with no head control and no words. ENMG showed signs of myopathy, and a muscle biopsy specimen from *m. vastus lateralis* showed myopathic changes with mild fibrosis, fiber size variation, internal nuclei, and predominance of type 1 fibers (90%). Although she was the monozygotic twin of her healthy sister, there are several mechanisms by which they can be genetically discordant, including post-zygotic gene mutation, differential DNA methylation, and mtDNA variants [Silva *et al.* 2011]. However, trio WES did not reveal any clear abnormal findings. Another patient (II-49, Table 6B) with mild fiber-type disproportion also remained unsolved.

Muscle biopsy samples of 14 patients had only mild non-specific changes (small lipid droplet clusters, filamentous bodies, cytoplasmic bodies, Z-streaming, single denervated fibers, atrophic type 2 fibers, or delay in muscle fiber maturation) or normal histology. Most of these patients presented with pyramidal signs or muscular hypotonia without muscle weakness. This group included patients with a 19p13.3 microdeletion syndrome (II-1), DeSanto-Shinawi syndrome (II-2), *BCS1L*-related MD (II-22), and *SET*-related intellectual disability (II-30), see Tables 5 and 6B.

Four patients with non-specific histological changes had candidate variants. Patient II-35 (Table 6B) had a heterozygous VUS, c.986T>A, p.(Val329Glu), in the *ARFGEF1* gene, which has just recently been associated with a disease phenotype [Thomas *et al.* 2021]. Our patient had a similar but significantly more severe phenotype than the patients described by Thomas *et al.*; therefore, the disease causality of the *ARFGEF1* variant remains unclear. Patient II-41 (Table 6B) had a ~200 kb deletion on chromosome 9 containing seven genes, including *KLHL9*, associated with a distal myopathy phenotype [Cirak *et al.* 2010]. In addition, patient II-42 (Table 6B) harbored a *de novo* nonsense c.4405C>T, p.(Gln1469Ter) variant in the *TNRC6A* gene. Two other patients with *de novo* loss-of-function *TNRC6A* variants and a partially overlapping phenotype have been found through the GeneMatcher platform [Philippakis *et*

al. 2015]. *TNRC6A* encodes the GW182 protein, which is involved in miRNA-induced gene silencing [Niaz and Hussain 2018]. Interestingly, patients with GW182 autoantibodies showed neuropathy, ataxia, arthritis, rheumatologic diseases, and cancers [Bhanji *et al.* 2007]. Another patient with a *de novo* variant in the candidate gene *KIAA0408* (patient 6, Table 3D) overlapped with the study group of patients suspected of an MD, discussed in section 5.1. Lastly, four patients (II-43, II-51, II-53, and II-54, Table 6B) with normal muscle histology or non-specific changes remained unsolved.

In conclusion, the muscle histology, clinical symptoms, and genotype correlated well. Most patients with apparent muscle involvement without signs of pyramidal tract damage showed myopathic or dystrophic changes in the biopsied muscle. On the contrary, muscle histology of most patients with a predominant CNS involvement or pyramidal signs was normal or revealed only non-specific changes. While the muscle biopsy was not necessarily needed in many cases, it played a substantial role in confirming several genetic diagnoses.

Table 6. A summary of the clinical and myopathological features of the study group patients.

№	Phenotype	Myopathology
A. Patients with predominant peripheral nervous system involvement		
II-4	Family with AD *DYNC1H1*-related SMA-LED, three affected individuals. Proband (male): motor DD, flaccid lower paraparesis since 8y, cognitive disability, signs of spinal motor neuron damage on ENMG. Sister: motor DD, non-ambulatory at 7y, knee contractures. Mother: muscle weakness since childhood.	At 11y: advanced pathology with groups of very atrophic fibers of both fiber types and large type 1 fibers referring to a neurogenic process.
II-5	Male with DMD: motor DD, progressive predominantly proximal muscle weakness, marked pseudohypertrophy of calves and thighs, high CK (max. 26,985 U/L), partial blockade of the right bundle of His, epileptic discharges on EEG.	At 2y10m: dystrophic changes with a total absence of dystrophin C- and N-terminus and rod domain, decreased α-DG, and up-regulation of utrophin.
II-6	Symptomatic female DMD carrier: motor DD, exercise intolerance, high CK (max. 8,706 U/L).	At 1y2m: dystrophic changes with a mosaic absence of dystrophin C- and N-terminus and rod domain, decreased α-DG, and up-regulation of utrophin.
II-7	Male with AD *COL6A3*-related Bethlem myopathy: progressive predominant proximal muscle weakness since 3y, tense Achilles tendons and calves, skin lesions, elevated CK (max. 947 U/L).	At 4y: myopathic changes with a few regenerating fibers and normal laminin β1 expression.
II-8	Female with AD *COL6A1*-related Bethlem myopathy: progressive predominant proximal muscle weakness since 4y, short Achilles tendons, pseudohypertrophy of calves, mildly elevated CK, unilateral SNHL.	At 12y: myopathic changes with significant fatty tissue infiltration and normal laminin β1 expression.
II-9	Male with AD *COL6A1*-related Bethlem myopathy: hip joint dysplasia, progressive predominant proximal muscle weakness since childhood, pseudohypertrophy of calves.	At 41y: myopathic changes with decreased laminin β1 expression and 5 COX– fibers.
II-10	Male with LGMD R1 *CAPN3*-related: exercise intolerance, muscle pain, and cramps after viral infection at 5y, mild proximal muscle weakness, pseudohypertrophy of calves, high CK (max. 20,000 U/L).	At 9y: patchy dystrophic changes with focal necrosis and large groups of immature fibers.

№	Phenotype	Myopathology
II-11	Male with LGMD R1 *CAPN3*-related: calf muscle pain after a febrile illness at 5y, mild proximal muscle weakness, high CK (max. 19,000 U/L).	At 7y: patchy dystrophic changes with large groups of regenerating and immature fibers.
II-12	Female with LGMD R3 *SGCA*-related: progressive predominantly proximal muscle weakness since 6y, loss of ambulation at 12y, elevated CK (800 U/L).	At 10y: dystrophic changes with decreased α-SARC expression and mild up-regulation of utrophin.
II-13	Male with AD *LMNA*-related myopathy: motor DD, progressive predominantly proximal muscle weakness, no joint contractures or cardiac problems, elevated CK (max. 1,167 U/L).	At 3y: myopathic changes with minicores and nuclear anomalies.
II-14	Family with FSHD type 1, eleven affected individuals. Proband (female): exercise intolerance since childhood, progressive predominantly shoulder girdle weakness, mildly elevated CK.	At 43y: very small specimen with poor quality. No changes detectable.
II-15	Male with AR *RYR1*-related core myopathy: weak fetal movements, DD, swallowing difficulties, Gowers' sign, ophthalmoparesis, hip subluxation, normal CK.	At 9y: central core myopathy with central nuclei and type 1 fiber predominance and hypotrophy.
II-16	Male with AR *NEB*-related nemaline myopathy: motor DD, generalized muscle weakness, severe bulbar paralysis, facial weakness, normal CK.	At 1y1m: nemaline myopathy with type 1 fiber predominance.
II-17	Male with AD *MYH7*-related congenital myopathy: marked muscular hypotonia since 3m, proximal and neck extensor muscle weakness, bilateral ptosis, normal CK.	At 1y1m: FTD and possible fibers with abnormal myosin.
II-18	Female with AR *DYSF*-related distal myopathy: distal lower limb weakness since 12y, progression to tetraplegia, elevated CK (max. 2,000 U/L).	At 31y: dystrophic changes. At 46y: end-stage changes with absent dysferlin expression.
II-19	Female with a reversible COX deficiency myopathy: acute illness at 2m with respiratory problems, lethargy, muscular hypotonia, and elevated lactate. Improvement after treatment with vitamins. Decreased RCC I and IV activity from muscle.	At 3m: mitochondriopathy with many RRFs, abundant COX– fibers, and pathological mitochondria.
II-20	Female with *MT-TL1*-related MD: lower limb weakness and pain since childhood, muscle stiffness and fasciculations, type 2 diabetes, calcifications in basal ganglia, retinopathy, bilateral SNHL, normal CK. RCCs activity normal in muscle tissue.	At 56y: mitochondriopathy with some RRFs, pathological mitochondria, and ring fibers. No COX– fibers.

№	Phenotype	Myopathology
II-21	Male with MERRF syndrome: myoclonic seizures since 12y, progressive predominantly proximal muscle weakness, dysarthria, axonal sensorimotor neuropathy, bilateral SNHL, mildly elevated lactate and CK, died at 44y. Decreased RCC I, III, and IV activity from muscle.	At 42y: mitochondrial myopathy with many RRFs and pathological mitochondria. No COX– fibers.
II-23	Male with AR Becker myotonia: myotonia induced by cold and exercise since childhood, hypertrophic muscles, tongue fasciculations and tremor, mild CK ↑.	At 22y: normal histology, no tubular aggregates.
II-26	Female with AD *COL6A1*-related Bethlem myopathy: motor DD, progressive predominantly proximal muscle weakness, ankle, elbow, wrist, and finger contractures, mildly elevated CK.	At 47y: myopathic changes with decreased laminin β1 expression and 2 COX– fibers.
II-27	Female with a probable AD *COL6A1*-related Bethlem myopathy: predominant proximal muscle weakness since 12y, long finger flexor, elbow, and ankle contractions, mildly elevated CK.	At 63y: myopathic changes with normal laminin β1 expression and 5 COX– fibers.
II-28	Female with FSHD type 1: progressive proximal and facial muscle weakness since 12y, scapular winging, mild CK ↑.	At 20y: end-stage changes with ring fibers.
II-31	Male with SMA type III: proximal lower limb weakness since 16y, pseudohypertrophy of calves, CK ↑ (max. 1,800 U/L), myopathic changes on ENMG.	At 36y: dystrophic changes with uneven oxidative stains.
II-32	Female with LGMD R27 *JAG2*-related: progressive predominantly proximal muscle weakness since 5y, mildly elevated CK.	At 6y: myopathic changes.
II-33	Male with AR *CELSR1*-related disease: decreased fetal movements, arthrogryposis, no active movements, no swallowing reflex, pontocerebellar hypoplasia, high CK (3,517 U/L), died at 29 days of life.	At 24 days of life: dystrophic changes with acid phosphatase activity in some fibers and normal α-DG expression.
II-34	Male with a probable very mild BMD: pseudohypertrophy of calves, high CK (max. 8,000 U/L), no muscle weakness.	At 8y: mild myopathic changes with normal dystrophin and utrophin IHC.
II-37	Male (brother of II-37) with bilateral ptosis since 3m, mild distal weakness, exercise intolerance, arthralgia, normal CK, hemochromatosis, and cutaneous porphyria.	At 28y: normal histology.
II-38	Male (brother of II-36) with exercise intolerance, arthralgia, problems with writing since childhood, myalgia, muscle cramps, ophthalmoparesis, and liver steatosis.	At 39y: normal histology.
II-39	Male with muscle cramps in thighs and calves during exercise since 17y, alleviated by rest, worse in cold, bilateral ptosis, normal CK.	At 24y: mitochondriopathy with decreased COX activity in some fibers.

№	Phenotype	Myopathology
II-40	Female with muscular hypotonia and feeding problems after birth, progressive proximal muscle weakness, loss of ambulation, dysarthria, respiratory insufficiency, severe scoliosis, neck and shoulder girdle muscle atrophy, cardiomyopathy, normal CK.	At 13, 16, and 31y: severe myopathic changes with autophagic vacuoles, myofibrillar disorganization, granulofilamentous material, and pathological mitochondria.
II-44	Female with distal lower limb muscle cramps since childhood (primarily nocturnal), underweight (BMI 15 despite regular diet), scoliosis, migraine, normal CK.	At 20y: normal histology.
II-47	Male with DD, muscular hypotonia, toe walking, frequent falls, exercise intolerance, lactate ↑, normal CK.	At 6y: non-specific changes with a few internal nuclei, minicores, and a filamentous body.
II-48	Male with progressive lower limb weakness since 10y, loss of ambulation at 20y, normal CK.	At 18y: myopathic changes with mild FTD and uneven oxidative stains.
II-50	Female with progressive vision impairment since early childhood, unilateral ptosis since 12y, myogenic changes in facial and limb muscles on ENMG, normal CK.	At 33y: normal histology with one COX– fiber.
II-52	Male with exercise intolerance since 9y, episode of upper limb weakness, myalgia, and cramps after intense exercise at 16y with high CK (6,000 U/L), mild proximal muscle weakness, scoliosis, migraine.	At 24y: normal histology.
B. Patients with predominant central nervous system involvement		
II-1	Female with 19p13.3 microdeletion syndrome: mild DD, moderate ID, macrocephaly, dysmorphism, Gowers' sign, CK not measured.	At 11y: normal histology, single lipid accumulations and a filamentous body on EM.
II-2	Female with DeSanto-Shinawi syndrome: mild DD, mild ID, behavioral problems, epileptic discharges on EEG, dysarthria.	At 9y: normal histology, some Z-streaming and a filamentous body on EM.
II-3	Male with mevalonic aciduria: loss of all acquired skills after severe pneumonia at 1y8m → slow regain of skills, elevated CK (736 U/L), hypoplastic cerebellum, ataxia, tremor, muscular hypotonia.	At 2y8m: mild myopathic changes with some minicores.

№	Phenotype	Myopathology
II-22	Male with *BCS1L*-related MD: speech delay, ID, bilateral SNHL, lactate normal, CK not measured. Decreased RCC III activity in muscle tissue.	At 15y: non-specific changes with mild mitochondrial proliferation, single denervated fibers, Z-streaming. No COX– fibers.
II-24	Family with *SCN1A*-related DEE, three affected siblings. Proband (female): umbilical hernia, arthrogryposis, hip dysplasia, DD, ID, seizures, dysmorphism.	At 25 days of life: mild myopathic changes.
II-25	Male with Arts syndrome: muscular hypotonia since 2m, DD, exercise intolerance, hypomimic face, dysphagia, dysmorphism, frequent infections, epilepsy.	At 2y1m: non-specific changes with type 1 fibers being larger than type 2 fibers.
II-30	Male with AD *SET*-related ID: DD, muscular hypotonia, moderate ID, joint hyperlaxity, scoliosis, normal CK.	At 19y: non-specific changes with single denervated fibers, ring fibers, and several filamentous bodies.
II-35	Male with *ARFGEF1* variants: born in severe asphyxia, myoclonic seizures since the 2nd day of life, severe DD, spasticity, dystonia, spontaneous Babinski sign, bilateral ptosis, normal CK.	At 3y: non-specific changes with some internal nuclei.
II-36	Male with an *RYR2* variant: epilepsy since 13y, behavior problems, signs of myopathy on ENMG, lactate ↑, CK ↑ (2,397 U/L).	At 16y: myopathic changes with mild FTD.
II-41	Male with a 9p21.3 ~200 kb deletion: progressive spastic lower paraparesis since 3y, dystonia, normal CK.	At 12y: non-specific changes with single atrophic type 2 fibers and cytoplasmic bodies.
II-42	Male with a *TNRC6A* variant: juvenile idiopathic arthritis since 3y, osteoporosis with compression fractures, scoliosis, joint hyperlaxity, obesity, muscular hypotonia, dysarthria, autism, dysmorphic, normal CK.	At 9y: normal histology.
II-43	Female with epilepsy since 11y, progressive distal hemiparesis, dystonia, hemianopia, mild ID, normal CK.	At 31y: normal histology with one COX– fiber.
II-45	Male with polyneuropathy, cerebellar syndrome, cognitive dysfunction, psychiatric problems since childhood, tremor, dystonia, vertical vision loss, died at 47y.	At 43y: neurogenic atrophy with numerous internal nuclei, near type 2 uniformity, one COX– fiber.

№	Phenotype	Myopathology
II-46	Female born from a monozygotic twin pregnancy with apnea episodes at the 5th day of life, arthrogryposis, bilateral hip luxation, severe DD, spastic tetraplegia, dystonia, SNHL, optic atrophy, normal CK, died at 8y. Her twin sister is healthy.	At 7m: myopathic changes with type 1 fiber predominance, acid phosphatase activity in some fibers, and Z line streaming.
II-49	Male with generalized epilepsy since 15y, mild tremor, scoliosis, high CK (max. 5,000 U/L), no muscle weakness.	At 18y: myopathic changes with mild FTD, ring fibers, and uneven oxidative stains.
II-51	Female with generalized seizures since 14y, partial blockade of the right branch of His bundle, cerebral white matter lesions of unknown etiology, scoliosis, normal CK.	At 17y: normal histology.
II-53	Male with DD, dystonia, ataxia, Babinski sign, small optic discs, strabismus, horizontal nystagmus, dysmyelination, lactate ↑, mild CK ↑ (717 U/L).	At 1y2m: non-specific changes with many MyHCn positive fibers.
II-54	Male with severe DD, arthrogryposis, respiratory problems, spasticity, Babinski sign, hypospadias, agenesis of the corpus callosum, died at 4y3m.	At 1y4m: normal histology.
C. A patient with LHON		
II-29	Male with AR *DNAJC30*-related LHON: sudden visual impairment of left → right eye, typical changes on ophthalmological examination, no muscle problems.	At 16y: normal histology, no COX– fibers.

№ – patient identification number; AD – autosomal dominant; AR – autosomal recessive; BMI – body mass index; CK – creatine kinase; COX – cytochrome C oxidase; DD – developmental delay; DEE – developmental and epileptic encephalopathy; DG – dystroglycan; DMD/BMD – Duchenne/Becker muscular dystrophy; EEG – electroencephalography; EM – electron microscopy; ENMG – electro-neuromyography; FSHD – facioscapulohumeral muscular dystrophy; FTD – fiber-type disproportion; ID – intellectual disability; IHC – immunohistochemistry; kb – kilobase; LGMD – limb-girdle muscular dystrophy; LHON – Leber hereditary optic neuropathy; m – months; MD – mitochondrial disorder; MERRF – myoclonic epilepsy associated with ragged-red fibers; MyHCn – myosin heavy chain neonatal/fetal; RCC – respiratory chain complex; RRF – ragged-red fiber; SARC – sarcoglycan; SMA – spinal muscular atrophy; SMA-LED – SMA, lower extremity predominant; SNHL – sensorineural hearing loss; y – years

5.4 A novel hemizygous *PRPS1* variant causes an atypical Arts syndrome phenotype (Publication III)

This male patient (II-25, Tables 5B and 6B) was born at term with normal birth parameters, adaptation, and otoacoustic emission screening test. However, shoulder girdle hypotonia was noticed at the age of 2 months. Since 4 months of age, he has suffered from frequent viral and bacterial upper respiratory tract and inner ear infections. During his first year of life, acquired vitamin B12 deficiency and iron deficiency anemia were diagnosed and treated. His motor development was significantly delayed, with head control achieved at 7 months and independent walking at 2 years 3 months of age. By the age of 3 years, he presented with considerable exercise intolerance, oropharyngeal dysphagia, and dysarthria. His daily activities (e.g., walking, running, climbing the stairs, and eating) were disturbed due to trunk instability, lack of defense reflexes, and poor eye-hand coordination. At the age of 5 years, he was diagnosed with epilepsy.

At a physical examination at the age of 3 years, he was friendly, cooperative, and made eye contact. His height (0 SD), weight (−1 SD), and OFC (+2 SD) measurements revealed relative macrocephaly. He had generalized muscle hypotonia with decreased deep tendon reflexes and a hypomimic face with an open mouth and pronounced saliva flow, but the strength of his limb muscles was normal. In addition, he presented with a high forehead, bilateral epicanthus, high-arched palate, hyperplasia of gums, markedly worn teeth, *pectus excavatum*, and valgus feet (Figure 22A).

Based on the Wechsler Preschool and Primary Scale of Intelligence IV (WPPSI–IV) tests, his expressive speech was significantly deficient (no words), and fine motor skills, eye-hand coordination, and receptive speech were below the normal range. His brain MRI at 12 months of age showed small heterotopic knots of grey matter in the walls of the lateral ventricles and a tiny arachnoid cyst. ENMG at 5 months of age was normal, but at 7 and 12 months of age showed possible myopathic changes. Echocardiography (at 7 months), funduscopic examinations (at 5 months, 1 year 5 months, and 4 years 2 months), brainstem auditory evoked potential test (at 2 years 6 months), and otoacoustic emission tests (at 2 years 9 months and 4 years 1 month) were normal. As the patient's treating physician initially suspected a congenital myopathy, a muscle biopsy was done from the right lateral vastus muscle at the age of 2 years 1 month. The muscle histology revealed mild fiber size variation, with type 1 fibers slightly larger than type 2 fibers (Figure 23A and B). However, there were no central nuclei, cores, or nemaline rods.

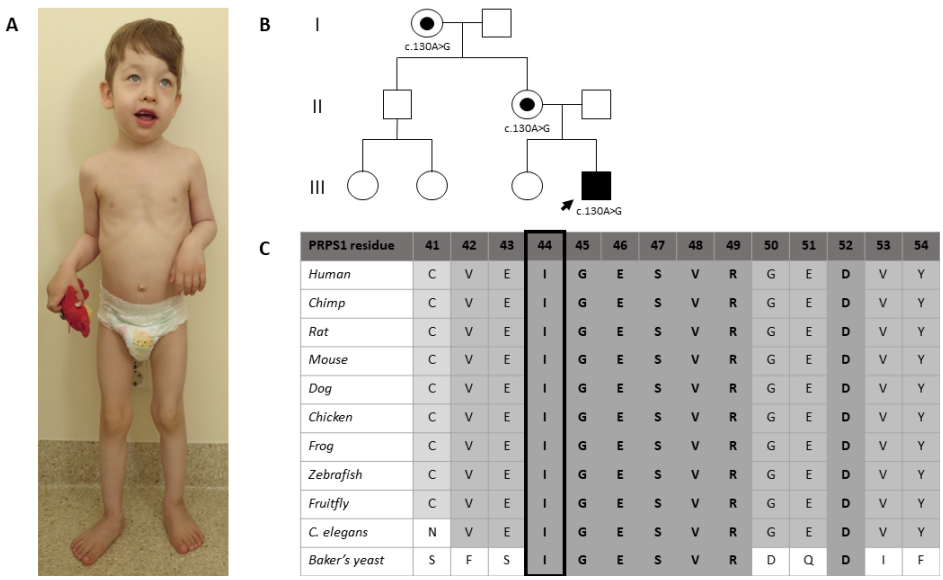
Mucopolysaccharides, oligosaccharides, transferrin isoelectric focusing (TIEF), acid α-glucosidase enzyme activity, serum and urinary amino acids, acylcarnitines, and very long chain fatty acids showed no significant changes. Serum organic acids showed some signs of MD (increased lactate, pyruvate, fumarate, and malate), but the lactate/pyruvate ratio was normal. Urine organic acids also showed subtle signs of a defect in energy metabolism (increased levels of

pyruvate, 3-OH-butyrate, sebacate, 4-OH-phenyllactate, and ketoglutarate), but other metabolites were also increased (4-OH-phenylacetate, glutarate, N-acetylaspartate, homovanillate, citrate, and methylmalonate). Initial urinary purine and pyrimidine analysis at 3 years of age was normal (Table 7).

Table 7. Purine and pyrimidine metabolite levels of patient II-25 at 3 and 4 years of age.

PP metabolite	At 3 years	At 4 years	Reference
Uric acid (S), µmol/L		123	106–325
Uric acid (U), mmol/mol Crea	356.2	348	165–618
Hypoxanthine (U), mmol/mol Crea	1.339	Traces	1–88.1
Xanthine (U), mmol/mol Crea	14.3	17.6	0–54.7

Crea – creatinine; PP – purine and pyrimidine; S – serum; U – urine



PRPS1 residue	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Human	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Chimp	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Rat	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Mouse	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Dog	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Chicken	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Frog	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Zebrafish	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
Fruitfly	C	V	E	I	G	E	S	V	R	G	E	D	V	Y
C. elegans	N	V	E	I	G	E	S	V	R	G	E	D	V	Y
Baker's yeast	S	F	S	I	G	E	S	V	R	D	Q	D	I	F

Figure 22. (A) The phenotype of patient II-25 at 4 years of age; note the myopathic face, *pectus excavatum*, and valgus feet (photograph published with permission from the patient's mother). (B) Family pedigree of the patient II-25 (arrow). (C) Conservation of the PRPS1 residues 41–54 across species; note the strict conservation of Ile-44 (black box).

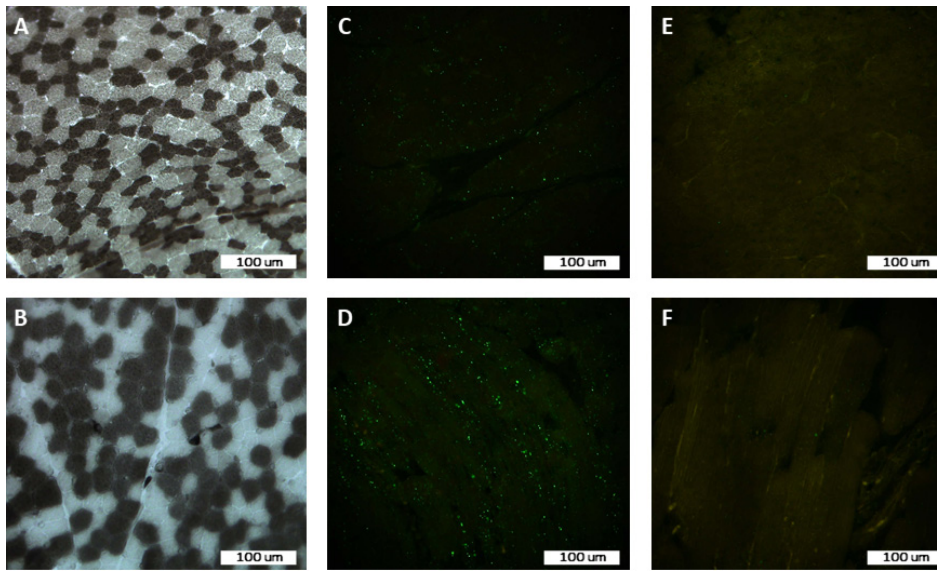


Figure 23. Muscle sections of patient II-25 with a *PRPS1* variant showing slightly larger type 1 fibers compared to type 2 fibers on (A) ATPase at pH 10.2 and (B) ATPase at pH 4.3, and (C and D) many diffuse anti-p62-positive punctae indicating increased autophagic flux, while muscle sections of (E and F) a control show single anti-p62-positive dots.

A gene panel (>4,800 genes) sequencing identified a hemizygous variant, c.130A>G, p.(Ile44Val), in the *PRPS1* gene (transcript NM_002764.3). The variant was confirmed by Sanger sequencing in the patient, and his mother and maternal grandmother were asymptomatic carriers (Figure 22B). The variant was a VUS according to the American College of Medical Genetics and Genomics variant interpretation guidelines [Richards *et al.* 2015]. It had not been reported before, was absent from the gnomAD database [Karczewski *et al.* 2020], and most *in silico* analyses predicted protein damaging effects.

Therefore, we measured the PRPS enzyme activity in the patient's erythrocytes, which was strongly reduced to 0.08 nmol/min/mg protein (compared to controls of 0.41–1.46 nmol/min/mg protein). This finding allowed us to reclassify the c.130A>G variant as likely pathogenic and confirm the diagnosis. In the literature, so dramatically decreased enzyme activity and loss-of-function variants in the *PRPS1* gene were described in Arts syndrome patients [Al-Maawali *et al.* 2015; Arts *et al.* 1993; de Brouwer *et al.* 2007; Maruyama *et al.* 2016; Synofzik *et al.* 2014]. These patients had congenital/early-onset sensorineural hearing loss, developmental delay, intellectual disability, muscle hypotonia and weakness, visual impairment, ataxia, susceptibility to infections, early lethality, peripheral neuropathy, seizures, and behavioral disturbances. On the other hand, patients with CMTX5 [Kim *et al.* 2007; Lerat *et al.* 2019; Meng *et al.* 2019], DFN2 [Cabanillas *et al.* 2018; Gandia *et al.* 2015; Kim *et al.* 2016; Liu *et al.* 2010; Robusto *et al.* 2015], or intermediate phenotypes with only

sensorineural hearing loss and peripheral neuropathy [Nishikura *et al.* 2019; Park *et al.* 2013; Robusto *et al.* 2015] have shown only a mild to moderate decrease in PRPS activity. Therefore, the severity of the *PRPS1*-related disorders largely correlates with the residual PRPS activity measured in erythrocytes or fibroblasts.

However, intrafamilial phenotypic variation was described in a Spanish family with nine affected males: all presented with prelingual severe-to-profound sensorineural hearing loss, but three males had additional features (global developmental delay, mild intellectual disability, seizures, and clubfoot) [Gandia *et al.* 2015]. Furthermore, nine male patients with hyperuricemia and gout (i.e., PRPS superactivity) also harbored specific point mutations in the *PRPS1* gene, and most of these patients additionally presented with developmental delay, intellectual disability, muscle hypotonia, sensorineural hearing loss, peripheral neuropathy, and frequent infections [Moran *et al.* 2012; Porrmann *et al.* 2017; Roessler *et al.* 1993]. Interestingly, while patients with PRPS superactivity did show increased PRPS activity in fibroblasts, those with additional neurological features had a marked decrease in PRPS activity in erythrocytes [Becker *et al.* 1995]. Moreover, two patients with hyperuricemia and congenital sensorineural hearing loss, developmental delay, and severe respiratory infections had normal PRPS activity in fibroblasts and decreased activity in erythrocytes [Moran *et al.* 2012]. This phenomenon could be explained by a destabilizing effect of the variants resulting in increased lability of PRPS in postmitotic cells.

Our patient presented with marked skeletal muscle hypotonia, developmental delay, and susceptibility to infections, features characteristic of Arts syndrome. However, at the age of 5 years, our patient had not (yet) developed hearing loss, described as early-onset in all Arts syndrome patients. Also, it has been shown that PRPS1 is expressed in cochlear and vestibular hair cells and spiral ganglion neurons in mice [Liu *et al.* 2010], and zebrafish models with prps1a and prps1b mutants revealed reduced numbers of inner ear hair cells resulting in significant loss of hearing in zebrafish [DeSmidt *et al.* 2020; Pei *et al.* 2016]. One explanation could be that the residual activity of the PRPS enzyme is higher in nucleated cells than in erythrocytes, and our patient may develop hearing loss later in life.

Two case reports of patients with loss-of-function *PRPS1* variants showed reduced urinary hypoxanthine [Al-Maawali *et al.* 2015; de Brouwer *et al.* 2007]. Our patient's first urinary purine and pyrimidine analysis was normal, so we performed new measurements from a follow-up urine sample (Table 7). Indeed, hypoxanthine was below our lower reference cutoff of 1 mmol/mol Crea. However, as our laboratory's experience shows that hypoxanthine values are frequently below this cutoff value, it is debatable whether this finding should be regarded as pathological. Furthermore, variable reference ranges for purine and pyrimidine metabolites have been set during the validation of different analysis methods, with some studies marking only the upper reference

cutoff for the majority of purine and pyrimidine metabolites [Hartmann *et al.* 2006; la Marca *et al.* 2006; Monostori *et al.* 2019; Vidotto *et al.* 2003].

Interestingly, a *Drosophila* model carrying two Arts syndrome variants and showing locomotive defects revealed dysfunctions in basal autophagy and lysosome homeostasis and hypersensitivity to oxidative stress. In addition, accumulation of lipid droplets in pupal eyes and protein aggregation in the brains of the mutant flies were observed [Delos Santos *et al.* 2019]. Therefore, we studied the expression of CASA proteins in our patient's muscle tissue. We found diffuse punctae in many muscle fibers on p62 IHC representing prominent autophagosomes within the sarcoplasm, which were not present in a control muscle (Figure 23C–F). This finding refers to an increased autophagic flux as the p62 protein is an autophagy receptor transporting cargo to autophagosomes [Arndt *et al.* 2010]. However, other CASA protein antibodies (anti-LC3, anti-HSP70, and anti-BAG3) showed no staining reaction. Therefore, the increase in p62 probably does not reflect defective macroautophagy or CASA-related autophagy but may be related to microautophagy. Further investigations regarding autophagy processes in patients with *PRPS1* variants could bring new insights.

In conclusion, this case demonstrates the challenges in clinical diagnostics. While the patient's phenotype showed signs of muscle disease and the metabolic analyses referred to an MD, we finally reached a diagnosis of PRPS1 deficiency. However, the patient has not (yet) developed hearing loss, the main early-onset feature of Arts syndrome.

5.5 Biallelic variants in the *JAG2* gene cause a novel type of muscular dystrophy (Publication IV)

Patient II-32 (Tables 5B and 6A) is an 11-year-old girl of Estonian origin. She was born from a twin pregnancy without complications. Her early psychomotor development was normal: she started walking at the age of 14 months and acquired the ability to run and navigate the stairs. The disease-onset was at about 3 years of age when she presented with decreased stamina and frequent falls. By 5 years of age, she had developed pronounced valgus feet, weak ankles, toe-walking, Gowers' sign, and exercise intolerance. According to the Wechsler Intelligence Scale for Children IV (WISC-IV) tests at 6 years, her cognitive skills were on the borderline level, but the results differed by subtest. Perceptual reasoning was age-appropriate, verbal comprehension and processing speed were on the marginal level, and working memory subtests were below the age-appropriate level. However, the results were considerably affected by the patient's insecurity and socio-emotional sensitivity.

During the next six years, she presented slowly progressive muscle weakness, predominantly proximal and axial, and muscle atrophy with a limb-girdle pattern. She lost the ability to run, stand on one foot, squat, and navigate the stairs independently. At the last follow-up at 11 years of age, she was ambulatory with a waddling gait and toe-walking, and her feet were internally

rotated. She had left- more than right-sided proximal more than distal lower more than upper extremity, hip girdle, back, and neck (flexion more than extension) muscle weakness, mild bladder sphincter weakness, muscle atrophy, scoliosis, ankle and elbow contractures, and decreased deep tendon reflexes. She did not have facial or bulbar muscle weakness. Her intellect, vision, and hearing were normal.

Her CK was 204 U/L (reference <149 U/L), but lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase concentrations in blood were normal. Urinary organic acids, serum acylcarnitines and TIEF, acid α-glucosidase, and OXPHOS enzyme analyses from muscle were also normal. ENMG at 6 years of age showed possible myogenic involvement in the right-side medial vastus and deltoid muscles, but at 8 years of age, the ENMG showed ubiquitous myogenic involvement. Echocardiography revealed mild mitral valve prolapse, which was clinically non-significant. mtDNA sequencing from blood, *SMN1* gene deletion/duplication analysis, sequencing of a large gene panel, and, subsequently, diagnostic child-parent trio WES were carried out with no significant findings.

At the age of 6 years, two muscle biopsies were performed from the right and left lateral vastus muscles. Both specimens showed moderate fiber size variability, fibrosis, foci of fat cells in peri- and endomysium, hypercontracted fibers, and Z-streaming. The ultrastructure of the left vastus muscle also revealed lots of electron-dense material of unknown origin (Figure 24). There were no internal nuclei, rods, cores, type 1 fiber predominance, or fiber-type disproportion. IHC for merosin, dystrophin C-terminus, α-, β-, and γ-sarcoglycans, and markers of protein aggregation and autophagy (p62, LC3, HSP70, BAG3 antibodies) was normal.

Reanalysis of the trio WES data revealed a heterozygous *de novo* candidate variant in the *JAG2* gene: c.2473C>T, p.Arg825Cys. This variant was absent from the gnomAD database [Karczewski *et al.* 2020], and PolyPhen-2 [Adzhubei *et al.* 2010] predicted it to be probably damaging. The *JAG2* gene was not associated with human Mendelian diseases, so we submitted our candidate gene to the GeneMatcher platform [Philippakis *et al.* 2015]. Our patient's phenotype matched with the patients of several other submitters. Through direct introductions between collaborators, 23 patients from 13 unrelated families from Estonia, USA, Morocco, Iran, France, Poland, United Kingdom, Sri Lanka, United Arab Emirates, and Egypt were finally gathered.

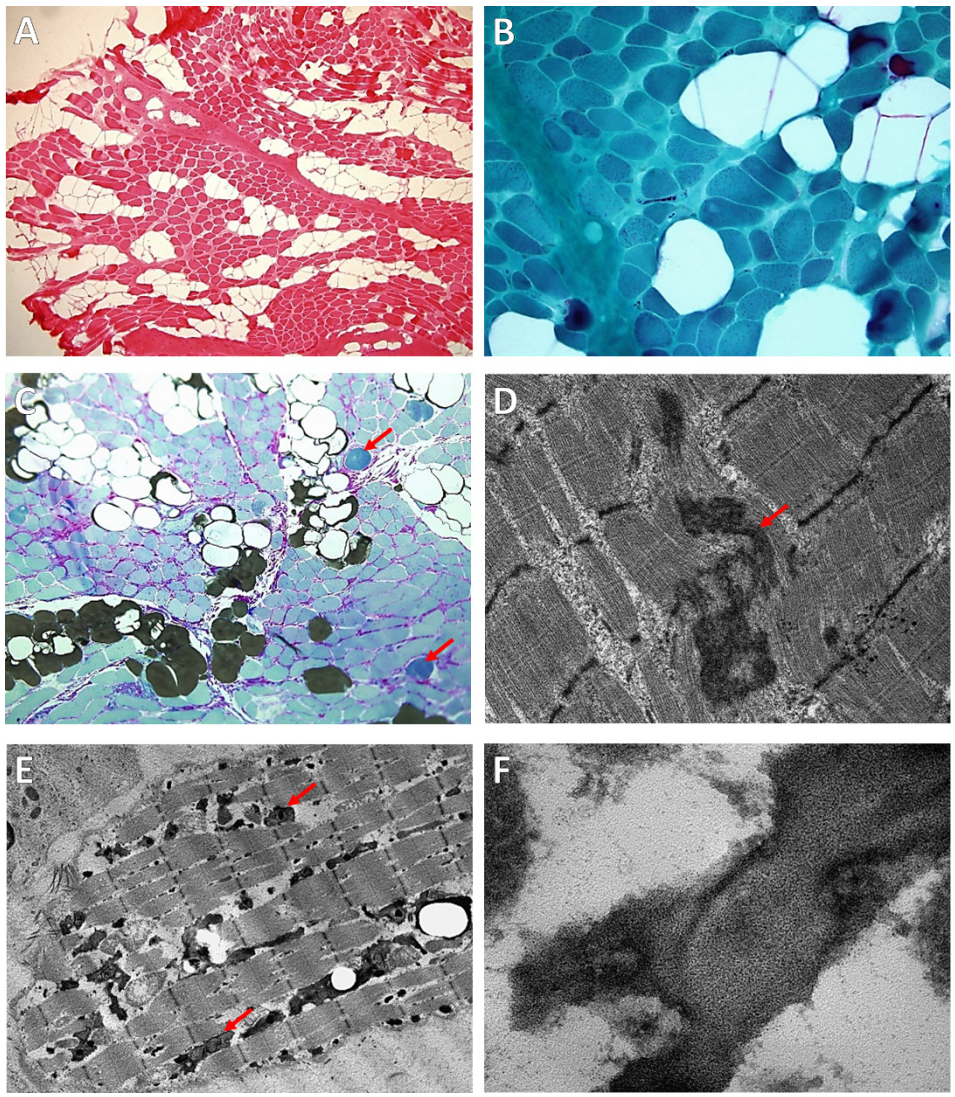


Figure 24. Muscle sections of patient II-32 with *JAG2* variants revealing fiber size variation, fibrosis, and fat cell infiltration on (A) HE and (B) mGT, (C) fibrosis of the endomysium, many fat cells accumulated between myofibers, and some hyper-contracted fibers on semi-thin section stained with methylene-blue and alkaline fuchsin (arrows), and ultrastructure of (D) Z line streaming and (E) electron-dense material with (F) showing higher magnification.

However, all other identified patients harbored biallelic variants in the *JAG2* gene. Homozygous missense variants were found in eight families. The other patients had compound heterozygous variants, including two missense, one nonsense, two frameshift, and one in-frame deletion variant, and one patient had inherited a maternal 3.2 Mb terminal deletion at 14q32.33, encompassing *JAG2*.

We performed a CMA analysis to exclude a deletion of *JAG2*; however, no aberrations were found. Nevertheless, an extensive search revealed a second variant in *JAG2*, c.283A>G, p.Thr95Ala, inherited from the mother. This variant is present in one individual with an allele frequency of 0.00003285 in the gnomAD database [Karczewski *et al.* 2020], and PolyPhen-2 [Adzhubei *et al.* 2010] and SIFT [Ng and Henikoff 2001] predict it to be probably damaging/ deleterious. Subsequently, we detected that the *de novo* c.2473C>T, p.Arg825Cys variant is located on the paternal chromosome, thus confirming that the variants are *in trans*. However, we cannot exclude germline mosaicism in the father. Autosomal recessive inheritance with one *de novo* variant has significant implications for genetic counseling, but it is challenging to uncover and requires manual data curation. Statistically, *de novo* recessive variants should rarely be detected in the clinic [Zlotogora 2004]. However, in Gaucher disease, for example, three cases have already been described [Hagege *et al.* 2017; Saranjam *et al.* 2013]. In addition, a study by Papuc *et al.* [Papuc *et al.* 2019] discovered three autosomal recessive cases with one inherited and one *de novo* variant, constituting 8% of all solved cases.

The *JAG2* gene encodes Jagged2, which belongs to the family of Notch ligands. The other Notch ligands have all been associated with monogenic disorders. The *JAG1* gene is associated with Alagille syndrome 1 (MIM# 118450), CMT, axonal, type 2H (MIM# 619574), and tetralogy of Fallot (MIM# 187500). Variants in *DLL1* cause neurodevelopmental disorder with nonspecific brain abnormalities and with or without seizures (MIM# 618709). The *DLL3* is associated with autosomal recessive spondylocostal dysostosis (MIM# 277300) and *DLL4* with Adams-Oliver syndrome 6 (MIM# 616589). Table 8 summarizes the phenotype of the 12 male and 11 female patients with biallelic *JAG2* variants. Six families were consanguineous. The age range of the patients at the last evaluation was 5–53 years. Muscle weakness was progressive in all but one affected individual.

Fourteen patients had a diagnostic skeletal muscle biopsy. All the specimens showed myopathic or dystrophic changes, including increased fiber size variation, internal nuclei, split, lobulated, whorled, and moth-eaten fibers, core-like areas, myofiber necrosis, phagocytosis, mild to marked endomysial fibrosis, and various degrees of fatty infiltration.

MRI of the lower limb muscles was performed on 11 participants, including our patient. A distinct pattern of muscle involvement was found with central areas of less affected muscle in the vastus lateralis, intermedius, and medialis (Figure 25A). This pattern of muscle involvement on MRI is similar to patients with Ullrich CMD (MIM: 254090) and Bethlem myopathy (MIM# 158810) [Mercuri *et al.* 2005]. Our patient also showed a focal area of fatty infiltration in the anterior tibial muscles (Figure 25B). Overall, proximal muscle groups were more severely affected than distal muscles, which showed only mild degeneration. However, no single muscle group appeared to be completely spared.

Table 8. Summary of the clinical features of patients with *JAG2* variants.

Clinical feature	Nr of patients	Clinical feature	Nr of patients
Disease-onset:		Facial weakness	4/23
- Infancy	5/23	Ptosis	4/23
- Childhood	9/23	Rigid spine/neck	7/23
- Adolescence / young adulthood	9/23	Scoliosis	12/23
		Contractures	13/23
Limb muscle weakness:		Muscle atrophy	8/23
- Proximal upper limb	23/23	Muscle hypertrophy	6/23
- Distal upper limb	15/23	Low FVC (41–84%)*	13/20
- Proximal lower limb	23/23	ID/speech delay/ASD	8/23
- Distal lower limb	18/23	Cardiac disease	5/23
Loss of ambulation (at 8–18 years)	8/23	Elevated CK (1–4x upper limit of normal)	10/22
Neck weakness	19/23	Myopathic ENMG	16/19

* none needed respiratory support

ASD – autism spectrum disorder; CK – creatine kinase; ENMG – electroneuromyography; FVC – forced vital capacity; ID – intellectual disability; Nr – number

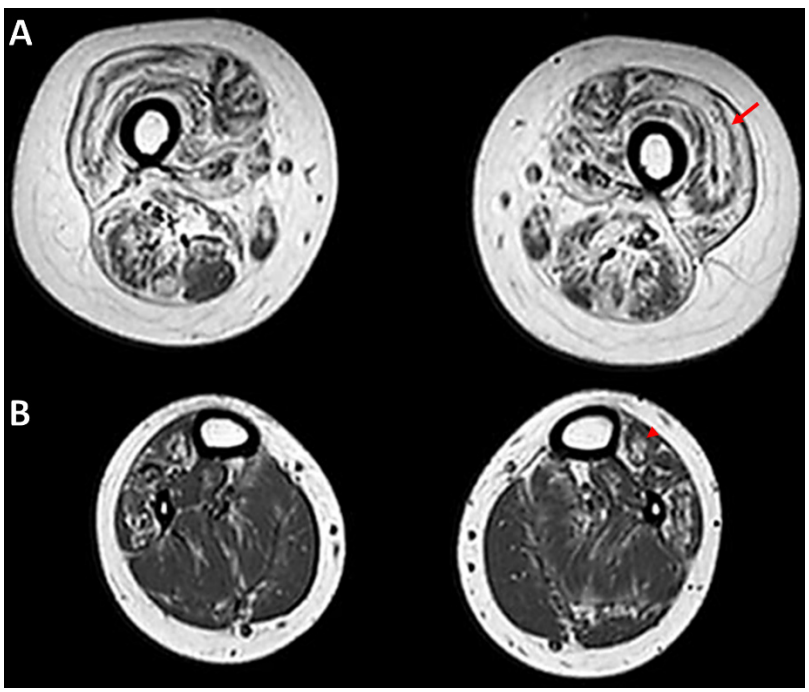


Figure 25. Muscle MRI of patient II-32. Axial chemical shift-encoded fat images of the (A) thighs and (B) calves showing a core of preserved muscle within the *m. vastus lateralis* (arrow), *m. vastus intermedius*, and *m. vastus medialis* and a focal area of degeneration in the *m. tibialis anterior* (arrowhead).

The transcriptome analysis of our patient identified three statistically significantly downregulated genes: *PAX7* (Z-score=-8.57, *p*adj=2.19x10^{-6}), *MYF5* (Z-score=-8.54, *p*adj=3.2x10^{-4}), and *CADM2* (Z-score=-7.16, *p*adj=7.26x10^{-4}). Of note, *PAX7* and *MYF5* downregulation was not observed in 12 other patients with muscle diseases or 100 control samples. The *PAX7* gene encodes a transcription factor indispensable for postnatal muscle growth and regeneration. The *MYF5* gene encodes a myogenic regulatory factor activated by *PAX7* [von Maltzahn *et al.* 2013].

During postnatal myogenesis or in response to exercise or skeletal muscle injury, satellite cells enter the cell cycle and become myoblasts, which first proliferate and then differentiate and fuse into myofibers. Both the *PAX7* gene and the Notch signaling pathway maintain the quiescence of satellite cells postnatally, and abrogation of either *PAX7* or Notch components result in precocious myoblast differentiation and depletion of the satellite cell pool [Mourikis and Tajbakhsh 2014; von Maltzahn *et al.* 2013]. Biallelic *PAX7* variants found in five patients with myopathic features resulted in a complete absence of anti-PAX7 staining on IHC in the biopsied muscle, referring to depletion of satellite cells [Feichtinger *et al.* 2019]. In addition, deficiency of two other muscle disease genes associated with Notch signaling, *POGLUT*, which encodes an enzyme that glycosylates the Notch receptors, and *MEGF10*, which encodes a transmembrane protein that interacts with NOTCH1, showed significantly reduced satellite cell count compared to controls [Li *et al.* 2021; Servian-Morilla *et al.* 2020].

Therefore, we performed additional IHC on our patient's muscle tissue and discovered that the number of anti-PAX7 and anti-myogenin (expressed in differentiated myocytes) positive cells did not differ between the patient and age-matched control. In addition, anti-MyHCd and anti-MyHCn revealed only single immature fibers. However, regenerating muscle fibers are a characteristic finding in muscular dystrophies. Also, muscle specimens of DMD patients showed an increase in satellite cells compared to controls, and patients with defective *DMD*, *DYSF*, and *FKRP* genes showed upregulation of *PAX7* mRNA levels [Dadgar *et al.* 2014; Kottlors and Kirschner 2010].

In conclusion, we presented the detailed clinical, imaging, pathological, and genetic data of a new muscle disease entity, LGMD R27 *JAG2*-related (MIM# 619566). Our findings indicated that the pathomechanism of this disease could be linked to satellite cell dysfunction.

6. CONCLUSIONS

1. We compared the genetic and myopathological findings in patients with a clinical suspicion of a mitochondrial disorder (Publication I).
   1.1. Muscle biopsy specimens revealed characteristic histopathological changes in six patients with variants in the nuclear MD-associated genes *POLG* and *SLC25A4*, an mtDNA gene *MT-ATP6*, SMA-associated gene *SMN1*, the *MYH2* gene causing a myosin myopathy, and the *EPG5* gene causing Vici syndrome.
   1.2. All patients with clinical symptoms indicating muscle involvement presented with some degree of myopathic histopathological changes, while all four patients with non-specific myopathological features had a primary CNS involvement and upper motor neuron signs.
   1.3. The muscle sample of the patient with *TSPOAP1*-related dystonia and spastic hemiparesis showed mild myopathic changes.
   1.4. In four patients diagnosed with a disease not classified as MD, an association with mitochondrial dysfunction was found either as part of the pathogenesis of the disease or as a secondary pathological finding.
   1.5. The muscle biopsy played an important role in confirming the genetic diagnosis in several patients by revealing typical histopathological changes or biochemical abnormalities supporting the pathogenicity of novel variants in known genes and enabling the analysis of tissue-specific mtDNA alterations.
2. We delineated the phenotype and pathomechanism of defective *SPATA5* (Publication II).
   2.1. The *SPATA5*-related neurodevelopmental disorder is an early-onset disease characterized by developmental delay, severe speech delay, hearing loss, epilepsy, microcephaly, brain anomalies, visual impairment, spasticity, and gastrointestinal problems.
   2.2. All types of variants have been reported, with an in-frame deletion c.989_991del, p.(Thr330del) being the most frequent.
   2.3. We showed that SPATA5 deficiency impairs mitochondrial fusion and fission, decreases the production of ATP, and hinders axonal growth of the rat primary cortical neurons.
   2.4. We concluded that the pathomechanism of *SPATA5*-related disease is associated with mitochondrial dysfunction.
3. We analyzed the genetic and myopathological findings of a muscle biopsy cohort of patients with a childhood-onset disorder.
   3.1. The combined diagnostic yield of different genetic analyses performed during routine clinical diagnostic workup was 43%. By actively contacting the genetically unsolved patients and performing WES (except in one patient with a typical FSHD phenotype whose DNA was sent for specific testing and another patient whose diagnosis was reached by

MLPA analysis of the *SMN1* gene), the diagnostic yield increased to 61% (35/57).

3.2. The phenotype of the study group patients was heterogeneous: 35 patients had a predominant PNS involvement, and 18 had a predominant CNS involvement. The diagnostic yield was much higher in the PNS involvement group (25/35, 71%) compared to the CNS involvement group (7/18, 39%).

3.3. The muscle histology, clinical symptoms, and genotype correlated well. Most patients with apparent muscle involvement showed myopathic or dystrophic changes in the muscle, with nine biopsies demonstrating specific histopathological features. On the contrary, muscle histology of most patients with a predominant CNS involvement or pyramidal signs was normal or revealed only non-specific changes.

3.4. Although the muscle biopsy was not always needed to confirm the diagnosis, it played a substantial role in the diagnostic process. Muscle biopsy enabled the detection of specific structural changes or altered protein expression on muscle histology, to discover mtDNA variants present only in muscle tissue, and to perform transcriptome studies.

4. We described a patient with a novel hemizygous *PRPS1* variant causing an atypical Arts syndrome phenotype (Publication III).

4.1. We presented a boy with marked muscular hypotonia, developmental delay, exercise intolerance, hypomimic face, dysphagia, epilepsy, and susceptibility to infections in whom we found a novel variant in the *PRPS1* gene. The disease causality was confirmed by the low enzyme activity of PRPS in erythrocytes.

4.2. While our patient had several features characteristic of Arts syndrome, the most severe phenotype in the spectrum of *PRPS1*-related diseases, he did not present with hearing loss, which was early-onset in all other reported Arts syndrome patients.

4.3. The patient's muscle histology showed relatively non-specific changes, including diffuse punctae of p62 in many muscle fibers indicating an increased autophagic flux.

5. We described a novel type of muscular dystrophy caused by defects in the *JAG2* gene (Publication IV).

5.1. We presented a girl with progressive predominantly proximal muscle weakness since 5 years of age, mildly elevated CK, and myopathic changes in the muscle. She harbored a maternally inherited variant on one *JAG2* allele and a *de novo* variant on the other.

5.2. An international collaboration resulted in establishing a new gene-disease association by gathering 23 patients with a similar phenotype and biallelic variants in the *JAG2* gene, including our patient. The patients presented with a limb-girdle type muscle weakness and a distinct pattern of muscle involvement on MRI with less affected central areas in vastus lateralis, intermedius, and medialis muscles.

5.3. The muscle transcriptome and satellite cell IHC data of our patient indicated that the pathomechanism of this disease could be linked to satellite cell dysfunction.

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WEB RESOURCES

GeneTable of Neuromuscular Disorders is an online version of the gene table printed annually in the Journal of Neuromuscular Disorders, which cross-references all the allelic phenotypes and links to external databases: https://www.musclegenetable.fr/.

Online Mendelian Inheritance in Man® (OMIM) contains referenced overviews of all known Mendelian disorders and over 16,000 genes, freely available and updated daily: https://omim.org/.

The Genome Aggregation Database (gnomAD) aggregates exome and genome sequencing data from various large-scale sequencing projects: https://gnomad.broadinstitute.org/.

The Human Protein Atlas is a Swedish-based program aimed at mapping all the human proteins in cells, tissues, and organs: https://www.proteinatlas.org/.

VarSome is a search engine aggregating and processing information regarding human genomic variants: http://varsome.com.

SUMMARY IN ESTONIAN

Lapseeas alanud pärilike neuromuskulaarsete haiguste molekulaargeneetiliste ja morfoloogiliste uuringutulemuste võrdlus

Aastal 1836 kirjeldasid itaalia arstid G. Conte ja L. Gioja kahte venda lihashüpertroofia ja progresseeruva nõrkusega ning oletasid, et vendade haigus võis olla põhjustatud lihases endas esinevast muutusest [Tyler 2003]. Veidi hiljem kirjeldas inglise arst Edward Meryon (1809–1880) samuti mitmeid lihashüpertroofia ja süveneva paralüüsiga patsiente. Meryon uuris nende haigete lahangutelt saadud koeproovide histoloogiat ja nägi, et närvikoed olid säilitanud oma terviklikkuse, kuid lihaskiud olid lagunenud ning muutunud granulaarseks ja rasvkoeliseks massiks [Emery and Emery 2011; Meryon 1852].

Prantsuse neuroloog Guillaume Benjamin Amand Duchenne (1806–1875) andis olulise panuse neuromuskulaarsete haiguste valdkonna arengusse. Aastal 1842 alustas ta oma kliinilises praktikas elektrofüsioloogiliste uuringute tegemist, kasutades enda leiutatud kaasaskantavat seadeldist, mis võimaldas üksikuid lihaseid ja närve elektriliselt stimuleerida. Lisaks leiutas ta instrumendi (tuntud kui Duchenne'i troakaar), mis võimaldas teha patsientidele perkutaanset lihasbiopsiat, ning ta oli üks esimesi arste, kes kogus patsientide ja histoloogiliste preparaatide pilte. Duchenne avaldas kogumiku, kus kirjeldas detailselt paljude pseudohüpertroofilise lihasparalüüsiga (praegu tuntud kui Duchenne'i lihasdüstroofia, ing k *Duchenne muscular dystrophy* ehk DMD) patsientide kliinilisi, elektrofüsioloogilisi ja neuropatoloogilisi leide haiguse erinevates staadiumites [Parent 2005; Tyler 2003; Waclawik and Lanska 2019].

Aastal 1954 avaldasid inglise neuroloogid John Walton ja Frederick Nattrass esimese lihasdüstroofiate klassifikatsiooni, kus kirjeldati seitset erinevat alatüüpi [Walton and Nattrass 1954]. Hiljem lisandusid mitmed uued lihashaiguste alagrupid, mis põhinesid lihaskoe histopatoloogilistel leidudel, näiteks glükogeeni akumulatsioon, nemaliinkehad ja südamikud [Kakulas 2008]. Esimene kliiniliste, biokeemiliste ja morfoloogiliste tunnuste kirjeldus patsiendist, kelle haiguse põhjuseks oli viga mitokondriaalsetes ensüümides, pärineb aastast 1962 [Luft *et al.* 1962]. Seda haiguslugu illustreerisid ka esimesed fotod parakristalliinsete ja lipiidsete inklusioonide ning kontsentriliste kristadega mitokondritest. Düstrofiini [Kunkel *et al.* 1985] ja paljude teiste lihasvalkude avastamine sillutas omakorda teed immunohistokeemia arenguks.

Geneetika kiire evolutsioon sai alguse Mendeli pärilikkusseadustest, kromosoomiteooriast, arusaamast, et geenid koosnevad desoksüribonukleiinhappest ehk DNA-st, ja DNA sekveneerimismeetodite leiutamisest (näiteks Sangeri sekveneerimine) ning arenes kaasaegseks molekulaardiagnostikaks koos järgmise põlvkonna sekveneerimise ja multi-oomikaga [Gayon 2016; Giani *et al.* 2020; Hasin *et al.* 2017]. Geneetika võidukäik on muutnud neuromuskulaarsete haiguste klassifikatsioonisüsteemi rohkem geenipõhiseks, kusjuures praeguseks

on erinevate neuromuskulaarsete fenotüüpidega seostatud juba üle 600 geeni (www.musclegenetable.fr).

Eestis alustas lihasbioptaatide valgus- ja elektronmikroskoopiliste uuringute tegemist emeriitdotsent Andres Piirsoo 1993. aastal koostöös lasteneuroloogi emeriitprof. Tiina Talvikuga, kes oli DMD epidemioloogiliste uuringute eestvedajaks Eestis. Tookordse uurimusega kaardistati Eesti DMD-ga patsientide kliinilised tunnused [Talkop *et al.* 1999]. DMD levimus Eesti meespopulatsioonis 1. jaanuaril 1998 oli 1:7,837 ja DMD esinemissagedus aastatel 1986–1990 oli 1:5,652 [Talkop *et al.* 2003]. Prof. Katrin Õunapi juhtimisel on läbi viidud põhjalikke uuringuid mitokondriaalse haiguse (MH) kahtlusega patsientidel. Esimeses projektis jõuti järeldusele, et lastel on varajase algusega MH-de sõeltestimiseks sobiv meetod seerumi laktaaditaseme mõõtmine. Aastatel 2003–2009 oli MH-de levimus Eestis 1:20,764 elussünni kohta [Joost *et al.* 2012]. Teises uuringus hinnati kogu eksoomi sekveneerimise (ing k *whole exome sequencing* ehk WES) efektiivsust MH kahtlusega patsientidel, mis oli 57%, kusjuures MH diagnoos kinnitus 14%-l [Puusepp *et al.* 2018b].

Käesoleva uuringu eesmärgid

1. Analüüsida lihaskoes esinevaid histopatoloogilisi muutusi mitokondriaalse haiguse kahtlusega patsientidel (I artikkel);
2. Iseloomustada *SPATA5* geeni variantide seost mitokondriaalse düsfunktsiooniga (II artikkel);
3. Avastada uusi haigusseoselisi patogeenseid variante lapseeas alanud lihashaiguse kahtlusega Eesti patsientidel;
4. Uurida *PRPS1* geeni varem kirjeldamata hemisügootse variandi kliinilist ja funktsionaalset mõju (III artikkel);
5. Kirjeldada uudse lihashaigusseoselise geeni *JAG2* bialleelsetest variantidest põhjustatud fenotüüpi, lihaskoe histopatoloogiat ja transkriptoomi profiili (IV artikkel).

Patsientide ja meetodite lühikirjeldus

Alates jaanuar 2014 kuni märts 2016 viisime läbi teadusprojekti, mille raames uurisime patsiente, kellel oli kliiniliselt kahtlus lapseeas alanud MH-le, kuid selget geneetilist diagnoosi polnud. Esiteks valisime uuringugruppi sobivad isikud retrospektiivselt 181 patsiendi hulgast, kellel oli kliinilise geneetika keskuses, Tartu Ülikooli Kliinikumis (TÜKis) säilitatud fibroblastide rakukultuur ajavahemikus 2003–2013. Teiseks selekteerisime kandidaadid prospektiivselt patsientide seast, kes olid projekti toimumise ajaperioodil suunatud meditsiinigeneetiku vastuvõtule TÜKi. Kokku osales teadusprojektis 28 patsienti, kellest 16-l olid olemas ka külmutatud lihaskoe preparaadid. Me analüüsisime WES-i efektiivsust – tulemused on kokku võetud dr. Karit Reinsoni doktoritöös [Reinson 2018]. Teiseks uurisime lihaskoe histopatoloogilisi muutusi nendel uuringugrupi patsientidel, kellele oli tehtud lihasbiopsia – tulemusi kirjeldatakse ja analüüsitakse käesolevas väitekirjas. Täiendavalt viisid prof. Allen Kaasik ja tema töögrupp läbi funktsionaalsed katsed roti närvirakkudel, et

uurida vigase *SPATA5* geeni mõju mitokondrite dünaamikale, energia tootmisvõimele ja aksonite kasvupotentsiaalile.

Teine uuringugrupp moodustus patsientidest, kellele oli Eestis ajavahemikus 2013–2016 tellitud lihasbiopsia histoloogiline analüüs ja kellel oli kahtlus lapseeas (<18 aasta vanuselt) alanud pärilikule neuromuskulaarsele haigusele. Projekti kaasamise kriteeriumitele vastas 64 patsienti, kellest seitse jäid uuringust välja (patsient ei soovinud geneetilisi uuringuid, ei soovinud osaleda teadusprojektis või ei olnud kättesaadav). Kahekümne neljal patsiendil oli geneetiline diagnoos selgunud juba rutiinse kliinilise diagnostika käigus. Erinevate teadusprojektide raames tegime meie geneetilisi analüüse 33-le patsiendile.

Kõik molekulaargeneetilised testid tehti vere lümfotsüütidest eraldatud DNA-st. Viiele patsiendile tegime TruSight One paneeli (Illumina Inc., San Diego, CA, USA) sekveneerimise analüüsi, mis kattis >4,800 monogeensete haigustega seotud geeni. Kahekümne neljale patsiendile tellisime GenomeScan'ist (Leiden, Holland) WES-i ning saadud andmeid analüüsisime Seqr platvormil (https://github.com/broadinstitute/seqr). Neljale patsiendile oli kliinilise diagnostika raames tellitud WES Eesti Geenivaramust (Tartu Ülikool), kus haigusseoselisi muutusi ei leitud. Kuid koostööprojekti raames teostati Massachusettsi Tehnoloogia Instituudi ja Harvardi Broadi Instituudis (Cambridge, MA, USA) nende patsientide WES-i andmete kordusanalüüs. Kõikide kandidaatvariantide kinnitamiseks ja perekondliku segregatsioonianalüüsi tegemiseks kasutasime Sangeri sekveneerimismeetodit. Lisaks saatsime ühe patsiendi DNA fatsioskapulohumeraalse lihasdüstroofia tüüp 1 geneetiliseks analüüsiks Leideni Ülikooli Meditsiinikeskusesse Hollandis.

PRPS1 geeni hemisügootse variandiga patsiendile tegime puriinide-pürimidiinide analüüsi kasutades vedelikkromatograafia-tandem-mass-spektromeetriat (Waters, Milford, MA, USA) ning André van Kuilenburgi labor (Amsterdam UMC, Holland) mõõtis fosforibosüül-pürofosfaat süntetaasi aktiivsust erütrotsüütides. *JAG2* bialleelsete variantidega patsiendile tellisime lihaskoest eraldatud RNA sekveneerimise Broadi Instituudist ja analüüsisime andmeid arvutiprogrammi R paketi OUTRIDER-ga [Brechtmann *et al.* 2018].

Me uurisime mõlema uuringugrupi kõikide patsientide olemasolevaid valgusmikroskoopia preparaate, elektronmikroskoopia võrke ja nendest tehtud digitaalselt arhiveeritud fotosid. Tehtud oli nii histoloogilisi, ensüümhistokeemilisi kui ka immunohistokeemilisi värvinguid. Täiendavalt tegime vajalikke lisavärvinguid nende patsientide materjalile, kelle külmutatud lihasbioptaat oli meile kättesaadav.

Peamised tulemused ja järeldused

1. Võrdlesime mitokondriaalse haiguse kahtlusega patsientide geneetilisi ja lihaskoe histopatoloogilisi leide (I artikkel).
   1.1. Iseloomulikud lihaskoe histopatoloogilised muutused esinesid kuuel patsiendil, kellel leidsime haigusseoselised variandid MH-ga seotud tuuma geenides *POLG* ja *SLC25A4*, mitokondriaalses DNA-s, spinaalse

lihasatroofiaga seotud *SMN1* geenis, müosiini müopaatiat põhjustavas *MYH2* geenis ja Vici sündroomi põhjustavas *EPG5* geenis.

1.2. Kõigil lihashaaratusele viitavate kliiniliste sümptomitega patsientidel esinesid müopaatilised patomorfoloogilised muutused, samas kui kõigil neljal mittespetsiifiliste histoloogiliste leidudega patsientidel esines primaarne kesknärvisüsteemi (KNS-i) haaratus ja ülemise motoneuroni kahjustuse tunnused.

1.3. *TSPOAP1*-seoselise düstoonia ja spastilise hemipareesiga patsiendi lihaskoes esinesid kerged müopaatilised muutused.

1.4. Neljal patsiendil, kellel diagnoositi haigus, mis ei klassifitseeru MH alla, leiti seos mitokondriaalse düsfunktsiooniga kas haiguse patogeneesi osana või sekundaarse patoloogilise leiuna.

1.5. Lihasbiopsia mängis olulist rolli mitme patsiendi geneetilise diagnoosi kinnitamisel, tuues esile tüüpilised histopatoloogilised muutused või biokeemilised kõrvalekalded, mis toetasid varem kirjeldamata geenivariantide haigusseoselisust, ja võimaldas analüüsida koespetsiifilisi mitokondriaalse DNA muutusi.

2. Me kirjeldasime vigase *SPATA5* geeniga seotud fenotüüpi ja patomehhanismi (II artikkel).

2.1. *SPATA5*-seoseline haigus on varases eas algav tõbi, mida iseloomustavad mahajäämus psühhomotoorses arengus, kõne arengu oluline hilistumine, kuulmislangus, epilepsia, mikrotsefaalia, ajuanomaaliad, nägemiskahjustus, spastilisus ja seedetrakti probleemid.

2.2. Kirjeldatud on erinevat tüüpi geenivariante, kuid kõige sagedamini esineb raamisisene deletsioon c.989_991del, p.(Thr330del).

2.3. Me näitasime, et SPATA5 valgu puudulikkus kahjustab mitokondrite dünaamikat, vähendab ATP tootmist ja takistab roti närviraku aksonite kasvu.

2.4. Me saame järeldada, et SPATA5-seoselise haiguse patomehhanism on seotud mitokondriaalse düsfunktsiooniga.

3. Me analüüsisime lapseeas alanud neuromuskulaarse haiguse kahtlusega patsientide geneetilisi ja lihaskoe morfoloogilisi leide.

3.1. Rutiinse kliinilise töö käigus tehtud geneetiliste analüüside kombineeritud diagnostiline efektiivsus oli 43%. Võttes aktiivselt ühendust geneetilise leiuta patsientidega ja tehes neile WES analüüsi (välja arvatud ühel tüüpilise fatsioskapulohumeraalse lihasdüstroofia fenotüübiga patsiendil, kelle DNA saadeti spetsiifiliseks testimiseks, ja ühel patsiendil, kelle diagnoos kinnitus *SMN1* geeni deletsioonide/ duplikatsioonide analüüsiga), tõusis diagnostiline saagikus 61%-ni.

3.2. Uuringugrupi patsientide fenotüüp oli heterogeenne: 35-l patsiendil oli peamiselt haaratud perifeerne närvisüsteem (PNS) ja 18 patsiendil oli domineeriv KNS-i haaratus. Diagnostiline saagikus oli PNS-i kahjustusega patsientidel (25/35, 71%) palju suurem kui KNS-i haaratusega haigetel (7/18, 39%).

3.3. Lihaskoe histoloogia, kliinilised sümptomid ja genotüüp korreleerusid hästi. Enamikul selge lihashaaratusega patsientidel esinesid lihaskoes müopaatilised või düstroofilised muutused, kusjuures üheksal juhul oli näha spetsiifilisi histopatoloogilisi tunnuseid. Teisalt, enamiku peamiselt KNS-i haaratusega patsientide lihaskoe histoloogia oli normaalne või näitas ainult mittespetsiifilisi muutusi.

3.4. Kuigi diagnoosi kinnitamiseks ei olnud alati lihasbiopsiat vaja, mängis see diagnostilises protsessis olulist rolli. Lihasbiopsia võimaldas tuvastada spetsiifilisi struktuurseid või valkude ekspressiooni muutusi, avastada ainult lihaskoes esinevaid mitokondriaalse DNA variante ja teha transkriptoomi uuringuid.

4. Me kirjeldasime patsienti, kellel on uudne hemisügootne *PRPS1* geeni variant, mis põhjustab atüüpilist Artsi sündroomi fenotüüpi (III artikkel).

4.1. Kõnealune patsient oli väljendunud lihashüpotoonia, psühhomotoorse arengu mahajäämuse, füüsilise koormuse talumatuse, hüpomiimilise näo, düsfaagia ja epilepsiaga ning infektsioonidele väga vastuvõtlik poiss, kellel leidsime *PRPS1* geenis varem kirjeldamata variandi. Haigusseoselisust kinnitas fosforibosüül-pürofosfaadi süntetaasi väga madal aktiivsus erütrotsüütides.

4.2. Kuigi meie patsiendil oli mitmeid Artsi sündroomile (*PRPS1*-ga seotud haigustest kõige raskem fenotüüp) iseloomulikke tunnuseid, ei esinenud tal kuulmislangust, mis on kõigil teistel raporteeritud Artsi sündroomiga patsientidel tekkinud juba varases lapseeas.

4.3. Patsiendi lihaskoe histoloogia näitas suhteliselt mittespetsiifilisi muutusi, sealhulgas difuusseid p62-positiivseid täpikesi paljudes lihaskiududes, mis viitasid suurenenud autofaagilisele aktiivsusele.

5. Me kirjeldasime uut lihasdüstroofia alavormi, mis on seotud *JAG2* geeniga (IV artikkel).

5.1. Me esitlesime Eestist pärit tüdrukut, kellel tekkis alates 5. eluaastast progresseeruv, valdavalt proksimaalne lihasnõrkus. Tema kreatiini kinaasi tase oli veidi kõrgenenud ja lihaskoe histoloogial olid näha müopaatilised muutused. Me leidsime tal ühel *JAG2* geeni alleelil emalt päritud variandi ja teisel alleelil uustekkese (*de novo*) variandi.

5.2. Tänu rahvusvahelisele koostööle kinnitus *JAG2* geeni defektide haigusseoselisus. Kokku koguti 23 patsienti erinevatest riikidest, kellel oli sarnane fenotüüp ja bialleelsed variandid *JAG2* geenis. Kõigil isikutel esines jäseme-vöötme tüüpi lihasnõrkus ning magnetresonantstomograafia uuringul oli näha iseloomulikku lihaste haaratuse mustrit, kus *vastus lateralis, intermedius* ja *medialis* lihaste tsentraalsed piirkonnad olid vähem haaratud.

5.3. Meie patsiendi lihaskoe transkriptoomi ja immunohistokeemia analüüsid näitasid, et *JAG2*-seoselise lihasdüstroofia patomehhanism võib olla seotud satelliitrakkude düsfunktsiooniga.

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- Professor Werner Stenzel has been guiding me in the world of myopathology and neuromuscular disorders. I deeply value the vast knowledge and skills he has taught me and the friendly, welcoming environment he always creates.

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PUBLICATIONS

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List of publications

1. Mroczek, M.; Inashkina, I.; Stavusis, J.; Zayakin, P.; Khrunin, A.; Micule, I.; Kenina, V.; Zdanovica, A.; Zidkova, J.; Fajkusova, L.; Limborska, S.; van der Kooi, A. J.; Brusse, E.; Leonardis, L.; Maver, A.; Pajusalu, S.; Õunap, K.; **Puusepp, S.**; Dobosz, P.; Sypniewski, M.; Burnyte, B.; Lace, B. (2022). *CAPN3 c.1746-20C>G variant is hypomorphic for LGMD R1 calpain 3-related*. Human Mutation.

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Publikatsioonide nimekiri

1. Mroczek, M.; Inashkina, I.; Stavusis, J.; Zayakin, P.; Khrunin, A.; Micule, I.; Kenina, V.; Zdanovica, A.; Zidkova, J.; Fajkusova, L.; Limborska, S.; van der Kooi, A. J.; Brusse, E.; Leonardis, L.; Maver, A.; Pajusalu, S.; Õunap, K.; **Puusepp, S.**; Dobosz, P.; Sypniewski, M.; Burnyte, B.; Lace, B. (2022). *CAPN3 c.1746-20C>G variant is hypomorphic for LGMD R1 calpain 3-related*. Human Mutation.
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mitochondrial DNA copy number. The American Journal of Human Genetics, 99 (4), 860−876.
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DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer**. Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar**. Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik**. Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu**. Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar**. Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus**. Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar**. Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
9. **Agu Tamm**. On metabolic action of intestinal microflora: clinical aspects. Tartu, 1993.
10. **Katrin Gross**. Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
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