# Genetic Testing for Neuromuscular Disorders (for New Jersey Only)

**Policy Number**: CS165NJ.G
**Effective Date**: October 1, 2024

Ü Instructions for Use

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**Related Policies**

- Chromosome Microarray Testing (Non-Oncology Conditions) (for New Jersey Only)
- Genetic Testing for Cardiac Disease (for New Jersey Only)
- Whole Exome and Whole Genome Sequencing (Non-Oncology Conditions) (for New Jersey Only)

## Application

This Medical Policy only applies to the state of New Jersey.

## Coverage Rationale

**Multi-gene targeted panel testing (5 or more genes) for the diagnosis of any of the following suspected Neuromuscular Disorders is proven and medically necessary**:

- Congenital myopathy, distal myopathy, metabolic myopathy (e.g., glycogen storage disease), or myofibrillar myopathy; or
- Hereditary ataxia; or
- Hereditary peripheral neuropathy; or
- Hereditary spastic paraplegia; or
- Muscular dystrophy (e.g., limb girdle muscular dystrophy, congenital muscular dystrophy including, but not limited to, dystroglycanopathy); or
- Mitochondrial disease [e.g., Kearns-Sayre syndrome (KSS), Leber hereditary optic neuropathy (LHON), Leigh syndrome, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome] in individuals with all of the following:
  - Mitochondrial testing ordered by, or in consultation with, a board-certified medical geneticist, developmental pediatrician, or neurologist; and
  - High degree of suspicion of having a mitochondrial disease based on medical history, family history, laboratory, or other clinical tests; and
  - The clinical presentation does not support use of single gene or targeted genetic analysis; and
  - The individual has clinical features consistent with a mitochondrial disease such as one of the following conditions:
    - Proximal weakness; or
    - Muscle cramping, fatigue, or exercise intolerance; or
    - Progressive external ophthalmoplegia; or
    - Sensorineural hearing loss

**Multi-gene comprehensive neuromuscular disease test panels targeting multiple conditions (e.g., muscular dystrophy and mitochondrial disease) are unproven and not medically necessary due to insufficient evidence of efficacy.**

**Note**: Whole Exome and Whole Genome Sequencing are addressed in the Medical Policy titled Whole Exome and Whole Genome Sequencing (Non-Oncology Conditions) (for New Jersey Only).

## Definitions

**Comparative Genomic Hybridization (CGH)**: CGH is a technology that can be used for the detection of genomic copy number variations (CNVs). Tests can use a variety of probes or single nucleotide polymorphisms (SNPS) to provide copy number and gene differentiating information. All platforms share in common that tumor (patient) and reference DNA are labelled with dyes or fluorescing probes and hybridized on the array, and a scanner measures differences in intensity between the probes, and the data is expressed as having greater or less intensity than the reference DNA (Piluso et al. 2011).

**Neuromuscular Disorders (NMD)**: A group of inherited diseases that represent a number of conditions that result from impairment of nerves that control the muscles, or direct impairment of the muscles (Piluso et al. 2011).

**Next Generation Sequencing (NGS)**: High-throughput DNA sequencing of large numbers of genes in a single reaction (Efthymiou et al. 2016).

**Variant of Unknown Significance (VUS)**: A variation in a genetic sequence that has an unknown association with disease. It may also be called an unclassified variant (Efthymiou et al. 2016).

**Whole Exome Sequencing (WES)**: About 1% of a person's DNA makes protein. These protein-making sections are called exons. All the exons together are called the exome. WES is a DNA analysis technique that looks at all of the exons in a person's DNA at one time rather than gene by gene (MedlinePlus, 2021).

**Whole Genome Sequencing (WGS)**: WGS determines the sequence of all the nucleotides in a person's entire DNA including the protein-making (coding) as well as non-coding DNA elements (MedlinePlus, 2021).

## Applicable Codes

The following list(s) of procedure and/or diagnosis codes is provided for reference purposes only and may not be all inclusive. Listing of a code in this policy does not imply that the service described by the code is a covered or non-covered health service. Benefit coverage for health services is determined by federal, state, or contractual requirements and applicable laws that may require coverage for a specific service. The inclusion of a code does not imply any right to reimbursement or guarantee claim payment. Other Policies and Guidelines may apply.

| CPT Code | Description |
|---|---|
| *0216U | Neurology (inherited ataxias), genomic DNA sequence analysis of 12 common genes including small sequence changes, deletions, duplications, short tandem repeat gene expansions, and variants in non-uniquely mappable regions, blood or saliva, identification and categorization of genetic variants |
| *0217U | Neurology (inherited ataxias), genomic DNA sequence analysis of 51 genes including small sequence changes, deletions, duplications, short tandem repeat gene expansions, and variants in non-uniquely mappable regions, blood or saliva, identification and categorization of genetic variants |
| *0417U | Rare diseases (constitutional/heritable disorders), whole mitochondrial genome sequence with heteroplasmy detection and deletion analysis, nuclear-encoded mitochondrial gene analysis of 335 nuclear genes, including sequence changes, deletions, insertions, and copy number variants analysis, blood or saliva, identification and categorization of mitochondrial disorder-associated genetic variants |
| 81440 | Nuclear encoded mitochondrial genes (e.g., neurologic or myopathic phenotypes), genomic sequence panel, must include analysis of at least 100 genes, including *BCS1L, C10orf2, COQ2, COX10, DGUOK, MPV17, OPA1, PDSS2, POLG, POLG2, RRM2B, SCO1, SCO2, SLC25A4, SUCLA2, SUCLG1, TAZ, TK2, AND TYMP* |

| CPT Code | Description |
|---|---|
| 81448 | Hereditary peripheral neuropathies (e.g., Charcot-Marie-Tooth, spastic paraplegia), genomic sequence analysis panel, must include sequencing of at least 5 peripheral neuropathy-related genes (e.g., *BSCL2, GJB1, MFN2, MPZ, REEP1, SPAST, SPG11, SPTLC1*) |
| 81460 | Whole mitochondrial genome (e.g., Leigh Syndrome, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes [MELAS], myoclonic epilepsy with ragged-red fibers [MERFF], neuropathy, ataxia, and retinitis pigmentosa [NARP], Leber hereditary optic neuropathy [LHON]), genomic sequence, must include sequence analysis of entire mitochondrial genome with heteroplasmy detection |
| 81465 | Whole mitochondrial genome large deletion analysis panel (e.g., Kearns-Sayre syndrome, chronic progressive external ophthalmoplegia), including heteroplasmy detection, if performed |
| 81479 | Unlisted molecular pathology procedure |



Codes labeled with an asterisk (*) are not on the State of New Jersey Medicaid Fee Schedule and therefore may not be covered by the State of New Jersey Medicaid Program.

# Description of Services

Technologies used for genetic testing of Neuromuscular Disorders (NMD) vary, and can include, but are not limited to, tests that evaluate variations in the genes, such as chromosome microarray and Next Generation Sequencing (NGS), as well as others that assess the gene products, such as gene expression arrays and microRNA analysis. The number of genes evaluated can range from a single gene to the whole exome or genome of an individual. Results of genetic testing may assist individuals and healthcare providers with determining a diagnosis, prognosis, and identification of appropriate clinical interventions (Savarese et al., 2016; Piluso et al., 2011; and Ghaoui et al., 2015). This policy addresses genetic test panels including five or more genes for NMD. Neuromuscular diseases that typically present with a cardiomyopathy and are caused by a variant in a cardiomyopathy gene are addressed in the Medical Policy titled Genetic Testing for Cardiac Disease (for New Jersey Only) and those associated with Whole Exome Sequencing are addressed in the Medical Policy titled Whole Exome and Whole Genome Sequencing (for New Jersey Only).

# Clinical Evidence

## Neuromuscular Disorders (NMD)

NMD are a heterogenous group of conditions that are caused by impaired muscles and nerves that control the muscles. Examples of NMD include muscular dystrophies, nerve conduction disorders such as Charcot-Marie-Tooth (CMT), motor neuron disease (MND), hereditary spastic paraplegia (HSP), spinal muscular atrophies (SMA), and neuromuscular junction disease (myasthenic syndromes). Common symptoms include muscle weakness, cramps, numbness, respiratory and cranial nerve palsies. Many of these disorders are inherited, and over 500 genes are implicated in causing NMD (Efthymiou et al., 2016).

Ebert et al. (2024) performed a retrospective review to examine the clinical usefulness and detection rate of genetic testing for individuals with suspected NMD at a single, large neuromuscular center. Genetic testing results were obtained for all individuals who had undergone multi-gene panel testing for NMDs through a single genetic testing laboratory (n = 192). These records were reviewed to determine whether genetic testing results confirmed a specific NMD diagnosis, including cases where a variant of uncertain significance (VUS) was identified. A positive result, defined as a pathogenic mutation, a VUS, or both, was identified in 77.1% of participants and a definitive diagnosis was conferred in 35.9%. The most common indication for testing was suspected neuropathy (53.3%), and myopathy was the indication with the highest diagnostic yield (48.7%). The main study limitation was that the testing included in this study was performed by a single genetic testing laboratory (Invitae) even though some genetic tests ordered at the center were performed at other laboratories (those results were excluded). Overall, the authors assert that their results bolster the existing evidence supporting the use of genetic testing to aid in the diagnosis and management of NMD.

In a retrospective chart review Rosenberg et al. (2023) evaluated genetic testing practices, including genetic test selection/genes analyzed, and diagnostic results of adults and children seen in a NMD clinic over a 3-year period. The primary objective was to improve genetic testing decisions and counseling for individuals with NMD, since an inaccurate and/or delayed diagnosis can negatively impact appropriate medical treatment. The study included 155 individuals with a suspected diagnosis of NMD. The authors noted that they focused on a clinical presentation of elevated CPK levels and muscle weakness since these are the most common findings associated with a referral to the NMD clinic. In total, 26

separate genetic tests were used, with test yields ranging from 0% to 66%. Over half of the participants had a comprehensive neuromuscular panel which included 110 to 211 genes known to be associated with muscular dystrophies, inherited myopathies, and congenital myasthenic syndrome. Overall, 262 individual tests were ordered (average of 1.7 tests per individual) and 21% of participants received a genetic diagnosis. The most revealing symptom associated with a diagnostic result was an elevated CPK with or without muscle weakness; all individuals diagnosed with Duchenne, Emery-Dreifuss, limb-girdle, or Becker muscular dystrophies, along with several congenital myopathies, had elevated CPK levels. Additionally, while muscle weakness was a symptom in about half of individuals diagnosed with a muscular dystrophy, all individuals who were diagnosed with a myopathy had this symptom. This led the researchers to conclude that the presence of one or both of these symptoms should trigger providers to consider genetic testing and careful clinical assessment for these disorders. Study limitations included small sample size, limited data (retrospective review of pertinent medical records), and lastly, composition including mostly White males, limiting generalizability to populations with greater diversity. As a result of this study, the authors determined that diagnostic approaches are often led by cost and insurance coverage in addition to clinical suspicion of disease, indicating that the diagnostic journey can be long and difficult for impacted individuals. Emotional support and coping resources are recommended for these individuals and their families.

In an observational study, Schuermans et al. (2023) evaluated the diagnostic yield of exome sequencing (ES) and multigene panel testing in individuals with adult-onset neurologic disorders, including neuromuscular disorders. A total of 1,411 individuals were tested using ES-based multigene panel testing. Panels for ataxia and spasticity, leukoencephalopathy, movement disorders, paroxysmal episodic disorders, neurodegeneration with brain iron accumulation, progressive myoclonic epilepsy, and amyotrophic lateral sclerosis were created and a total of 725 genes associated with Mendelian inheritance were included overall. Genetic diagnosis was identified in 10% of the total cases, including 71 different monogenic disorders. The highest diagnostic yield was seen in individuals demonstrating ataxia or spastic paraparesis (19%) and varied based on individual phenotype. The majority of diagnoses found included disorders with autosomal dominant inheritance (62%), and the genes that most often showed variation were *NOTCH3* Z (n = 13), *SPG7* (n = 11) and *RFC1* (n = 8). The authors concluded that ES-based molecular testing can be successfully and efficiently used to diagnose adult-onset neurologic diseases but point out some technological limitations and recommend further studies assessing other technologies (such as genome sequencing) that could be used to assist with diagnosis of rare neurological diseases.

In a prospective, multicenter study evaluating clinical utility and diagnostic yield of a targeted gene panel for inherited neuromuscular disorders (INMD), Barbosa-Gouveia et al. (2022) used comprehensive gene-panel analysis and next-generation sequencing (NGS) to evaluate 268 individuals (both children and adults) with a suspected diagnosis of INMD. Three versions of the multi-gene testing panel were designed during the three year study period, with progressive addition of genes to the panel, resulting in an exponential increase in diagnosis rate. The first version (278 genes) yielded a diagnosis rate of 31% while the third (324 genes) yielded a diagnosis rate of 40%. Mean diagnostic rate over the entire 3 year study period was 36%. Most common diagnoses included muscular dystrophies/myopathies (68.4%) and peripheral nerve diseases (22.5%). TTN, RYR1 and ANO5 were the most common causative genes found and contributed to nearly 30% of diagnosed cases. The authors assert that in the case of INMDs, reaching a definitive diagnosis requires identification of specific variants in disease-causing genes. They recommend comprehensive gene-panel testing of all neuromuscular disease-related genes, including those most commonly implicated, in individuals with suspected INMD.

In a 2021 publication, Nicolau et al. provided guidelines that outlined a methodology for genetic testing of muscle and neuromuscular junction disorders. The authors indicate that the individual's phenotype sets the guiding approach for genetic testing. Phenotypes suggesting myopathy that require targeted testing (i.e., myotonic dystrophies, facioscapulohumeral muscular dystrophy, oculopharyngeal muscular dystrophy, dystrophinopathies, oculopharyngodistal myopathy and mitochondrial myopathies) must be identified as a first step. For remaining individuals, the researchers suggest a gene panel encompassing a large number of genes related to congenital myasthenic syndromes (CMSs) and myopathies, including copy number variation (CNV) analysis. Specific focus should be placed on the avoidance of missing potentially treatable neuromuscular conditions such as Pompe disease or CMSs. Unfortunately, according to this article, many individuals will remain without molecular diagnosis even after testing due to such factors as disorders that are not amenable to detection via next-generation sequencing (NGS) or acquired disorders mimicking inherited myopathies. The researchers state that techniques including exome, genome and RNA sequencing will likely play a greater role in the investigation of undiagnosed affected individuals in the near future.

Bowen et al. (2021) reported the clinical findings of a no-charge, sponsored NGS program called "SMA Identified". Eligible individuals had either a confirmed or suspected diagnosis of spinal muscular atrophy (SMA), or a family history of SMA. The study took place over a 2 year period. A total of 2459 individuals underwent testing with an NGS-based approach looking for sequence and copy number of SMN1 and SMN2. Participants were then categorized according to their test results as follows: diagnostic (two pathogenic SMN1 variants), nearly diagnostic (SMN1 exon-7 deletion with variant of

uncertain significance [VUS] in SMN1 or SMN2), indeterminate VUS (one VUS in SMN1 or SMN2), carrier (heterozygous SMN1 deletion only), or negative (no pathogenic variants OR VUS in SMN1 or SMN2). Analysis was completed based on clinician reported clinical findings and genetic modifiers. Diagnostic yield for diagnostic and nearly diagnostic (combined) was 31.3% (n = 771/2459). Clinical presentation and age of onset of symptoms were variable across individuals and dependent on SMN2 copy number. The most common genetic etiology was homozygous deletions (96.2%). The authors concluded that use of a high-yield panel test early in evaluation of individuals who have or are at higher risk for having SMA may lead to earlier interventions in affected individuals.

Winder et al. (2020) aimed to evaluate the diagnostic yield of genetic testing for hereditary neuromuscular disorders by creating a comprehensive data set after analysis of 25,356 unrelated individuals with NGS-based gene panels (not exome-based), testing subsets of 266 genes. The panels used in the study were designed using published literature which addressed associations between genes/disorders and genotype-phenotype associations as well as mode(s) of inheritance and differential diagnoses. Participants were enrolled in the study if there was a suspicion of NMD; a definitive diagnosis was determined in 5,055 (20%) of the participants. Typically, genetic studies do not include CNV analysis; however, in this study, the CNVs accounted for up to 39% of the significant variants found. Multi-gene testing addressed differential diagnoses in at least 6% of individuals with positive results. This large study provided additional direction for clinical providers who use genetic tests to diagnose neuromuscular diseases.

Wu et al. (2018) evaluated a group of 169 individuals who had been referred to a Canadian neuromuscular clinic using an NGS-based panel of 163 to183 muscular dystrophy-related genes. Participants were classified by individual reason for referral, including: 1) muscle weakness (n = 135), 2) recurrent rhabdomyolysis (n = 18), or 3) unexplained hyperCKemia. Individuals were excluded if they were suspected of having an acquired or inflammatory cause for their symptoms like a statin-induced myopathy, or had classic features of a single gene NMD, such as myotonic dystrophy or Duchenne muscular dystrophy. ACMG guidelines were used to interpret variants; variants identified in participants before the publication of the ACMG guidelines underwent re-interpretation in 2017. Pathogenic and likely pathogenic variants were considered in the calculation of the detection rate. Overall, pathogenic, and likely pathogenetic variants were identified in 61 (36%) of participants. In the cohort that presented with muscle weakness (n = 135), causative variants were found in 50 (37%). The detection rate in individuals with pediatric-onset symptoms (n = 47) was 38%. In individuals with recurrent rhabdomyolysis (n = 18), causative variants were found in six (33%). Sixteen participants had idiopathic hyperCKemia, and five (31%) had candidate variants identified. The authors noted that clinicians should be aware of the limitations of NGS, and that clinical examination and other diagnostic tools such as electromyography and muscle biopsy are still an important part of the diagnostic process. NGS may be subject to laboratory-specific limitations in detecting a variety of variant types including copy number variants, regulatory sequence variants, trinucleotide repeat expansions, and deep intronic mutations.

Nishikawa et al. (2017) studied the clinical utility of targeted NGS panels designed to identify inherited muscle diseases associated with muscular dystrophy, congenital myopathy (CM), metabolic myopathy (MM), and myopathy with protein aggregations/rimmed vacuoles (MFM). They analyzed blood samples on 188 individuals who had blood and muscle biopsies submitted to their lab in 2014 and 2015. Genes for the panels were identified from the 2013 gene table of monogenic NMD, and the target gene numbers were 65 (muscular dystrophy), 41 (CM), 45 (MM), and 36 (MFM). The authors did not combine the genes into one large panel for cost and time efficiency purposes. To analyze the muscular dystrophy panel, 65 individuals who had muscle biopsies and clinical findings suspicious for muscular dystrophy were recruited. Likely causative mutations were found in 30 participants (46%), and the genotype correlated with clinical findings. Sixty-five participants were analyzed for the CM panel. Causative mutations were found in 17 (26%), and an additional 13 individuals had variants that were consistent with their phenotype, but not enough data existed in the literature to be able to designate the mutations as pathogenic. Ten individuals were analyzed for the MM panel (30%). Causative mutations were found in three participants. The MFM panel was used for 48 participants who had histological profiles in biopsied muscle tissue consistent with MFM. Causative mutations were found in 12 (25%). Overall, the diagnostic yield was 33% for all 188 participants. The authors noted that additional genes and data that might have changed some variant classifications were found after the analysis was complete, so panels need to be updated on an ongoing basis. Their final conclusion was that a NGS panel in combination with histological, mRNA, and protein analysis is useful and efficient for determining a genetic diagnosis in individuals with muscle disease.

Five hundred and four individuals and an additional eighty-four unaffected family members from the Italian Network of Congenital Myopathies and the Italian Network of Limb-Girdle Muscular Dystrophy were studied by Savarese et al. (2016) using an NGS platform designated as MotorPlex. MotorPlex is made up of 93 genes that are considered causes of nonsyndromic myopathies that typically cannot be diagnosed clinically. Eighty-five percent of the participants were Italian, and 60% were male. All participants were classified according to their primary clinical presentation as LGMD (51%), CM (32%), distal myopathy (3.8%), isolated hyperCKemia (3.4%), MM (1.2%), or “other” (8.6%). Most cases were sporadic, but 96 were familial. Bioinformatic filters took into account population frequency and current variant annotation. Variants

were further scrutinized based on clinical presentation, age of onset, and segregation analysis in family members when appropriate. As a result, 218 (43.3%) cases obtained a diagnosis, and 160 individuals had candidate variants identified that were interesting, but unproven. LGMD genes were considered causative of the phenotype in 115 participants. In 30% of diagnosed cases the phenotype was atypical for that gene, expanding the understanding of the disease phenotype. The authors noted that some of the unsolved cases could be due to variants in genes not yet identified as causing NMD, and that ancillary tests such as comparative genomic hybridization (CGH) to detect CNVs may be a necessary subsequent step. The conclusion of the study was that NGS may become a universal first-tier step in diagnosing heterogeneous conditions such as NMD.

### *Clinical Practice Guidelines*

### American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM)

AANEM developed a position statement regarding the utility of genetic testing in neuromuscular disease (NMD) (Kassardjian et al. 2016). The goal of the statement was to generally endorse genetic testing as a component of diagnosing NMD, but not to endorse a specific test or testing algorithm. The authors provided a consensus opinion from an expert panel that highlighted the benefits of genetic testing including reduced time to diagnosis, avoidance of unnecessary testing, improved surveillance and monitoring, family testing and family planning, and better access to research and clinical trials. The authors note that recommendations and guidelines exist that direct the selection of appropriate genetic tests and referenced AANEM guidelines for limb-girdle muscular dystrophies (Narayanaswami et al. 2014; reaffirmed 2022), congenital muscular dystrophy (Kang et al. 2015; reaffirmed 2021) and facioscapulohumeral muscular dystrophy (Tawil et al. 2015; reaffirmed 2021).

## Metabolic Myopathies

Metabolic refers to the chemical processes in the body that utilize nutrients and energy to provide healthy functioning and growth. Metabolic myopathies are genetic disorders in which the metabolic processes for the muscles have been interrupted and can result in muscle weakness, exercise intolerance, or muscle pain. There are three primary categories of metabolic myopathies: glycogen-storage diseases (GSD), disorders of fatty oxidation, and mitochondrial myopathies (American College of Rheumatology, 2023 and Tarnopolsky, 2016).

### *Glycogen Storage Diseases*

Glycogen storage diseases (GSDs) that may cause metabolic myopathies and have overlapping symptoms include GSD type 2 (Pompe disease), GSD type 3 (Debrancher Deficiency), GSD type 4 (Andersen's disease), GSD type 5 (McArdle's disease), GSD type 7 (Tarui disease), and GSD type 9 (Phosphorylase Kinase Deficiency). Identifying the correct diagnosis is important because some GSDs have treatment available, such as Late Onset Pompe Disease (Lilleker et al., 2018). Symptoms often start in the second or third decade of life with muscle cramps that occur during the first few minutes of exercise. Many individuals may not see their physician at the onset of symptoms because they avoid exercise or they modify exercise by starting off slow, then ramping up activity as aerobic metabolism takes over and blood born energy is delivered to the muscle. In particular, individuals with McArdle disease report that exercise gets easier after a few minutes of activity, known as the second wind-phenomenon, and they feel better and less symptomatic after a high carbohydrate meal. Individuals with other forms of metabolic myopathies do not experience a second wind phenomenon and report that they feel worse with a high carbohydrate meal and better after fasting. Some affected individuals will experience dark urine due to the presence of muscle derived proteins. The classic diagnostic test is a forearm exercise test including pre-and post-exercise measurements of lactic acid and ammonia. This has a very high sensitivity and specificity for the presence of a glycogenic defect, with the possible exception of phosphorylase b kinase deficiency, which can be further evaluated with an aerobic cycling test. Serum CK is usually elevated in McArdle disease but is typically normal in other glycogen storage diseases. EMG is often normal, and muscle biopsy may show high glycogen, absent phosphorylase, or absent phosphofructokinase. If these tests suggest McArdle syndrome, or muscle biopsy is suggestive of a particular GSD, targeted genetic testing is suggested to confirm the diagnosis. For example, on muscle biopsy, central cores suggest RYR1 or CACNA1S mutations, abnormal dystrophin staining suggests a dystrophinopathy, ragged red fibers point to a mitochondrial disorder, and membrane bound glycogen suggests Pompe disease. NGS panels may be beneficial in reaching a diagnosis (Lilleker et al., 2018 and Tarnopolsky, 2016).

Sniderman King et al. (2023) reported on data from the Lantern Project, a program offering diagnostic assistance to individuals with suspected Pompe disease and LGMD as well as other lysosomal storage and neuromuscular disorders. Included in this article was information specific to an acid α-glucosidase (GAA) enzyme assay as well as GAA sequencing and lastly, the Focused Neuromuscular Panel, which includes GAA. A total of 140 individuals in the project have been confirmed to have Pompe disease. The most common symptom reported at the time of testing was proximal muscle weakness (58 individuals) and elevated creatinine kinase (present in 29 individuals) was the most common laboratory result. Molecular results supported diagnosis in 128 individuals. The authors assert that these findings further support the

use of testing with enzymatic and genetic methods to aid in the diagnosis of Pompe disease and indicate that the use of multigene NGS panels allow the critical differentiation between Pompe disease and other LGMDs.

The genetic lab at Centro de Diagnóstico de Enfermedades Moleculares in Madrid, Spain, reported on its experience with NGS for GSD (Vega et al., 2016). Blood samples from 47 participants suspected of having a GSD were analyzed. Two methods were employed. Sixteen individuals were evaluated using a panel of 111 GSD related genes. Twelve of these individuals, plus an additional 39, were analyzed by the TrueSightOne gene panel which represents all of the known disease-causing genes described in the Online Inheritance of Man (OMIM) database as of 2013. Variants were filtered by population frequency, phenotype, and inheritance pattern. Genes with potentially pathogenic mutations were assessed in the context of the individual phenotype according to OMIM criteria. Variants that met these criteria were confirmed by Sanger sequencing. In the first testing group, five of 12 individuals received a genetic diagnosis (30%). In the second group, 18 of 43 individuals were found to have pathogenic mutations; 14 were in GSD-related genes and four were in non-GSD genes. Eleven mutations had never been reported before and were confirmed through segregation analysis. The authors concluded that the combination of clinical findings, biochemical test results, and NGS can provide an efficient and accurate means of making a genetic diagnosis.

Lévesque et al. (2016) studied the clinical utility of a targeted NGS panel to diagnose Late Onset Pompe Disease (LOPD). Pompe disease is an autosomal recessive disease caused by a defect in the GAA gene, resulting in a deficiency of acid alpha-glucosidase. The classic infantile form presents early in life with general muscle weakness, cardiomyopathy, and respiratory distress. The disease is treatable with enzyme replacement therapy, but without treatment, it is a fatal disease. LOPD can present at any age after infancy with limb-girdle weakness but is most commonly identified in adulthood. Affected individuals may also have rigid spine syndrome, scoliosis, and low body mass, as well as nocturnal hypoventilation due to diaphragmatic weakness. Because of the low incidence of LOPD and the overlap of symptoms with other neuromuscular disease, this treatable condition is often not diagnosed until 10 years after the first onset of symptoms. The authors developed an NGS panel comprised of 77 genes representing muscle disorders that have a clinical overlap with LOPD. Twenty individuals with Pompe disease and known mutations were used to determine the sensitivity of the assay, and all mutations were accurately identified. Positive gene results were confirmed by measuring GAA activity. GAA activity level was measured using tandem mass spectrometry, and 15 individuals with Pompe disease were used as positive quality controls. Forty-nine healthy controls were used to establish normal GAA activity. This pilot study included 34 individuals (seven children and 27 adults) suspected of having an inherited muscle disorder, but in whom the etiology could not be determined. Most (71%) had undergone a muscle biopsy, and 15 (44%) had at least one single gene test performed, but still did not have a diagnosis. Using the NGS panel, a genetic diagnosis was found in 32% of participants. One case of LOPD was found, confirmed by GAA activity testing. The remaining cases were various forms of LGMD, including three individuals with atypical presentations. The authors concluded that targeted muscle gene panels utilized as a first-tier diagnostic test have the potential to reduce the time to diagnosis. They also note that challenges exist with the high number of VUS identified and the limited performance of bioinformatics tools for analyzing CNVs but anticipate that these issues will be resolved as NGS-based technology continues to advance.

Savarese et al. (2016) described the clinical validity of a targeted NGS panel (MotorPlex) for NMD in 504 individuals with LGMD (51%), congenital myopathy (CM) (32%), distal myopathy (3.8%), isolated hyperCKemia (3.4%), metabolic myopathy (MM) (1.2%) and other (8.6%). Within this subset are 275 individuals with a clinical presentation of LGMD and hyperCKemia that includes LOPD within the differential diagnosis reported in a subsequent publication focusing on LOPD (Savarese et al., 2018). Ultimately, 16 individuals from nine unrelated families were diagnosed with LOPD. All individuals had the common c. 32 13T > G variant in the GAA gene with a second, already known mutation on the other allele. The symptoms in this cohort were primarily proximal weakness and fatigability. Exercise intolerance, myalgia, and contractures were less common. Some participants had atypical symptoms that likely confounded the clinical diagnosis, such as dysphagia, pseudohypertrophy, and calf hypertrophy. The authors concluded that with decreasing costs and technological improvements, NGS panels are likely to become important in first-tier diagnostic testing in the near future.

## *Fatty Acid Oxidation (FAO) Disorders*

Disorders of fatty acid oxidation (FAO) can result in three different presentations; hepatic, sudden infant death from hypoketotic hypoglycemia from catabolic events or cardiac disease, and a mild adult onset form. The hepatic form is severe, often lethal, and is triggered in the neonatal or infancy time period by a catabolic state, such as from frequent infections. Infants may also present with dilated or hypertrophic cardiomyopathy. These conditions may be treatable through dietary restriction of long chain triglycerides and supplementation of medium chain triglycerides, so are included in newborn screening programs. Diagnosis can be tricky, however, and may require in vivo loading tests using sunflower oil and phenylbutyrate or fasting tests. Mass spectrometry of the acyl carnitine pathway remains the gold standard for newborn screening and other diagnostic tests. Enzyme testing in lymphocytes can confirm the diagnosis, and genetic testing of the specific gene can identify the molecular problem (Houten et al., 2016). The adult onset or mild form presents with exercise-induced myalgia and may have pigmenturia within 24 hours of exercise due to rhabdomyolysis and delayed

onset of muscle soreness. Symptoms may result from prolonged fasting, or prolonged exercise, especially if illness is present, too. In affected children, it is common to see pigmenturia during fever or fasting because of illness, or when vomiting. The exercise induced symptoms are not noted until the teen years. In these individuals, CK levels are usually normal except during rhabdomyolysis. Hyperkalemia and hypoketotic hypoglycemia can occur during rhabdomyolysis as well, and in some this might result in kidney failure. The best diagnostic test for individuals suspected of having a mild fatty oxidation disorder is a mass spectrometry analysis for acyl carnitine. A false negative can happen if the testing is performed during a non-stressed period. Targeted genetic testing can confirm a diagnosis based on the mass spectrometry results, and if acyl carnitine results are not informative, or targeted genetic testing is negative, panel genetic testing may yield additional information. For example, LPIN1 deficiency can cause rhabdomyolysis with fever or other illness but does not cause exercise-related symptoms (Tarnopolsky, 2016). In general, most FAO disorders are diagnosed through mass spectrometry and other metabolic testing, but in some cases additional genetic testing, which can include exome or genome analysis, may help diagnosis unexpected phenotypes (Houten et al., 2016).

Valencia et al. (2016) examined the utility of a customized NGS panel of 26 genes in twelve children with acute liver failure and elevated blood molar lactate/pyruvate of indeterminate etiology. The participants were selected from a retrospectively identified cohort of 74 individuals with acute liver failure because their fixed and frozen liver samples were available for additional analysis and had indeterminate etiology. The 26 genes evaluated included 15 nuclear genes involved in mitochondrial disorders and six genes associated with FAO defects. Hepatic DNA was analyzed. Five participants were found to have significant genetic variants. Two had genetic variations in the RRM2B gene, not previously associated with acute liver failure. Both had patchy micro- and macro-vesicular steatosis and reduced respiratory chain complex activity, and good post-liver transplant outcomes. One infant with severe lactic acidosis was a compound heterozygote for variants in ACAD9, associated with isolated complex I deficiency. Two participants had abnormal mitochondria by electron microscopy, and VUS in the POLG and DGUOK genes. Both had developed acute liver failure after drug exposure. The authors conclude that targeted NGS helped expand the understanding of genes involved in the spectrum of pediatric acute liver failure.

## Hereditary Ataxia

Ataxia is lack of muscle control or coordination of voluntary movements, and is a symptom found in a number of NMDs. It is also the primary feature of a heterogeneous group of disorders such as Friedreich's ataxia and many spinocerebellar ataxias (SCAs). Over 50 genes have been implicated in ataxias (Sandford and Burmeister, 2014).

In a 2021 publication, Benkirane et al. documented their evaluation of the efficacy of molecular diagnoses for inherited ataxia and related diseases. In this study, the researchers analyzed 366 unrelated consecutive individuals with ataxia or related disorders that had not yet been diagnosed by using clinical exome-capture sequencing. Analysis was performed via an in-house pipeline combining variant ranking and CNV searches. Variant interpretation was done according to ACMG/Association for Molecular Pathology (AMP) guidelines. A molecular diagnosis was established in 46% of the test subjects. In addition, 35 individuals who were mildly affected with causative variants in genes classically associated with severe presentations were identified. Such cases were explained by hypomorphic variants and rarely suspected mechanisms such as C-termination truncations and translation reinitiation. Two genes, PEX10 and FASTKD2 are potential candidates for translation reinitiation, which accounted for mild disease presentation. The authors concluded that a significant fraction of phenotypic overlap and clinical heterogeneity is explained by hypomorphic variants that are not readily predictable and difficult to identify.

Hadjivassiliou et al. (2017) prospectively examined 1500 individuals presenting with cerebellar ataxia at the Sheffield Ataxia Centre in the United Kingdom over a period of twenty years. Each participant underwent extensive workups that were repeated at six month to yearly intervals. Baseline assessments included, but were not limited to, full blood count, erythrocyte sedimentation rate (ESR), vitamin B12, folate, vitamin E, copper, urea, electrolytes, thyroid function, anti-GAD antibodies, celiac testing, HLA typing, and immunoglobulin analysis. Genetic testing was limited to what was available at the time. Expanded NGS panels were available after June 2014. Mitochondrial testing was only performed when indicated. Overall, there were 295 individuals with a familial ataxia, and 1205 individuals with a sporadic form. In those with a familial ataxia, a genetic diagnosis was confirmed in 58% of included subjects. In those with sporadic ataxia, 13% were found to have a genetic diagnosis. Since June 2014, 146 participants had genetic testing using the NGS panel; fifty-four of these had a dominant family history, 17 had a family history consistent with recessive inheritance, 33 had sporadic early-onset ataxia, and 42 had sporadic late-onset ataxia. Positive results were found in 32% of participants. Of note, none of the individuals with an episodic ataxia type 1 EA2 mutation had episodic ataxia, but rather had a progressive form. Additionally, four participants had an SPG7 mutation that helped identify a related phenotype of slurred speech, ataxia, mild spastic paraparesis, and proximal weakness. This led to the genetic testing of SPG7 in 58 additional individuals with the same phenotype. Twenty-eight of these (48%) were found to be positive. The authors concluded that in their cohort, the potential for a genetic diagnosis was present in 30% of cases, which included the 13% diagnosed with a genetic disease in the sporadic cases, and the familial cases. The diagnostic yield of NGS testing when introduced was

32% overall, but 46% in the cases with a dominant family history of disease. The authors also noted that in the sporadic ataxia group, if genetic testing was applied only to those who had other diagnoses ruled out, the diagnostic yield became 35%. Because genetic testing is expensive, the authors recommended a selection criterion to increase the diagnostic yield that includes brain imaging, family history, routine lab tests, age of onset, physical exam, and then targeted genetic testing based on those results, if possible. If the targeted genetic testing is negative, or the other clinical tests do not point to a specific genetic diagnosis, then the NGS panel test should be considered.

To understand the value of NGS in diagnosing genetic ataxias, Nemeth et al. (2013) identified 50 individuals from unrelated families in the UK that did not have a genetic diagnosis of ataxia, but ataxia was their primary symptom. All participants had negative genetic testing for the gene expansion found in spinocerebellar ataxias 1–3, 6, 7, Friedreich's ataxia, and for mtDNA abnormalities. Multiple standard biochemical tests were performed to rule out other metabolic diseases. Targeted NGS including 118 genes, 42 of which were associated with the primary phenotype and the remaining considered "good candidate genes" based on their function, was completed. The overall detection rate was 18% and varied from 8.3% in those with an adult-onset progressive disorder to 40% in those with a childhood- or adolescent-onset progressive disorder. Those that had an adolescent-onset and a positive family history had a detection rate of 40%. The authors noted difficulties in variant interpretation which are being addressed with updated bioinformatics tools and the need to confirm positive variants with Sanger sequencing and functional testing. Some individuals who did not receive a genetic diagnosis may have variants in genes that were not included in the analysis, as the list of genes that are implicated in ataxia continues to grow.

## Inherited Peripheral Neuropathies (IPN)

Inherited peripheral neuropathies (IPN) occur in about 1 in 2500 people. They are a heterogeneous group of disorders caused by over 90 genes. The main subtypes include Charcot-Marie-Tooth (CMT) disease, hereditary sensory and autonomic neuropathy (HSAN), hereditary motor neuropathy (HMN), and hereditary neuropathy with liability of pressure palsy (HNPP) (Antoniadi et al., 2015).

CMT is a common neuromuscular disorder affecting approximately 16 per 100,000 individuals overall (Theadom et al., 2019). Classical symptoms include slow, progressive distal muscle weakness, muscle atrophy, and sensory loss over time in the distal limbs. Electrodiagnostic testing has been used to classify CMT as demyelinating or axonal. One gene variation can result in multiple phenotypes, and all forms of inheritance have been reported. Many genes have been associated with CMT. Traditional Sanger sequencing is cost-prohibitive in investigating all the genes associated with CMT, making targeted NGS an attractive option.

Examining the impact of targeted NGS panels on the molecular diagnosis of CMT disease in standard clinical practice and demonstrating the importance and limitations of the use of NGS related to the diagnosis of CMT was the focus of a retrospective study by Ceylan et al. (2023). Molecular techniques including multiplex ligation probe amplification (MLPA), NGS and WES were used to identify variations related to CMT disease. After molecular evaluation with MLPA, 25 of 64 individuals with suspected CMT disease (39%) were positively diagnosed. Duplication in PMP22 was seen in 14 participants and PMP22 deletion was seen in 11 participants. Fifty individuals had NGS with targeted gene panels specific to CMT and 36% of those had pathogenic or likely pathogenic variants. Lastly, five individuals with normal NGS results underwent WES; diagnostic yield for those who had WES was reported to be 80%. The authors determined that in this study, targeted NGS panel use was diagnostic in approximately one-third of participants after exclusion of PMP22 deletion/duplication assessment. They advocate for an algorithmic molecular approach for genetic evaluation along with genetic counseling and pedigree analysis and further study to uncover additional information related to the etiology of CMT.

IPNs have been associated with a variety of genomic variants which include large duplication and/or deletion and repeat expansion; this has made molecular diagnosis difficult. In a large case series, Ando et al. (2022) sought to pinpoint genetic features in a group of Japanese individuals with IPNs. Clinical information was obtained for 2695 individuals with IPN in Japan; no individuals with a finding of PMP22 CNV were included in this case series. Several technologies were used for genetic evaluation including DNA microarrays, NGS-based gene panels, WES, CNV analysis and RFC1 repeat expansion analysis. Overall, 909 suspected IPNs and pathogenic or likely-pathogenic variations were detected. For individuals with early-onset disease, MFN2 was the most frequent finding. GJB1 and MPZ were most frequently identified as the cause of middle- and late-onset disease and GJB1 and MFN2 were most common in demyelinating and axonal subtypes. Overall, the most commonly identified genes linked to IPNs were MFN2, GJB1, MPZ and MME. CNVs in MPZ and FJB1 genes and RFC1 repeat expansions were also detected. The authors concluded that completing a comprehensive genetic evaluation for individuals with suspected IPNs revealed genetic origins in this case series. They recommend further study focused on clinical features and their relationship to genetic variations to continue to aid in development of best practices for assessment of affected individuals in order to obtain early diagnosis.

Volodarsky et al. (2021) performed comprehensive sequencing and copy number analysis of 34 CMT-associated genes in a cohort of 2517 individuals with suspected CMT. The researchers identified a large number of pathogenic variants that were novel, as well as variants of unknown significance in CMT-related genes. Overall diagnostic yield was found to be 15% in males and 21% in females. The authors feel that this study expanded the mutational continuum of CMT-related genes and supported clinical utility of comprehensive sequencing and copy number NGS-based testing in individuals suspected of having CMT.

Vogt et al. (2020) evaluated the clinical utility of genetic screening in individuals who presented with neuropathy without a confirmed etiology in a neuromuscular clinic. The testing consisted of an inherited neuropathy panel (72-81 genes) using NGS-based technology. Of the 200 participants screened, 30 had pathogenic mutations and 83.3% of the positive mutations were found in PMP22, TTR and GJB1. In four individuals, the determination of pathogenic mutation altered management. Two individuals undergoing treatment for demyelinating autoimmune neuropathy were diagnosed with CMT subtypes. The researchers determined that in this small study, although a minority of individuals with unsuspected inherited neuropathies tested positive, screening did alter management in a small percentage.

Between 2010 and 2015, individuals with inherited motor neuropathies seen at the genetic neuropathy clinic at Newcastle Hospitals NHS Foundation in Northern England) were evaluated by NGS or WES (Bansagi et al., 2017) to study prevalence, genetic basis, and clinical presentation of these neuropathies. Genetic testing to rule out common mutations for PMP22 deletion/duplication, MFN2, and GJB1 was performed for all participants, and neurological and electrophysiological tests were used to identify candidates. One hundred and five unique individuals from 73 families were ultimately included in the study, with symptoms including distal motor neuropathy (n = 64), axonal neuropathy (n = 16), or complex disease impacting the motor nerves (n = 25). Variant classification was based on the 2013 Association for Clinical Genetic Science Practice Guidelines. Twelve index participants were diagnosed from the NGS panel (26%) and 18 index participants from WES (45%). Candidate gene sequencing based on clinical presentation alone found a genetic diagnosis in only five of the 105 participants (4.7%). Overall, causative mutations were found in 26 of 73 families, resulting in a 35.6% detection rate. The diagnostic rate in the distal motor neuropathy group was 32.5% and a likely causative mutation was found in an additional 10%, which was higher than what was reported previously. Many of the positive genes were the same between the distal motor neuropathy and axonal neuropathy group, suggesting that disease classifications may need to change.

Nam et al. (2016) researched the clinical utility of a 73-gene targeted NGS panel in 78 Korean families affected with IPN (89 affected and 46 unaffected individuals). Fifteen individuals were already known to have the common PMP22 duplication variant in CMT1. In addition, 300 healthy Korean controls were included in the analysis. Clinical information collected included age of onset, motor and sensory impairments, deep tendon reflexes, muscle atrophy, and nerve conduction studies. Variants were filtered using population frequency and pathogenicity scores from various bioinformatics tools. Putative causative variants were confirmed using Sanger sequencing. In the 15 individuals with a known PMP22 duplication, the gene panel results for read depth for PMP22 and TEKT3 in the duplicated region, when compared to 5 non-duplicated health controls, were significantly higher at 1.49 and 1.47, respectively. This demonstrates that this panel can detect the common CMT1 variant. In the remaining study participants, 15 pathogenic or likely pathogenic variants in eight genes were identified in 25 individuals from 17 families. Eight mutations had not been previously identified as pathogenic but were segregated with impacted individuals in each family group. In this remaining group, the diagnostic yield was 27%. If the common PMP22 duplication is taken into account, 32 of 78 families could be diagnosed using this panel.

In a pilot study of 22 unrelated Chinese individuals diagnosed with CMT, the common PMP22 duplication was analyzed first, and detected in eight participants. The remaining individuals underwent targeted NGS of 44 genes. Genetic variants were classified using ACMG standards. Eleven individuals were found to have a total of ten possible pathogenic variants, including seven previously reported variants. The three novel variants identified underwent functional testing. Two were found to be likely pathogenic and one likely benign. The authors concluded that NGS has the potential to make a more rapid and precise diagnosis for individuals with CMT and that functional analysis of novel variants is critical (Li et al., 2016).

Antoniadi et al. (2015) explored the clinical validity of a targeted NGS panel evaluating 56 genes associated with IPN. From July 2013 to December 2014, 448 samples referred from neurologists (67%) or geneticists (33%) meeting clinical criteria were evaluated. The inclusion criteria included a diagnosis of idiopathic peripheral neuropathy with progressive weakness in hands/wrists, feet/ankles, or associated pes cavus or finger contractures, and/or peripheral sensory loss with supportive nerve conduction studies and an absence of other non-genetic causes or central nervous system involvement. Most participants (70%) were over the age of 18. Variants were classified using the Association of Clinical Genetics Science Practice Guidelines. Genetic diagnosis was made in 137 (31%) participants, involving 195 pathogenic variants in 31 genes. Nearly half of those diagnosed had a pathogenic variant in a gene not previously available for testing, or in a

gene whose primary clinical association was not IPN. The authors concluded that this approach overcomes the limitations of a sequential single gene approach and is an efficient tool for obtaining a genetic diagnosis in IPN.

## *Clinical Practice Guidelines*

### American Academy of Neurology (AAN), American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM), and American Academy of Physical Medicine and Rehabilitation (AAMPR)

Distal symmetric polyneuropathy (DSP) is the most common variety of neuropathy. Since the evaluation of this disorder is not standardized, the available literature was reviewed to provide evidence-based guidelines regarding the role of laboratory and genetic tests for the assessment of DSP in a report of the AAN, AANMEM and AAMPR (England et al. 2009, reaffirmed 2022).

- Screening laboratory tests may be considered for all individuals with polyneuropathy (Level C). Those tests that provide the highest yield of abnormality are blood glucose, serum B12 with metabolites (methylmalonic acid with or without homocysteine), and serum protein immunofixation electrophoresis (Level C). If there is no definite evidence of diabetes mellitus by routine testing of blood glucose, testing for impaired glucose tolerance may be considered in distal symmetric sensory polyneuropathy (Level C).
- Genetic testing should be conducted for the accurate diagnosis and classification of hereditary neuropathies (Level A). Genetic testing may be considered in individuals with cryptogenic polyneuropathy who exhibit a hereditary neuropathy phenotype (Level C). Initial genetic testing should be guided by the clinical phenotype, inheritance pattern, and electrodiagnostic features and should focus on the most common abnormalities which are CMT1A duplication/HNPP deletion, Cx32 (GJB1), and MFN2 mutation screening.
- There is insufficient evidence to support routine genetic testing in individuals with cryptogenic polyneuropathy without a classical hereditary phenotype.

The majority of hereditary DSPs are variants of Charcot Marie Tooth (CMT) disease, which has a wide range of phenotypic expression and can be caused by de novo mutations, so a family history may not always be informative. When a hereditary DSP is suspected, the authors recommend a stepwise approach based on family history and electrodiagnostic test results as follows:

- Electrodiagnostic testing
  - Demyelinating
    - Positive family history, autosomal dominant
      - PMP22 duplication, first tier
      - PMP22 and MPZ testing, second tier
      - EGR2 and LITAF testing, third tier
    - Positive family history, autosomal recessive
      - PRX and GDAP1 testing
    - Positive family history, X-linked
      - GJB1 testing
    - Negative or uninformative family history
      - PMP22 duplication and GJB1 testing, first tier
      - MPZ and PMP22 sequencing, second tier
      - EGR2, LITAF, PRX, and GDAP1 testing, third tier
  - Axonal
    - Positive family history, autosomal dominant
      - MFN2 testing, first tier
      - MPZ testing, second tier
      - RAB7,GARS, NEFL, and HSPB1 testing, third tier
    - Positive family history, autosomal recessive
      - GDAP1 testing
    - Positive family history, X-linked
      - GJB1 testing
    - Negative or uninformative family history
      - MFN2 and GJB1 testing, first tier
      - MPZ sequencing, second tier
      - RAB7,GARS, NEFL, HSPB1, and GDAP1 testing, third tier

The authors concluded that there was insufficient evidence to support routine genetic testing for individuals with cryptogenic polyneuropathy without a classical hereditary phenotype.

## Hereditary Spastic Paraplegias (HSP)

Hereditary Spastic Paraplegias (HSP) are a group of genetic diseases characterized by spastic paralysis of the legs, typically caused by selective distal axonal degeneration. They are rare, chronic disorders that occur in about 1 to 9 in 100,000 people and present in childhood and young adulthood. The typical clinical picture is of a slowly progressive, symmetrical, spastic paraplegia. Minor sensory abnormalities (such as absent vibration sensation) and neurological bladder involvement, are common. Arm involvement is not usually seen, and if present, it is minimal and does not extend beyond hyperreflexia and minor weakness (e.g., difficulty unscrewing a tight bottle top). HSP is categorized into the subtypes of “pure” and “complex.” The pure form is most common in European populations and can be autosomal dominant or recessive. The complex form is typically autosomal recessive and is more commonly found in populations with a high rate of consanguinity. Over 70 genes have been identified for HSP (Hensiek et al., 2015).

Iqbal et al. (2017) researched the use of NGS in the diagnosis of 105 hereditary ataxia (HA) and HSP probands identified through the HA and HSP registry in the Department of Neurology, Oslo University Hospital. HA and HSP have phenotypic overlap. HA is characterized by progressive limb and gait ataxia, loss of coordination and disturbances of speech and oculomotor control, and HSP is characterized by progressive spasticity and weakness of the lower limbs; the weakness often being mild relative to the spasticity. The HA/HSP registry has 446 probands, and 77 HSP and 41 HA individuals had a molecular diagnosis at the time of the study. Of those without a genetic diagnosis, 48 individuals with HSP and 58 individuals with HA were selected for NGS, and eight individuals with a known diagnosis were included as a positive control. Variants were classified per the joint consensus recommendations of the ACMG and AMP and were confirmed by Sanger sequencing. The NGS panel identified all eight positive controls. In the test group, 12 individuals with HSP had pathogenic or likely pathogenic variants, and two had VUS. Eight individuals with HA had pathogenic or likely pathogenic variants, and eight had VUS. Overall, 19% had a definitive molecular diagnosis.

Chrestian et al. (2016) conducted a multi-center observational study of individuals who met the clinical criteria for the diagnosis of HSP in Alberta, Ontario and Quebec from 2012-2015 and reported on the genetic test results. Five hundred and twenty-six individuals with HSP were identified during this time period. DNA testing was conducted on peripheral blood samples. Fifty-one families representing 108 individuals had WES, and the variants were filtered against all known HSP genes. Thirty-seven individuals, a cohort from Ontario, had NGS of 51 HSP related genes. Individuals with cerebellar signs were screened for mutations in FXN, SACS, and the common spinocerebellar ataxias (SCAs 1–8) prior to being included in the study. Overall, 150 (28.5%) of individuals from 58 families had a confirmed genetic diagnosis. Mutations from 15 different genes were identified, and the most common were in SPAST (78%), ALT1 (16%), and SPG11 (8%). Genotype/phenotype correlations were noted with SPAST mutations (SPG4) and were more likely to have a later age of onset but also have bladder dysfunction. SPG11 mutations were more strongly associated with the presence of learning dysfunction and cognitive deficits.

Kara et al. (2016) investigated the genetic cause of disease in a series of 97 index cases with complex spastic paraplegia referred to a tertiary referral neurology center in London for diagnosis or management. Individuals enrolled had clinical details and DNA available prior to 2015. Only the proband was included in the analysis when a family had more than one affected member. Inclusion criteria included slowly progressive HSP as the primary clinical finding along with at least one other neurological feature; peripheral neuropathy, cognitive decline, epilepsy, skeletal/bony abnormalities, visual problems, parkinsonism, dystonia, or ataxia. Acquired and metabolic causes of HSP were ruled out. Participants were classified by their symptoms as severe, moderate, or mild. Sanger sequencing of the SPG11 gene was conducted. In those who were negative for SPG11 or had only one mutation identified, NGS was employed using the TrueSightOne platform including analysis of 4813 genes. Filtering and variant analysis focused on a subset of genes related to spastic paraplegia, neurodegeneration, ataxia, peripheral neuropathy, Parkinson’s disease, and pallidopyramidal syndromes. Except for one case without available DNA, Sanger sequencing was used to confirm identified variants. A likely pathogenic variant was found in 48 of 97 participants (49%). Mutations in SPG11 were the most common, found in 30 participants. No copy number variants of SPG11 were identified with NGS. Numerous VUS were detected, which is a frequent issue with high-throughput NGS studies.

## Muscular Dystrophies

### *Congenital Muscular Dystrophies (CMDs)*

CMDs are disorders of muscle weakness and hypotonia that manifest in the first two years of life. The prevalence is variable and not all geographies have epidemiological data. In European populations, about 1 in 100,000 individuals are affected. Serum creatine kinase (CK) levels are often, but not always, elevated. Muscle biopsy usually shows abnormalities such as necrosis, regenerating fibers, variable fiber size, and increased perimysial and endomysial connective tissue. The three major categories of CMD are collagenopathies, such as Ullrich CMD and Bethlem myopathy, merosinopathies (merosin-deficient CMD), and dystroglycanopathies. Collagenopathies involving muscular dystrophy are typically associated with the COL6A1, COL6A2, COL6A3 genes and have recessive as well as dominant forms of

inheritance. The age of onset and severity can vary widely, but involves a combination of progressive muscle weakness, joint hypermobility, and contractures. Merosinopathies are also known as laminin α2 related CMDs are caused by defects in the LAMA2 gene and feature congenital weakness, elevated CK levels, and brain magnetic resonance imaging (MRI) evidence of white matter signal abnormalities. Dystroglycanopathies include Walker Warburg syndrome, muscle-eye-brain disease, Fukuyama congenital muscular dystrophy, and congenital muscular dystrophy 1C and 1D. Multiple genes are responsible for these disorders. Originally it was thought that each individual dystroglycanopathy was a distinct disorder that could be defined solely by clinical findings and mutations in a specific gene. However, it is now known that there can be significant phenotypic overlap in clinical findings and variable disease severity. Common symptoms remain, however, within dystroglycanopathies; typically, these include muscular dystrophy, elevated CK, and abnormal muscle biopsy. Most have some degree of ocular and brain abnormality as well. (Kang et al., 2015; reaffirmed 2021; Bönnemann et al., 2014, Jobling et al., 2014 and Martin et al., 2005).

O'Grady et al. (2016) researched the use of a targeted NGS panel vs. candidate gene sequencing for individuals with CMD who were identified retrospectively and prospectively through clinical records and the Institute for Neuroscience and Muscle Research Biospecimen Bank. Individuals were identified from a 35-year period, and were included for study if there was evidence of muscle weakness and hypotonia in the first two years of life, and clinical features were consistent with CMD. Only the proband from a family was included when a sibling was identified in the cohort. Exclusion criteria included identification of structural changes in skeletal muscle diagnostic of a congenital myopathy, or if the case was identified from many years prior and re-contact could be considered insensitive. A total of 123 individuals with CMD were included. Participants underwent histological studies for laminin-a2, glycosylated a-dystroglycan, and collagen VI. Microarray analysis was performed, and candidate gene sequencing was driven by the histological classification and clinical phenotype and included analysis of FKRP, LARGE, POMT1, POMT2, FKTN, and POMGNT1, the 3 collagen VI genes, LAMA2, SEPN1, LMNA, DNM2, and ACTA1. This approach yielded a firm genetic diagnosis in 39 (32%) of participants; two additional participants had a probable diagnosis. The remaining undiagnosed individuals were offered additional genetic analysis. Targeted NGS was performed with a research-based 45 gene panel, a commercial 336 gene panel, or WES. Twenty-eight participants who were identified clinically from 1993 or later, consented to additional studies. Two participants had the 45 gene panel, four had the 336 gene panel, and one had both panels. The remaining 21 had WES. Eleven of this cohort had causative variants identified. Overall, 59 of the 123 (48%) probands had a genetic diagnosis established by this study. The authors felt this data supported NGS as a first-line tool for genetic evaluation of individuals with CMD, with muscle biopsy reserved as a second-tier investigation.

## Clinical Practice Guidelines

### *American Academy of Neurology (AAN) and American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM)*

In many situations, CMD can be diagnosed clinically based on a characteristic phenotype, histological results, and other clinical tests (Kang et al., 2015; reaffirmed 2021). However, genetic diagnoses are beneficial to the affected individual, as they often enable physicians to provide more accurate prognoses and facilitate genetic counseling and family-planning discussions and may enable individuals to become more aware of future clinical trials for which they may be eligible. In 2015 (reaffirmed 2021), Kang et al. published an evidence-based guideline for the AAN and AANEM that included the use of genetic testing in the evaluation and diagnosis of CMD, and make the following recommendations:

- When available and feasible, physicians might order targeted genetic testing for specific CMD subtypes that have well-characterized molecular causes (Level C).
- In individuals with CMD who either do not have a mutation identified in one of the commonly associated genes or have a phenotype whose genetic origins have not been well characterized, physicians might order whole-exome or whole genome sequencing when those technologies become more accessible and affordable for routine clinical use (Level C).

## *Limb Girdle Muscular Dystrophies (LGMD) and Myofibrillar Myopathies (MFM)*

LGMDs are a relatively rare group of diseases impacting up to .43 per 100,000 individuals. Incidence can vary by ethnicity (Narayanaswami et al., 2014; reaffirmed 2022). LGMD are characterized by proximal muscle weakness (shoulders, upper arms, pelvic area, and thighs), muscle wasting, and myopathic or dystrophic myopathological features (Kuhn et al., 2016). There are many subtypes of LGMD which can vary with age of onset, severity, and additional co-morbidities such as weakness of the heart muscles (MedlinePlus, 2019). There are at least thirty genes associated with LGMD; seven are autosomal dominant, and twenty-three are autosomal recessive (Kuhn et al., 2016). LGMD are classified according to inheritance pattern. LGMD1 are autosomal dominant, and LGMD2 are recessive. Further subtyping is delineated using a letter. In their most recent guidelines, the American Academy of Neurology (AAN) identified LGMD1A-LGMD1F, and LGMD2A-LGMD2S (Narayanaswami et al., 2014; reaffirmed 2022).

Nallamilli et al. (2023) evaluated the data from “The Lantern Project” (also referenced in Sniderman King, 2023) with the main focus on the genes associated with LGMD, LGMD subtypes and other myopathies. The author’s objective was to develop a comprehensive NGS-based multi gene panel known as the Lantern Focused Neuromuscular Panel that would detect both sequence variants and CNV in one assay. Molecular diagnosis was determined in 19.6% (1266) of 6473 cases. Major genes contributing to LGMD included CAPN3 (5.4%, 68), DYSF (4.0%, 51), GAA (3.7%, 47), ANO5 (3.6%, 45), and FKRP (2.7%, 34). Genes of other overlapping MD subtypes identified included PABPN1 (10.5%, 133), VCP (2.2%, 28), MYOT (1.2% 15), LDB3 (1.0%, 13), COL6A1 (1.5%, 19), FLNC (1.1%, 14), and DNAJB6 (0.8%, 10). In 95 cases (7.5%) different sizes of CNVs including single exon, multi-exon, and whole genes were identified in DMD, EMD, CAPN3, ANO5, SGCG, COL6A2, DOK7, and LAMA2. The authors note that these findings support the use of comprehensive NGS panels such as the Lantern Focused Neuromuscular Panel including CNV analysis in addition to evaluation of standard LGMD genes to aid in the diagnosis of LGMD subtypes as well as other myopathies with a similar clinical presentation.

In a retrospective evaluation, Çavdarlı et al. (2023) assessed the diagnostic rate of a 47-gene, NGS-based panel (created by the research team) to identify genetic variations in a population of 146 individuals in Turkey (ages 6 months to 67 years) suspected to have a neuromuscular disorder based on clinical examination, laboratory findings and imaging. Individuals who had been diagnosed with dystrophinopathy based on genetic evaluation of dystrophin by MLPA were excluded. The genes included in the panel targeted variations related to muscular dystrophy and myopathies that have been suggested for first-tier testing. Based on the study results, 67 individuals were found to have a genetic basis for their disorder, correlating to a diagnostic yield of 46%. Twenty-three genes showed variations associated with neuromuscular disorders; these included CAPN3(11), DYSF(9), DMD(8), SGCA(5), TTN(4), LAMA2(3), LMNA(3), SGCB(3), COL6A1(3), DES (2), CAV3(2), FKRP(2), FKTN(2), ANO5, COL6A2, CLCN1, GNE, POMGNT1, POMGNT2, POMT2, SYNE1, TCAP, and FLNC. Novel variants were identified in 16 genes. Indeterminate results were found in 27 participants, including those with VUS, only one heterozygous variant for an autosomal recessive disease, and individuals with two variants in different genes. Based on the results of the study, the authors assert that targeted NGS testing is a viable option for molecular diagnosis of neuromuscular conditions such as muscular dystrophy and could reduce the need for WES.

Winckler et al. (2022) examined the diagnostic yield of an NGS panel made up of 39 genes to be used as a first-tier test for diagnosing individuals with genetic myopathies. This cross-sectional study took place in Brazil and included 51 cases where genetic myopathies were suspected based on clinical findings. In this study, the diagnostic yield of the NGS panel was found to be 52.9%; when candidate variants were included in the evaluation, the diagnostic yield increased to 60.8%. LGMD was identified in 12/25 individuals (48%), 7/14 individuals (50%) with congenital muscular diseases were identified, and 7/10 (70%) with muscular dystrophy including prominent joint contractures were diagnosed. The researchers indicate that these results show that the customized NGS panel studied produced high diagnostic yields when used early in the exploration of gene-related myopathies, which could result in earlier diagnosis and potential treatments.

Kuhn et al. (2016) examined the clinical utility of an NGS panel for LGMD in a group of 58 German individuals who were suspected to have LGMD. The panel focused on 23 genes known to cause LGMD and 15 genes known to cause a similar phenotype. The age of onset ranged from 3 to 63 years of age. Four individuals had autosomal dominant forms of disease, and sixteen had affected siblings, suggesting autosomal recessive inheritance. X-linked inheritance was most likely in two participants. The remaining individuals were considered to have sporadic cases. All participants had a muscle biopsy that confirmed myopathic or dystrophic changes, but LGMD immunohistochemistry or immunoblotting was not possible on the remaining sample. NGS was performed on the 38 targeted genes with an average 20X coverage. All pathogenic variants and VUS were confirmed by Sanger sequencing. Disease-causing mutations that explained the phenotype were found in 19 of 58 participants (33%). In 28% of those with autosomal recessive disease, only a single pathogenic mutation was found. Additional sequencing and CNV analysis on the relevant gene to identify another pathogenic mutation, consistent with recessive inheritance, was negative. VUS were found in 10% of participants, and the remainder had no mutations identified.

Monies et al. (2016) studied an NGS panel of 759 genes associated with neurological disorders in individuals from 50 families presenting with muscle weakness affecting the pelvic girdle and shoulder, of which 36 had an autosomal recessive form of inheritance. These families were identified through the Neurosciences Clinic of King Faisal Specialist Hospital and Research Centre, Saudi Arabia. Variants were analyzed and classified using the ACMG and AMP guidelines. Thirty-eight families (76%) received a genetic diagnosis from this study. Thirty-four had LGMD related mutations, and four had novel genetic variants not usually associated with LGMD. Families with negative results had follow up WES, but no additional variants were found. The authors concluded that their panel was sensitive, cost-effective, and rapid; significantly assisting the clinical practice.

Ankala et al. (2015) reported on the design and validation of several NGS panels at Emory Genetics Lab (EGL), developed to help expedite the diagnosis of NMD, including LGMD. The authors report that in their experience, clinicians

must go through an extensive diagnostic workup in order to determine a small gene list to pursue for NMD, and individuals may opt out of the process before a diagnosis is finalized. A combination of a targeted NGS panel and a targeted CGH test to identify copy number variants may reduce the burden of invasive tests. From October 2009 to May 2014, the authors analyzed the data for LGMD single gene and NGS/CGH panels to determine the difference in clinical utility. Exome analysis was also compared for 20 random samples to determine how well the exome covered the NMD genes in the study. In this timeframe, 343 LGMD single gene tests were ordered which included 250 sequencing tests and 93 CGH tests. The diagnostic yield was 19% overall. It was very low for CGH, with only eight positives of 93 tested. Ninety-six individuals had an LGMD eleven-gene NGS panel with a diagnostic yield of 26%. Eighty-one had a broad NGS panel that covered 41 genes for NMD, including genes that can cause overlapping phenotypes with LGMD, Emery-Dreifuss muscular dystrophy (EDMD), metabolic myopathies, congenital myopathies, dystrophinopathies, and congenital disorders of glycosylation (CDG). The diagnostic yield of the NMD panel was 46%. The authors also compared WES to the LGMD and NMD panel. Based on the low coverage of WES for some key NMD genes, they concluded that WES would miss variants in five key LGMD genes, whereas the NMD panel would miss one.

## Clinical Practice Guidelines

### *American Academy of Neurology (AAN) and American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM)*

When presented with an individual with a possible LGMD or other distal myopathies like MFM, the AAN and AANEM (Narayanaswami et al., 2014: reaffirmed 2022) recommend referring to a specialized neuromuscular center for evaluation, management, and diagnosis because of the complex nature of NMDs and the need for a multi-disciplinary team. They recommend utilizing an approach focused on a clinical evaluation to narrow down the possible forms of LGMD or other muscular dystrophies. Their evidence-based review found that utilizing information such as pattern of muscle weakness, hypertrophy, or atrophy of certain muscle groups, cardiac or respiratory involvement, muscle biopsy findings, electromyogram (EMG) results, and creatinine kinase (CK) serum levels, can narrow down the differential to just a few disorders. Verification of the specific disorder through genetic testing is recommended, as this will direct the most efficient care path and identify necessary prophylactic interventions, such as the correct timing for placing a pacemaker or the monitoring interval for cardiorespiratory function.

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with limb girdle weakness and probable autosomal dominant inheritance:

- If cardiomyopathy, respiratory involvement, EMG with myotonic or “pseudomyotonic” discharges, foot drop, and myofibrillar myopathy on muscle biopsy are present; test for mutations in the genes desmin (LGMD1E), myotilin (LGMD1A), DNAJB6 (LGMD1D), ZASP, filamin C, αB-crystallin, and titin.
- If rippling muscles and percussion-induced rapid contractions are present; test for mutations in the caveolin-3 gene (LGMD1C).
- If early humeroperoneal weakness, contractures (neck, elbows, knee, ankle), and cardiomyopathy are present; test for mutations in the lamin A/C gene (LGMD1B or AD-EDMD).
- If distal weakness, myotonic discharges on EMG, past or family history of Paget disease, frontotemporal dementia, or motor neuron disease are present; test for mutations in VCP (hIBMPFD).
- If no clinical features suggest a specific form of dystrophy, or if initial genetic testing is not informative, perform a muscle biopsy to direct further genetic testing (such as immunohistochemistry/immunoblotting for various sarcolemmal proteins, calpain-3, or features of myofibrillar myopathy) or to exclude an alternative diagnosis (e.g., a metabolic myopathy, mitochondrial myopathy, congenital myopathy, or inflammatory myopathy).

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with limb girdle weakness and probable autosomal recessive inheritance:

- If scapular winging but no calf hypertrophy, and normal cardiorespiratory function are present; test for mutations in calpain-3 (LGMD2A). Individuals of English, French, Spanish, Italian, Portuguese, or Brazilian descent may have a higher pretest probability of this disorder.
- If calf atrophy and weakness (i.e., inability to stand on toes) are present; test for mutations in anoctamin-5 (LGMD2L) or dysferlin (LGMD2B).
- If the onset of symptoms is in the teens or early twenties or the affected individual is from Asia, clinicians should assess for dysferlin mutations first and, if negative, test for anoctamin-5 mutations. If the onset of symptoms is in the 30s or later or the affected individual is of English or northern European ancestry, clinicians should assess for anoctamin-5 mutations first and, if negative, test for dysferlin mutations.
- If muscle biopsy immunohistochemistry showing reduction in 90 α-, β-, γ-, or δ-sarcoglycans is present; test for mutations in the sarcoglycan genes, SGCA, SGCB, SGCG, and SGCD (LGMD2C–2F).
- If the affected individual is of Hutterite descent; test for mutations in TRIM32.

- If scapular winging, calf hypertrophy, and early cardiorespiratory involvement are present; test for mutations in FKRP.
- If mental retardation is present; test for mutations in genes that cause primary or secondary deficiency of α-dystroglycan, POMT1, POMT2, FKTN, FKRP, LARGE1, POMGNT1, and ISPDI genes (LGMD2K, LGMD2M, LGMD2N, LGMD2O, and LGMD2P).
- If epidermolysis bullosa or pyloric atresia; test for mutations in plectin, PLEC.
- If no other specific clinical features are identified, or the muscle biopsy does not inform genetic testing, clinicians should perform a dried blood spot test for α-glucosidase (acid maltase) deficiency or Pompe disease.

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with limb girdle weakness and probable X-linked inheritance:
- If male, perform testing for mutations in the dystrophin (DMD) gene.
- If female, test for DMD gene mutations or perform a muscle biopsy and immunostaining for dystrophin to assess for a mosaic pattern of staining. If positive, confirm diagnosis with DMD gene testing.

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with humeroperoneal weakness and probable autosomal dominant inheritance:
- If early cardiac involvement and no joint laxity are present; perform genetic testing for mutations in the lamin A/C gene (AD-EDMD, LGMD1B).
- If joint laxity, protuberant calcaneus, and no cardiac involvement are present; test for mutations in the collagen VI gene (Bethlem myopathy).

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with humeroperoneal weakness and probable autosomal recessive inheritance:
- If congenital onset, joint laxity, protuberant calcaneus, and no cardiac involvement are present; test for mutations in the collagen VI gene (Ullrich myopathy).

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were provided for those with humeroperoneal weakness and probable X-linked inheritance:
- If joint laxity, protuberant calcaneus, and no cardiac involvement are present; test for mutations in the emerin (EMD) gene.

If humeroperoneal weakness and suspected muscular dystrophy with early cardiac involvement and no joint laxity are present, and there are no mutations found in the lamin A/C or emerin gene, clinicians should perform muscle biopsy to delineate characteristic abnormalities that direct further genetic testing (evidence level B, expert consensus based on moderate evidence).

If late adult onset of index finger and wrist extensor weakness, followed by atrophy and weakness of hand muscles, and muscle biopsy showing rimmed vacuoles are present; a diagnosis of Welander distal myopathy is most likely and should be confirmed through genetic testing for Welander myopathy (evidence level B, expert consensus based on moderate evidence).

The following phenotype and genotyping recommendations (evidence level B, expert consensus based on moderate evidence) were made for individuals with suspected distal muscular dystrophy and probable autosomal recessive inheritance:
- If early onset of calf weakness is present; test for mutations in the anoctamin-5 and dysferlin genes.
- If early onset (< 30 years of age) of progressive foot drop is present in individuals who are of Japanese or Middle Eastern Jewish descent; test for GNE mutations (AR-hIBM).
- If none of the clinical features above are noted, clinicians should perform a muscle biopsy to direct further genetic testing.

In individuals with muscular dystrophy who have proximal as well as distal weakness, clinicians should use specific clinical features (e.g., rippling muscles, cardiomyopathy, atrophy of specific muscle groups, irritability on EMG) and biopsy features (myofibrillar myopathy [MFM], reduction of emerin immunostaining, presence of rimmed vacuoles) to guide genetic testing, which may include mutations in the genes causing the various forms of MFM; LGMD2B (dysferlin), LGMD2L (anoctamin-5), LGMD2J (titin), LGMD1C (caveolin-3), and EDMD (emerin and lamin A/C).

In individuals with suspected muscular dystrophy in whom initial genetic testing, muscle biopsy, and dried blood spot test for Pompe disease do not provide a diagnosis, clinicians may obtain genetic consultation or perform parallel sequencing

of targeted exomes, whole exome sequencing, whole genome sequencing, or NGS to identify the genetic abnormality (Level C, expert consensus based on modest evidence).

## Mitochondrial Disease

Mitochondria are organelles, and hundreds to thousands of mitochondria are found in each cell. Mitochondrial Myopathies (MM) are a group of genetic disorders with a primary defect in electron transport chain function, resulting in abnormal energy production from fat and carbohydrate oxidation pathways. Symptoms such as muscle weakness, muscle cramping, or pain often appear during periods where these pathways are relied upon most, such as endurance sports activities, illness, or periods of fasting. The mitochondria have their own DNA (mtDNA) that exists in a double stranded circle, and multiple copies of mtDNA can occur in each mitochondrion. mtDNA codes for about 37 genes, but the transcription, translation, and function of the mitochondrial DNA is dependent on a number of nuclear genes. Therefore, MM are a constellation of diseases that can have their root cause in either the mitochondrial or the nuclear DNA (Tarnopolsky 2016).

Because each cell can have multiple copies of normal and abnormal mitochondria, called heteroplasmy, mitochondrial based diseases are known to have a wide range of phenotypic expression. DNA testing can be challenging as a result, but the advent of NGS allows for better detection of heteroplasmy in blood, ranging from 1-10% depending on the methodology and tissue type (Parikh et al. 2015).

The prevalence of most types of MMs is unknown; however, mitochondrial disease is one of the most common groups of genetic diseases with a minimum prevalence of greater than 1 in 5000 in adults. Examples of MM with neuromuscular manifestations include, but are not limited to, the following:

- Kearns-Sayre syndrome (KSS) is a mitochondrial disorder characterized by the onset of progressive external ophthalmoplegia (PEO) younger than age 20, pigmentary retinopathy, heart block, and cerebellar ataxia. There is wide phenotypic expression, and some may experience myopathy, deafness, dysphagia, hypoparathyroidism, diabetes, and dementia (Chinnery, 2021).
- Chronic progressive external ophthalmoplegia (CPEO) is characterized by external ophthalmoplegia, bilateral ptosis, and mild proximal myopathy (Chinnery, 2021). This is often the canonical symptom representing mitochondrial disease. It can be caused by a mutation or large rearrangement of mitochondrial DNA that accumulate throughout life in the skeletal muscle and cause disease. Nuclear genes that interact with the mitochondria such as SPG7 have also been implicated in the disease (Pfeffer et al., 2014).
- Progressive external ophthalmoplegia (PEO) is part of a spectrum of disorders, including CPEO, and has an unknown prevalence. Similar disorders include ataxia neuropathy spectrum and KSS. They are typically clinical diagnoses that are made through history and examination. Imaging studies, blood and cerebral spinal fluid tests, electromyography of the limbs, and muscle biopsy can help refine the differential diagnosis if there is doubt. About 50% of PEO is inherited and caused by mutations in mtDNA and nuclear genes such as POLG1, POLG2, ANT1, Twinkle, RRM2B, DNA2, and OPA1. The remaining 50% is sporadic, and mtDNA testing often shows an accumulation of a single large mtDNA deletion. The size of the deletion may be associated with disease severity (McClelland et al., 2016).
- MERRF (myoclonic epilepsy with ragged red fibers [RRF]) is a multisystem disorder with a childhood onset that presents with myoclonus followed by generalized epilepsy, ataxia, weakness, and dementia. Additional findings can include hearing loss, short stature, optic atrophy, cardiomyopathy, pigmentary retinopathy, and lipomatosis. Diagnosis is usually clinical and based on the presence of four primary features; myoclonus, generalized epilepsy, ataxia, and RRF identified in a muscle biopsy. Genetic testing can be used to confirm a diagnosis, and often the genetic variants in MERRF are found in white blood cells. However, because of mitochondrial heteroplasmy, it is possible that the mutation is not detectable in blood and another tissue type should be tested if blood is negative (DiMauro and Hirano, 2015).
- MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) is a multisystem disorder with onset typically in childhood although it can begin at any age. The first symptoms can be exercise intolerance or proximal limb weakness, followed by generalized tonic-clonic seizures, recurrent headaches, anorexia, and recurrent vomiting. Seizures manifest as stroke like episodes which may involve transient blindness or hemiparesis. Over time, the recurrent seizures may result in deafness, impaired motor abilities, and vision and intellectual capabilities. Diagnosis is usually clinical and based on presentation of stroke-like episodes, typically before age 40 years, encephalopathy with seizures and/or dementia and mitochondrial myopathy, evidenced by lactic acidosis and/or RRF on muscle biopsy. In addition, two of the three following symptoms are also required for diagnosis; normal early psychomotor development, recurrent headache, or recurrent vomiting. Genetic testing can be used to confirm a diagnosis (DiMauro and Hirano, 2013).
- Leigh syndrome has onset of symptoms in the first year of life, often after a viral infection. Clinical manifestations include hypotonia, spasticity, movement disorders, cerebellar ataxia, and peripheral neuropathy. Cardiomyopathy may occur as well. About 50% of children die by age 3 as a result of respiratory or cardiac failure. A diagnosis is usually

accomplished clinically through characteristics features on brain imaging, typical neuropathologic changes, and similar symptoms in an affected sibling. The detection of a pathogenic variant in one of the 14 mitochondrial genes that are known to be involved in mtDNA-associated Leigh syndrome can confirm the diagnosis (Thorburn et al., 2023).

- NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa) first appears in childhood or early adulthood and is characterized by proximal neurogenic muscle weakness with sensory neuropathy, ataxia, and pigmentary retinopathy. Learning disabilities may also be present. Diagnosis can be suspected through clinical means but may require DNA testing to confirm a diagnosis. The suggested approach is to look for two common variants that cause NARP in the MT-ATP-6 gene, and if negative consider mtDNA genome sequencing (Thorburn et al., 2023).

Select articles below review the experience of researchers and clinical labs with targeted NGS and the clinical validity for diagnosing mitochondrial diseases.

In a retrospective, multi-center study, Wu et al. (2023) analyzed the diagnostic yield of dual genomic sequencing along with mitochondrial disease criteria (MDC) in a pediatric population. Enrollees included 503 children aged less than 18 years with an unknown neuromuscular disorder or a multisystem progressive disease that was suspected to be related to a mitochondrial abnormality. The children underwent dual genomic analysis and the results were evaluated in terms of potential relationship of the variants to clinical features seen in the children and also to previously reported clinical features, leveraging the ACMG classification of variants and segregation patterns. If the genes were associated with mitochondrial diseases in the literature, they were classified as “mitochondria-related.” If not, they were classified as “non-mitochondria-related.” Overall, causative variants were detected in 177/503 (35.2%) of participants. Mitochondrial-related variants were found in 46 individuals (9.1%). Of these, 25 individuals had nuclear DNA variants, 15 had mitochondrial DNA variants and six had dual genomic variants. When MDC was applied, 15.2% of individuals with mitochondrial-related variations were found “unlikely to have mitochondrial disorder.” Additionally, 4.5% of individuals with non-mitochondrial related variants and 1.43% with no genetic abnormality were classified with MDC as “probably has a mitochondrial disorder.” The authors propose using MDC to guide dual genomic sequencing, particularly in cases where an individual is assessed as “possibly having a mitochondrial disorder” and “probably having a mitochondrial disorder.”

Mavraki et al. (2023) published United Kingdom consensus guidelines developed by “a working group of clinical scientists from the NHS Highly Specialized Service followed by national laboratory consultation. The guidelines highlight the current technologies and methods recommended for evaluation of mitochondrial DNA and nuclear-encoded genes in individuals with suspected mitochondrial diseases. The guidelines state that nuclear gene analysis can be comprehensively performed through the use of multi-gene panels with NGS. Genes included in the panels are based on associated clinical phenotype data and may include a comprehensive mitochondrial disorder nuclear gene panels or targeted nuclear gene panels. Consultation with a genetic counselor is recommended to assist with determining testing recommended for family members.

In a 2018 article, Witters et al. detailed their retrospective study which aimed to validate the diagnostic value of mitochondrial disease criteria (MDC). The study included a multicenter cohort with genetically confirmed primary mitochondrial disease (MD). A total of 136 individuals were studied, of which 91 had nuclear DNA (nDNA) mutations. Of these, 51% had definite MD according to MDC, and 33% had probable MD. Muscle biopsy was performed in 63 (47%) of the participants. The researchers found that those with (nDNA) mutations versus mitochondrial DNA mutations were younger (6.4 ±9.7 versus 19.5 ±17.3 years) and had higher MDC scores. Using a cutoff of 6.5/8 on the MDC scale, sensitivity to diagnose individuals with nDNA mutations was 72.5% and positive predictive value was 69.5%. In the group of subjects with nDNA mutations, the researchers found that whole exome sequencing was better able to diagnose individuals with lower scores compared to Sanger sequencing; 7/8 individuals diagnosed with possible MD by MDC were diagnosed by whole exome sequencing. The authors concluded that MDC continue to be very useful in clinical diagnosis of MD, assisting with decision-making regarding muscle biopsy and aiding in interpretation of whole exome sequencing results.

Plutino et al. (2018) utilized NGS in a cohort of 80 individuals who were clinically diagnosed with mitochondrial disease tests to determine the clinical validity of targeted panel approach to a genetic diagnosis. The participants were diagnosed through clinical, biochemical, and histological analysis. They included 24 children and 56 adults, 38 males and 32 females. Participants first underwent mtDNA testing, and if negative, a custom nuclear gene panel was run. Single deletions and point mutations in the mtDNA were identified using XL-PCR and NGS and confirmed by Southern blot. The custom panel was comprised of 281 genes known to be involved in mitochondrial disease and were analyzed by NGS and confirmed with Sanger sequencing. Variant filtering and interpretation focused on rare mutations that were predicted to be missense, frame-shift, stop-gain, stop-loss, or splice site variants. Pathogenic variants were found in mtDNA in the first step in one child and 14 adults. The remaining 65 participants had the targeted NGS panel and an additional five children and three adults achieved a genetic diagnosis for an overall diagnostic rate of 29%. The authors compared their panel testing results to other studies involving WES and larger panels with reported diagnostic rates of 8-24% and concluded

that larger gene panels are not necessary in mitochondrial diseases because of their high heterogeneity, the ongoing discovery of novel genes, and genes that may not appear to be related to mitochondrial function could lead to secondary respiratory chain deficiency.

Lilleker et al. (2018) suggested an approach to identifying and diagnosing metabolic myopathies that focuses on the following priorities; identify those that might have a 'genuine' metabolic myopathy, determine clinically the most likely biochemical process, identify which individuals need a further work up such as a muscle biopsy or genetic test, identify those individuals with conditions for which treatment is available, and offer genetic counseling to the affected individuals and family members, as appropriate. To meet these goals, the authors recommend obtaining a thorough history, which might include obtaining historical medical records to determine how symptoms and lab results change over time, development, and exercise history. Some symptoms could be attributed by the affected individual or family to something normal, such as "growing pains," when in fact this is a subtle, yet important, clue to a diagnosis. It is important to ask specific and targeted questions to prevent missing possible symptoms. A physical exam, including neurological elements, is key. If a metabolic myopathy is suspected, the early involvement of an experienced multidisciplinary team will be important to help tailor further investigations and reduce the time to diagnosis and treatment. This team may help further rule out "pseudomyopathies" and recommend further CK testing, muscle biopsy, skin biopsy, exercise testing, or EMG. Enzymatic testing based on history, symptoms, and specialist analysis may lead to diagnosis or identify the most likely pathway that is affected in the affected individual. Genetic testing is proving to be a valuable tool in diagnosing metabolic myopathies, and targeted genetic testing can help confirm a diagnosis. There is a shift towards NGS panel testing as a first-tier diagnostic investigation of choice by some; however genetic testing sometimes produces results that are difficult to interpret. The authors conclude that the diagnostic workup and management of individuals with metabolic myopathies is complex; early referral to a specialist neuromuscular multidisciplinary clinic is strongly recommended.

The Neurological Institute C. Besta in Milan, Italy reported on its experience using a combination of targeted NGS and WES in a cohort of 125 individuals strongly suspected to have mitochondrial disease (Legati et al. 2016). The participants were divided into respiratory chain complex groups based on their histological findings, mtDNA testing, and biochemical testing results; complex I (n = 5), complex II (n = 18), complex III (n = 15), complex IV (n = 21), complex V (n = 5), multiple defects (n = 26), CoQ10 deficiency (n = 3), mtDNA deletions or depletion determined by Sanger sequencing (n = 8) and pyruvate dehydrogenase (PDH) complex defects (n = 14). DNA was extracted from blood and underwent targeted NGS sequencing for 132 genes associated with mitochondrial disease for all participants. Overall, targeted NGS found causative mutations in 19 individuals (15%). Two had defective complex I, two had defective complex II, two had defective complex III, two had defective complex IV, two had defective PDH complex, six had multiple defects, one had mtDNA depletion and two were biochemically undefined. Additionally, 27 participants had candidate genetic variants that were suspicious but not conclusively pathogenic. Ten participants with negative panel results were selected for WES based on the accuracy of family history, clinical description, parental consanguinity, and availability of other family members for testing. Variants found on WES were confirmed by Sanger sequencing. Six participants who had undergone WES had pathogenic mutations (60%) that were confirmed by Sanger sequencing and family segregation studies. The authors concluded that the approach of a targeted NGS panel followed by WES in select individuals was powerful and predicted that if used as a first line test, the detection rate would be about 25%, but noted that the choice of which approach may be best for these disorders depends on several institutional factors, such as availability of funding, space, personnel, and bioinformatics expertise.

## Clinical Practice Guidelines

### *Mitochondrial Medicine Society (MMS)*

There are no published consensus-based practice parameters for initiating diagnosis or management of individuals with mitochondrial diseases. In 2015, Parikh et al. reviewed the literature on mitochondrial disease and made the following consensus-based recommendations for the diagnosis and management of these individuals:

- Massively parallel sequencing/NGS of the mtDNA genome is the preferred methodology when testing mtDNA and should be performed in cases of suspected mitochondrial disease instead of testing for a limited number of pathogenic point mutations.
- Individuals with a strong likelihood of mitochondrial disease because of a mtDNA mutation and negative testing in blood, should have mtDNA assessed in another tissue to avoid the possibility of missing tissue-specific mutations or low levels of heteroplasmy in blood; tissue-based testing also helps assess the risk of other organ involvement and heterogeneity in family members and to guide genetic counseling.
- Heteroplasmy analysis in urine can selectively be more informative and accurate than testing in blood alone, especially in cases of MELAS due to the common m.3243A > G mutation.
- mtDNA deletion and duplication testing should be performed in cases of suspected mitochondrial disease via NGS of the mtDNA genome, especially in all individuals undergoing a diagnostic tissue biopsy.

- If a single small deletion is identified using polymerase chain reaction–based analysis, then one should be cautious in associating these findings with a primary mitochondrial disorder.
- When multiple mtDNA deletions are noted, sequencing of nuclear genes involved in mtDNA biosynthesis is recommended.

- When a tissue specimen is obtained for mitochondrial studies, mtDNA content (copy number) testing via real-time quantitative polymerase chain reaction should strongly be considered for mtDNA depletion analysis because mtDNA depletion may not be detected in blood.
  - mtDNA proliferation is a nonspecific compensatory finding that can be seen in primary mitochondrial disease, secondary mitochondrial dysfunction, myopathy, hypotonia, and as a by-product of regular, intense exercise.
- When considering nuclear gene testing in individuals with likely primary mitochondrial disease, NGS methodologies providing complete coverage of known mitochondrial disease genes is preferred. Single-gene testing should usually be avoided because mutations in different genes can produce the same phenotype. If no known mutation is identified via known NGS gene panels, then WES should be considered.

## U.S. Food and Drug Administration (FDA)

This section is to be used for informational purposes only. FDA approval alone is not a basis for coverage.

Laboratories that perform genetic tests are regulated under the Clinical Laboratory Improvement Amendments (CLIA) Act of 1988. More information is available at: https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/IVDRegulatoryAssistance/ucm124105.htm. (Accessed May 21, 2024)

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## Policy History/Revision Information

| Date | Summary of Changes |
|---|---|
| 10/01/2024 | **Coverage Rationale**<br>• Added language to clarify multi-gene *comprehensive* neuromuscular disease test panels targeting multiple conditions (e.g., muscular dystrophy and mitochondrial disease) are unproven and not medically necessary<br>**Supporting Information**<br>• Updated *Description of Services*, *Clinical Evidence*, and *References* sections to reflect the most current information<br>• Archived previous policy version CS165NJ.F |

## Instructions for Use

This Medical Policy provides assistance in interpreting UnitedHealthcare standard benefit plans. When deciding coverage, the federal, state or contractual requirements for benefit plan coverage must be referenced as the terms of the federal, state or contractual requirements for benefit plan coverage may differ from the standard benefit plan. In the event of a conflict, the federal, state or contractual requirements for benefit plan coverage govern. Before using this policy, please check the federal, state or contractual requirements for benefit plan coverage. UnitedHealthcare reserves the right to

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