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# Investigation of hereditary muscle disorders in the genomic era

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**Abstract**
Identifying the genetic basis of inherited muscle disease is the single most important step to accurately guide patient care. A timely and accurate diagnosis is crucial for patients with neuromuscular disorders and their families. Advances in genomics are transforming the way we diagnose and treat many inherited diseases and their integration into clinical practice has reduced the diagnostic odyssey for patients with limb-girdle muscular dystrophies and myopathies. This review proposes a new, less invasive diagnostic algorithm that incorporates next generation sequencing (NGS) into neuromuscular clinics, reserving muscle biopsies for the "difficult to diagnose" patients. We discuss the importance of accurate history taking and detailed phenotyping, followed by initial screening investigations and exclusion of the common neuromuscular disorders. Once sufficient clinical and screening information has been obtained, NGS would be considered an appropriate next step, with a targeted neuromuscular panel usually favoured in view of the lower cost and less difficulties with variant data compared to whole exome and whole genome sequencing. Using this diagnostic paradigm will enable a greater number of patients to achieve an accurate and timely diagnosis, receive appropriate disease-specific treatments and gain access to informed family planning.

## Introduction

Many patients with limb-girdle muscular dystrophies and inherited myopathies often remain undiagnosed or are misdiagnosed for long periods of time due to the phenotypic heterogeneity of these disorders. The traditional diagnostic pathway has relied on a stepwise process of clinical assessment and multiple investigations that are performed prior to proceeding to a muscle biopsy. Histologic and biochemical assessment of a muscle biopsy has remained the historical "gold-standard" for diagnosing the muscular dystrophies and myopathies.[1,2] Based on the muscle biopsy findings and the clinical phenotype, Sanger sequencing of candidate genes would be subsequently performed, usually one gene at a time. A lack of clear genotype-phenotype correlation meant many genes often needed to be sequenced to identify the causative gene and pathogenic variants. Sanger sequencing a large number of individual genes is time consuming, laborious and prohibitively expensive. Moreover, often large genes such as titin (*TTN*) with 363 exons, were not entirely Sanger sequenced routinely due to its size and complexity. Thus, only a few *TTN* mutations were reported prior to the advent of next generation sequencing (NGS).[3]

Using this traditional sequential pathway, the diagnostic rate for the limb-girdle muscular dystrophies remained low as reported in a review of a large Australasian limb-girdle muscular dystrophy (LGMD) cohort for whom 65% of families remained without a genetic diagnosis, despite numerous investigations at an expert neuromuscular centre.[4]

## Integration of NGS technology into clinical practice for the diagnosis of Neuromuscular Disorders: *Benefits and Ongoing Challenges*

Implementation of NGS into clinical practice has transformed how we investigate and deliver health care to myopathy and muscular dystrophy patients. NGS, also known as massively parallel sequencing, enables high-throughput DNA sequencing of large numbers of genes simultaneously. There are three methods of DNA sequencing technologies available; Neurogenetic Subexomic Supercapture (NSES), also known as targeted neuromuscular panel, whole exome sequencing (WES) and whole genome sequencing (WGS).[5,6]

NGS has been shown to be efficacious[4,7,8] and also cost-effective.[9] NGS has also facilitated the discovery of novel disease genes[10] and allowed us to expand the phenotype of known disease genes.[11-13] In a cohort of Australasian LGMD patients that had been previously extensively investigated, the use of NSES or WES had enabled a diagnosis to be achieved in 45% of these families. Other studies have shown a similar diagnostic rate for the limb-girdle muscular dystrophies, myopathies and the congenital myopathies.[7,14] The inclusion of family members or "trios" for WES yielded a better diagnostic rate of 60% compared to 40% diagnosis in cases where the proband was only included for WES.[4] The inclusion of 'trios' allows filtering and stratifying identified variants based on familial segregation with disease. Moreover, including family members highlights variants that might have been interpreted as unlikely candidates or simply overlooked when a large amount of data is generated with the initial bioinformatics analysis.[4]

Despite our best efforts to improve the diagnostic yield of neuromuscular patients using

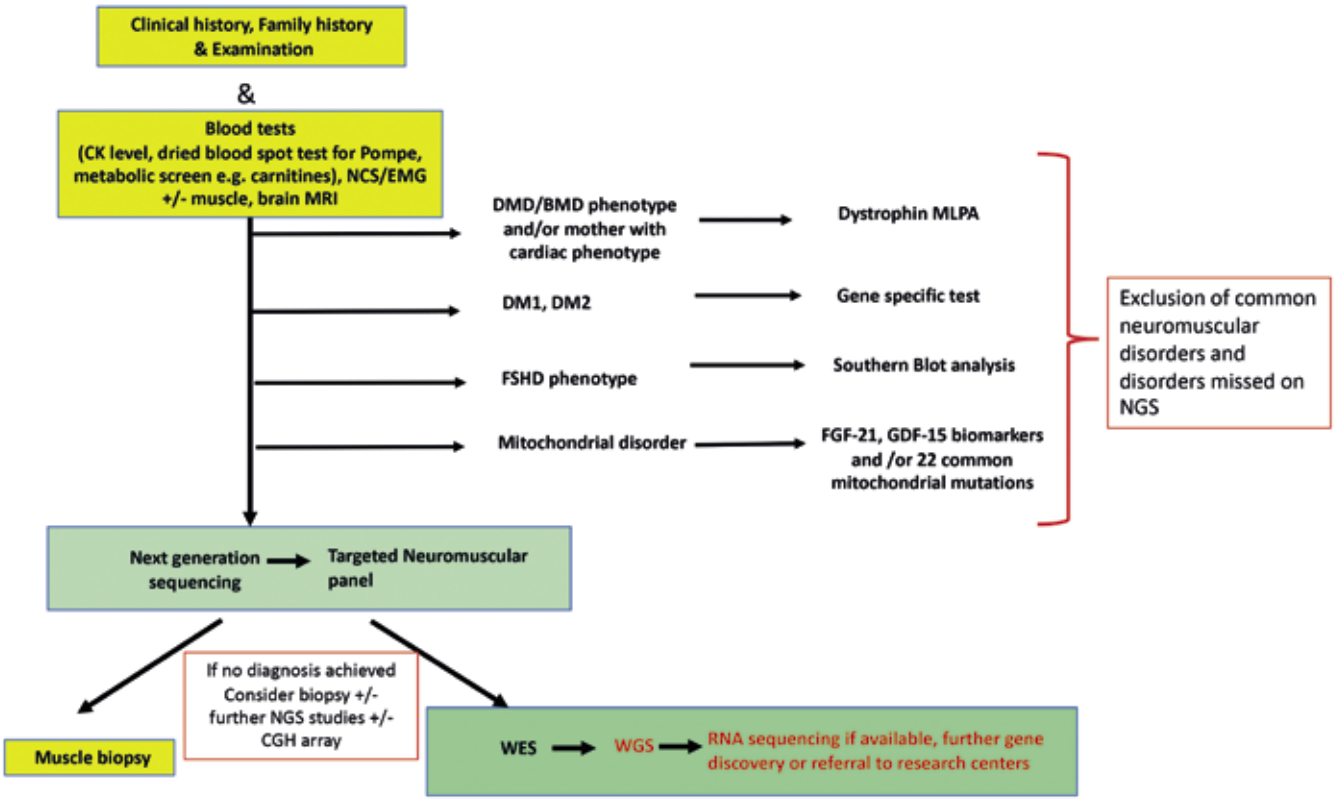


Figure 1: Proposed diagnostic algorithm for limb-girdle muscular dystrophy and myopathy. CK=creatine kinase, NCS=nerve conduction studies, EMG= electromyography, MRI=magnetic resonance imaging, DMD=Duchenne muscular dystrophy, BMD=Becker's muscular dystrophy, MLPA= Multiplex Ligation-dependent Probe Amplification, DM1=myotonic dystrophy type 1, DM2=myotonic dystrophy type 2, FSHD=facioscapulohumeral muscular dystrophy, FGF-21=Fibroblast growth factor 21, GDF15=growth and differentiation factor 15, WES=whole exome sequencing, WGS=whole genome sequencing, NGS=next generation sequencing, CGH=comparative genomic hybridisation microarray.

NGS technology, the diagnostic rate for the adult dystrophies and myopathies remains under 50%.[4] This may in part be due to the large number of challenges and limitations relating to the use of NGS technology that we need to be aware of when utilising this technology.

## Limitations of NGS

### *1. Common neuromuscular disorders are missed by NGS*

Not all coding regions are well covered with NGS platforms potentially missing variants in those regions. On average 10% of the entire exome lacks the required 20x coverage or reads. This can occur in large genes with repetitive regions such as titin (*TTN*) and nebulin (*NEB*), in genomic regions with high GC-content,[15-17] in the promoter region and the 5′ untranslated region (5′UTR) regions which are also poorly covered with targeted capture and WES.[18]

Standard NGS technology such as targeted panels and WES will not detect disorders that arise from repeat expansions. Detection of repeat expansions is currently performed with polymerase chain reaction–based assays[19] or with Southern blots for large expansions and repetitive sequences such as the D4Z4 repeats in facioscapulohumeral muscular dystrophy (FSHD) type 1.[20] The most common neuromuscular disorders such as the myotonic dystrophies are also due to repeat expansions. These genetic changes represent a potential pitfall of using NGS and may possibly account for a proportion of our undiagnosed myopathy patients, especially if clinicians are not suspecting these disorders and thus have not ordered the correct gene-specific assay.

Other complex genetic abnormalities such as structural variants (insertions/deletion) and copy number variations (CNVs) are also poorly detected by NGS.[17] To detect these variants, comparative genomic hybridisation (CGH) microarrays need to be performed as an ancillary test. CGH microarray may also be performed in individuals suspected to have recessive inheritance but exome or targeted panel sequencing only found one causative mutation.[14,21] Recently, several CNV analysis tools for NGS data have been developed and are in use for routine diagnosis.[22,23]

### *2. Challenges in analysis of the variant data generated by NGS*

NGS produces a large amount of variant data which requires analysis and correlation with clinical phenotype to accurately interpret their significance.[4,7,24] Assigning pathogenicity to variants and our ability to interpret their functional and clinical impact is also a challenge.[24,25] Moreover, proving genetic variants are pathogenic can be a long and difficult process. In particular, this applies when variants are in non-essential splice sites or missense variants not previously linked to disease. There can also be many "variants of uncertain significance" (VUS) that are identified. Any rare variant has the potential to be pathogenic even if bioinformatics tools predict that the variant is benign. Functional studies are often critical to prioritise and follow up candidate variants. These functional studies are often done under research activities, rather than as part of standard diagnostic laboratory practices. Abnormal splicing events in disease genes, for example, deep intronic variants which create novel splice sites or activate a cryptic splice sites, are increasingly becoming recognised as an important mechanism of disease.[26-28] The reliance on additional investigations such as a CGH array and RNA sequencing to detect variants missed by NGS is becoming a necessary step to increase our diagnostic rate.

### *3. Other disorders that may be missed with NGS; Mitochondrial and Methylation*

There is also poor coverage of the mitochondrial genome through targeted capture and in standard whole-exome capture kit to either provide accurate variant data of the complete mitochondrial DNA (mtDNA) genome sequence, or reliably detect low levels of heteroplasmy.[29] Modified exome kits have been developed and together with WGS are likely to improve the diagnosis of mitochondrial disorders. WGS can sequence both nuclear DNA (nDNA) and mtDNA simultaneously and provides high levels of mtDNA coverage (>30, 000 reads), allowing even low

levels of heteroplasmic mtDNA mutations (<1%) to be detected and reliably quantitated.[29] If a clinician is suspecting a mitochondrial disorder, WGS should be considered as the preferred diagnostic test.

DNA methylation changes are also not picked up by NGS[17] and further research is required to further understand whether such changes can be pathogenic and how to effectively screen for them.

**A Proposed Diagnostic Pathway**

A stepwise process is required for investigating patients with suspected hereditary muscle disorders taking into account the limitations of NGS technology. It remains essential for the initial patient evaluation and non-invasive investigations to be implemented as previously described.[30] We propose the following diagnostic paradigm to investigate patients with inherited muscle disorders (Figure 1). Our paradigm emphasises the importance of accurate history taking and accurate phenotyping. Physicians need to look for clues on examination of a patient presenting with muscle weakness to direct them towards specific disorders. These may include contractures, skin changes, pattern of muscle weakness and/or wasting and other organ involvement, (such as cardiomyopathy).

A detailed family and past medical history may provide clues about inheritance patterns, involvement of other organs, or the presence of diabetes or deafness which may point to a mitochondrial disorder. Following initial assessment, the appropriate screening investigations are recommended to be undertaken including a CK level, dried blood spot test for Pompe disease, Thyroid function tests, neurophysiology studies (nerve conduction studies [NCS] and electromyography) and lower limb muscle MRI. In particular clinical circumstances, other blood tests may be required for example to help rule out an autoimmune muscle condition, or if the history points towards a metabolic muscle disease (such as a fasting carnitine profile).

The next step is to exclude common neuromuscular disorders that are missed by NGS such as FSHD type 1, myotonic dystrophy type 1 (DM1) and type II (DM2), spinal muscular atrophy (SMA which should be clear on NCS) and Duchenne and Becker's muscular dystrophy. For suspected female Duchenne carriers or Becker's muscular dystrophy, a dystrophin MLPA would be required. In cases where a mitochondrial disorder is suspected, the clinician may consider requesting mitochondrial biomarkers on serum tests[31] such as FGF21 or GDF15, and/or screening for the common mitochondrial disorders prior to proceeding to WGS.

Once the common neuromuscular disorders are excluded, then requesting NGS testing would be considered an appropriate next step and deferring invasive investigations such as a muscle biopsy for the "difficult to diagnose cases", and where a candidate gene has not been identified. NSES is usually favoured in view of the lower cost to WES and WGS. Moreover with NSES, there are less variant data generated in addition to VUS's and incidental findings.[32] In cases where a diagnosis is not achieved despite NSES, then referral for WES for the affected proband and preferably the parents (trio) may further aid the diagnostic process.[4,10] A trio exome however may be costly and discussion with a local genetics service would be recommended.

Should a diagnosis remain elusive despite NSES or WES, a clinician may at this point consider liaising with a neuromuscular centre or research laboratory regarding further diagnostic or research testing. A muscle biopsy may be considered or alternatively WGS, and/or RNA sequencing (RNA-seq), which requires access to muscle tissue (as the preferred tissue).

WGS has increasingly been used where a diagnosis had not been achieved with NSES or WES. WGS has the added advantage of improved identification of disease-causing copy number and structural variations, repeat expansions, non-exonic regulatory and splicing variations and better coverage of the mitochondrial genome. Evidence for an added diagnostic benefit for WGS over WES in paediatric childhood diseases has been conflicting.[33-35] One of the main challenges of using WGS is the vast amount of genomic data that is generated and also difficulties in variant interpretation of the genomic data. There is often difficulty in the validation of non-coding variants or coding changes that impact RNA expression.[32] Previous studies have shown that RNA-seq is valuable for the interpretation of coding as well as non-coding variants, and can provide a substantial diagnosis rate in patients for whom exome or whole genome analysis has not yielded a molecular diagnosis. RNA-seq has the potential to detect structural variants such as inversions or translocations in known genes that have likely inferred pathogenicity.[26,27,36] RNA-seq has also been shown to identify splice altering variants in both exonic and deep intronic regions that may be missed on WES and WGS thereby improving our diagnostic rate.[37] In two cohorts of rare, undiagnosed muscle disorders, RNA-seq analysis from muscle biopsies achieved a diagnosis in 35% and 36% of cases.[26,36] The application of WGS combined with RNA-seq may further increase the diagnostic rate in these patients by improving our ability to interpret variants[26] and potentially identify new disease genes. A significant challenge with the study of RNA-seq in neuromuscular disease is its limited availability as a diagnostic test, as currently it is mainly accessible on a research basis.

Finally, it is important to note that WES, WGS and RNA sequencing are not accredited in all laboratories around Australia and liaising with the local genetics service, research labs and neuromuscular centres may offer guidance on further testing or research inclusion if available in the "difficult to diagnose case." The Australasian Neuromuscular Network (ANN) website provides information on the neuromuscular gene tests that are available in NATA accredited laboratories in addition to other resource information for health professionals (https://www.ann.org.au/).

**Conclusions and Recommendations**

Identifying the genetic basis of muscle disorders is the single most important step to accurately guide patient care. A timely and accurate diagnosis is crucial for patients with neuromuscular disorders and their families. It enables us to provide them with better and more accurate prognostic information, as well as predict and prevent associated complications, such as heart involvement. We cannot yet cure these families or treat most inherited myopathies and dystrophies, but we can prevent the family from having further affected children as they are able to access pre-implantation genetic testing. Moreover, entry criteria for clinical trials are often dependent on the genotype being known, especially now with the emergence of gene therapies for various muscular disorders.[38-41]

Implementation of NGS technologies into clinical practice has transformed the diagnostic pathway, replacing sets of multiple and invasive investigations with a simple blood test and ensuring appropriate use of genetic testing to allow earlier interventions and personalised medical management. Integration of NGS in our neuromuscular clinics has paved the way for a new, less invasive and more cost-effective diagnostic algorithm to be incorporated into neuromuscular clinics worldwide. NGS has enabled a greater number of patients to achieve accurate and timely diagnosis, receive appropriate disease-specific treatments and gain access to informed family planning.

Ongoing improvements in sequencing coverage of DNA and RNA sequencing are likely to further improve the diagnostic yield for our patients and also identify new disease genes. This, in turn, can lead to insights into disease pathogenesis and the potential for identification of new targets for future therapies, which can have a lasting impact on the quality of life and improving morbidity and mortality of patients.

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is an Adult Neurologist at the Royal Adelaide Hospital with subspecialty qualifications in neuromuscular and neurogenetic disorders. After completing a combined neuromuscular and neurophysiology fellowship at the Royal North Shore hospital in Sydney in 2012, she completed a PhD in neurogenetics and neuromuscular disorders at the Children's hospital at Westmead and through the University of Sydney in 2017. Dr Ghaoui's main research interests include using next generation sequencing to establish the genetic diagnoses in previously undiagnosed families with myopathies and limb-girdle muscular dystrophies and in the treatment of muscle disorders. She is the South Australian member of the Mito Foundation Scientific and Medical Advisory Panel. She is also part of the working group and the state Co-Lead for the neuromuscular disorders flagship of the Australian genomics health alliance (AGHA) project, which is focused on studying the diagnostic yield from single gene testing as standard of care, targeted panel analysis and whole genome and RNA-sequencing. She has been recently awarded an early career research fellowship that will enable her to examine the utility of RNA sequencing as a complementary diagnostic tool in a cohort of families with inherited myopathy or limb-girdle muscular dystrophy (LGMD) that have remained undiagnosed despite next generation sequencing (NGS).

## *Merrilee Needham, FRACP PhD,*

Photograph by Josh Wells

is the current Foundation Chair in Neurology, a joint position between Fiona Stanley Hospital, Murdoch University and Notre Dame University Australia. She was the Head of the Neurology unit at Fiona Stanley Hospital from 2015-2019 and is the current Head of Neuromuscular and Myositis research at the Perron Institute, Western Australia. She is a Consultant Neurologist at Fiona Stanley Hospital and Fremantle Hospital in addition to being the Director of Research for the South Metropolitan Health Service. Professor Needham has a passion for helping people suffering with neuromuscular disorders with a particular interest in Inclusion Body Myositis (IBM). A vital part of providing the best care possible is partnering with her patients in a research programme to understand their diseases better, identify treatment targets and facilitate participation in clinical trials. She has established a translational and experimental research programme, diagnosing and managing patients over time as well as biobanking them. The laboratory programme is performing immunological studies to better understand the role of the immune system in IBM and other forms of myositis, how inflammation links to the ultimate degeneration of muscle and identify new treatment targets. This programme now supports a full-time Immunologist and Clinical Research Manager, and is currently hosting three honours students and two PhD candidates.

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