The phenotyping and mutational profile of inherited muscle diseases in a South African cohort



By

Dr Sérgio Miguel Alves, MBChB

ALVSER001

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Department of Medicine, Faculty of Health Sciences,

University of Cape Town

Supervisor: Prof Jeannine Heckmann, PhD

Co-supervisor: Dr Melissa Nel, PhD

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Declaration

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Abstract

Introduction: Inherited muscle diseases (IMD) are a clinically and genetically heterogeneous group of disorders causing variable muscle weakness. In South Africa, accurate diagnosis is hindered by limited access to genetic testing, resulting in prolonged diagnostic odysseys and many patients remaining without a definitive diagnosis. Between 2020 and 2025, probands were enrolled in the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD) study to provide definitive molecular diagnoses through comprehensive genetic testing unavailable locally.

Aim: To describe the phenotypic and genetic findings of a South African IMD cohort who underwent locally available genetic testing, if appropriate, and locally unavailable genetic testing.

Methods: Patients with suspected IMD attending Groote Schuur Hospital, University of Cape Town, were phenotyped and categorised by predominant weakness pattern: limb-girdle, distal, extraocular muscle (EOM), myotonia-related, myasthenic-like with fatigable weakness and periodic or exercise-related muscle symptoms. Tier 1 testing included locally available testing for Dystrophinopathies (DMD), Myotonic Dystrophy type 1 (DM1) and mitochondrial DNA (mtDNA) screening if clinically indicated. Thereafter, undiagnosed patients proceeded to tier 2 testing which included *PABPN1* repeat expansion testing (Oculopharyngeal Muscular Dystrophy, OPMD) or *D4Z4* contraction testing (Facioscapulohumeral Muscular Dystrophy, FSHD1) if clinically indicated or next generation sequencing (NGS).

Results: Of 89 probands with suspected IMD, 55 (62%) obtained genetic diagnoses although only 16 (18%) were solved via tier 1 testing. Locally solved cases included 8 dystrophinopathies (88% with childhood onset), 5 DM1 (none with African ancestry) and 3 adults with EOM paralysis due to a single large mtDNA deletion. Despite locally available dystrophinopathy screening, median diagnostic delay was 10 years. Tier 2 testing provided diagnoses for 53% (39/73) of cases remaining undiagnosed or untested by tier 1. Targeted testing identified FSHD1 (n=8) and OPMD (n=5) in 13 of 17 cases tested, with OPMD predominating in older adults (>50 years). Notably, FSHD1 was absent in patients of African ancestry. NGS, the most frequently employed tier 2 modality, resulted in genetic diagnoses in 41% of those tested (26/64). Frequently identified conditions via NGS included Myotonia Congenita (*CLCN1*, n=4), Desminopathies (*DES*, n=3) and a large heterozygous *NEB* deletion

in three South African admixed families with autosomal dominant inheritance of a distal myopathy. Of seven cases with a prioritised variant of uncertain significance (VUS) in disease-associated genes, six (7%) were reportable and probably solved, while one was inconclusive and considered possibly solved. Eight probands harboured variants novel to ClinVar, with four classified as VUS.

Conclusion: This study represents the largest adult single-centre heterogeneous IMD cohort reported in a diverse South African population. Despite limited tier 1 testing, an overall diagnostic yield of 62% was achieved with tier 2 testing contributing 53% of diagnoses. Prolonged diagnostic delays despite available dystrophinopathy screening suggest barriers beyond laboratory access. This study characterises a unique South African mutational landscape including ancestry-dependent disease distribution in which FSHD1 and DM1 predominated in European, and less so in admixed ancestry patients, but were absent in those of African ancestry. These findings underscore the inadequacy of current IMD testing in South Africa and the need for expanded genomic testing capacity.

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List of abbreviations

Abbreviation	Term
AAO	age at onset
ACMG	American College of Medical Genetics and Genomics
AD	autosomal dominant
ADOA	autosomal dominant optic atrophy
AR	autosomal recessive
AV	atrioventricular
B/L	bilateral
BMD	Becker muscular dystrophy
bp	base pairs
CCD	central core disease
CK	creatine kinase
CMS	congenital myasthenic syndrome
CNM	centronuclear myopathy
COIN	Clinical Omics and Informatics Unit
CPEO	chronic progressive external ophthalmoplegia
CPT2	Carnitine palmitoyltransferase II
DGC	dystrophin-glycoprotein complex
DM1	Myotonic dystrophy type 1
DMAT	Distal myopathy with anterior tibial onset
DMD	Duchenne muscular dystrophy
DMPK	Dystrophia myotonica protein kinase
ECG	electrocardiography
EDMD	Emery-Dreifuss muscular dystrophy
EDMD1	Emery-Dreifuss muscular dystrophy type 1
EDMD2	Emery-Dreifuss muscular dystrophy type 2
EMG	electromyography
EOM	Extraocular muscle
FKRP	fukutin-related protein
FSHD	Facioscapulohumeral muscular dystrophy
FSHD1	Facioscapulohumeral muscular dystrophy type 1
FSHD2	Facioscapulohumeral muscular dystrophy type 2
FVC	forced vital capacity
GAA	α-Glucosidase
GNE	UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase
GSD	glycogen storage disease
GSH	Groote Schuur Hospital
GWAS	genome-wide association studies
HIC	high-income countries
HPO	Human Phenotype Ontology
HREC	Human Research Ethics Committee
ICGNMD	International Centre for Genomic Medicine in Neuromuscular Diseases
IMD	inherited muscle diseases
IOPD	infantile-onset Pompe disease

IQR	interquartile range
IU/L	International units per litre
kb	kilobases
LG	limb-girdle
LGMD	Limb-girdle muscular dystrophy
LL	lower limb
LMIC	low- and middle-income countries
LOPD	late-onset Pompe disease
LP	likely pathogenic
MLPA	multiplex ligation-dependent probe amplification
MRC	Medical Research Council
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
NARP	Neuropathy, ataxia, and retinitis pigmentosa
N/R	not reported
NCS	nerve conduction studies
NGS	Next generation sequencing
NHLS	National Health Laboratory Service
NMD	neuromuscular diseases
OPMD	Oculopharyngeal muscular dystrophy
P	pathogenic
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
PFGE	pulsed-field gel electrophoresis
RBBB	right bundle branch block
REDCap	Research Electronic Data Capture
RyR1	ryanodine receptor type 1
SAMRC	South African Medical Research Council
SCM	sternocleidomastoid
SD	standard deviation
SFN	small fibre neuropathy
SNV	single nucleotide variant
UCL	University College London
UCMD	Ullrich congenital muscular dystrophy
UCT	University of Cape Town
UDP	uridine diphosphate
UL	upper limb
ULN	upper limit of normal
VCP	valosin-containing protein
VLCAD	Very long-chain acyl-CoA dehydrogenase
VUS	variant of uncertain significance
WES	whole exome sequencing
WGS	whole genome sequencing
XL	X-linked

Chapter 1: Background

1.1. Inherited muscle diseases

Inherited muscle diseases (IMD) are a genetically and clinically heterogeneous group of disorders characterised by progressive weakness affecting skeletal muscle. Within the spectrum of IMD, a distinction can be made between inherited myopathies, due to defects in the contractile apparatus causing specific structural abnormalities without progressive fibre degeneration, and inherited muscular dystrophies which arise from defects in the muscle membrane or proteins, leading to muscle fibre necrosis and regeneration (Cardamone, Darras & Ryan, 2008). With over 500 genes implicated in IMD, the genetic diversity underlying these conditions creates substantial diagnostic complexity (Dowling, Weihl & Spencer, 2021). Clinically, IMD present with variable symptom patterns including proximal weakness affecting hip and shoulder girdle muscles, distal weakness in the feet or hands and presentations with facial weakness, eye muscle involvement or further systemic involvement (McDonald, 2012).

Inherited muscle diseases demonstrate genetic heterogeneity, where different genetic variants cause similar clinical phenotypes. This can be classified as either allelic, locus or phenotypic heterogeneity (Zaganas et al., 2020). Allelic heterogeneity refers to different variants within the same gene producing similar phenotypes, whereas locus heterogeneity refers to variants in distinct genes producing clinically overlapping phenotypes and is exemplified by limb-girdle muscular dystrophy (LGMD), for which approximately 29 different genetic loci have been identified (Straub et al., 2018). Phenotypic heterogeneity refers to the same genetic variant producing variable clinical manifestations with differences in age at symptom onset, clinical severity and progression of disease across individuals. This interplay produces the broad clinical spectrum characteristic of inherited muscle diseases.

To organise IMD into clinically useful categories, they can be classified by inheritance pattern (autosomal dominant, autosomal recessive, X-linked or mitochondrial), age at onset of symptoms, predominant pattern of weakness, and increasingly, by the primary molecular defect e.g. dystrophies, mitochondrial or metabolic myopathies, channelopathies and congenital myopathies (Mathis et al., 2018). This classification approach is useful as it guides both diagnostic strategy and clinical management. However, the genetic complexity of IMD, where phenotypic boundaries are blurred by locus heterogeneity and variable expressivity, means that conventional diagnostic strategies are often insufficient.

The global burden of IMD remains incompletely characterised, although systematic meta-analyses of population-based epidemiological studies provide robust estimates. The worldwide prevalence of inherited muscular dystrophies is estimated at 16.14 per 100,000, with varied prevalence for different conditions (Mah et al., 2016). Duchenne and Becker muscular dystrophy (DMD/BMD) were reported to have a prevalence of 4.78 and 1.53 per 100,000 males respectively (Mah et al., 2014). The prevalence of various other IMD include 8.26 per 100,000 for myotonic dystrophy, 1.63 per 100,000 for LGMD and 0.99 per 100,000 for congenital muscular dystrophies. These figures are likely underestimates due to limited clinical awareness among non-specialist clinicians leading to missed diagnoses, phenotypic variability resulting in atypical presentations or misclassification, individuals with attenuated features remaining undiagnosed and, particularly for Africa, limited access to genetic testing in low- and middle-income countries (LMIC) (Juntas Morales et al., 2021; Oliveira et al., 2022; Raga et al., 2022). African populations are markedly excluded from genetic databases and epidemiological studies, creating a critical knowledge gap regarding IMD burden and genetic diversity in these populations (Raga et al., 2022). As a result, the clinical utility of precision genomic medicine is currently inequitably distributed, with African patients facing particularly severe barriers to diagnosis and specialised care.

The genetic and clinical heterogeneity outlined above creates substantial diagnostic complexity that cannot be resolved through clinical evaluation alone. In South Africa, where neuromuscular expertise and molecular diagnostics are constrained, this leads to prolonged diagnostic delays and underdiagnosis of IMD. Comprehensive genomic approaches that allow simultaneous investigation of multiple candidate genes are therefore essential. The following sections highlight key IMD subgroups that illustrate this heterogeneity and the diagnostic challenges they pose in the South African context.

1.2. Literature search strategy

A structured literature search was conducted to identify published evidence relevant to the clinical, genetic and contextual scope of this dissertation. PubMed was searched for articles using MeSH terms: "inherited muscle diseases" or "inherited myopathies" or "hereditary myopathies" or "inherited neuromuscular disease" or "muscular dystrophy" or "myotonic dystrophy" or "dystrophinopathy" or "Duchenne muscular dystrophy" or "Becker muscular dystrophy" or "Facioscapulohumeral muscular dystrophy" or "limb-girdle muscular dystrophy" or "distal myopathy" or "mitochondrial myopathy" or "congenital myopathy" or "myopathy" and "sub-Saharan Africa". Google Scholar was additionally searched using

relevant free-text terms including disease-specific names such as "dysferlinopathy", "FSHD" and "calpainopathy" combined with "Africa" or "sub-Saharan Africa". Reference lists of identified articles were manually searched for further relevant studies. The primary search had no lower date restriction to preserve foundational studies, with an upper limit of February 2026.

Literature was selected based on relevance to the clinical, genetic, epidemiological or health systems aspects of IMD in sub-Saharan Africa and South Africa. Where African-specific data were limited or unavailable, international literature was incorporated to provide the necessary clinical and genetic context. Articles were excluded if they described acquired myopathies, lacked a molecular genetic diagnosis, reported no clinical phenotype or were preprints or letters to the editor. Selected literature is synthesised thematically, organised sequentially by disease group according to established genetic classification frameworks, with critical discussion of diagnostic approaches and contextualisation within the sub-Saharan African setting.

1.3. Genetic heterogeneity of IMD

1.3.1. Muscular dystrophies

1.3.1.1. Dystrophinopathy

Duchenne muscular dystrophy (DMD), the commonest childhood muscular dystrophy (Limback et al., 2022), is an X-linked recessive condition caused by mutations in the *DMD* gene encoding dystrophin, a crucial cytoskeletal protein within the dystrophin-glycoprotein complex (DGC) that maintains the structural integrity of muscle fibres (Rando, 2001). Symptoms typically manifest between 2-3 years with progressive muscle weakness, cardiac muscle dysfunction, respiratory insufficiency, loss of ambulation by the second decade and ultimately, premature death. Becker muscular dystrophy (BMD), a milder form of dystrophinopathy, exhibits variable clinical presentation and progression determined by the reading frame rule, whereby in-frame deletions or variants preserve the *DMD* mRNA reading frame, producing truncated but partially functional dystrophin, whereas out-of-frame mutations in *DMD* disrupt the reading frame, resulting in absent or non-functional dystrophin (Nicolas et al., 2015).

Genetic analyses of dystrophinopathy patients reported from seven sub-Saharan African countries (Hallwirth Pillay et al., 2007; Peddareddygari et al., 2012; Uwineza et al., 2014; Dekker et al., 2019; Wonkam-Tingang et al., 2020; Oliveira et al., 2022) demonstrated that most harbour deletions in exons 8-21 and 46-50, consistent with well-described deletion

hotspots in *DMD* globally (Chen et al., 2014). However, two reported cases deviated from this pattern, namely an Angolan participant with an exon 2 duplication and a Cape Verdean individual with a c.8217+1G>A mutation in intron 55 (Oliveira et al., 2022). In South Africa, *DMD* deletion/duplication screening via multiplex ligation-dependent probe amplification (MLPA) is available through the National Health Laboratory Service (NHLS), but point mutation detection requires referral for further sequencing which remains largely research based.

1.3.1.2. Facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is a common autosomal dominant muscular dystrophy with a prevalence of approximately 4.4 per 100,000 people, characterised by progressive, asymmetric muscle weakness primarily affecting the facial, shoulder and arm muscles (Orrell, 2011). FSHD1, caused by *D4Z4* repeat contraction at the 4q35 locus, accounts for the majority of cases, while FSHD2 is due to a combination of a heterozygous pathogenic *SMCHD1* variant and the presence of a permissive 4qA allele (de Greef et al., 2010).

In South Africa, FSHD has not been described in patients of African ancestry, with reported cases limited to individuals of European or admixed ancestry (Giardina et al., 2024). Notably, a case report documented a South African admixed ancestry family harbouring a compound heterozygous configuration with *D4Z4* repeat deletions of 24 kb and 34 kb, the first such case documented in South Africa (Olckers et al., 2012). Clinical suspicion for FSHD remains higher for individuals of European ancestry in the South African setting, however African ancestry does not exclude FSHD testing, particularly given how understudied IMD are in African populations.

1.3.1.3. Myotonic dystrophy

Myotonic dystrophy type 1 (DM1) is the commonest adult-onset muscular dystrophy globally (Udd & Krahe, 2012). It is an autosomal dominant multisystem disease characterised by myotonia, progressive proximal and distal muscle weakness with prominent face and eye muscle weakness as well as cardiac muscle dysfunction.

DM1 is caused by the expansion of a CTG trinucleotide repeat in the *DMPK* gene. The *DMPK* CTG repeat expansion is believed to have arisen after migration out of Africa with large normal CTG repeats (>18 repeats) being less frequent in African populations (Goldman, Ramsay & Jenkins, 1994; Krause, Seymour & Ramsay, 2018). This finding was recently

supported in a large population study on repeat expansion disorders which illustrated that the 99.9th percentile of *DMPK* repeat sizes was 39 in Europeans compared to 30 in Africans (Ibañez et al., 2024). This may explain the near absence of DM1 in African populations. Only a single Nigerian family was confirmed with DM1 following PCR and Southern Blot analysis (Krahe et al., 1995). The repeat expansion identified in this case was thought to represent an independent *de novo* mutational event and was not associated with the European ancestral haplotype.

1.3.1.4. Oculopharyngeal muscular dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is a distinctive form of myopathy characterised by gradually progressive ptosis, dysphagia and proximal muscle weakness. It is a late-onset disease, typically manifesting in the fifth or sixth decade of life (Brais, 2009). OPMD is an autosomal dominant condition caused by trinucleotide (GCN) expansions in the *PABPN1* gene with pathogenic expansions containing ≥12 GCN repeats, compared to the normal wild-type allele of 10 GCN repeats. In South Africa, OPMD has been documented in only one Afrikaner family with European ancestry and a *PABPN1* GCN_{15} mutation (Schutte et al., 2015).

1.3.1.5. Emery-Dreifuss muscular dystrophy

Emery-Dreifuss muscular dystrophy (EDMD) typically presents in the first decade of life and is characterised by progressive muscle weakness and atrophy, early contractures and cardiac muscle dysfunction. Weakness often presents in a humero-peroneal pattern involving proximal upper limb and distal lower limb (Heller et al., 2020). EDMD is caused by mutations in genes encoding nuclear envelope proteins, with the most common genetic causes being *EMD* (X-linked recessive), *FHL1* (X-linked recessive) and *LMNA* (autosomal dominant). Less common disease-causing genes include *SYNE1*, *SYNE2*, *TTN*, *SUN1*, *SUN2* and *TMEM43*.

1.3.2. Limb-girdle muscular dystrophy

Limb-girdle muscular dystrophy (LGMD) represents a clinically and genetically heterogeneous group of IMD characterised by progressive weakness affecting the proximal muscles of the pelvic and shoulder girdles, with phenotypic variability ranging from mild adult-onset weakness to severe presentations in childhood (Bouchard & Tremblay, 2023). The group encompasses two major inheritance patterns, namely autosomal recessive forms (LGMDR, formerly designated as LGMD2) and autosomal dominant forms (LGMDD,

formerly designated as LGMD1), with the recessive variants being more prevalent and accounting for over 90% of all LGMD cases (Nigro & Savarese, 2014). Currently, there are 29 genes implicated as disease-causing for LGMD, although the exact number continues to evolve as new genetic loci are identified (Straub et al., 2018).

1.3.2.1. Calpainopathy

Calpainopathy (LGMD R1) is the commonest LGMD that is caused by variants in the *CAPN3* gene encoding for calpain-3 protein. Calpain-3 is a muscle specific calcium-dependent protease that maintains calcium homeostasis and sarcomere integrity (Angelini et al., 2010). While primarily an autosomal recessive disease, there have been rare reports of autosomal dominant calpainopathy (LGMD D4). Age at symptom onset is variable and is divided into early (< 12 years), typical (12-30 years) and late onset (> 30 years) (Bardakov et al., 2025).

Clinical features include symmetric proximal muscle weakness, contractures, calf pseudohypertrophy and scapular winging (Bardakov et al., 2025). Phenotypes can be divided into the more common Leiden-Möbius pelvifemoral phenotype, characterised by pelvic girdle weakness, and the Erb scapulohumeral form which primarily affects the shoulder girdle. The Erb scapulohumeral form tends to have later onset and a milder disease course. In South Africa, calpainopathy remains underreported with limited genetic confirmation.

1.3.2.2. Desminopathy

Desminopathy is a form of myofibrillar myopathy caused by *DES* gene variants that encode for desmin, that was previously classified as a LGMD, but has since been categorised as a myofibrillar myopathy owing to the primary weakness pattern (Straub et al., 2018). Desminopathy typically presents with adult-onset distal weakness affecting peroneal muscles coupled with cardiac involvement, although it can also present with proximal or scapuloperoneal weakness. It is primarily inherited in an autosomal dominant pattern. However, cases with autosomal recessive inheritance have been reported and usually present with more severe weakness and earlier onset (Van Spaendonck-Zwarts et al., 2011).

1.3.2.3. Dysferlinopathy

Dysferlinopathy refers to a group of autosomal recessive muscle diseases caused by variants in the *DYSF* gene that encodes for dysferlin protein. Dysferlin is important for muscle membrane repair and defective dysferlin leads to chronic damage, inflammation and

progressive muscle weakness. Dysferlinopathy typically presents with young adult-onset progressive muscle weakness and markedly elevated creatine kinase (CK) levels (Anwar & Yokota, 2024). Less common presentations include distal myopathy with anterior tibial onset (DMAT) and asymptomatic hyperCKaemia. Importantly, the traditional binary classification into LGMDR2 and Miyoshi myopathy is increasingly regarded as an oversimplification.

Data from the international Jain Foundation Clinical Outcome Study demonstrated that these subtypes represent a continuous spectrum of proximal and distal muscle involvement rather than discrete entities, with no clinically relevant difference in proximal-to-distal involvement between the two diagnostic categories (Consortium et al., 2021). The same *DYSF* pathogenic variants can produce both proximal and distal predominant phenotypes, even within the same family, highlighting the marked inter- and intrafamilial phenotypic variability associated with dysferlinopathy (Anwar & Yokota, 2024). Dysferlinopathy is therefore best conceptualised as a single disease entity spanning a proximal-to-distal phenotypic spectrum, with implications for clinical trial enrolment and therapeutic access that should not be stratified by phenotypic subtype alone (Consortium et al., 2021).

1.3.2.4. Collagen VI-related myopathy

Collagen VI-related myopathy comprises a spectrum of disease ranging from severe Ullrich congenital muscular dystrophy (UCMD), one of the most common congenital muscular dystrophies seen globally, to Bethlem myopathy which is the least severe (Yonekawa & Nishino, 2015). Collagen VI-related myopathy is caused by mutations in *COL6A1*, *COL6A2* and *COL6A3,* encoding for collagen VI that is found in the extracellular matrix of skeletal muscle and has an important role in maintaining structural support of tissues, with pathogenic variants leading to collagen VI deficiency (Bönnemann, 2011).

UCMD presents with delayed motor milestones, proximal muscle weakness with joint contractures, distal joint hyperlaxity, spinal deformities and respiratory failure (Yonekawa & Nishino, 2015). Bethlem myopathy is the milder primarily autosomal dominant form of collagen VI-related myopathy, presenting with childhood-onset mild proximal muscle weakness and joint laxity. As the disease progresses, contractures develop with loss of ambulation occurring later during adulthood as compared to UCMD (Bönnemann, 2011).

1.3.3. Distal myopathies

Distal myopathies present with distal limb weakness that can vary in terms of onset and severity, often with progression to proximal muscle weakness. There are almost 30 genes

associated with distal myopathies including *GNE*, *TTN*, *MYOT*, *FLNC*, *NEB*, *ANO5* and *MYH7*, among others (Savarese et al., 2020).

1.3.3.1. GNE myopathy

GNE myopathy is an autosomal recessive condition caused by variants in the *GNE* gene, encoding for the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) enzyme that is essential for sialic acid synthesis (Savarese et al., 2020). Typical onset is in early adulthood and presents with gait abnormalities, frequent falls or foot drop secondary to weakness of the anterior tibialis with gradual progression to involve proximal muscles, but mostly spares the quadricep muscles (Park et al., 2012).

GNE myopathy has been extensively characterised in affected populations from Japan, South Asia and the Middle East (Baskar et al., 2024), but remains under-recognised in sub-Saharan Africa. A Malian case with a novel homozygous *GNE* p.Gly613Glu variant identified by whole exome sequencing (WES) represents the first genetically confirmed diagnosis in the region, underscoring diagnostic gaps in African populations (Kotioumbé et al., 2024).

1.3.3.2. Distal nebulin myopathy

The *NEB* gene encodes for nebulin which is a large protein that is fundamental in actin filament stabilisation and efficient muscle contraction (Sagath et al., 2025). Pathogenic *NEB* variants produce a phenotypic spectrum ranging from severe nemaline myopathy caused by biallelic loss-of-function variants to milder distal myopathies arising from heterozygous in-frame deletions reflecting the relationship between residual nebulin function and clinical severity (Skrypnyk et al., 2023).

Distal nebulin myopathy presents with childhood-onset distal weakness starting with ankle dorsiflexion weakness and finger extensor weakness, followed by gradual progression to proximal muscle and neck flexor weakness with retained ambulation (Udd, 2014). While pathogenic *NEB* variants typically cause autosomal recessive nemaline myopathies, three studies identifying large autosomal dominant *NEB* deletions, presenting with milder, predominantly distal phenotypes distinct from the typical autosomal recessive presentations have been reported (Sagath et al., 2025).

1.3.4. Congenital myopathies

Congenital myopathies typically present from birth with hypotonia and gradually progressive muscle weakness (Raga et al., 2022). While previously classified by muscle biopsy

histopathology, current classification integrates genetic, clinical and histopathological features.

1.3.4.1. RYR1-related myopathy

RYR1-related myopathies are the commonest form of congenital myopathy caused by pathogenic *RYR1* variants, encoding for the ryanodine receptor type 1 (RyR1) that is responsible for mediating excitation-contraction coupling in skeletal muscle (Leão et al., 2020). This may cause a broad clinical and histopathological spectrum spanning both dominant and recessive inheritance patterns. Autosomal dominant *RYR1* variants are classically associated with central core disease (CCD) and susceptibility to malignant hyperthermia, characterised by proximal muscle weakness with variable age at onset and lifelong anaesthetic risk (Chang et al., 2022). Autosomal recessive variants produce a wider phenotypic range including centronuclear myopathy, multiminicore disease and congenital fibre-type disproportion, often presenting with more severe neonatal-onset weakness and bulbar involvement.

In South Africa, local *RYR1* genetic testing is restricted to two South African founder variants associated with autosomal recessive centronuclear myopathy, capturing only a small fraction of pathogenic *RYR1* variants (Table 1.1) (Raga et al., 2022). While comprehensive genetic testing via WES would identify the broader spectrum of *RYR1* variants, including those causing autosomal dominant RYR1-related myopathy, such testing remains limited in the South African setting and is largely research-based.

1.3.4.2. SELENON-related myopathy

SELENON-related myopathy (previously *SEPN1*) is an autosomal recessive congenital myopathy characterised by early-onset axial weakness, progressive rigid spine with scoliosis and disproportionate respiratory insufficiency despite preserved ambulation (Villar-Quiles et al., 2020). The *SELENON* gene encodes selenoprotein N which plays a role in muscle tissue protection against oxidative stress. Affected individuals typically present with infantile hypotonia, neck flexor weakness and severe diaphragmatic involvement leading to respiratory failure in childhood or adolescence. To date, SELENON-related disease has been identified in only two South African patients, documented in a retrospective cohort study published as a MPhil thesis (Oshi, 2021).

1.3.5. Metabolic myopathies

Metabolic myopathies comprise a wide spectrum of rare inherited disorders of cellular energy metabolism affecting skeletal muscle with approximately 30 genes implicated (Urtizberea, Severa & Malfatti, 2023). These disorders are classified into glycogen storage diseases (GSD), lipid storage disorders and mitochondrial myopathies, presenting with myalgia, exercise intolerance, rhabdomyolysis, muscle weakness as well as cardiac and respiratory dysfunction. Common lipid storage disorders include Carnitine palmitoyltransferase II deficiency due to *CPT2* mutations and Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) deficiency due to *ACADVL* mutations (Yoo, 2021). Common forms of GSD affecting muscle include Pompe disease and McArdle disease due to *GAA* and *PYGM* mutations respectively, as well as Phosphorylase kinase deficiency due to mutations in *PHKA1*, *PHKA2*, *PHKB* and *PHKG2* genes (Roscher et al., 2014). Among GSD affecting skeletal muscle, Pompe disease (Glycogen storage disease type II) is the most common and severe, warranting further discussion (Urtizberea, Severa & Malfatti, 2023).

Pompe disease (GSD II) is an autosomal recessive condition caused by α-glucosidase (GAA) deficiency due to *GAA* variants and presents as either infantile-onset Pompe disease (IOPD) or late-onset Pompe disease (LOPD) depending on the degree of GAA enzyme deficiency (Turaça et al., 2015). IOPD may be more severe and presents with hypotonia, hypertrophic cardiomyopathy and respiratory dysfunction that may be fatal if not treated. LOPD may present with varied onset and severity of symmetric limb-girdle muscle weakness and respiratory dysfunction.

1.3.6. Mitochondrial myopathies

Mitochondrial myopathies represent a genetically heterogeneous group of disorders resulting from defective oxidative phosphorylation due to mutations in mitochondrial DNA (mtDNA) or nuclear genes affecting mitochondrial function (Wen et al., 2025). These disorders preferentially affect high energy tissues, manifesting with exercise intolerance, pathological fatigability and predominantly proximal muscle weakness. Chronic progressive external ophthalmoplegia (CPEO) represents the most common presentation and is characterised by slowly progressive bilateral ptosis, ophthalmoparesis and proximal muscle weakness (Erhunmwunse & Joshi, 2025). The preferential involvement of extraocular muscles reflects their exceptionally high mitochondrial density and continuous contractile activity.

Mitochondrial diseases are characterised by heteroplasmy which is the coexistence of wild-type and mutant mtDNA, with clinical symptoms typically manifesting when mutant mtDNA reaches 60-90% (Tuppen et al., 2010). This is referred to as the phenotypic threshold. Critically, heteroplasmy levels differ markedly across tissues in that mtDNA mutations may be present at high levels in muscle (25-96%)(Pancrudo et al., 2007; Hardy et al., 2016), but low or undetectable levels in blood (0-29%)(Leung et al., 2018). Consequently, blood testing frequently yields false-negative results in mitochondrial myopathy, necessitating muscle biopsy for definitive diagnosis.

1.3.6.1. Large mtDNA deletions

Single large mtDNA deletions account for approximately 40-70% of CPEO cases, typically occurring sporadically (Heighton et al., 2019). Deletion size inversely correlates with age of onset, with longer deletions associated with earlier and more severe phenotypes (Zhao et al., 2024). The clinical spectrum ranges from isolated CPEO to Kearns-Sayre syndrome with symptom onset before 20 years and progressive external ophthalmoplegia (PEO), retinopathy, cerebellar ataxia and cardiac conduction defects. Beyond large mtDNA deletions, mitochondrial myopathies may result from mtDNA point mutations affecting oxidative phosphorylation complexes such as *MT-ND4*, *MT-CYB*, *MT-CO1* and *MT-ATP6* or from nuclear gene mutations encoding mitochondrial proteins such as *POLG*, *TWNK* (formerly *C10orf2*), *OPA1* and *RRM2B* impairing mtDNA maintenance or mitochondrial function (Wen et al., 2025).

1.3.6.2. Nuclear gene defects

Nuclear gene mutations affecting mtDNA maintenance or mitochondrial function account for a substantial proportion of adult-onset mitochondrial myopathy, typically presenting with autosomal dominant CPEO or multisystem phenotypes (Wen et al., 2025). The commonest single-gene cause is *POLG* variants, producing a range of phenotypes from severe autosomal recessive Alpers-Huttenlocher syndrome, typically presenting with neurodevelopmental regression, hepatic failure and intractable seizures to adult-onset CPEO (Wen et al., 2025). *TWNK* variants typically cause adult-onset ptosis, ophthalmoparesis, proximal muscle weakness and exercise intolerance (Bermejo-Guerrero et al., 2022). *RRM2B* variants, encoding the RRM2B protein (p53R2), typically cause severe infantile or milder adult-onset presentations depending on inheritance pattern, with autosomal dominant variants retaining partial enzyme activity (Pitceathly et al., 2012; Munro et al., 2025). *OPA1* variants cause

autosomal dominant optic atrophy (ADOA) with approximately 20% of patients developing ADOA-plus syndrome characterised by optic neuropathy, sensorineural hearing loss, ataxia and muscle weakness (Lenaers et al., 2021).

In South Africa, NHLS mitochondrial genetic testing is restricted to large mtDNA deletion screening in muscle tissue, without access to comprehensive mtDNA sequencing or nuclear gene panels. The diagnostic utility of open muscle biopsy is constrained by its invasiveness, requirement for surgical theatre space and limited sensitivity for low-level heteroplasmy (<15%) using traditional deletion screening methods (Leung et al., 2018). Additionally, false-negative results may occur early in the disease course. Consequently, mitochondrial myopathies caused by mtDNA point mutations or nuclear gene defects remain largely undiagnosed in the South African healthcare setting (Meldau et al., 2022).

1.3.7. Myotonia congenita

Myotonia congenita is an inherited channelopathy caused by *CLCN1* variants that encodes the chloride channel CLC-1 (Wang et al., 2025). Loss of CLC-1 function impairs the resting membrane potential stabilisation, resulting in sustained muscle fibre excitability and delayed relaxation following voluntary contraction which is the clinical hallmark of myotonia. The condition can be inherited as autosomal dominant (Thomsen disease) or autosomal recessive (Becker disease), with clinical features including myotonia improving with exercise (warm-up phenomenon), myalgia and muscle hypertrophy. Becker disease is more common and typically more severe than Thomsen disease (Gowda, Sajjan & Srinivasan, 2025). As there are no targeted *CLCN1* panels currently available in South Africa, WES is currently utilised as the first-line genetic test in the research setting where available.

1.3.8. Congenital myasthenic syndrome

Congenital myasthenic syndromes (CMS) are inherited disorders of neuromuscular transmission characterised by fatigable weakness affecting bulbar, extraocular, facial or limb muscles typically presenting at birth or early childhood (Ohno et al., 2023). There are approximately 35 causative genes implicated with most subtypes following autosomal recessive inheritance, although some with autosomal dominant inheritance have been observed. The most frequently identified variants are in the *CHRNE* gene, producing variable phenotypes from mild ptosis, ophthalmoplegia, bulbar or limb weakness to generalised myasthenia (Finsterer, 2019). Given the extensive genetic heterogeneity, single-gene *CHRNE* testing carries the risk of missing non-*CHRNE* CMS subtypes. WES or

comprehensive CMS panels are therefore preferred when molecular confirmation is pursued (Engel et al., 2015).

In South Africa, targeted single-gene *CHRNE* testing is not routinely available through the NHLS. Next generation sequencing (NGS), when accessible through research collaborations or specialised referral pathways, are most appropriate given the lack of population-specific data to guide targeted testing and as it enables simultaneous interrogation of all CMS-associated genes (Kulsirichawaroj et al., 2025).

1.3.9. Population-specific genetic considerations

The genetic architecture of IMD varies considerably across populations, which has important implications for diagnostic strategy design (Raga et al., 2022). As noted above, there are ancestry-dependent disease distributions in which FSHD and DM1 predominate in European ancestry, but have not been reported in those of African ancestry. Within South Africa, specific founder variants have been identified that are enriched in the local population, but uncommon or absent in other populations. Examples include *FKRP* variants causing LGMD R9 and *RYR1* variants associated with centronuclear myopathy, which were identified through targeted screening informed by local case observations (Raga et al., 2022). While founder variant testing may improve diagnostic yield within the population where they are prevalent, such targeted approaches have limited applicability across diverse ancestries and risk missing non-founder pathogenic variants even within the same population, supporting the use of comprehensive genetic testing such as NGS when feasible.

1.4. Barriers to equitable genomic diagnosis of IMD in South Africa

The disease groups and genetic heterogeneity outlined above occur within a health system and research environment that is markedly different from high income countries (HIC) in which most IMD data have been generated (Raga et al., 2022). In South Africa and the broader sub-Saharan African region, structural barriers at the global, regional and local levels such as global database under-representation, regional research capacity gaps and local infrastructure limitations, profoundly shape access to genomic diagnosis for IMD.

1.4.1. Under-representation of African populations in genomic databases

Over the past two decades, significant advancements have been made in understanding the genetic underpinnings of IMD. However, the majority of published data on pathogenic

variants in inherited myopathies originates from European cohorts, leading to an under-representation of African data that impacts the classification of gene variants in these populations (Wilson et al., 2023). For individuals of African ancestry, variants may be disproportionately classified as variants of uncertain significance (VUS) or incorrectly flagged as pathogenic due to their novelty in European-centric databases. This increases the rate of false-positive results and complicates variant prioritisation (Sharo et al., 2023).

Beyond the technical challenges, this disparity reflects a broader inequity in global genomics research. Studies have shown that over 80% of participants in genome-wide association studies (GWAS) are of European ancestry, whereas only around 3% are of African ancestry, despite Africa harbouring the greatest human genetic diversity (Popejoy & Fullerton, 2016). This exclusion perpetuates a cycle in which African-specific variants remain under-recognised. As a result, variant interpretation tools and diagnostic algorithms are effectively optimised for European populations and the clinical utility of genomic medicine is comparatively lower for African patients (Dennis & Lee, 2024).

1.4.2. Research capacity and data gaps in sub-Saharan Africa

Data on IMD from sub-Saharan Africa remain extremely limited compared with European and North American cohorts, with available data disproportionately skewed towards North African countries where longstanding collaborations with Mediterranean neurology groups and higher consanguinity rates result in more frequent observations of autosomal recessive disorders (Aderinto, AbdulBasit Opeyemi & Amao, 2023). Among 54 African countries, only 17 have published on the genetics of neurological diseases (Aderinto, AbdulBasit Opeyemi & Amao, 2023). Of these countries, 12 have reported patients with inherited myopathies in sub-Saharan Africa confirmed by molecular testing (Figure 1.1), represented by 21 published articles (Krahe et al., 1995; Hallwirth Pillay et al., 2007; Wilmshurst et al., 2010; Ravenscroft et al., 2011; Olckers et al., 2012; Peddareddygari et al., 2012; Kerr et al., 2013; Uwineza et al., 2014; Esterhuizen et al., 2014; Schutte et al., 2015; Jalali-Sefid-Dashti et al., 2018; Amin et al., 2019; Dekker et al., 2019; Wonkam-Tingang et al., 2020; Oliveira et al., 2022; Wilson et al., 2023; Gao et al., 2024; Kotioumbé et al., 2024; Maiga et al., 2024; Sagath et al., 2025; Schoonen et al., 2025). These publications are dominated by dystrophinopathies, while other conditions such as LGMD, congenital myopathies and distal myopathies are each described in only a few cases, most as single case reports or small series geographically concentrated in South Africa. This markedly underestimates the true burden and spectrum of IMD on the

continent and underscores the need for further phenotypic and genetic characterisation in African populations.

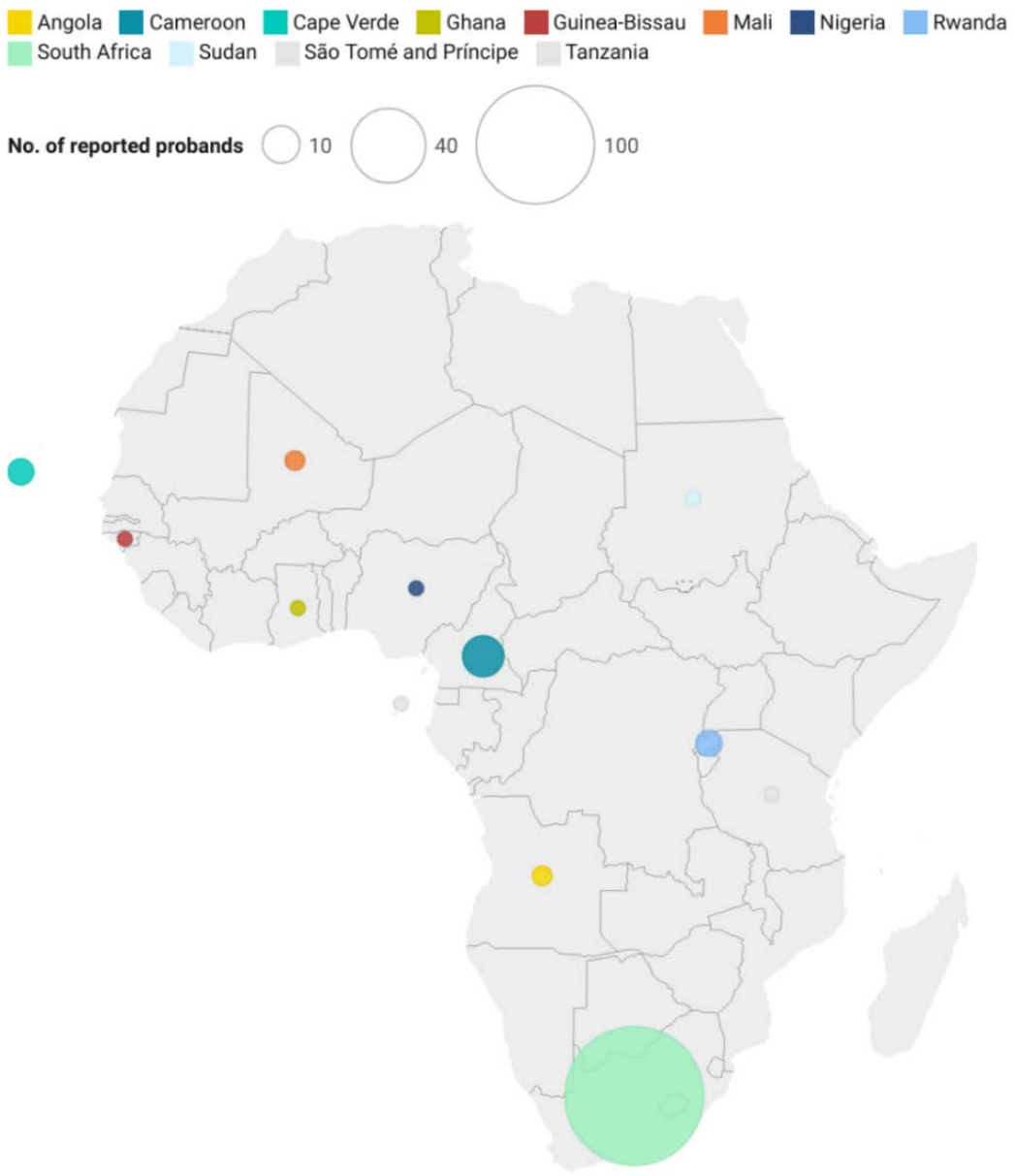


Figure 1.1 Geographic distribution of published research on inherited muscle diseases in sub-Saharan Africa. Circles indicate the number of reported probands with confirmed molecular diagnoses of inherited muscle diseases in twelve sub-Saharan African countries, with circle size proportional to the number of probands. Countries are colour-coded as shown in the key.

1.4.3. Diagnostic infrastructure for IMD in South Africa

Genetic testing and diagnosis of IMD in South Africa face multiple obstacles. Healthcare resource allocation is constrained by the country's quadruple burden of disease, encompassing communicable diseases, non-communicable diseases, maternal and child health challenges as well as trauma-related morbidity, appropriately prioritising high-impact interventions over rare inherited conditions (Rotimi et al., 2014)(Coovadia et al., 2009). Additional barriers include the scarcity of specialist neurologists with neuromuscular and genomic expertise, geographic concentration of tertiary centres in urban areas while many patients reside in rural or outlying areas and the paucity of African genomic data complicating pathogenic variant classification (Raga et al., 2022).

Genetic testing for IMD locally available via the NHLS in South Africa is very limited (Table 1.1) (National Health Laboratory Service, 2015). Biochemical and enzyme-based testing for metabolic myopathies is provided by the Centre for Human Metabolomics at North-West University, although this is predominantly research-based and not routinely accessible through public healthcare pathways (Meldau et al., 2016).

The limited genetic testing available through the NHLS was prioritised based on disease prevalence and evidence derived predominantly from European populations. However, emerging local data increasingly demonstrate that the diagnostic yield of these targeted tests may be suboptimal for African populations, where disease prevalence, founder variant spectra and phenotype-genotype correlations may differ substantially from reference populations (Mahungu et al., 2023; Dennis & Lee, 2024). This mismatch between available testing infrastructure and local disease architecture underscores the need to investigate more comprehensive testing strategies such as targeted gene panels or WES, that can simultaneously interrogate diverse genetic aetiologies without reliance on population-specific assumptions, while also building the evidence base for understanding IMD genetic architecture in African populations.

Table 1.1 Genetic tests available for inherited muscle diseases via National Health Laboratory Service (NHLS) in the public healthcare setting.

Test name (DNA Analysis – PCR/Sanger sequencing)	**Inherited muscle disease**
Dystrophin screen (*DMD* del/dup MLPA screen)	Duchenne/Becker muscular dystrophy (DMD/BMD)
Myotonic Dystrophy type I – *DMPK* CTG expansion assay	Myotonic dystrophy type 1 (DM1)
FKRP-related muscular dystrophy* Tests for *FKRP* variants: *c.1100T>C (p.Ile367Thr)*[#] *c.826C>A (p.Leu276Ile)*[a]	Congenital muscular dystrophy type 1C & LGMDR9 FKRP-related
RYR1-related centronuclear myopathy* Tests for *RYR1* variants: *c.8342_8343del (p.Ile2781fs)* *c.14524G>A (p.Val4842Met)*[b]	Centronuclear myopathy (CNM) & Central core disease (CCD)

Table 1.1 *These refer to very rare highly selected gene variant screening. [#] Founder mutation in South African Afrikaner population (Mudau, Essop & Krause, 2016). [a] Founder variant in Danish and German populations (Walter et al., 2004; Sveen, Schwartz & Vissing, 2006). [b] This variant occurs as part of a biallelic South African-specific founder haplotype and occurs in *cis* with *RYR1* c.10348-6C>G (Schoonen et al., 2025). This *RYR1* c.10348-6C>G variant is not routinely tested for in South Africa. DMPK: Dystrophia myotonica protein, FKRP: Fukutin-related protein, MLPA: multiplex ligation-dependent probe amplification.

Targeted gene panels for LGMD, and single-gene testing for OPMD or FSHD, are not available locally. These specialised tests and WES remains inaccessible for routine clinical use and are only available through research collaborations (Van Der Merwe, Ramesar & De Vries, 2022). Access to international laboratories is constrained by prohibitive costs and complex logistics, reinforcing reliance on limited locally available testing thereby contributing to persistent underdiagnosis (Campbell et al., 2023). The South African Medical Research Council (SAMRC) has addressed this limitation by establishing a dedicated NGS platform offering WES at R3,000-R5,000 depending on service platform, representing significant cost reduction compared to previous international options, although access remains primarily research-based rather than routine clinical service (Qiang et al., 2025)(South African Medical Research Council, 2025).

While WES is now accessible through SAMRC research platforms, a critical implementation barrier extends beyond sequencing availability, preventing translation into routine clinical care. The disconnection between clinicians with access to patients, bioinformatics experts who can interpret data and genetics services for curation, result return and counselling prevents research-accessible sequencing from benefiting clinical populations.

1.4.4. Clinical consequences

Prolonged diagnostic delays are characteristic of IMD globally, yet this diagnostic odyssey is markedly more prolonged in LMIC. Whereas the mean diagnostic delay for IMD is approximately 4.3 years in Europe, sub-Saharan African studies report delays averaging 19.7 years (Spuler et al., 2011; Oliveira et al., 2022). This disparity is particularly striking for well-characterised conditions such as DMD which shows mean delays of 2.5 years in the USA and 4.7–7 months in Northern and Western Europe (Ciafaloni et al., 2009; Hiebeler et al., 2023), compared to 7.5 years reported in Cameroon (Wonkam-Tingang et al., 2020). Individual case examples from sub-Saharan Africa illustrate these disparities such as a Guinea-Bissau patient with calpainopathy experiencing a 19 year delay from symptom onset to molecular diagnosis, highlighting the significant barriers to timely genetic testing in resource-limited settings (Oliveira et al., 2022). Dysferlinopathy diagnostic delays in HIC have improved from 20.5 years in the 1970's to a mean of 3.1 years in recent years, reflecting improving recognition and availability of genetic testing (Harris et al., 2016). In contrast, prolonged diagnostic delays remain in resource-constrained settings, highlighting diagnostic inequities. These pronounced inequalities reflect the convergence of multiple limiting factors endemic to resource-constrained settings including limited genetic testing infrastructure concentrated in isolated urban centres, low disease awareness among non-specialist clinicians and fragmented referral pathways that delay clinical recognition and investigation (Oliveira et al., 2022).

The consequences of diagnostic delays extend far beyond patients themselves. Missed genetic counselling prevents at-risk family members from accessing carrier testing (Germain et al., 2021). Diagnostic uncertainty leads to missed therapeutic windows such as timely corticosteroid initiation periods for DMD patients (Ciafaloni et al., 2009). Exclusion from clinical trials and emerging targeted gene therapies occurs as molecular confirmation is now prerequisite for participation and this therapeutic gap disproportionately affects resource-limited populations lacking diagnostic infrastructure (Girisha & Moosa, 2024). The

psychosocial burden of diagnostic uncertainty, compounded by delayed access to multidisciplinary care, further impacts quality of life (Phillips et al., 2024).

1.5. Conventional diagnostic approaches to IMD

Within this constrained context, clinicians in South Africa still rely predominantly on conventional diagnostic approaches. The diagnosis of IMD begins with comprehensive history-taking and detailed neuromuscular-specific examination to characterise the clinical phenotype including age at symptom onset, pattern of weakness, presence of multisystem involvement and mode of inheritance. This clinical assessment guides selection of targeted investigations, which typically include CK muscle enzyme levels, electromyography (EMG), nerve conduction studies (NCS) and muscle biopsies, typically directed by a specialist neurologist (Nilipour, 2019).

Muscle biopsy remains an essential diagnostic tool, particularly for conditions where histopathological or biochemical analysis provides valuable diagnostic information such as mitochondrial myopathies or when immunohistochemical protein expression analysis can guide targeted genetic testing. However, open muscle biopsy is invasive, subject to sampling error and requires specialised histopathological expertise for accurate interpretation (Bergmann, Weis & Probst-Cousin, 2009). In South Africa, due to resource constraints, access to facilities allowing for high-quality open muscle biopsy and specialised histopathologists is limited, thereby influencing decisions regarding when this investigation is pursued.

Modern diagnostic practice in specialist facilities in HIC integrates detailed clinical phenotyping and targeted investigations with NGS (Beecroft et al., 2020). Detailed clinical assessment remains essential as it defines the phenotype that guides genetic testing interpretation and informs selection of appropriate testing strategy. Genetic confirmation, when obtained, complements clinical findings to confirm diagnosis and guide clinical decision-making, prognostication and genetic counselling.

1.6. Genomic sequencing: Evolution from single gene to comprehensive testing

As noted earlier, the extensive genetic and phenotypic heterogeneity of IMD, combined with the limitations of traditional diagnostic approaches outlined above, necessitated development of molecular genetic testing strategies (Hunter et al., 2015). Traditional approaches have evolved from single-gene testing to next generation sequencing encompassing multi-gene

panels, WES and whole genome sequencing (WGS). This section reviews this evolution and the capabilities and limitations of NGS approaches in IMD diagnosis.

1.6.1. Traditional genetic testing methods

In high-income countries, targeted panels capable of investigating up to 500 genes have replaced sequential single-gene Sanger sequencing as the first-line genetic diagnostic approach for IMD since 2015, offering superior diagnostic yield and cost-effectiveness (Ghaoui et al., 2015; Ng et al., 2022). Targeted single-gene testing is reserved for conditions with high clinical specificity or well-established population-specific founder variants. When panels are uninformative, WES or WGS follows as second-tier testing (Ghaoui et al., 2015).

In contrast, genetic testing infrastructure in South Africa is far more limited with few tests for IMD available via the NHLS and advanced genetic testing not routinely accessible (Table 1.1) (Mahungu et al., 2023). While founder variants have been identified in specific South African populations, comprehensive genetic characterisation of IMD in African populations is insufficient to inform population-specific testing strategies. Targeted gene panels are unavailable through public healthcare and WES access is limited to research collaborations or international referral. Given these constraints and the genetic heterogeneity of IMD, WES represents the most comprehensive diagnostic strategy when molecular testing is feasible (van Niekerk et al., 2024).

1.6.2. Next generation sequencing (NGS)

Next generation sequencing, particularly WES, has emerged as a transformative diagnostic tool for IMD globally, interrogating approximately 20,000 protein-coding genes of the human genome simultaneously, enabling diagnosis without requiring a specific candidate gene hypothesis, particularly advantageous for IMD where clinical features alone cannot reliably distinguish between differential diagnoses (Vinkšel et al., 2021).

Targeted gene panels represent an alternative NGS approach, interrogating a curated set of disease-specific genes (typically 40-500 genes) with high coverage depth (Ng et al., 2022). While panels offer lower cost, faster analysis and simpler interpretation than WES, they require prior clinical hypothesis and may miss causative variants in genes not included on the panel, limiting utility in diagnostically heterogeneous IMD cohorts where the candidate gene list continues to expand. Consequently, unbiased approaches such as WES are increasingly preferred for initial IMD evaluation, with targeted panels reserved for confirmation testing or specific clinical scenarios where the genetic basis is narrowly defined.

Large-scale studies report WES diagnostic yields of 37-65.2% in heterogeneous neuromuscular disease cohorts, varying with disease severity, age at onset, phenotype specificity and ancestry (Ghaoui et al., 2015; Harris et al., 2017; Babić Božović et al., 2021; Kulsirichawaroj et al., 2025)(Lee et al., 2024). A study of 86 myopathy patients without previous genetic testing achieved a 52% diagnostic yield, with unspecified myopathies contributing 35% (Babić Božović et al., 2021). Patients with conditions that could be diagnosed via single-gene testing such as DMD, FSHD and DM1, were excluded. WES is cost-effective compared to sequential single-gene testing, reducing time to diagnosis and overall healthcare expenditure (Schofield et al., 2017).

Whole genome sequencing interrogates both coding and non-coding regions and can detect structural variants, deep intronic variants and repeat expansions not captured by WES (Ewans et al., 2022). However, across Mendelian and rare-disease cohorts, short-read WGS has so far provided only a modest incremental diagnostic yield over high-quality WES pipelines, while incurring substantially higher costs and analytic complexity (Ewans et al., 2022). The majority of pathogenic IMD variants reside in coding regions well-captured by WES. Emerging long-read WGS shows promise for structural variants and repeat expansions, but remains predominantly research-based (Owusu & Savarese, 2023). For most IMD diagnostic scenarios, WES represents the optimal first-line approach, with WGS reserved for WES-negative cases where specific non-coding or structural variants are suspected.

1.6.3. Technical limitations of NGS

Despite its substantial impact, NGS has three major technical limitations, namely the inability to accurately size repeat expansions, poor detection of deep intronic or structural variants and variable coverage across GC-rich regions (Table 1.2) (Ng et al., 2022).

Short-read NGS cannot accurately size tandem repeat expansions exceeding read length, responsible for over 60 inherited diseases including DM1 (*DMPK* CTG repeats) and OPMD (*PABPN1* GCN expansions) (Hujoel et al., 2025). While computational tools such as ExpansionHunter can identify some expansions from short-read data, performance depends on sufficient coverage and allele size within discriminable range (Dolzhenko et al., 2019). Additionally, WES has inherently poor sensitivity for structural variants due to the targeted capture approach, whereas short-read WGS, despite having similar read length constraints, can detect more structural variants due to its genome-wide coverage, although both remain substantially inferior to long-read sequencing (Yang, 2020; Kosugi & Terao, 2024).

Table 1.2 Comparison of technical features and diagnostic capabilities across NGS platforms for inherited muscle diseases (Yeow et al., 2025)(Ng et al., 2022)(Slatko, Gardner & Ausubel, 2018).

Feature	**Targeted gene panels**	**WES**	**short-read WGS**	**long-read WGS**
Genome space	Selected genes (40-500)	Coding exons (~1-2% genome)	Whole genome	Whole genome
Read length	50-300bp (short-read)	50-300bp (short-read)	50-300bp (short-read)	10-100kb+
Repeat expansions	Not detected	Not detected	Limited detection	Accurately sized
Deep intronic variants	Not captured	Not captured	Captured	Captured
Structural variants	Limited	Limited	Partial	High sensitivity
Coverage uniformity	High (focused genes)	Variable	Variable	Improved
Cost	Lowest	Moderate	High	Highest
Bioinformatic complexity	Low	Moderate	High	Very high

Table 1.2 WES: Whole exome sequencing, WGS: Whole genome sequencing, bp: base pairs, kb: kilobases.

Secondly, WES targets only protein-coding exons, missing deep intronic and structural variants. While short-read WGS interrogates the entire genome theoretically enabling detection of these variant classes, the incremental diagnostic yield of short-read WGS over WES has been modest in Mendelian disorders, suggesting that most clinically actionable IMD variants reside in coding regions well-captured by WES (Lelieveld et al., 2015; Ewans et al., 2022).

With regards to coverage uniformity, both WES and short-read WGS show reduced coverage in GC-rich regions and repetitive domains. For example, genes such as *TTN*, *NEB*, *RYR1*, and *SMN1*/*SMN2* have suboptimal coverage or mapping ambiguity, resulting in false-negative variant calls even when the causative gene appears adequately covered based on average sequencing metrics (Oliveira et al., 2016; Barbitoff et al., 2020). Long-read sequencing improves coverage uniformity and has successfully identified causative variants in previously unsolved neuromuscular cases, particularly through detection of repeat expansions, structural variants, and deep intronic variants inaccessible to short-read platforms, but remains expensive and predominantly research-based, limiting accessibility in

resource-constrained settings such as South Africa (Owusu & Savarese, 2023; Yeow et al., 2025).

1.6.4. Variant classification

Beyond technical detection limitations, interpretation of detected variants requires a standardised framework and presents its own challenges. The American College of Medical Genetics and Genomics (ACMG) established a five-tier classification system for variants, namely Pathogenic and Likely Pathogenic, Variant of Uncertain Significance (VUS), Likely Benign and Benign (Richards et al., 2015). The classification guideline utilises a semi-quantitative system that weighs different evidence types by strength. Pathogenic variants are genetic changes strongly demonstrated to be disease-causing, supported by very strong or multiple strong criteria. Likely pathogenic variants have a high probability of being disease-causing, but with less definitive evidence than pathogenic classification.

Variants of uncertain significance (VUS) lack sufficient available evidence for definitive classification as pathogenic or benign, typically having isolated or conflicting supporting evidence (Joynt et al., 2022). Within the VUS category, some groups further distinguish ‘strong VUS’ for variants that narrowly miss the likely pathogenic threshold, but with suspicious features likely to be reclassified as pathogenic with additional evidence. While the ACMG framework has improved variant interpretation consistency, under-representation of African populations in reference databases results in a higher proportion of variants classified as VUS, complicating interpretation of findings and limiting definitive diagnosis (Chen, Facio, et al., 2023).

1.7. Capacity building

Against this background of limited routine genetic testing, but expanding research NGS capacity, initiatives such as the International Centre for Genomic Medicine in Neuromuscular Disease (ICGNMD) and the SAMRC NGS platform have created opportunities to apply comprehensive genomic approaches to South African IMD cohorts.

The International Centre for Genomic Medicine in Neuromuscular Disease, established in 2019 as an MRC-funded transcontinental partnership, addresses diagnostic access disparities by providing WES and bioinformatic support to neuromuscular clinicians in LMIC, linking clinical expertise in resource-limited settings with centralised sequencing capacity (Wilson et al., 2023). The ICGNMD partnership includes 18 centres across Brazil, India, South Africa, Turkey, Zambia, Netherlands and the UK. South African patients with suspected inherited

muscle diseases analysed in this dissertation were enrolled through ICGNMD at the University of Cape Town, with clinical assessment performed locally and WES conducted via the ICGNMD.

1.8. Problem statement and study rationale

Despite major advances in genetic testing for inherited muscle diseases (IMD), their application to African populations remains constrained by critical gaps in both epidemiological knowledge and diagnostic infrastructure. A systematic review of published literature identified only 12 countries in sub-Saharan Africa that have reported research on patients with IMD containing detailed phenotype and genetic information, highlighting the profound paucity of data from sub-Saharan African populations. This scarcity of epidemiological data is compounded by the marked under-representation of African genomes in global reference and disease variant databases, which inflates VUS rates and undermines confidence in pathogenicity assessment. Furthermore, local testing options in South Africa are restricted to targeted genetic tests, with limited institutional access to NGS. These converging barriers contribute to prolonged diagnostic delays and systematic underdiagnosis of IMD in affected individuals of African ancestry.

This study therefore seeks to characterise the phenotype and mutational profile of patients with inherited muscle diseases in a South African cohort, thereby addressing these critical knowledge gaps and contributing to the evidence base for genetic diseases in African populations.

1.9. Aims and Objectives

1.9.1. Aim:

This retrospective cohort study aims to extract, classify and analyse clinical and genetic data collected between 2020 and February 2025, in order to characterise the phenotypic and mutational spectrum of inherited muscle diseases in a South African patient cohort.

1.9.2. Objectives:

- To analyse the clinical data of a cohort of patients with suspected inherited muscle diseases, to determine the spectrum of clinical phenotypes in our setting.

- To determine the diagnostic yield of genetic testing performed in this cohort between 2020 and February 2025, and concurrently to characterise the mutational spectrum of variants identified including variant types, inheritance patterns, diagnostic rates by testing modality (single-gene testing, WES, short-read WGS) and overall frequency of molecular diagnoses.
- To examine phenotype-genotype associations across disease subgroups in the cohort in order to identify testing patterns that could inform future recommendations for a tiered genetic testing strategy for inherited muscle diseases in the South African context.

Chapter 2: Methods

2.1. Patients and data collection

Participants were opportunistically recruited from neuromuscular specialist clinics within the Division of Neurology, Department of Medicine, Groote Schuur Hospital (GSH) in Cape Town, South Africa between 2020 and February 2025. Clinical data, including a detailed neuromuscular history, examination findings and results of investigations, were systematically collected using standardised templates by Professor JM Heckmann and the Neurology Research Group.

The study was approved by the institutional Human Research Ethics Committee (HREC 136/2025, R030/2018) and written informed consent was obtained using a detailed informed consent document. The de-identified clinical data of participants with suspected inherited muscle diseases were extracted from the REDCap database.

2.2. Study population

As of end February 2025, there were 230 participants with suspected inherited neuromuscular diseases enrolled in the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD) parent study (Figure 2.1). The data of patients suspected clinically of having inherited muscle diseases were extracted (n=103). The remaining 127 were excluded from this study due to alternate neurological diagnoses, unaffected relative status or withdrawal of consent.

Of the 103 participants for whom data were extracted, 89 probands were included in subsequent analyses. Thirteen affected relatives and one proband with significant small fibre neuropathy overlap were excluded (Figure 2.1).

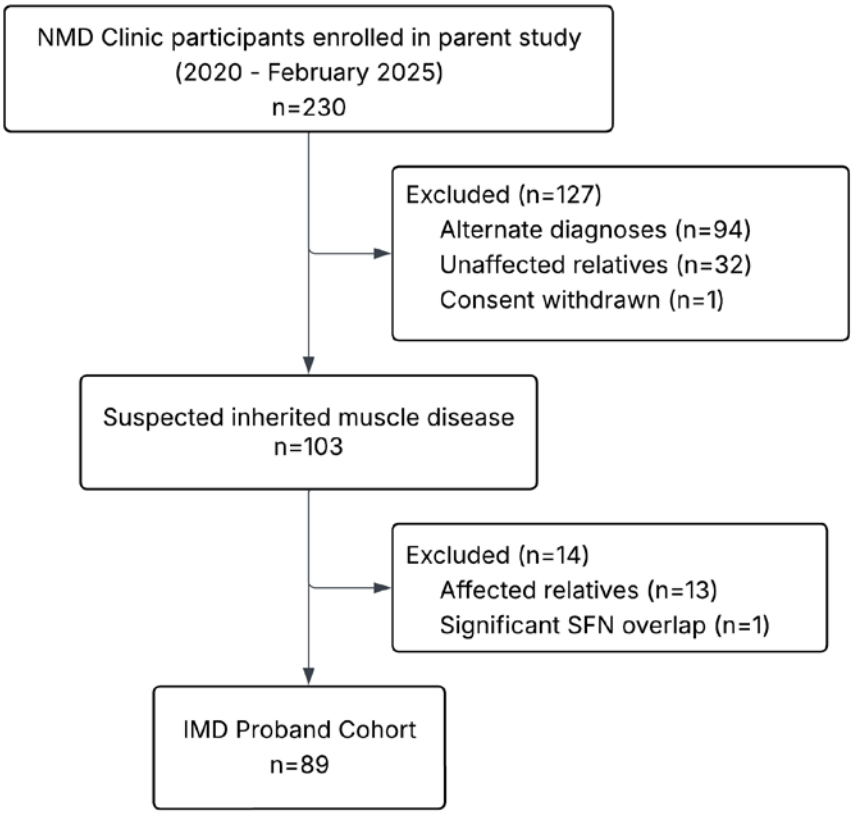


Figure 2.1. Flow diagram of participants included in the study with reasons for exclusion. 'Alternate diagnoses' refers to patients with inherited neuropathies and neurodegenerative diseases e.g. Spinocerebellar ataxia, Charcot-Marie-Tooth disease, Inclusion Body Myositis. IMD: Inherited muscle disease, NMD: Neuromuscular diseases clinic, SFN: Small fibre neuropathy.

2.2.1. Inclusion criteria

All adult and adolescent patients who attended the NMD clinic at GSH from 2020 to end February 2025 suspected of having an inherited muscle disease (IMD). IMD was suspected in patients presenting with unexplained muscle weakness (proximal, distal or mixed distribution), facial weakness or bulbar signs, family history suggestive of neuromuscular disease, elevated serum CK or electrophysiological or histopathological findings consistent with myopathy or muscular dystrophy.

2.2.2. Exclusion criteria

Clinical and electrophysiological findings indicating an alternate diagnosis.

2.3. Ethical considerations

All participants provided written informed consent prior to enrolment. During the consenting process participants were informed of the purpose of the study, any potential risks and the implications of the results as well as how the information will be disseminated and utilised. Consent was obtained in participants' preferred language by trained research staff. It was ensured that participants understood that the genetic testing was done for research purposes and not as part of a diagnostic service. Participants retained the right to withdraw consent at any time without consequence. Pre- and post-test genetic counselling was provided by a dedicated genetic counsellor. The principles as per the ‘Declaration of Helsinki’ have been respected throughout the study including data management.

Ethics approval was granted by the University of Cape Town (UCT), Faculty of Health Sciences, Human Research Ethics Committee (HREC 136/2025). The study forms part of a parent study on inherited neuromuscular diseases, which received prior ethics approval (HREC R030/2018).

2.3.1. Data management and safety

Participant data were de-identified using study codes e.g. IC_SGS_00001. Identifying data were stored separately from research data in a locked research room with restricted access. De-identified data were managed using REDCap with role-based access controls ensuring only authorised research staff could access specific datasets.

2.3.2. Risks and benefits to participants

This study analyses existing clinical observational data and correlates clinical findings with the results of genomic analyses. No further procedures or interventions were performed.

Potential risks included breach of privacy of patient information and psychological implications of genetic results. Participants could experience psychological distress from unexpected or uncertain genetic findings, including incidental findings or variants of uncertain significance (VUS) that lack clear clinical utility. However, the psychological burden of results disclosure was mitigated through comprehensive pre- and post-test counselling provided by a dedicated genetic counsellor, ensuring participants received appropriate support.

The benefit of participation was access to genetic testing otherwise unavailable in this clinical setting in South Africa. Those participants that received confirmed genetic diagnoses were able to name their condition and shorten their diagnostic odyssey.

2.4. Clinical assessment

All participants underwent comprehensive neuromuscular examination by a specialist neurologist. The de-identified clinical data were entered into REDCap. A minimum of five Human Phenotype Ontology (HPO) terms were assigned to each case. Muscle strength was graded according to the Medical Research Council (MRC) grading scale ranging from 0 (no movement) to 5 (normal muscle strength) (Beenakker et al., 2001). Muscle weakness was defined as; Mild: 4/5, Moderate: 3/5 and Severe: < 3/5.

2.5. Special investigations

Relevant special investigations were documented as part of the clinical assessment. Serum CK levels were measured via the NHLS of South Africa or via private laboratories. The laboratory reference range for CK was 20-180 IU/L for females and 20-200 IU/L for males, as stated on patient laboratory reports. Elevated CK was defined as being greater than the respective upper limit of normal (ULN) for males and females. Assessment of cardiac function included electrocardiography (ECG), followed by echocardiography by a cardiologist if appropriate who then made the diagnosis of cardiomyopathy or conduction defects. Forced vital capacity (FVC) was measured via spirometry to assess lung function. Respiratory insufficiency was defined as FVC < 60% of the predicted value.

Electrophysiology was performed and documented in the database as either normal or abnormal. Nerve conduction abnormalities were documented as axonal, demyelinating or mixed. Electromyography (EMG) abnormalities were documented as myopathic, neurogenic, neuromuscular transmission disorder or other.

Muscle biopsies were performed when clinically indicated and documented in the database as either normal, abnormal or not done. When abnormalities were present, histopathology and immunohistochemistry were used to evaluate for features of muscular dystrophy, congenital myopathy and mitochondrial disease. Muscle ultrasound and Magnetic Resonance Imaging (MRI) are not typically performed in our setting.

2.6. Genetic testing and variant classification

Genetic testing was performed as part of the parent study prior to the commencement of the present analysis and followed a tiered approach. Genetic testing and bioinformatics analysis were performed by Dr Melissa Nel and the Clinical Omics and Informatics Unit (COIN) at the University of Cape Town (UCT).

Tier 1 comprised locally available genetic tests including *DMD* MLPA, *DMPK* repeat expansion assay and mitochondrial DNA (mtDNA) deletion testing. Patients without a molecular diagnosis following tier 1 testing, or in whom tier 1 testing was clinically inappropriate, proceeded to tier 2 testing.

Tier 2 testing referred to all genetic testing done that was not available locally. This testing was facilitated via the ICGNMD consortium. *PABPN1* repeat expansion testing for Oculopharyngeal muscular dystrophy (OPMD) was performed at University College London (UCL) in the United Kingdom (UK). Pathogenic *D4Z4* contraction testing for Facioscapulohumeral muscular dystrophy (FSHD) was performed at the Leiden University Medical Centre in the Netherlands using pulsed-field gel electrophoresis (PFGE) and Southern blotting. Whole exome sequencing (WES) and short-read whole genome sequencing (WGS) were performed for comprehensive genomic analysis. While these tests generally followed sequentially, the testing approach was not strictly stepwise as there were cases that underwent short-read WGS directly without prior WES. This was based on clinical availability rather than specific clinical or technical rationale.

This pragmatic approach reflects the real-world constraints of testing access in a resource-limited research setting, where genetic testing was largely facilitated through an international research consortium prior to the commencement of this analysis, and the selection of modality was not within the direct control of the present study. Although this introduces some heterogeneity into the testing pathway, it reflects the operational reality of IMD diagnostic research in sub-Saharan Africa and is discussed further in the Limitations section.

Variant curation and classification were performed by COIN according to American College of Medical Genetics and Genomics (ACMG) 2015 guidelines (Richards et al., 2015). Variants were assigned to the five standard ACMG categories. For this study, only pathogenic, likely pathogenic or VUS were included in the analysis and reporting. In this study, VUS identified via exome sequencing and subsequently evaluated and prioritised

through the COIN computational pipeline under the direction of Dr Melissa Nel were selected for reporting.

2.7. Data extraction

The data collection component of the study had mostly concluded as part of the parent study. Participants' phenotypic and genetic findings were recorded in their physical clinical folder and in a digital document which was then de-identified and uploaded to REDCap. Following review of the participants digital data, a folder review was conducted to further supplement the participants' data as needed.

The applicable data fields from REDCap were exported to a de-identified electronic Microsoft Excel spreadsheet. Components of the data collected and analysed included: Age at symptom onset, age at confirmed molecular diagnosis for cases considered solved, sex (male/female), self-assigned ancestry, inheritance pattern/family history of disease, first noted and predominant symptoms, loss of independent walking, loss of ambulation, systemic involvement, clinical findings, Human Phenotype Ontology (HPO) terms, biochemical tests, nerve conduction studies (NCS), EMG and muscle biopsies if done.

For the purposes of standardised data extraction and analysis, the following functional outcomes were operationally defined:

- Age at symptom onset: Age at which first symptoms attributable to their muscle disease was noted by patient or family.
- Loss of independent walking: Unable to walk unassisted. Requires physical assistance or assistive devices.
- Loss of ambulation: Uses wheelchair as primary mobility aid.

Clinical findings were systematically documented and included dysmorphic features, scapular winging, spinal abnormalities, cognitive dysfunction, ocular signs, bulbar signs, facial weakness, muscle atrophy, pattern of limb muscle weakness, contractures, fatigability, presence of myotonia, cerebellar signs and sensory loss.

Following extraction from REDCap, data were cleaned using a standardised protocol. The following checks were completed:

- Continuous variables were assessed for biologically implausible values. For example, ensuring age at onset 0-90 years.

- Logical inconsistencies were identified and resolved by folder review. For example, age at loss of ambulation preceding age at symptom onset.
- Free-text entries for ethnicities, symptoms and clinical signs were standardised to predefined categories.
- A consistency check was conducted and if an issue was present it was resolved by folder review. For example, if motor ability was reported as ‘Transported in a manual wheelchair in all settings’ then age at loss of ambulation should not be blank.
- Data that was absent by omission was handled by recoding blank to ‘Absent’. This assumption was based on the fact that ‘No’ was a selectable option that was left blank alongside pathological options, suggesting that the data capturer did not engage with the field as there was likely nothing to report. The clinical convention of documenting abnormal findings while omitting negative findings was also considered. Cases in which this occurred underwent folder review and were found to be consistent with their clinical folders.

2.8. Phenotypic categorisation

Participants were classified into broad clinical categories based on first symptom noted and predominant pattern of weakness documented at study enrolment: limb-girdle, extraocular muscle (EOM), periodic, myotonia-related muscle symptoms, exercise-related muscle symptoms and myasthenic-like syndrome with fatigable weakness.

Participants presenting with predominantly shoulder or pelvic girdle weakness were classified as ‘limb-girdle weakness’. Those with weakness of the levator palpebrae superioris, rectus and oblique muscles were classified as ‘EOM weakness’. Participants with episodic muscle weakness were classified as ‘periodic weakness’. Participants who presented with muscle weakness worsening with repetitive use and improving with rest were classified as ‘myasthenic-like syndrome with fatigable weakness’. Those with inability of the muscles to relax quickly following contraction were classified as ‘myotonia-related muscle symptoms’. Lastly, those with myalgia, muscle weakness or rhabdomyolysis following periods of exercise were classified as ‘exercise-related muscle symptoms’.

Discrepancies were resolved through consensus discussion with the study supervisor, Professor JM Heckmann, who is a specialist neurologist. Cases with overlapping features e.g.

limb-girdle weakness with prominent extraocular involvement, were classified according to the predominant presenting complaint and objective clinical findings.

2.9. Data analysis

Exploratory data analysis was done to assess the clinical phenotypes and mutational profile of the whole cohort as well as by phenotype subgroups. These subgroups are noted in the section above. Raw data were collated and analysed using Microsoft Excel. Categorical variables were expressed as frequencies and percentages and compared using Chi-square test. Two-tailed p-values were used unless specifically testing directional hypotheses. Statistical significance was set at $p < 0.05$. Key data processing was done using Microsoft Excel and statistical analyses done using Open Epi v.3.01 (Dean AG, Sullivan KM, 2013) with figure generation using Prism GraphPad v.10.5.0 (GraphPad Software, 2023) and Datawrapper (GmbH, 2025). Results were summarised and presented primarily using descriptive statistics including percentages, mean with standard deviation (SD) for parametric distributed data or median with interquartile ranges (IQR) for non-parametric distributed data. These statistics were used to illustrate the demographic information, age at onset, age at confirmed molecular diagnosis, clinical characteristics and genetic analysis. The summarised results are presented in tables and graphs.

For each phenotypic subgroup, causative genes were tabulated and frequencies calculated e.g. limb-girdle weakness. The proportion of solved cases attributable to each gene was calculated. Clinical features such as age at symptom onset, pattern of weakness and systemic involvement were compared descriptively between genetically solved and unsolved cases, as well as between different genetic diagnoses within phenotypic subgroups. Clinical and genetic data were summarised to describe phenotypic features associated with specific genes and to consider the diagnostic utility of clinical classification in predicting genetic aetiology within the South African context.

The overall diagnostic yield was calculated for the entire cohort and stratified by phenotypic subgroup. For this study, diagnostic yield was defined as the proportion of probands in whom pathogenic or likely pathogenic (P/LP) variants were identified in genes with established association to the patients' clinical phenotype. Based on COIN variant curation, cases with reportable VUS in disease-associated genes were categorised as 'probably solved' (i.e. 'strong VUS' as defined in Background section 1.5.4.), while those with inconclusive VUS were classified as 'possibly solved'.

For clarity, neither 'probably solved' nor 'possibly solved' constitutes a confirmed molecular diagnosis. These categories are reported separately to transparently convey the proportion of cases with clinically plausible but unproven genetic explanations, a finding of particular relevance to a South African cohort in which elevated VUS rates are expected given African genomic under-representation in reference databases.

Chapter 3: Results

This chapter presents the demographic, phenotypic and mutational profile of 89 probands from a heterogeneous South African cohort with inherited muscle diseases (IMD) opportunistically recruited from a neurology service at the University of Cape Town (UCT). Due to limited testing locally available to patients suspected of having IMD, UCT joined a research consortium with the objective of improving the molecular genetic diagnostics for patients as part of a research study conducted between 2020 and February 2025.

Eighty-nine probands were included and categorised by their initial symptoms and predominant pattern of weakness into limb-girdle weakness (n=45), muscle weakness with prominent extraocular muscle (EOM) weakness (n=19), myotonia-related symptoms (n=10), distal weakness (n=5), myasthenic-like syndrome with fatigable weakness (n=4), exercise-related symptoms (n=4) and periodic weakness (n=2). In 55 (62%) probands, a definitive molecular diagnosis was obtained, while 27 (30%) remained unsolved.

There were 51 (57%) males and 38 (43%) females. The self-reported genetic ancestry of the cohort was diverse, comprising individuals of African ancestry (14%), South African admixed ancestry (40%), European ancestry (42%) and Asian ancestry (3%).

3.1. Clinical features

Median age at onset (AAO) of symptoms was 17 years (IQR, 8-35) with 54% manifesting symptoms in childhood/adolescence (n=48, < 20 years) and 46% with AAO during adulthood (n=41, ≥ 20 years). Median age at which molecular diagnosis was confirmed was 46 years (IQR, 29-58). Median diagnostic delay between AAO and confirmed molecular diagnosis was 16.5 years (IQR, 8-32).

Thirty-two (36%) probands reported a positive family history of muscle disease with five (6%) probands being born of a consanguineous relationship. 60% of the cohort (n=53) had loss of independent walking with median age of 26 years (IQR, 10-48). Eleven probands (12%) were wheelchair-bound at the time of study enrolment. The median age at loss of ambulation was 34 years (IQR, 11-48).

3.2. Pathway to molecular genetic diagnosis

3.2.1. Local testing (Tier 1)

Locally available genetic testing yielded molecular diagnoses in 18% (16/89) of the cohort. Among the 55 solved cases, local testing accounted for 29% (16/55) and included *DMD* MLPA, *DMPK* expansion assay and mitochondrial DNA (mtDNA) deletion testing. Those solved via locally available testing showed variants in *DMD* (n=8), *DMPK* (n=5) genes and single large mitochondrial DNA deletions (*mtDNA*, n=3). The most frequently identified gene confirmed locally was *DMD*. The median time from symptom onset to confirmed molecular diagnosis in this group was 10 years (IQR, 7-17).

3.2.2. Further testing (Tier 2)

Of the 55 confirmed molecular diagnoses, 39 (71%) were diagnosed via genetic testing not available in South Africa (tier 2). These tier 2 diagnoses represented 53% (39/73) of patients who remained undiagnosed or untested after tier 1 testing. Tier 2 testing included *PABPN1* repeat expansion analysis, FSHD genetic testing (See Methods section 2.6), whole exome sequencing (WES) and whole genome sequencing (WGS) available in the research setting. The median time from symptom onset to confirmed molecular diagnosis in this group was 22 years (IQR, 9-35).

Among those undergoing tier 2 testing as part of the research study, WES was the most frequently employed modality, contributing 39% (24/62) of the diagnoses in this group. Targeted analysis for repeat expansions (*PABPN1)* and FSHD genetic testing (*D4Z4* repeat contraction) contributed 76% (13/17), while short-read WGS solved for 18% (2/11) of those tested. Overall, tier 2 testing yielded a diagnosis in 53% (39/73) of the patients who remained undiagnosed or untested after locally available genetic testing, with next generation sequencing (WES and WGS) accounting for 41% (26/64) of the diagnoses in those tested (Figure 3.1). Additionally, seven probands harboured prioritised variants of uncertain significance (VUS) in disease-associated genes. Six (7%) were reportable and classified as probably solved and one as inconclusive and possibly solved.

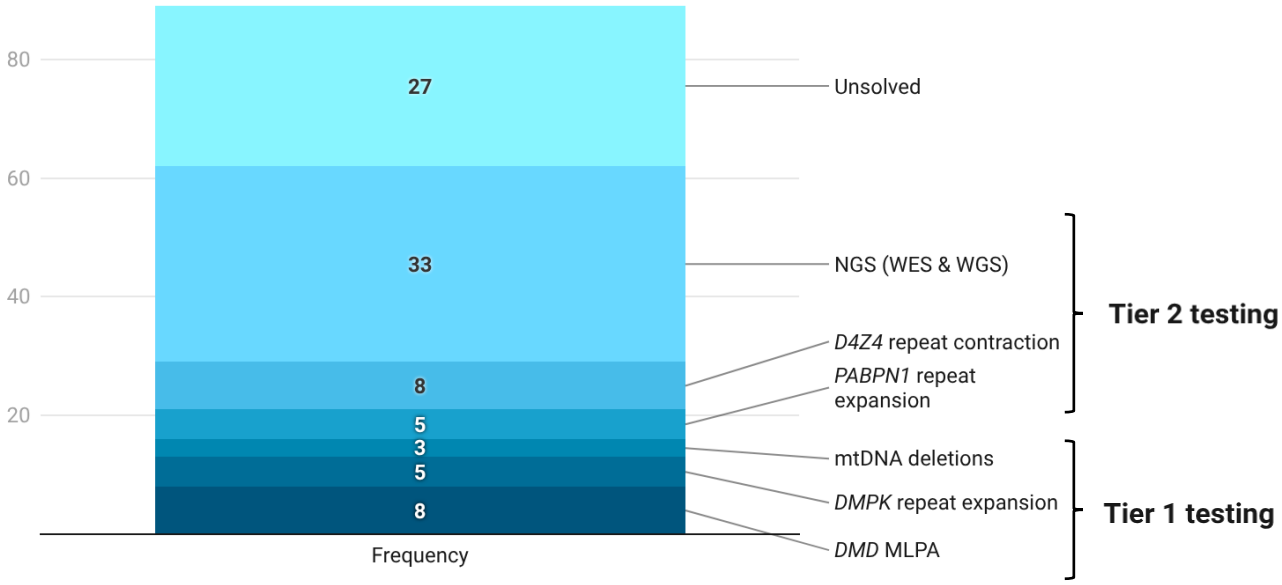


Figure 3.1 Stratification of diagnostic outcomes by genetic testing tier in a South African inherited muscle diseases cohort. Next generation sequencing (NGS) comprised of whole exome sequencing (WES) and whole genome sequencing (WGS). Cases with reportable and inconclusive VUS identified by NGS are included within the NGS bar. Unsolved denotes probands in whom no pathogenic, likely pathogenic or reportable VUS was identified.

The *D4Z4* repeat contraction (n=8) found in FSHD was identified as the commonest gene identified via tier 2 testing. The *PABPN1* gene (n=5) was identified as the next commonest. Myotonia congenita was diagnosed in four probands with pathogenic variants in the *CLCN1* gene (n=4) identified on WES. Desminopathy (*DES*) and distal nebulin myopathy (*NEB*) was diagnosed in three probands each. Collagen VI-related myopathy was found in three probands with *COL6A1* (n=1) and *COL6A2* (n=2) genes identified. *DYSF*, *CAPN3* and *RRM2B* genes were noted in two probands each. The remaining confirmed diagnoses were associated with single genes and include *SELENON*, *RYR1*, *OPA1*, *TWNK*, *GAA*, *PHKA1*, *EMD*, *LMNA*, *MT-ATP6* and *GNE* genes (Figure 3.2A). Of the variants identified, six were reportable VUS classified as probably solved, namely variants in *CAPN3*, *COL6A2*, *CLCN1*, *TWNK*, *MT-ATP6* and *PHKA1*. One *RRM2B* variant was classified as an inconclusive VUS and considered possibly solved.

Overall, 42% of probands (37/89) had a genetically confirmed muscular dystrophy. Within this muscular dystrophy group, autosomal dominant forms including FSHD, OPMD and EDMD2 accounted for 51%, while autosomal recessive forms, namely calpainopathy, desminopathy and dysferlinopathy comprised 19%. X-linked recessive conditions such as dystrophinopathy and EDMD1 comprised the remaining 30% (11/37).

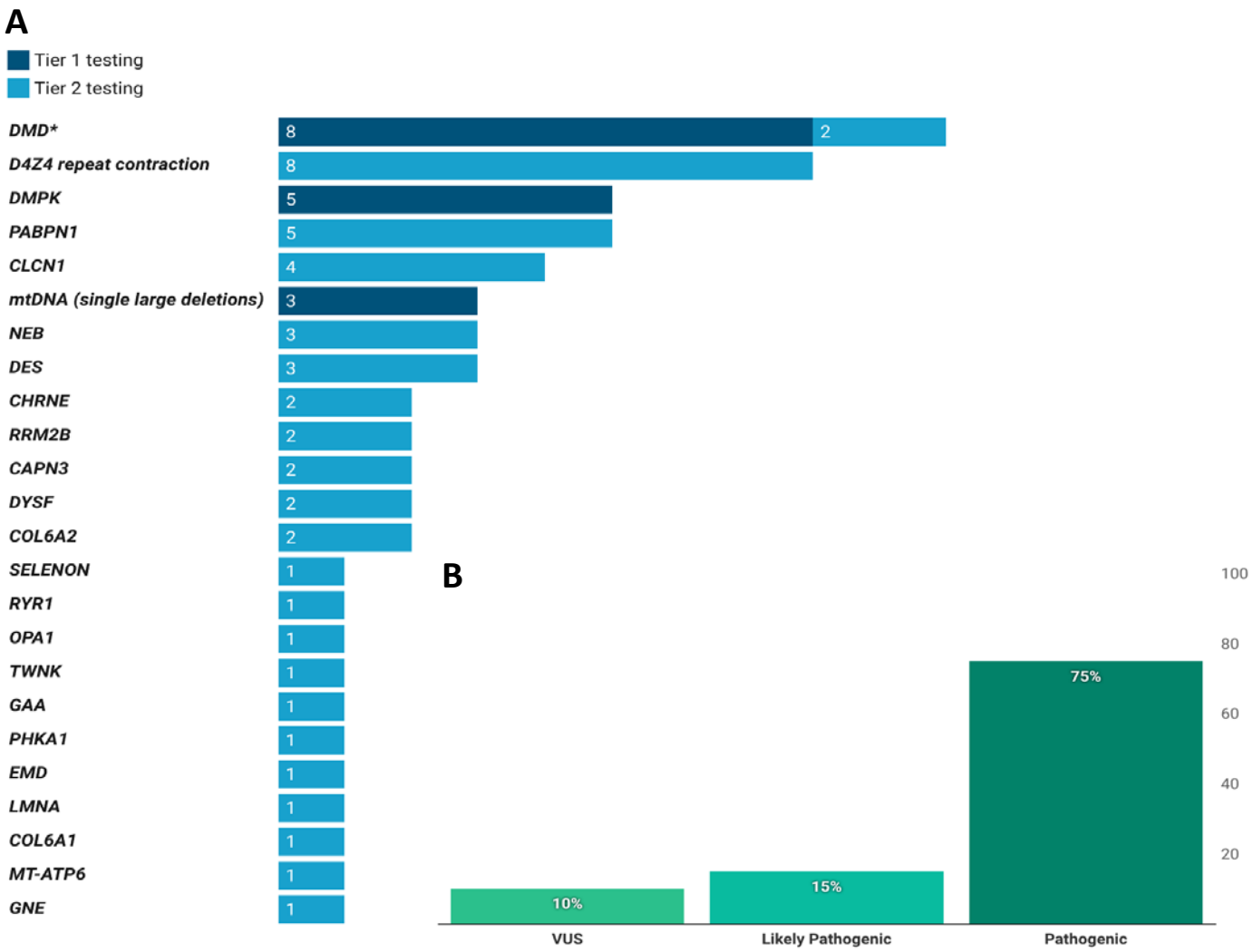


Figure 3.2 **A**: Total number of genes identified in cohort by tier 1 vs tier 2 testing. **B**: Classification of identified variants according to The American College of Medical Genetics and Genomics (ACMG) guidelines with probability of pathogenicity. *Two patients with DMD had false negatives via *DMD* MLPA.

Of the variants identified, 75% were classified as pathogenic, 15% as likely pathogenic and 10% as VUS (Figure 3.2B). VUS arise when identified variants are unable to be classified definitively as pathogenic based on currently available data. In this study, only VUS occurring in genes with an established association with the probands' phenotype and prioritised by the Clinical Omics and Informatics Unit (COIN) curation pipeline were considered reportable and included in the probably or possibly solved categories (See Methods section 2.6). 'Probably solved' refers to cases with a reportable VUS in a disease-associated gene, where the variant was prioritised through computational curation but does not meet the threshold for a confirmed pathogenic or likely pathogenic classification. 'Possibly solved' refers to cases with an inconclusive VUS where confidence in pathogenicity

is lower. Four probands were found to have VUS that were novel to ClinVar. Two of these probands were of African ancestry and two were of European ancestry (Table 3.1).

There was one likely pathogenic and three pathogenic variants that were reported as novel to ClinVar in our cohort. These were *EMD* X:154380743-154381208del, *SELENON* c.301+2T>C (p.Gly315Ser), *DMD* c.503del (p.Ala168Valfs) and *RYR1* c.1580T>G (p.lle527Ser) respectively. Despite being novel, these variants were classified as likely pathogenic or pathogenic due to their functional effect being consistent with known disease mechanisms. The first three are null variants that cause loss of function. The other is the *RYR1* variant that changes a critical amino acid and is located within the C-terminal region that is a known hotspot region for other pathogenic variants causing central core disease (Leão et al., 2020).

Table 3.1 Variants of uncertain significance that are novel to ClinVar and associated clinical phenotypes identified in a South African cohort with IMD.

Ancestry	**Phenotype**	**Gene**	**Inheritance**	**Variant**	**ACMG Classification**
African	LG weakness	*PHKA1*	XL	c.1283G>A (p.Arg428His)	VUS
African	LG weakness	*CAPN3*	AD	c.1240T>G (p.Cys414Gly)	VUS
European	EOM weakness	*TWNK*	AD	c.1117C>G (p.Leu373Val)	VUS
			AD	c.1124A>T (p.Glu375Val)	VUS
European	Distal weakness	*COL6A2*	AD	c.1179+2T>C	VUS

Table 3.1 Summary of probands with IMD in whom VUS were novel to ClinVar. For each case ancestry, predominant phenotype, gene, inheritance pattern, variant and ACMG classification is shown. AD: autosomal dominant, EOM: Extraocular muscle weakness, LG: Limb-girdle, VUS: variant of uncertain significance, XL: X-linked recessive.

Eight probands harbouring variants novel to ClinVar were discovered in our cohort (Supplementary table 1). A pathogenic *DYSF* p.Tyr1433Ter variant was previously reported (Jalali-Sefid-Dashti et al., 2018). Five of these variants were identified in individuals of African or South African admixed ancestry.

3.2.3. Cases remaining without a molecular genetic diagnosis after NGS

Twenty-seven probands remain without molecularly confirmed diagnoses despite locally available testing and WES or short-read WGS. Of these probands, 11 had limb-girdle weakness, eight had myopathy with or without EOM weakness alone and there was one proband each that had distal weakness and myotonia-related symptoms. Four cases who had exercise-related symptoms and two cases with periodic weakness remained without a molecular genetic diagnosis.

Those unsolved in the limb-girdle weakness group were categorised into four groups based on their clinical features, namely limb-girdle weakness with contractures, scapulopelvic weakness, complex neurological signs and atypical distribution. The limb-girdle weakness with contractures phenotype group had three probands who presented with limb-girdle weakness, upper and lower limb contractures with one who had keloids and another with cardiomyopathy. There were four in the scapulopelvic weakness group who had proximal upper limb weakness, scapular winging and proximal lower limb weakness. The complex neurological signs group had two probands who both had cognitive impairment and one with clumsiness. Lastly, two were noted to have atypical distributions with one that had prominent axial weakness and the second had a hand tremor with hand weakness.

Eight probands from the EOM weakness group remained unsolved. Three had clinical symptoms of CMS with fatigable ptosis and ophthalmoparesis with two that had facial weakness and proximal lower limb weakness. Secondly, there was a proband with congenital bilateral ptosis, diplopia and scoliosis without ophthalmoparesis. The following two had clinical features in keeping with OPMD, namely ptosis, ophthalmoplegia and dysphagia. Neither had proximal limb weakness. Another proband had congenital bilateral ptosis and scoliosis with progressive ophthalmoplegia. She also had pectus excavatum. Finally, one had ptosis, ophthalmoloplegia and infantile seizures alongside pes cavus, hammertoe, ankle contractures and pelvic girdle weakness.

One unsolved proband, thought to have myotonia congenita, was WES negative. He initially noted myotonia with warm-up phenomenon at 12 years. He had myalgia, muscle stiffness and exercise-induced leg cramps. He reported that his brother was also affected and noted handgrip myotonia. Lastly, there was one with predominantly distal lower limb weakness remaining unsolved. She had neck flexor, proximal and distal lower limb muscle weakness

with anterior compartment more affected in the distal lower limbs. No facial muscle weakness was observed.

Comparing the probands with confirmed genetic diagnoses versus those without molecular confirmation, there was no significant difference in proportions between African, European or South African admixed ancestry probands (p=0.41).

Tier 2 testing showed variation in diagnostic yield across age categories (Figure 3.3). In the childhood- and adolescent-onset group (<20 years), there were similar proportions of cases with confirmed genetic diagnoses versus those who remained without a genetic diagnosis. In the adult-onset group (20-50 years), probands with confirmed molecular diagnoses were more prevalent than those without a genetic diagnosis. Late-onset disease (>50 years) was uncommon and most affected probands received a confirmed molecular diagnosis. The predominant phenotype in this late-onset group was characterised by prominent EOM weakness with *PABPN1* repeat expansions, single large mtDNA deletions and variants in *RRM2B* and *MT-ATP6* genes being identified.

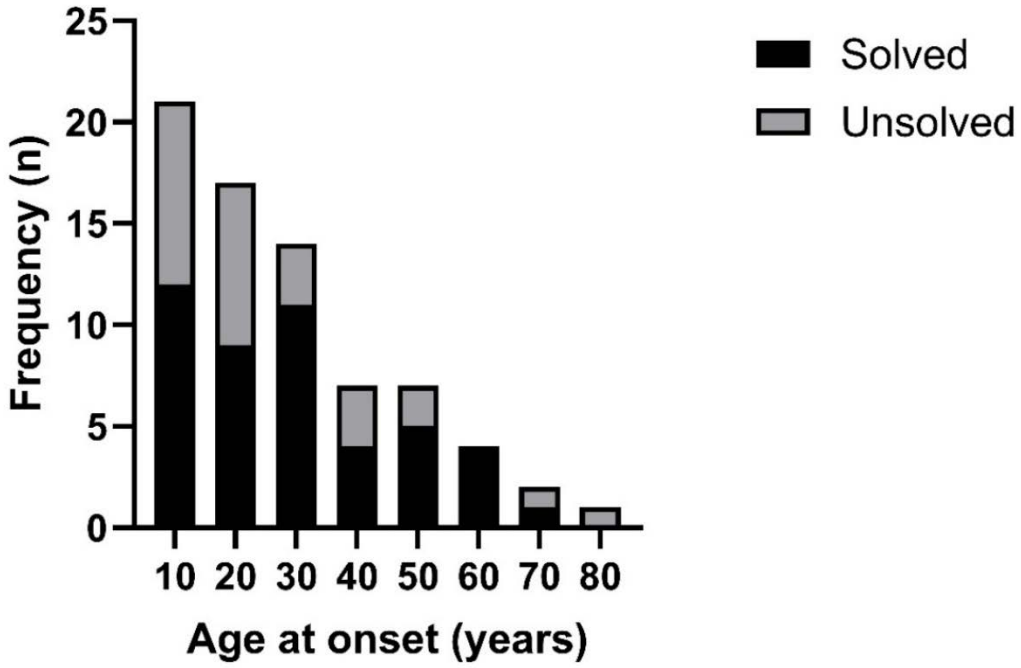


Figure 3.3 Distribution of tier 2 genetic testing outcomes by age at symptom onset. Histogram depicting the frequency of probands with molecular diagnoses confirmed by tier 2 testing (solved) vs those without genetic confirmation (unsolved) across age at onset categories. Included in the dataset are those who underwent *PABPN1* repeat expansion, FSHD1 genetic testing for *D4Z4* repeat contractions, WES and WGS.

3.3. Limb-girdle weakness

Of the 89 probands, 45 (51%) were classified as limb-girdle (LG) weakness. There was a slight male predominance in this group with 29 (64%) (p=0.089). The participants were predominantly of European and South African admixed ancestry with 16 of European ancestry (36%), 17 of South African admixed ancestry (38%), 11 of African ancestry (24%) and 1 of Asian ancestry (2%).

3.3.1. Clinical features

Fourteen probands (31%) reported a positive family history of muscle disease with two (4%) being born of a consanguineous relationship. The median AAO was 14 years (IQR, 6-27) with 62% manifesting symptoms during childhood/adolescence (n=28, < 20 years) and 38% during adulthood (n=17, >20 years). The median age at which molecular diagnosis was confirmed was 37 years (IQR, 23-52). Therefore, the median diagnostic delay to confirmed molecular diagnosis was 16 years (IQR, 8-29).

Thirty-seven probands (82%) with limb-girdle weakness had loss of independent walking with a median age of 18 years (IQR, 7-34). Eleven (24%) probands were wheelchair-bound with median age at loss of ambulation of 34 years at time of study enrolment (IQR, 11-48).

Three probands (7%) were reported to have hearing loss and three separate probands (7%) were reported to have cognitive dysfunction or learning difficulties. On inspection, muscle atrophy affecting proximal upper and lower limb muscles was observed in 76% of the subgroup (n=34). Muscle hypertrophy was observed in 29% (n=13). Twenty-six probands (58%) showed signs of spinal abnormalities including hyperlordosis (n=18), scoliosis (n=9) and kyphosis (n=4). Scapular winging was observed in 47% of probands (n=21) with 90% being bilateral. Contractures were observed in 42% of probands (n=19), affecting the lower limbs (n=15), upper limbs (n=9) and spine (n=3), with probands demonstrating contractures in multiple regions. Cranial nerve examination revealed facial weakness in 22% (n=10) and bulbar dysfunction, namely dysphagia or dysarthria, in 16% (n=7). Ocular abnormalities were noted in 7% (n=3).

Motor examination revealed the defining feature of this group, namely limb-girdle muscle weakness affecting shoulder and/or pelvic girdle muscles. Thirty-seven probands (82%) had combined shoulder and pelvic girdle weakness at time of study enrolment. Weakness severity ranged from mild to severe (MRC grade 0-4/5), with functional impact ranging from mild difficulty with activities of daily living to complete loss of ambulation. Notably, there was

substantial overlap between proximal and distal muscle involvement. Among those combined limb-girdle weakness, 76% (28/37) also demonstrated distal lower limb weakness and 59% (22/37) had distal upper limb weakness, indicating a proximal-to-distal gradient of disease progression. Six probands (16%) had isolated proximal weakness without distal involvement at time of study enrolment.

Systemic examination revealed cardiac dysfunction in 38% of probands (n=17) with cardiomyopathy being most frequent (47%). Fourteen (31%) were noted to have respiratory dysfunction primarily due to neuromuscular respiratory weakness (71%).

Within the limb-girdle weakness group, CK results were available on 38 (84%) with a median CK ~3x ULN (581 U/L, IQR,310-2932). EMG was conducted on 25 cases and EMG was abnormal in 96% of those that were conducted (n=24). The predominant findings on EMG were in keeping with myopathic abnormalities (n=22). Muscle biopsies obtained from 22 probands were found to be abnormal in all.

3.3.2. Genetic findings

Thirty-four probands with limb-girdle weakness received a confirmed molecular diagnosis (76%). The eight (24%) locally solved (tier 1 testing) were all childhood-onset cases confirmed to have *DMD* variants which will be discussed further below. Among the tier 2 diagnoses (76%; 26/34), childhood-onset probands (n=13/21) achieved diagnostic resolution comparable to adult-onset probands (n=13/13).

Amongst those with predominant limb-girdle weakness, autosomal dominant conditions were most frequently identified. The commonest gene identified via tier 2 testing was the *D4Z4* repeat contraction found in FSHD1 (n=8) and autosomal dominant desminopathy (*DES*, n=2) was the next commonest genetic diagnosis (Figure 3.4). Other cases with autosomal dominant aetiologies included Emery-Dreifuss muscular dystrophy type 2 (EDMD2) (*LMNA*, n=1), COL6A1-related Bethlem myopathy (n=1), RYR1-related myopathy (n=1) and OPA1-related autosomal dominant optic atrophy plus syndrome (ADOA plus, n=1).

Autosomal recessive conditions included calpainopathy (*CAPN3*, n=2) and dysferlinopathy (*DYSF*, n=2). Single patients were identified with autosomal recessive forms of desminopathy (*DES*, n=1), COL6A2-related myopathy (n=1), GNE myopathy (n=1) and rigid spine syndrome (*SELENON*, n=1). X-linked recessive diagnoses included *EMD* deletion-related EDMD1 (n=1) and PHKA1-related glycogen storage disease (n=1).

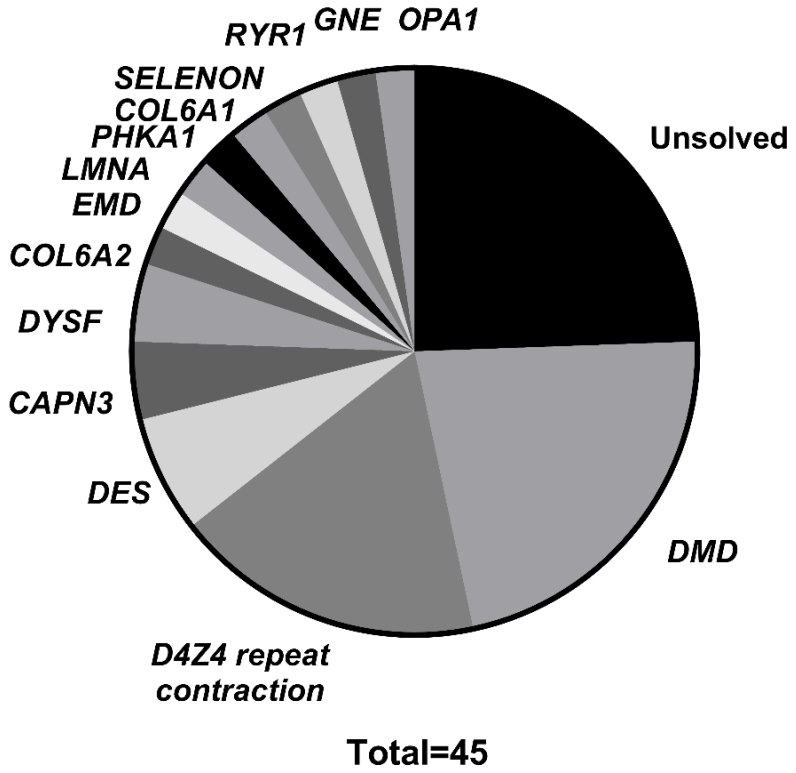


Figure 3.4 Genetic heterogeneity in limb-girdle weakness cohort: confirmed genetic diagnosis and gene distribution in a cohort of 45 probands. Unsolved cases (24%; 11/45) remained without molecular diagnosis despite WES and short-read WGS.

I will now briefly discuss the phenotypes and variants identified in these cases below.

3.3.3. Dystrophinopathy

In the LG weakness cohort, ten male probands were found to have dystrophinopathy with pathogenic variants reported below. Eight probands with LG phenotype were solved via locally available *DMD* MLPA and two were falsely negative on MLPA, but were subsequently identified with pathogenic variants in the dystrophin gene by WES and WGS.

Probands were predominantly of African ancestry (60%), with two of European ancestry (20%) and two of South African admixed ancestry (20%). None of the cases had a family history of muscle disease. The median AAO was six years old (IQR, 5-16). Median age at confirmed molecular diagnosis was 21 years (IQR, 14.5-28.5) translating into a median diagnostic delay of 10 years (IQR, 7.5-16.5). Eight (80%) of the probands had loss of independent walking by the time a genetic diagnosis was made, with a median age at loss of independent walking of 9.5 years (IQR, 5.5-18). Three probands were wheelchair-bound at time of study enrolment with mean age at loss of ambulation of 11 years (SD=2)

Table 3.2 Duchenne muscular dystrophy and Becker muscular dystrophy diagnostic criteria.

Dystrophinopathy type	AAO (years)	Age at loss of ambulation (years)	Genetic findings
Duchenne (DMD)	≤ 5	<13	• Out-of-frame del/dup with disrupted reading frame • Minimal to nil functional dystrophin produced
Becker (BMD)	> 5	≥16	• In-frame del/dup with preserved reading frame • Partially functional dystrophin produced

Table 3.2 Diagnostic criteria used to classify probands as DMD or BMD as defined by the European Neuromuscular Centre (ENMC) (Emery & Centre., 1997). AAO: Age at symptom onset, BMD: Becker muscular dystrophy, DMD: Duchenne muscular Dystrophy, del: deletion, dup: duplication.

Apart from delayed motor ability or proximal lower limb muscle weakness before the age of seven, one proband (10%) reported exercise intolerance, muscle cramps and exercise-induced myoglobinuria as the first noted symptoms at the age of 16 years. At the time of study enrolment, 60% of the probands had associated muscle atrophy of the proximal upper and lower limbs. Scapular winging was present in 30% of probands. Six probands had spinal abnormalities, namely scoliosis (30%), hyperlordosis (20%) and kyphosis (10%). Contractures were observed in 60% with predominantly ankles and knees affected, although two also had elbow flexion contractures. Facial weakness and dysphagia were observed in 10% of cases. Learning difficulties was reported in one case.

All but two cases had calf pseudohypertrophy noted on examination, one of whom had BMD with mild muscle involvement and the other had very advanced disease with widespread muscle atrophy. Five (50%) probands had both proximal upper and lower limb weakness and three (30%) had only proximal lower limb weakness (Table 3.3).

Clinical information regarding cardiac dysfunction was not available for one person. The remaining probands had cardiomyopathy (n=8) or a right bundle branch block (RBBB) (n=1). Respiratory dysfunction was noted in two probands at time of study enrolment with one having a Forced Vital Capacity (FVC) 30% of predicted value. CK levels were available for seven cases with a median CK of 9076 IU/L (IQR, 2885-11,352). Muscle biopsies were abnormal in three cases with absent dystrophin staining. However, since the availability of local dystrophin gene testing, few cases have muscle biopsies performed.

Table 3.3 Clinical features present on examination at study enrolment in probands diagnosed with dystrophinopathy.

Clinical Phenotype	AAO (years)	Age at diagnosis	Wheelchair-bound	Muscle atrophy	Calf pseudohypertrophy	Contractures	Pattern of weakness	Systemic involvement
BMD	18	28	No	Proximal LL (Quads)	Present	B/L ankles	Proximal LL, Distal UL & LL	Cardiac
BMD	12	37	No	Proximal UL & LL (SCM, quads)	Present	B/L ankles	Proximal UL & LL, Axial	Cardiac
BMD	26	29	No	Proximal UL & LL (Pec, hamstrings, quads)	Absent	Nil	Proximal LL	Cardiac
BMD	16	21	No	Nil	Present	Nil	Proximal LL	Cardiac
DMD	1	15	No	Proximal UL (Biceps)	Present	Nil	Proximal UL & LL, Distal LL	Cardiac
DMD	3	12	Yes	Generalised	Present	Elbows, knees & ankles	Generalised	Cardiac & Respiratory
DMD	6	24	Yes	Generalised	Present	Elbows, hips, knees & ankles	Generalised	Cardiac & Respiratory
DMD	5	N/R	N/R	N/R	N/R	Elbows	Generalised	Cardiac
DMD	6	21	N/R	N/R	Present	Large joints	Generalised	N/R
DMD	6	14	Yes	Proximal UL & LL (SCM, quads)	Absent	Knees & Ankles	Generalised	Cardiac

Table 3.3 Generalised atrophy/weakness refers to severe widespread muscle involvement of proximal and distal upper and lower limbs. Age at diagnosis refers to confirmed genetic diagnosis.

AAO: Age at symptom onset, BMD: Becker Muscular Dystrophy, B/L: bilateral, DMD: Duchenne Muscular Dystrophy, LL: lower limb, N/R: not reported, pec: pectoralis major, quads: quadriceps femoris, SCM: sternocleidomastoid, UL: upper limb.

Three of four probands had phenotypes in keeping with BMD and the remainder were DMD according to the European Neuromuscular Centre (Table 3.2). One patient had a pseudometabolic presentation and reported muscle cramps, exercise-induced weakness and myoglobinuria with onset at 16 years. The muscle weakness was mild (MRC grade 4/5) only in the proximal lower limbs with a positive Gower's sign and calf muscle pseudohypertrophy.

Young males presenting with pelvic girdle weakness, calf pseudohypertrophy or cardiac involvement in the overall cohort underwent *DMD* MLPA (n=19). Of these patients, *DMD* MLPA was positive in 53% (8/19) of cases. Among those confirmed with DMD, two cases were falsely negative for *DMD* via MLPA. The one proband had a splice region deletion, c.4519-2_4523, that spanned intron 32 and exon 33 of the *DMD* gene predicted to cause skipping of exon 33 detected by WES. The other was *DMD* negative on MLPA and had absent dystrophin staining on muscle biopsy. He underwent WGS which detected a pathogenic c.503del (p.Ala168Valfs) frameshift variant, novel to ClinVar, in the *DMD* gene on exon 6 (Table 3.4). WGS was performed directly following negative MLPA results, without intermediate exome sequencing. One proband with *DMD* deletions identified on MLPA was found to have multiple exon deletions with apparent dropout of exons 14, 15 and 18.

Table 3.4 Characterisation of dystrophin gene deletions/variants in DMD/BMD cohort.

Dystrophinopathy type	**Diagnostics method**	**Mutation type**	**Position and/or sequence variation**
BMD	MLPA	Deletion	Exons 45 - 47
BMD	MLPA	Deletion	Exons 48 & 49
BMD	MLPA	Deletion	Exons 45 - 49
DMD	MLPA	SNV	c.620T>G (Exon 7)
DMD	MLPA	Deletion	Exon 50
DMD	MLPA	Deletion	Exons 8,9,11,12,13,16,17,19,25,32,34
DMD	MLPA	Deletion	Exon 51
DMD	MLPA	Deletion	Exons 50 – 52
BMD	WES	Deletion	c.4519-2_4523del (Intron 32/Exon 33)
DMD	WGS	Frameshift	c.503del (Exon 6)

Table 3.4 BMD: Becker Muscular Dystrophy, DMD: Duchenne Muscular Dystrophy, del: deletion, MLPA: multiplex ligation-dependent probe amplification, SNV: Single Nucleotide Variant, WES: whole exome sequencing, WGS: whole genome sequencing.

This cohort of dystrophinopathy cases illustrates the phenotypic and genetic heterogeneity characteristic of DMD/BMD with mutations spanning exonic deletions, frameshift variant, splice-site deletion and nonsense SNV. Cardiac involvement was present in 89% of those with available clinical data consistent with the progressive multi-system nature of dystrophinopathy. Of the ten cases with molecularly confirmed *DMD*, MLPA was successful in 80% of cases with 20% requiring further testing, one solved via WES and the other via WGS.

3.3.4. Facioscapulohumeral muscular dystrophy (FSHD)

There were nine probands who were suspected of having FSHD based on characteristic clinical features described below. Since genetic testing for FSHD is not available in South Africa, whole blood samples were sent to the Leiden University Medical Centre in the Netherlands for testing with pulsed field gel electrophoresis (PFGE) (as per ICGNMD consortium arrangement) and *D4Z4* repeat contractions were identified, thus confirming the diagnosis of FSHD1 in eight of nine (89%) samples.

The eight confirmed FSHD1 probands, comprised six males (75%) and two females (25%), with half reporting multi-generation family history which supports autosomal dominant inheritance. Among the eight probands with FSHD, seven were of European ancestry (87.5%), representing 64% of European ancestry autosomal dominant muscular dystrophy in the cohort (7/11). One proband was of South African admixed ancestry (12.5%), representing 7% of admixed ancestry autosomal dominant muscular dystrophy (1/15). There were no FSHD cases identified in individuals of African ancestry.

Median age at symptom onset was 16 years old (IQR, 12-34) with median age at confirmed molecular diagnosis of 47.5 years (IQR, 31.5-59). Seven probands (87.5%) reported loss of independent walking with median age of 30 years (IQR, 16-47). Only one proband was wheelchair-bound at study enrolment with age at lost ambulation being 54 years. The first symptom reported by probands were mostly difficulty lifting arms above their head or proximal upper limb weakness (75%) and less commonly difficulty walking or frequent falls (25%).

Objectively, 62.5% had facial weakness (n=5) and all probands had bilateral asymmetrical scapular winging. Although three probands had facial-sparing at study enrolment, the asymmetrical weakness, adolescent or young adult symptom onset with gradual progression and scapular winging meant they were considered for FSHD testing with one having an

autosomal dominant family history. Six patients (75%) were found to have hyperlordosis. One proband had ankle contractures. All cases had muscle atrophy and weakness with predominantly moderate to severe proximal upper limb muscle weakness (MRC grade 2-3/5) and distal lower limb weakness (100%) ranging from mild to severe (MRC grade 2-4/5), axial and proximal lower limb weakness (87.5%) and distal upper limb weakness (62.5%). Seven cases were observed to have Beevor's sign, even in those three without observed facial weakness (Table 3.5). Creatine kinase levels in seven cases showed a median CK of 423 IU/L (IQR, 310-1341).

Although not all cases had facial weakness, Beevor's sign was present in 88% of cases. There was a high diagnostic confirmation rate (89%) in clinically suspected cases. Interestingly, there was predominantly European ancestry, with only one proband and his family with South African admixed ancestry.

Table 3.5 Phenotypic characteristics and distribution of atrophy and muscle weakness in the FSHD1 cohort.

AAO (years)	Muscle atrophy	Facial weakness	Scapular winging	Pattern of weakness	Symmetry	Beevor's sign
18	Generalised	Absent	Present	Proximal & Distal UL & LL, Axial	Asymmetric (R UL > L UL)	Present
10	Proximal UL & LL, Distal LL	Present	Present	Proximal & Distal UL & LL, Axial	Asymmetric (R > L)	Present
24	Proximal UL & LL, LL distal, Axial	Absent	Present	UL & LL proximal, LL distal, Axial	Asymmetric (R > L)	Present
14	Proximal UL & LL	Absent	Present	Proximal UL & LL, Distal LL, Axial	Asymmetric (R UL > L UL, L LL > R LL)	Present
14	Proximal UL & LL, Distal LL	Present	Present	Proximal & Distal UL & LL, Axial	Asymmetric (R > L)	Present
47	Proximal & Distal UL & LL	Present	Present	Proximal & Distal UL & LL	Asymmetric (R UL > L UL, L LL > R LL)	Absent
10	Proximal UL & LL	Present	Present	Proximal UL & LL, Distal LL, Axial	Asymmetric (R > L)	Present
44	Proximal UL	Present	Present	Proximal & Distal UL, Distal LL, Axial	Asymmetric (R > L)	Present

Table 3.5 Generalised atrophy refers to widespread muscle involvement of proximal and distal upper and lower limbs.

AAO: Age at symptom onset, L: left, LL: lower limb, R: right, UL: upper limb.

3.3.5. Desminopathy

Three cases of desminopathy (previously LGMD 1E) were identified by WES. Two of them were of South African admixed ancestry and one was of Asian ancestry. Two were males and one was female. One male was born of a consanguineous relationship and has a male first cousin who apparently has features of an unspecified muscular dystrophy.

The cases manifested with frequent falls, lower limb muscle weakness and difficulty climbing stairs with a mean age at onset of 32 years (SD=10) with loss of independent walking at 33 years (SD=10). None were wheelchair-bound at time of study enrolment. The mean age at which they received a confirmed molecular diagnosis was 39 years (SD=11).

One reported hearing loss. Two reported dysphagia and nasal speech was observed in two probands (Table 3.6). Two had spinal abnormalities namely, scoliosis and hyperlordosis and one had bilateral scapular winging. All three probands had muscle atrophy and weakness primarily affecting shoulder (MRC grade 4/5) and pelvic girdle muscles (MRC grade 2-4/5) with two also having mild distal upper (MRC grade 4/5) and mild to moderate distal lower limb weakness (MRC grade 3-4/5). Axial weakness was observed in all three probands. Two were noted to also have respiratory muscle weakness. All probands had EMG with myopathic features. CK levels were raised in these probands, but <5x ULN.

All three cases were noted to have cardiac dysfunction. One had a complete heart block with dilated cardiomyopathy and the second had a restrictive cardiomyopathy with heart block with both receiving pacemakers. The third was diagnosed with a myocardial infarction at 29 years old.

The case with consanguineous parents was found to have a homozygous likely pathogenic *DES* p.Gly456Arg variant. The other two cases were both found to have heterozygous known pathogenic *DES* variants, p.Arg406Trp and p.Arg454Trp.

Table 3.6 Clinical and genetic features of molecularly confirmed desminopathy cohort.

	LG 7	**LG 30**	**LG 31**
AAO (years)	44	25	28
Site of onset	Legs	Legs	Legs
Pattern of weakness	LG	LG	LG
Additional clinical features	Dysphagia	Nasal speech, dysphagia, ankle contractures	Nasal speech, facial weakness
Cardiac involvement	Moderate	Severe	Severe
Respiratory insufficiency	Present	Present	Absent
EMG	Myopathic	Myopathic	Myopathic
Inheritance	AR	AD	AD
Gene	*DES*	*DES*	*DES*
Variant	p.Gly456Arg	p.Arg406Trp	p.Arg454Trp

Table 3.6 Severity of cardiac involvement is defined according to disease-specific severity for desminopathy. Mild: Minor conduction delays with preserved systolic function, Moderate: Cardiomyopathy and arrythmias, Severe: Advanced heart failure or complete heart block (Feingold et al., 2017). AAO: Age at symptom onset, AD: autosomal dominant, AR: autosomal recessive, LG: limb-girdle.

The three cases identified with desminopathy, both autosomal dominant and recessive inheritance patterns, had dysphagia, limb-girdle muscle weakness and cardiac manifestations. The severity of the cardiac manifestations in this subgroup underscores the importance of comprehensive cardiac surveillance in desminopathy.

3.3.6. Emery-Dreifuss muscular dystrophy (EDMD)

Two probands, one male and one female of South African admixed ancestry presented with classic features of EDMD which include gradually progressive weakness, early-onset contractures and cardiac dysfunction. They each had a family history of muscle disease. One case had an X-linked recessive pattern with three maternal uncles affected and the other reported that her paternal great aunt had lower limb weakness, although paternal relatives were unavailable for confirmation. Both had age at onset during the first two years of life where an abnormal gait or delayed ability to walk was noted. They both had walking difficulties during childhood with one being wheelchair-bound by 15 years. They received confirmed genetic diagnoses at 35 and 27 years respectively.

Apart from muscle atrophy, weakness and contractures, they were both noted to have kyphosis and hyperlordosis. One case also had bilateral scapular winging. Contractures affected the elbows, ankles, neck and spine in both with one also having knee contractures. Weakness was observed in proximal upper limbs, proximal and distal lower limbs. The male patient had severe proximal upper limb (MRC grade 2/5) and distal lower limb weakness (MRC grade 1/5) with mild proximal lower limb weakness (MRC grade 4/5). The other had mild proximal upper limb, proximal lower limb and distal lower limb weakness (MRC grade 4/5). CK levels were <3x ULN in both probands. Both probands were found to have cardiac dysfunction requiring pacemaker insertion for paroxysmal atrial fibrillation and atrioventricular block (AV) with right bundle branch block (RBBB) respectively.

On WES, the female was found to have a likely pathogenic heterozygous p.Arg541Pro variant in the *LMNA* gene in keeping with EDMD2. Here the main features are autosomal dominant inheritance, both sexes affected, humeroperoneal weakness as well as cardiac abnormalities. The male patient with X-linked recessive inheritance harboured a hemizygous deletion of exons 5-6 in the *EMD* gene, novel to ClinVar, in keeping with EDMD1. Here the main features are X-linked recessive inheritance, males affected with humeroperoneal weakness and cardiac dysfunction.

The constellation of progressive humeroperoneal weakness, contractures and cardiac involvement seen in both cases are characteristic of EDMD and underscores the importance of early recognition and comprehensive multidisciplinary care for patients with EDMD.

3.3.7. Calpainopathy

Two male probands of South African admixed ancestry and African ancestry respectively were confirmed to have a calpainopathy (previously LGMD2A) on WES. The one case reported a family history of muscle disease with his half-brother and uncle both being wheelchair-bound by 40 years old. Both his half-brother and uncle died between 40-50 years (Figure 3.5). The other case had no family history of myopathy. Neither proband was born of a consanguineous relationship.

They both reported initial symptom being lower limb weakness with age at symptom onset at 35 years. Genetic diagnoses were made at 57 and 51 years old respectively. One had loss of ambulation at 51 and the other had loss of independent walking at 35 years old.

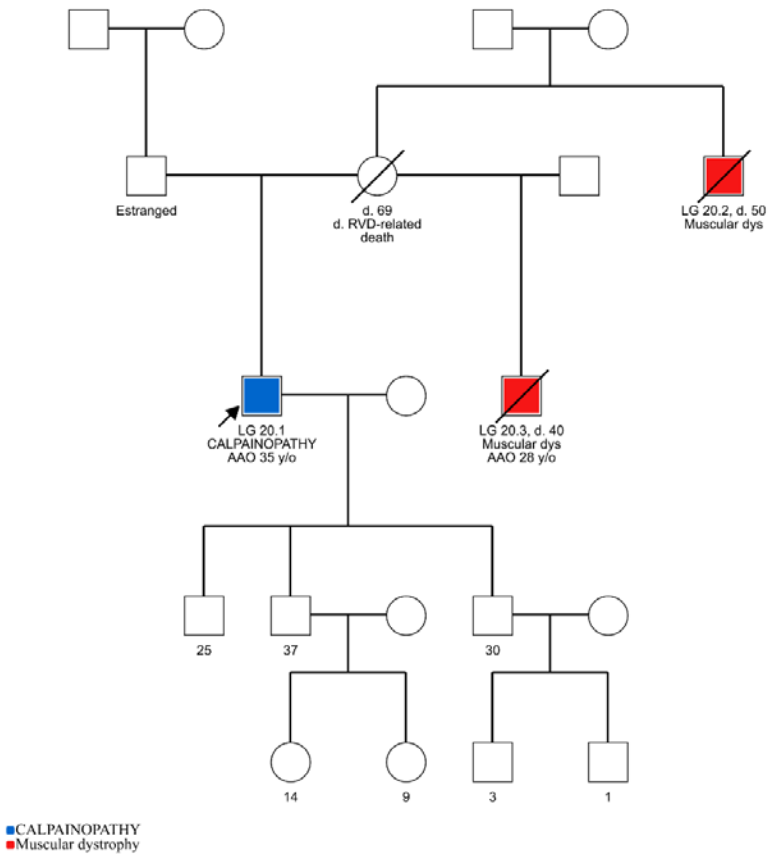


Figure 3.5 Pedigree of family discussed above. Symbols with red blocks were found to have features of muscular dystrophy without molecular confirmation. The blue block represents molecularly confirmed calpainopathy.

On examination, both had scapular winging with spinal abnormalities including hyperlordosis and the second had kyphosis, scoliosis and hyperlordosis. There were no contractures present. They both had proximal upper and lower limb muscle atrophy with symmetrical severe proximal upper (MRC grade 2/5) and mild lower limb weakness (MRC grade 4/5) as well as axial weakness. Quadriceps sparing was noted in one case, but with no distal weakness observed. The CK level was <2x ULN in one and >15x ULN in the other. The case with the lower CK level also had respiratory muscle weakness (FVC 54% of predicted value) suggesting more chronic burnt-out disease. Neither had any cardiac dysfunction.

They were both found to have calpainopathy consistent with autosomal recessive inheritance (LGMDR1). A homozygous likely pathogenic p.Ala133Thr variant in *CAPN3* was identified in LG 20.1 and is rare in population databases (gnomAD v4.1.0 allele frequency = 0.000016 in 62,498 alleles in remaining population)(Karczewski et al., 2020) and putative compound heterozygous *CAPN3* variants, comprising a pathogenic p.Arg489Gln and p.Cys414Gly VUS

were identified in the African patient. The p.Cys414Gly variant discovered in this case is a VUS that is novel to ClinVar. Pathogenicity could not be confirmed as functional and segregation data were unavailable.

The pedigree structure, specifically the pattern of affected maternal relatives, is strongly suggestive of X-linked inheritance. This lineage pattern is discordant with the autosomal recessive inheritance typically associated with calpainopathy, making pseudo-dominance an unlikely explanation for the broader family history. The possibility of two distinct genetic muscle conditions segregating in this family cannot be excluded. As these family members are deceased and unavailable for testing, this cannot be confirmed.

In conclusion, these two cases illustrate the phenotypic variability seen in calpainopathy. Their clinical courses, spanning over two decades of diagnostic uncertainty, highlight the impact of delayed diagnosis in under-resourced settings and the spectrum of genetic findings, including a *CAPN3* VUS novel to ClinVar. Notably, the family described demonstrates the diagnostic complexity that may arise when more than one IMD may be segregating.

3.3.8. Dysferlinopathy

Two probands, one female and one male of South African admixed and European ancestry respectively were found to have dysferlinopathy (LGMDR2, previously LGMD2B). Both have affected siblings with features of muscle disease consistent with autosomal recessive inheritance. Nil consanguinity was reported in either case. Age at symptom onset was at 25 and 17 years respectively. Both probands initially presented with pelvic girdle and proximal lower limb weakness. They received confirmed molecular diagnoses at 49 and 60 years respectively. The one had loss of independent walking at 37 years and loss of ambulation at 39 years old. The admixed family was previously reported (Jalali-Sefid-Dashti et al., 2018).

Contractures were evident in the female proband affecting elbows and ankles bilaterally. Muscle atrophy and weakness was present in both probands across proximal and distal upper and lower limbs. Lower limbs were more affected than upper limbs with severe hip flexion, extension, abduction, adduction and ankle plantarflexion weakness (MRC grade 1/5). No cardiac or respiratory dysfunction were noted.

The CK level was >17x ULN. Although muscle biopsy was performed in both probands, the findings were non-specific and in keeping with muscular dystrophy (marked variation in fibre size, fibre degeneration/regeneration and increase in endomyseal fibrous tissue). Dysferlin staining was absent in one case and inconclusive in the other. WES revealed homozygous

pathogenic *DYSF* p.Tyr1433Ter and p.Tyr1451Ter variants respectively. The homozygous *DYSF* p.Tyr1433Ter was reported as novel in this admixed family (Jalali-Sefid-Dashti et al., 2018).

These two dysferlinopathy cases illustrate the typical, posterior compartment distal leg weakness, yet variable clinical spectrum of dysferlinopathy observed in South African patients with LGMDR2.

3.3.9. Collagen VI-related myopathy (COL6-related myopathy)

Two female probands of South African admixed ancestry were found to have Collagen VI-related myopathy. There was no family history of disease or consanguinity. They noted their initial symptoms to be difficulty standing and climbing stairs with symptom onset at 8 and 29 years respectively. LG 11 became wheelchair-bound at 48 years old. They were molecularly diagnosed at 53 and 45 years respectively.

They were both observed to have contractures of either ankles or elbows. LG 11 was found to have bilateral scapular winging and LG 13 had hyperlordosis. LG 11 had proximal and distal upper limb atrophy and LG 13 had proximal upper limb wasting. Muscle weakness was present globally with severe proximal lower limb weakness (MRC grade 2/5) in both and severe proximal upper limb (MRC grade 2/5) and mild distal lower limb weakness (MRC grade 4/5) in LG 11. There was sparing of distal lower limb muscles in LG 13. Respiratory insufficiency was present in both with nil signs of cardiac dysfunction.

CK levels ranged between normal and mildly elevated (<2x ULN). WES identified a pathogenic heterozygous *COL6A1* p.Gly263Val variant in LG 11. LG 13 had pathogenic putative compound heterozygous variants, p.Arg468Ter and p.Arg876His, identified in the *COL6A2* gene.

These cases illustrate the genetic heterogeneity of COL6-related myopathy, with heterozygous and compound heterozygous variants identified within a single South African cohort, demonstrating a convergent clinical phenotype of progressive limb-girdle weakness with contractures and respiratory involvement.

3.3.10. GNE myopathy with predominant features of limb-girdle weakness

A proband and her brother were affected with similar muscle symptoms and were found to have autosomal recessive GNE myopathy (Figure 3.6). They were born of a consanguineous marriage.

The proband noted difficulty lifting her arms above her head at 25 years old. She was wheelchair-bound by 34 years. On examination at 43 years, she was noted to have bilateral ankle contractures. She had severe weakness affecting primarily proximal upper and lower limbs (MRC grade 0/5) with severe quadriceps weakness (MRC grade 1/5). She also had axial muscle weakness and global areflexia. CK was mildly elevated at <2.5x ULN. She had an abnormal EMG with myopathic features. The muscle biopsy showed an increase in muscle fibre variation, extensive fibroadipose tissue replacement, scattered angular atrophic fibres and no rimmed vacuoles reported.

Her brother first noted difficulty walking and a waddling gait at 23 years. He was wheelchair-bound by 38 years. On examination, muscle atrophy was noted in upper and lower limbs and pseudohypertrophy of the deltoid muscles bilaterally. He had left sided scapular winging. He had reduced tone globally and areflexia. He was weak with moderate to severe proximal upper limb weakness (MRC grade 2/5) and mild distal upper limb weakness (MRC grade 4/5). He had severe distal (MRC grade 0/5) and proximal lower limb weakness (MRC grade 1/5) with relative quadriceps-sparing (MRC grade 5/5). No contractures were noted.

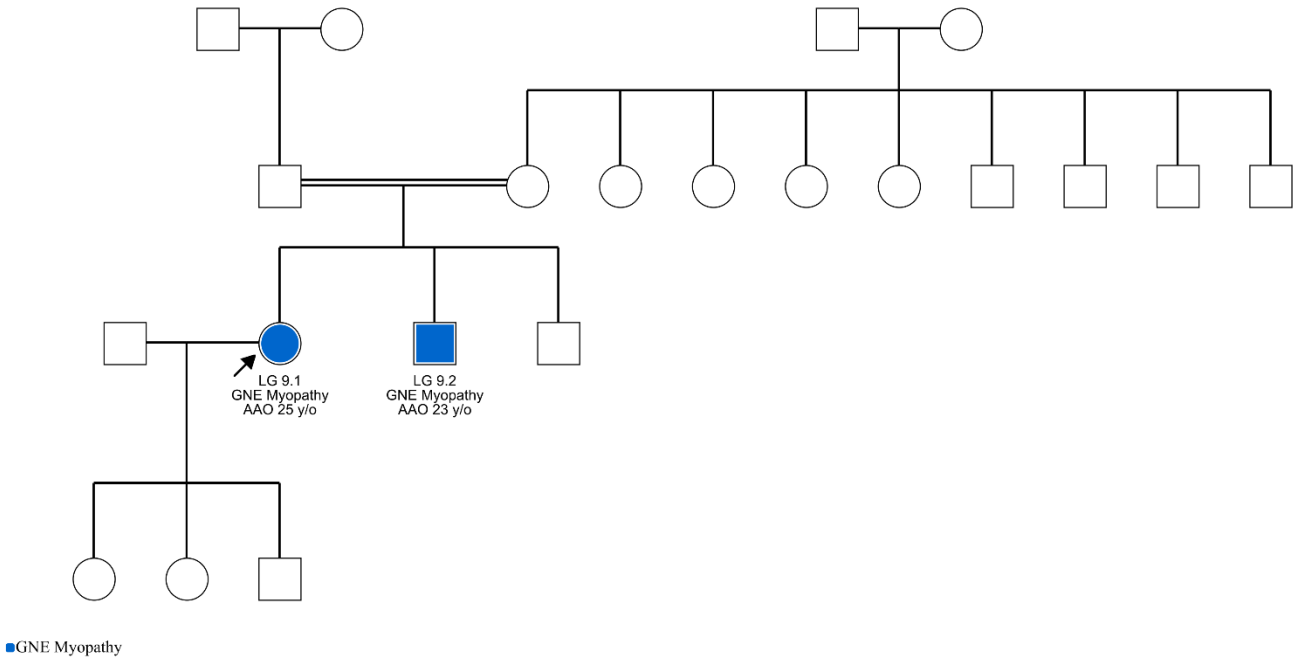


Figure 3.6 Pedigree of the above family. Blue symbols represent those diagnosed with GNE myopathy following WES.

WES identified a likely pathogenic homozygous *GNE* p.Met265Thr. This variant has been reported in affected individuals of European, Asian, and Middle Eastern ancestry, in both the homozygous and compound heterozygous states (No et al., 2013). While not a novel

variant, it spans the allosteric region of *GNE*. Heterozygous variants in this region have previously been reported to cause a different GNE-associated condition called Sialuria that does not typically present with weakness.

While GNE myopathy typically presents with distal weakness and particularly foot drop, this sibship was found to have predominantly proximal weakness with severe quadriceps weakness in the proband.

3.3.11. Glycogen storage disease IXd

A male of African ancestry reported difficulty walking due to lower limb weakness with onset at 11 years. He had no affected relatives. On examination, he was found to have hyperlordosis, scoliosis, bilateral ankle contractures and mild proximal lower limb weakness. He was noted to have a sensory neuropathy. There were no signs of cardiac or respiratory dysfunction present. His CK levels were normal (86 IU/L), and nerve conduction studies were normal. His EMG was found to be myopathic.

He was diagnosed with X-linked recessive glycogen storage disease IXd (GSD IXd) due to a *PHKA1* p.Arg428His VUS detected via WES that was novel to ClinVar. The clinical phenotype together with the identified *PHKA1* variant supported a working diagnosis of GSD IXd, although the VUS classification warrants cautious interpretation and ongoing re-evaluation as further evidence emerges.

3.3.12. RYR1-related myopathy

A male of South African admixed ancestry had been reported to be floppy and weak since birth with delayed motor milestones as he started walking at 2 years. Later, he noted fatigue when running. There were no affected relatives. On examination, he was noted to have facial weakness, high arched palate, scoliosis, ligamentous laxity and pes cavus. He had shoulder and pelvic girdle muscle weakness without scapular winging. Contractures and ophthalmoparesis were absent. There were no features of cardiac dysfunction present. He had a normal CK level and muscle biopsy was not performed.

A sample was sent for WES and he was found to have a heterozygous likely pathogenic *RYR1* p.Ile4898Thr variant, novel to ClinVar, associated with RYR1-related myopathy.

3.3.13. SELENON-related myopathy

A female of European ancestry reported that she had neck flexor weakness at birth. On examination at 35 years, she had ptosis, nasal speech, scapular winging, scoliosis and rigid spine. She had mild symmetrical proximal upper and lower muscle weakness (MRC grade 4/5) with neck flexor weakness. She was found to have respiratory failure, with an FVC of 37% of predicted, and pulmonary hypertension.

WES revealed two pathogenic putative compound heterozygous *SELENON* variants that have not been confirmed in *trans*. The first was a known pathogenic missense p.Gly315Ser variant in exon 7 and the other was a canonical splice site c.301+2T>C variant in intron 2 novel to ClinVar. The combination of infantile neck flexor weakness, rigid spine with scoliosis, respiratory dysfunction and biallelic pathogenic *SELENON* variants is in keeping with autosomal recessive SELENON-related myopathy (rigid spine muscular dystrophy).

3.3.14. Autosomal dominant optic atrophy plus syndrome (ADOA plus)

One male of European ancestry reported visual impairment at 28 years associated with proximal muscle weakness. Affected relatives were present across multiple generations consistent with autosomal dominant disease including his sister, father and paternal grandfather who were all reported to have abnormal gait and visual impairment. In addition, two brothers and their offspring (two nieces and one nephew) reported visual impairment. On examination, he was observed to have optic atrophy, proximal muscle atrophy, moderate proximal upper limb (MRC grade 3/5) and mild proximal lower limb weakness (MRC grade 4/5) as well as sensory neuropathy in a glove and stocking distribution.

He had a mildly elevated CK level (394 IU/L). WES identified a pathogenic heterozygous deletion of exons 2-5 in the *OPA1* gene consistent with ADOA plus syndrome.

3.4. Extraocular muscle (EOM) weakness in the setting of myopathy

The predominant feature in this category were ocular signs with or without non-ocular muscle weakness. Nineteen of the 89 probands were classified as having extraocular muscle (EOM) weakness in the setting of myopathy (21%). Of these, 53% were male (n=10). Nine probands were of South African admixed ancestry (47%), six were of European ancestry (32%), two were of African ancestry (11%) and two were of Asian ancestry (11%). Seven probands (37%) had a family history of a similar phenotype with muscle symptoms and EOM weakness.

Median age at onset of symptoms was 37 years old (IQR, 20-58). The median age at which probands received confirmed genetic diagnoses was 66 years old (IQR, 57-70). Median time to genetic diagnosis was 11 years (IQR, 7-18). Six of the probands (32%) had loss of independent walking at median age of 63 years (IQR, 54-68). Nil probands had loss of ambulation at time of study enrolment.

3.4.1. Clinical features

The most reported initial symptom was ptosis which was present in 84% (n=16). Eleven probands had bulbar signs with 9 (47%) reporting dysphagia and two (11%) having neck weakness.

One proband (5%) reported seizures and one (5%) had cognitive dysfunction. On examination, five probands (26%) had spinal abnormalities, namely scoliosis, kyphosis and hyperlordosis. Seventeen of the probands had ptosis (89%), 13 had ophthalmoparesis (68%) and eight (42%) had facial weakness.

Twelve of the probands (63%) had non-ocular muscle weakness with predominantly proximal lower limb (58%) and upper limb weakness (37%), followed by axial (26%) and then distal upper (21%) and lower limb muscle weakness (11%). Cardiac dysfunction and respiratory muscle insufficiency were observed in 16% and 32% of probands respectively. Ten probands (53%) had CK levels available with a median CK level of 229 IU/L (IQR, 157-454). Muscle biopsy was performed in six probands with no information available for one. The most common abnormal specific finding was ragged red fibres (n=3), while others demonstrated inflammatory infiltrates (n=1) or mild non-specific atrophy (n=1).

3.4.2. Genetic findings

Of the 19 probands classified as having EOM weakness with or without non-ocular muscle weakness, 12 (63%) had a confirmed genetic diagnosis while seven remained unsolved (37%). Three (25%) were solved via locally available mtDNA deletion testing (tier 1) and nine (75%) were solved via tier 2 testing with five solved via *PABPN1* repeat expansion assay and four solved via WES.

The most frequent genetic diagnosis was oculopharyngeal muscular dystrophy (OPMD) due to an expansion in *PABPN1*, which was found in 42% of those solved (n=5). The next most common were the single large deletions in *mtDNA* which was identified in 25% of the EOM weakness cohort (n=3). *RRM2B* gene variants were identified in two probands with one being

an inconclusive VUS and reported as possibly solved. The *TWNK* (n=1) and *MT-ATP6* (n=1) genes were identified in one case each.

3.4.3. Oculopharyngeal muscular dystrophy (OPMD)

There were eight probands suspected of having OPMD. Of these, five (63%) were genetically confirmed to have OPMD via *PABPN1* repeat expansion testing. Three were female (60%) and two were male (40%). Four were of South African admixed ancestry (80%) and one was of European ancestry (20%). Median age at onset was 52 years (IQR, 41-55). The median age at confirmed molecular diagnosis was 66 years old (IQR, 57-70) with a median diagnostic delay of 14 years (IQR, 11-17). All probands had a positive family history of disease consistent with autosomal dominant inheritance.

Ptosis was the initial symptom noted in all probands. On examination, kyphosis was noted in one patient. Two had ophthalmoplegia and four had dysphagia at time of study enrolment. Two cases had facial weakness and three had neck muscle weakness. Three patients were noted to have mild proximal weakness (MRC grade 4/5). Two had both proximal upper and lower limb weakness and one had only proximal lower limb weakness (Table 3.7). Two patients had respiratory dysfunction. Mean CK levels were >1.5 ULN.

Table 3.7 Summary of clinical and genetic findings in OPMD probands.

Sex	AAO (yrs)	Age at diagnosis (yrs)	Dysphagia	Ptosis	Ophthalmoplegia	Muscle weakness distribution	No. of GCN repeats
M	52	63	Present	Present	Absent	Mild facial weakness	14
F	44	54	Present	Present	Absent	Proximal and distal LL, neck flexor & facial weakness	14
M	55	62	Present	Present	Present	Neck flexor weakness	14
F	55	72	Absent	Present	Present	Mild proximal UL & LL	14
F	60	76	Present	Present	Absent	Mild proximal UL & LL, neck extensor weakness	No. of GCN repeats unavailable*

Table 3.7 MRC Power Grading - Mild: 4/5, Moderate: MRC 3/5, Severe: <3/5. *No. of GCN repeats unavailable for EOM 16 as results are pending, but diagnosis confirmed in her sister in Scotland.
AAO: Age at onset, M: male, F: female, UL: upper limb, LL: lower limb.

The *PABPN1* repeat expansion assay (performed at UCL, UK) revealed that four of the five probands were heterozygous for a *PABPN1* $(GCN)_{14}$ expanded allele and the other was awaiting diagnostic confirmation (delayed due to technical issues), but her sister had previously been molecularly confirmed in the UK with a *PABPN1* expansion and OPMD. In addition, two of these probands' *PABPN1* repeat expansions were independently confirmed on research re-analysis of existing WGS data using a specialised STR-genotyping pipeline (Mngadi et al., unpublished).

These findings indicate that OPMD represents a relatively common cause of ptosis with myopathy and dysphagia in South Africa. All the patients presented with cardinal features of OPMD with four carrying the $(GCN)_{14}$ repeat expansion in *PABPN1*, consistent with the molecular hallmark of OPMD. The marked diagnostic delay is likely due to historical unavailability of *PABPN1* testing in South Africa. The majority of probands (n=4/5) were of South African admixed ancestry and one was of European ancestry.

3.4.4. Mitochondrial myopathy

Seven of the probands were found to have mitochondrial myopathies as their cause of EOM weakness. Four were female (57%) and three were male (43%). Four were of South African admixed ancestry (57%) and three were of European ancestry (43%). The median age at symptom onset was 58 years (IQR, 25-59). Median age at confirmed molecular diagnosis was 66 years (IQR, 37-70). Median diagnostic delay was 8 years (IQR, 7-18).

The predominant symptom that was noted initially was ptosis (86%, n=6/7) with one proband noting dysphagia initially and noticing ptosis six years later. On examination, one proband had scapular winging with scoliosis. All probands in this subgroup had ophthalmoparesis. Three probands developed facial weakness (43%), five had bulbar signs, namely dysphagia (n=3) and neck muscle weakness (n=5) with one reporting dysarthria. Five (71%) probands developed muscle weakness, predominantly mild proximal upper and lower limb muscle weakness.

Chronic progressive external ophthalmoplegia (CPEO) due to a single large mitochondrial deletion on muscle mtDNA confirmed by tier 1 testing after clinical suspicion of this clinical diagnosis was detected in three probands. All three had bilateral ptosis and ophthalmoplegia. One was found to have pigmentary retinopathy. The other two had mild weakness of proximal upper and lower limbs (MRC grade 4/5).

The remaining four probands were solved by WES. The first proband presented with unilateral ptosis, dysphagia, upgaze palsy and diplopia. He was found to have MT-ATP6-related mitochondrial myopathy with a heterozygous p.Gly167Asp variant in the *MT-ATP6* gene that was previously reported (Wang et al., 2022). It is currently classified as a VUS.

The next proband presented with progressive external ophthalmoplegia, bilateral ptosis, facial muscle weakness and scapular winging. He had mild proximal upper and lower limb weakness (MRC grade 4/5). He later developed respiratory insufficiency, noted at the time of study enrolment, with FVC 60% of predicted value.

He was found to have adult-onset PEO with proximal myopathy and WES revealed two heterozygous VUS in *cis* in the *TWNK* gene, namely p.Leu373Val and p.Glu375Val which were both reported as novel to ClinVar. Genetic testing of his affected son confirmed both variants were inherited together. As both variants occur in *cis*, it is not possible to determine which variant is pathogenic without functional studies, although one is likely disease-causing.

The third proband presented with adult-onset ptosis, ophthalmoplegia, proximal lower limb weakness and fatigable weakness of proximal limb muscles. She had respiratory and cardiac dysfunction. WES revealed a homozygous *RRM2B* p.Pro149Leu inconclusive VUS that is considered possibly solved. Finally, one presented with ptosis, ophthalmoplegia, fatigable weakness, dysphagia and proximal upper and lower limb muscle weakness. She was confirmed to have a likely pathogenic heterozygous p.Ile224Ser variant in the *RRM2B* gene.

In conclusion, this sample of probands with prominent EOM dysfunction with ptosis and/or ophthalmoplegia and myopathy with symptom onset from adult to late adulthood, demonstrated a range of pathogenic mechanisms. The most frequent findings were large single mtDNA deletions associated with CPEO and *RRM2B*, *MT-ATP6* and *TWNK* variants. Of these variants, several were novel to ClinVar. Ocular features such as ptosis and ophthalmoparesis were predominant and were often accompanied by bulbar, facial and limb muscle weakness. Clinically fatigable weakness of limb muscles was observed in both probands harbouring *RRM2B* variants, but only one had a confirmed likely pathogenic variant.

3.5. Myotonia-related symptoms

Ten of the 89 probands were classified as having myotonia-related symptoms (11%). Seven probands (70%) were male. There were seven probands of European ancestry (70%) and three of South African admixed ancestry (30%) and notably none with African ancestry. Five

of the probands (50%) had a family history of similar disease corresponding with autosomal dominant inheritance.

The median age at symptom onset was 15 years (IQR, 9-30). Median age at confirmed molecular diagnosis was 44 years (IQR, 26-52). There was a median diagnostic delay of 17 years (IQR, 6.5-27.5). Four probands had loss of independent walking at a median age of 36 years old (IQR, 12-59). Nil probands had loss of ambulation.

3.5.1. Clinical features

The most prominent symptom initially was of myotonia and muscle stiffness with delayed relaxation in seven cases (70%). The other three each reported a different initial symptom including stiffness of legs, distal muscle weakness and difficulty walking after standing up. Six probands (60%) reported dysphagia and dysarthria. On examination, two probands (20%) were observed to have frontal balding, two (20%) had hyperlordosis and four (40%) had facial weakness. Four (40%) had ptosis. Myotonia was noted in all probands. Muscle weakness was present in four probands in predominantly the distal upper and lower limbs (75%), axial (50%) and one proband had proximal upper limb weakness (25%).

CK levels in four probands showed a median CK of 1.5x ULN (299 IU/L, IQR 197-553). Cardiac dysfunction was present in two probands. There was no respiratory insufficiency noted in the probands.

3.5.2. Genetic findings

Nine of the ten probands (90%) had a confirmed molecular diagnosis. One proband who initially reported myotonia with warm-up phenomenon remained unsolved following WES. Of those solved, five were confirmed to have Myotonic dystrophy type 1 (DM1) via locally available *DMPK* repeat expansion testing (tier 1). The other four probands were diagnosed with Myotonia congenita due to pathogenic variants in the *CLCN1* gene which were detected via NGS (tier 2 testing).

3.5.3. Myotonic dystrophy (DM1)

Of the five probands found to have DM1, three were of South African admixed and two were of European ancestry with no individuals of African ancestry present in the cohort. Four of them had a positive family history of disease in keeping with autosomal dominant inheritance. Two had childhood-onset (< 20 years) of symptoms and three had adult-onset (> 20 years) of symptoms. On inspection, frontal balding was present in four of the probands.

Four probands (80%) were noted to have ptosis. Besides the myotonia, they had predominantly mild distal upper and lower limb weakness (MRC grade 4/5) with facial muscle weakness. No cataracts were noted in any case. Two probands had cardiac conduction defects.

There were seven patients who were suspected of having myotonic dystrophy who underwent locally available *DMPK* repeat expansion testing and these five probands (71%, 5/7) were molecularly confirmed to have DM1 with CTG repeats > 50 considered pathogenic (tier 1).

Notably, both childhood- and adult-onset presentations were identified within this cohort with approximately half exhibiting cardiac conduction defects, highlighting the systemic nature of the disease.

3.5.4. Myotonia congenita

Myotonia congenita was diagnosed in four probands. They were all of European ancestry with three males and one female. All probands had childhood-onset of symptoms (<20 years). Muscle stiffness was reported by all probands with warm-up phenomenon present. On examination, skeletal muscle hypertrophy was observed in three probands (75%) and percussion myotonia was elicited in all probands.

They were mainly found to have pathogenic putative compound heterozygous variants in the *CLCN1* gene, although these were not confirmed in *trans*. One proband had a pathogenic heterozygous *CLCN1* p.Phe307Ser variant and a heterozygous c.2596-11C>G VUS (Table 3.8).

Table 3.8 Mutational profile of cohort confirmed with myotonia congenita.

Gene	Inheritance	Variant 1	ACMG Classification	Variant 2	ACMG Classification
CLCN1	AR	p.Phe307Ser	P	c.2596-11C>G	VUS
CLCN1	AR	p.Arg894*	P	p.Gly285Glu	P
CLCN1	AR	p.Arg894Ter	P	p.Pro480HisfsTer24	P
CLCN1	AR	c.1471+1G>A	P	p.lle527Ser	P

Table 3.8 AR: autosomal recessive, P: pathogenic, VUS: variant of uncertain significance.

These cases with myotonia congenita were defined by early childhood-onset, classic symptoms of muscle stiffness with warm-up phenomenon, skeletal muscle hypertrophy and percussion myotonia on examination. These clinical features combined with the putative

compound heterozygous *CLCN1* variants are consistent with autosomal recessive Becker disease.

3.6. Distal myopathy

Of the 89 probands, five were classified as distal myopathies due to predominantly distal lower limb weakness (6%). Four of the probands were of South African admixed ancestry (80%) and one was of European ancestry (20%) which included three females and two males. Four of these cases had confirmed genetic diagnoses with three probands with distal nebulin myopathy and one with collagen VI-related dystrophy. The fifth proband remains unsolved.

3.6.1. Distal nebulin myopathy

There were three probands from unrelated South African admixed families found to have autosomal dominant distal nebulin myopathy. Two were female and one was male. The mean age at symptom onset was 16 years (SD=11.3). Mean age at confirmed molecular diagnosis was 49 years (SD=5.7) with an average diagnostic delay of 33 years (SD=9.5). All three had loss of independent walking. None were wheelchair-bound at time of study enrolment.

DW 2.1 had 12 affected family members who all had features of distal myopathy (Figure 3.7A). Three of them had age at symptom onset during childhood/adolescence (<20 years) with two family members noting onset of symptoms in adulthood (≥20 years). DW 3.1 had six affected family members (Figure 3.7B). DW 5.1 had nine affected family members with distal weakness (Figure 3.7C).

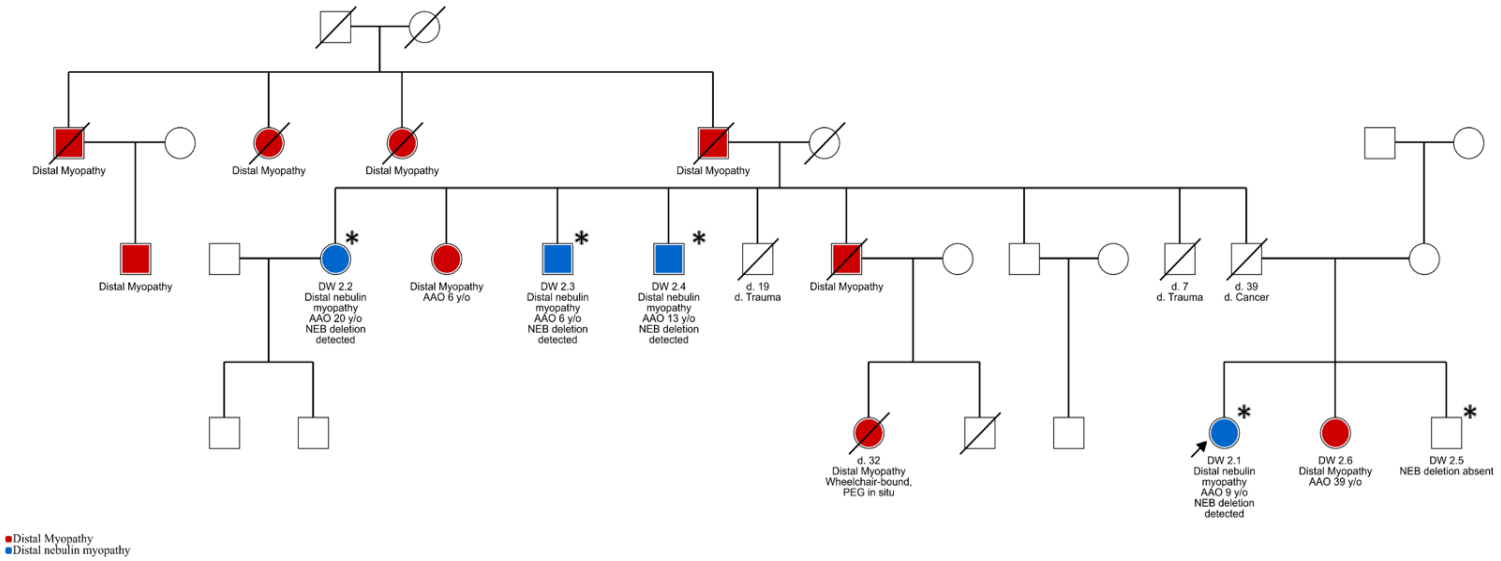


Figure 3.7A DW 2.1 Family Pedigree. All affected individuals are marked in red. Blue represents those with molecularly confirmed distal nebulin myopathy. *Patients who have undergone NGS for *NEB* deletions.

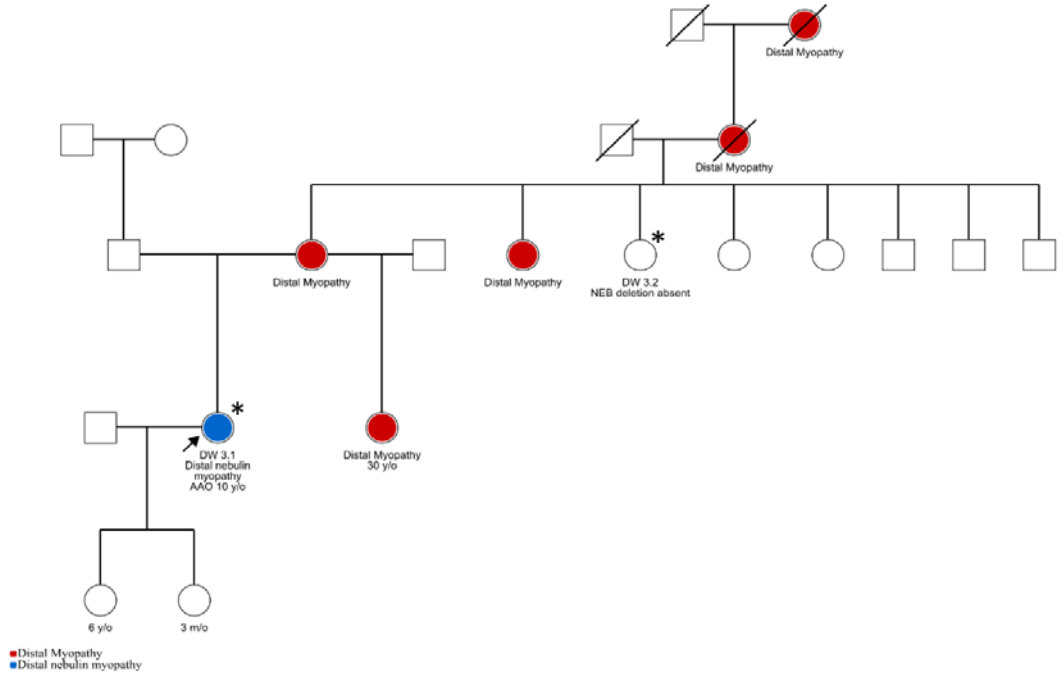


Figure 3.7B DW 3.1 Family Pedigree. All affected individuals are marked in red. Blue represents those with molecularly confirmed distal nebulin myopathy. *Patients who have undergone NGS for *NEB* deletions.

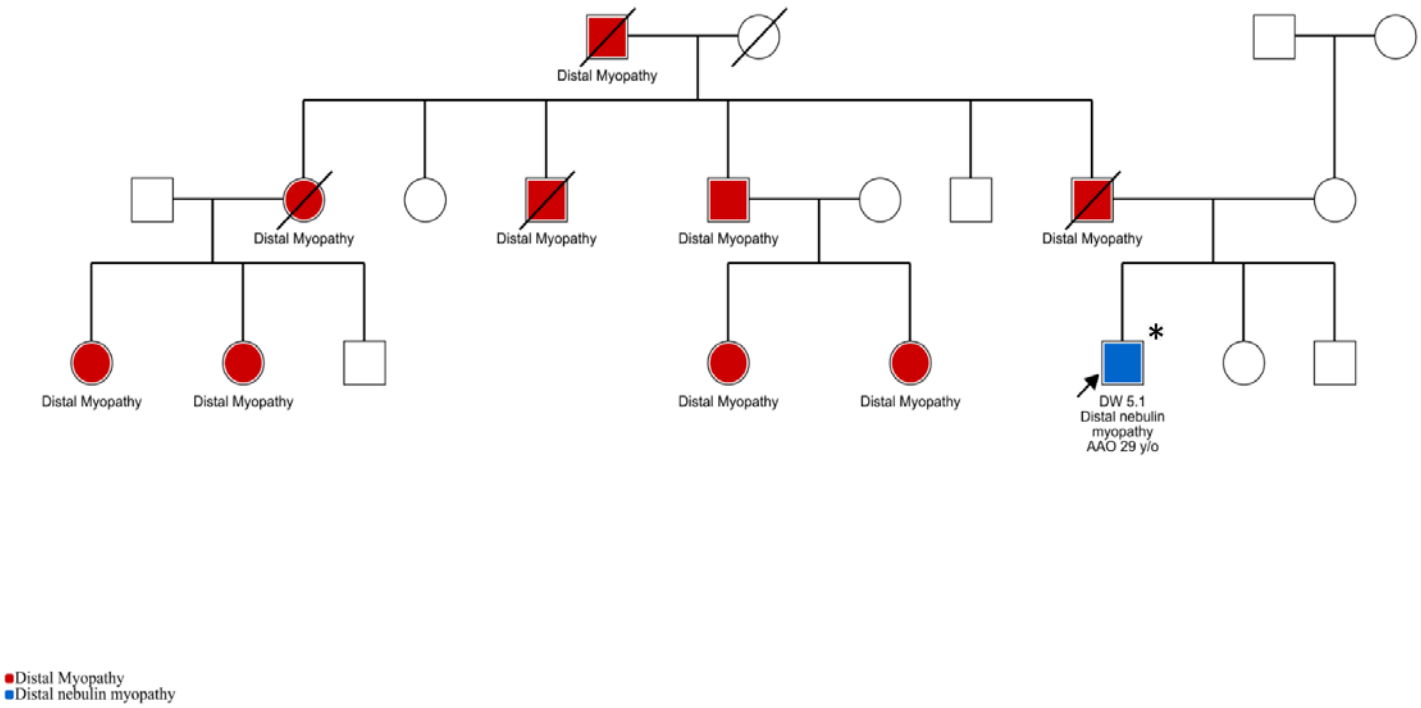


Figure 3.7C DW 5.1 Family Pedigree. All affected individuals are marked in red. Blue represents those with molecularly confirmed distal nebulin myopathy. *Patients who have undergone NGS for *NEB* deletions.

They all reported the first symptom noted as being lower limb weakness presenting as frequent falls, difficulty running and weakness in the legs for each proband respectively. One patient reported dysarthria. On inspection, DW 2.1 had facial weakness and right sided scapular winging. DW 3.1 had bilateral scapular winging and hyperlordosis. Distal upper and lower limb muscle atrophy was noted in all, with one proband (DW 2.1) also having proximal upper limb muscle atrophy. Neck flexor weakness was present in all three. All three exhibited moderate to severe weakness (MRC grade 0-3/5) predominantly affecting the distal lower and upper limbs, followed by proximal lower (67%) and upper limbs (67%), with anterior compartment involvement (foot drop) noted in the distal lower limbs (Table 3.9). They were all found to have small fibre neuropathy in a sock distribution with DW 2.1 having glove and sock distribution. Vibration and joint position sense were normal.

The CK levels were normal in all probands. EMG was performed in all probands and found to be purely myopathic for DW 2.1 and have mixed myopathic and neurogenic features for the remaining two. The muscle biopsies performed in family members years prior were non-contributory.

Using WES data, heterozygous *NEB* deletions in exons 14-77 were found in two families and segregated in both families with only affected cases inheriting the *NEB* deletion (5 affected and absent in 2 unaffected family members). In the third family a heterozygous *NEB* deletion of exons 13-75 was detected using WGS data (all courtesy of Dr Melissa Nel, COIN.

Table 3.9 Clinical and genetic features of distal nebulin myopathy cases.

	DW 2.1	**DW 3.1**	**DW 5.1**
Myopathy Type	Distal nebulin myopathy	Distal nebulin myopathy	Distal nebulin myopathy
AAO (years)	9	10	28
Site of onset	Legs	Legs	Legs & Hands
Leg compartment	Anterior	Anterior	Anterior
Weakest LL muscles	Ankle DF 3/5	Ankle DF 0/5	Ankle DF 2/5, toe extension 0/5
Inheritance	AD	AD	AD
Gene	*NEB*	*NEB*	*NEB*
Variant	deletion of exons 14-77	deletion of exons 14-77	deletion of exons 13-75

Table 3.9 AAO: Age at onset, AD: autosomal dominant, DF: dorsiflexion, LL: lower limb.

In conclusion, these three families demonstrate a multigenerational autosomal dominant distal nebulin myopathy phenotype. This cohort demonstrated variable age at symptom onset ranging from 6 to 39 years, predominantly distal lower limb weakness with gradual progression to proximal muscle weakness. A heterozygous large *NEB* deletion segregated with the clinical phenotype strengthening the phenotype-genotype association.

3.6.2. Collagen VI-related myopathy

One patient of European ancestry presented at 69 years with gradually progressive distal weakness. He reported that he initially noted frequent falls with symptom onset at 40 years. He was unable to walk independently by 50 years. He had no family history of muscle disease.

On examination, he was noted to have bilateral pes cavus, hammertoes and webbing of the 2nd and 3rd toes bilaterally. There were no contractures or joint hyperlaxity noted. He had marked forearm and peroneal atrophy. Mild neck flexion weakness was observed (MRC grade 4/5). He had predominantly lower limb posterior compartment weakness with mild weakness of upper and lower limbs (MRC grade 4/5). Extensor weakness was prominent in

the upper limbs, with finger extensors most severely affected (MRC grade 4^{-}/5), while knee flexor weakness predominated in the lower limbs (MRC grade 4^{-}/5). There was no respiratory dysfunction noted.

EMG was found to be myopathic. He had an abnormal muscle biopsy showing small aggregates of mitochondria with slightly enlarged scattered mitochondria as well as nemaline bodies with fewer Zebra bodies. WES identified a heterozygous *COL6A2* c.1179+2T>C VUS novel to ClinVar. This is a canonical splice site variant in intron 13 that is predicted to affect splicing, likely resulting in skipping of exon 13.

This case represents an unusual presentation of collagen VI-related myopathy. It is characterised by late-onset, gradually progressive distal muscle weakness, absence of contractures and joint hyperlaxity with preserved respiratory function into the seventh decade. The identification of a heterozygous *COL6A2* c.1179+2T>C variant novel to ClinVar, predicted to disrupt exon 13 splicing, expands the recognised phenotypic spectrum of COL6A2-related myopathies to include distal myopathy with a mild late-onset disease course.

3.7. Myasthenic-like syndrome with fatigable weakness

Four probands (5%) were clinically classified as having myasthenic-like syndrome with fatigable muscle weakness. They were all female and two were of European ancestry (50%) and two were of South African admixed ancestry (50%). Median age at symptom onset was one year old (IQR, 0-14). The mean age at molecular diagnosis was 49 years (SD=24.5). Mean diagnostic delay was 39 years (SD=34.9). One proband had delayed walking. There were no probands with loss of ambulation. One proband, from a consanguineous family, reported having a first cousin with symptoms suspicious for a myasthenic syndrome, but no further medical history for this relative was available. In addition, another case with clinical congenital myasthenia for more than three decades was of a consanguineous relationship but without affected siblings.

3.7.1. Clinical features

Probands reported that the first symptom noticed was weakness of proximal limbs that worsened with exercise and improved with rest (50%), waddling gait (25%) and fatigable ptosis (25%). The most frequently noted findings on exam were fatigable weakness (100%) and ptosis (100%). Apart from one individual with fatigable ptosis and scapular winging, the remainder had fatigable ptosis and limb muscle weakness in proximal upper and lower limbs.

FW 2 was the only case remaining unsolved on WES. She presented with fatigable ptosis, hyperhidrosis, diplopia, hyperlordosis, scoliosis and bilateral scapular winging. She was acetylcholine receptor antibody negative. Repetitive nerve stimulation demonstrated a decrement of 9.6%. There was subjective improvement in her symptoms while being treated with pyridostigmine.

3.7.2. Genetic findings

Three of the four probands (75%) had confirmed molecular diagnoses by WES. Two unrelated probands, born of consanguineous unions, were diagnosed with congenital myasthenic syndrome (CMS), while one had a metabolic myopathy clinically mimicking a myasthenic syndrome. FW 1 presented with fatigable proximal limb weakness, ptosis, ophthalmoplegia and facial weakness. She had abnormal repetitive nerve stimulation demonstrating >10% decrement in the abductor digiti minimi (ADM) with ulnar nerve stimulation and myopathic features on EMG.

FW 3 had ptosis, ophthalmoplegia and fatigable proximal limb weakness. She was acetylcholine receptor antibody positive. FW 1 and FW 3 were found to have autosomal recessive congenital myasthenic syndrome (CMS) due to pathogenic *CHRNE* variants*;* one a homozygous *CHRNE* p.Val150ThrfsTer29 variant and the other a homozygous *CHRNE* p.Gln398Ter variant.

The final proband (FW 4) had ptosis, facial muscle weakness, hyperlordosis, fatigable proximal limb weakness and axial muscle weakness. Due to the fatigability on examination, initial investigations were for autoimmune myasthenia gravis. The acetylcholine receptor antibody test was positive, but there was no response to immune therapies and in retrospect, this was likely a false positive. The progressive pelvic girdle weakness and raised CK levels (561 IU/L) prompted further investigation for a possible genetic cause, with differential diagnoses of IMD or CMS. Therefore, WES was performed and she was diagnosed with autosomal recessive Pompe disease. Two pathogenic variants in the *GAA* gene were identified, namely a heterozygous c.-32-13T>G and a heterozygous p.Arg854Ter variant. Sanger sequencing was performed on a blood sample from her unaffected mother which supported the *trans* configuration of these pathogenic variants in the *GAA* gene. None of the probands were noted to have macroglossia, ‘second-wind’ or ‘out-of-wind phenomena’. No cardiac, respiratory or liver dysfunction were noted.

This subgroup had prolonged diagnostic delays despite childhood onset with 50% arising from consanguineous unions. While three cases achieved a molecular diagnosis, one case with neurophysiological evidence of fatigable weakness remained unsolved by WES.

3.8. Chapter conclusion

This comprehensive phenotypic and genetic characterisation of 89 probands with inherited muscle diseases had a combined diagnostic yield of 62% with 53% being solved via tier 2 testing. Notable findings include the diverse genetic ancestry composition, overall median diagnostic delay from onset of symptoms to molecular diagnosis of 16.5 years, marked phenotypic and genetic heterogeneity and autosomal dominant *NEB* deletion families. While these results demonstrate the feasibility of implementing genomic medicine approaches in resource-constrained settings, it also highlights several challenges in this area. The implications of these findings for clinical practice and diagnostic strategies in South Africa as well as the limitations and future research opportunities will be discussed in the next chapter.

Chapter 4: Discussion

This study characterised the clinical phenotypes and mutational profile of inherited muscle diseases in a heterogeneous South African cohort of 89 probands, utilising a tiered diagnostic approach that incorporated both limited locally available genetic testing and advanced next generation sequencing technologies. To our knowledge, this study represents the largest reported adult cohort of heterogeneous IMD from a South African tertiary neuromuscular clinic. Among 89 patients, molecular diagnoses were established in 62%, with about one-third determined using locally available genetic tests including the *DMD* MLPA, *DMPK* repeat expansion assay and mtDNA deletion testing. However, genetic diagnoses in 53% (39/73) required genetic testing unavailable in the state sector in South Africa, including repeat expansion testing for *PABPN1*, FSHD genetic testing (See Methods section 2.6), WES and WGS. These figures illustrate both the diagnostic utility of targeted first-line testing and the substantial diagnostic gap in current South African neuromuscular services, highlighting the potential role of advanced genomic approaches in identifying inherited muscle diseases in our setting.

The following sections are organised by testing pathway rather than traditional disease class. Clinically recognisable phenotypes such as dystrophinopathy (*DMD* MLPA), myotonic dystrophy (*DMPK* repeat expansion), FSHD (*D4Z4* repeat contraction) and OPMD (*PABPN1* repeat expansion) that were amenable to single-gene, repeat expansion or repeat contraction testing, whether performed locally or via international laboratories, are discussed first, followed by the remaining IMD presentations that required NGS, integrating diagnostic delay, clinical features and molecular findings in each group.

Figure 4.1 presents a descriptive summary of the testing pathways utilised in this cohort, including diagnostic yields. The recognisable phenotypes demonstrated diagnostic yields ranging from 53% (DMD) to 89% (FSHD). These yields reflect the high positive predictive value of clinical pattern recognition in these disorders, supporting targeted testing for these specific presentations. However, important ancestry-specific considerations emerged, namely that FSHD and DM1 diagnoses were only confirmed in European and South African admixed ancestry individuals. The majority of patients required WES or WGS for molecular diagnosis for two principal reasons. First, many presented with heterogeneous phenotypes in which clinical features alone were insufficient to prioritise a specific gene among multiple candidates. Second, even when clinical features raised strong suspicion of specific genetic

causes such as calpainopathy or dysferlinopathy, targeted single-gene testing for these conditions was unavailable in South Africa, necessitating WES via research collaboration. These approaches achieved an overall diagnostic yield comparable to those reported in international cohorts (Babić Božović et al., 2021).

These patterns support a pragmatic hybrid approach combining targeted testing for a small subset of highly recognisable clinical patterns with NGS for most patients with non-specific or heterogeneous presentations, with consideration of locally documented ancestry-enriched elements where relevant. Given the modest size of this cohort, these patterns should be interpreted as exploratory observations on how clinical presentations and genetic findings aligned in this series, generating hypotheses for similar settings rather than providing formal guidance on diagnostic practice.

4.1. Recognisable phenotypes diagnosed by single-gene testing

Thirty-two patients presented with clinically distinctive phenotypes that enabled targeted gene-specific, repeat expansion or repeat contraction testing. There were four conditions in this group, namely dystrophinopathy, FSHD, OPMD, and DM1 (Figure 4.1). Although these conditions reflect diverse inheritance patterns, they share the defining feature that phenotype reliably predicted genotype, permitting efficient use of targeted molecular testing either available locally or via international research consortia.

Muscular dystrophies comprised almost half of the total cohort (42%) with dystrophinopathies, the commonest form of muscular dystrophy globally (Limback et al., 2022), being the most frequent. Autosomal dominant (AD) muscular dystrophies accounted for half of the muscular dystrophy group, including FSHD, OPMD and EDMD2. Autosomal recessive muscular dystrophies comprised 19% including calpainopathy, desminopathy and dysferlinopathy. Dystrophinopathies and Emery-Dreifuss muscular dystrophy type 1 (EDMD1), both with X-linked recessive inheritance patterns, comprised 30% (11/37).

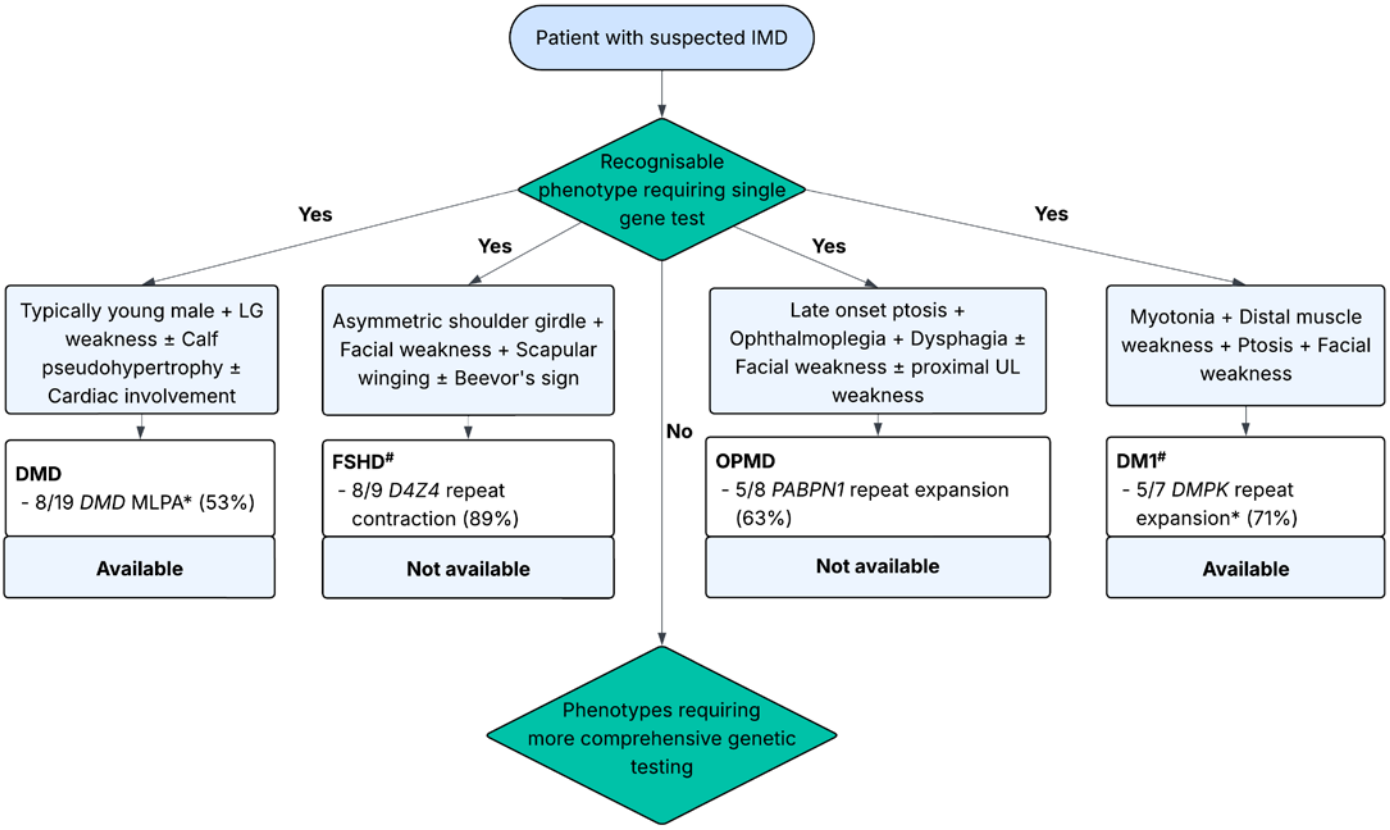


Figure 4.1 Schematic summary of the clinical presentations and corresponding genetic diagnoses identified in this South African cohort with inherited muscle diseases. The schematic illustrates that for certain highly distinctive phenotypes, targeted single-gene tests provide efficient molecular confirmation. However, other phenotypes may require comprehensive testing via NGS, either due to genetic heterogeneity with multiple possible candidate genes or unavailability of targeted test for clinically suspected genes. Diagnostic yields shown in brackets represent the proportion of genetically confirmed cases among those tested with each modality and the availability of each test in South Africa is indicated below the yield. *Tier 1 testing modalities: *DMD* MLPA and *DMPK* CTG repeat expansion were performed on blood-derived DNA. Mitochondrial DNA (mtDNA) deletion screening was performed on muscle biopsy tissue. #Ancestry-specific patterns are noted for FSHD and DM1, which were identified exclusively in European and admixed ancestry individuals in this cohort. DM1: Myotonic dystrophy, DMD: Duchenne muscular dystrophy, FSHD: Facioscapulohumeral muscular dystrophy, LG: limb-girdle, OPMD: Oculopharyngeal muscular dystrophy, UL: upper limb.

Within the muscular dystrophy group, only a few entities had sufficiently distinctive phenotypes with access to gene-specific testing. The classic DMD presentation of a young male with pelvic girdle weakness, calf pseudohypertrophy and cardiac involvement yielded a confirmed diagnosis in 53% via *DMD* MLPA (8/19 tested). The asymmetric scapular weakness, facial weakness and Beevor's sign, characteristic of FSHD, achieved 89%

confirmation. The recognisable myotonic phenotype in DM1 yielded a 71% diagnostic confirmation, while the distinctive late-onset ptosis, ophthalmoplegia and dysphagia presenting in OPMD achieved a 63% molecular confirmation. These single-gene tests are cost-effective, rapidly available and provide timely molecular confirmation enabling early access to multidisciplinary care, genetic counselling and family planning. By contrast, most limb-girdle and non-distinctive presentations showed overlapping clinical features that could implicate several candidate genes, necessitating broader genetic testing via WES or WGS. Furthermore, even when clinical features raised strong clinical suspicion for a specific condition such as calpainopathy or dysferlinopathy, single-gene testing for these conditions was unavailable in our setting and these cases therefore proceeded directly to WES.

This distribution of inheritance patterns can be compared with international cohorts, although direct comparison requires consideration of cohort composition. In Indian cohorts, autosomal recessive conditions account for up to 96% (39/41) of molecularly confirmed myopathies, reflecting high rates of consanguinity and founder mutations in genes such as *CAPN3* and *GNE*. The commonest genes identified in Indian cohorts were *GNE*, *DYSF* and *CAPN3* (Chakravorty et al., 2020; Khadilkar et al., 2022). In the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD) multinational cohort, which included South African participants, *DYSF*, *CAPN3* and *GNE* were the commonest genes identified in LGMD cases, although an explicit autosomal dominant to recessive ratio was not provided (Wilson et al., 2023). These comparisons highlight that the relative contribution of autosomal dominant and recessive muscular dystrophies in reported cohorts varies by region, reflecting not only population-specific founder effects and consanguinity rates but also socioeconomic factors that may limit family history ascertainment. This reinforces the need for region-specific data when interpreting IMD cohorts in South Africa, recognising that autosomal recessive conditions may present as apparently sporadic cases due to incomplete pedigree information.

4.1.1. Dystrophinopathy

Childhood-onset, well described conditions such as Duchenne Muscular Dystrophy (DMD) have been shown to have a mean delay between symptom onset and molecular diagnosis of 2.5 years in USA population-based surveillance studies with shortened delays in European countries with streamlined diagnostic pathways (4.7-7 months in Germany and Austria respectively)(Ciafaloni et al., 2009; Hiebeler et al., 2023). In our cohort, the median diagnostic delay for the DMD cohort was 10 years which is approximately 4-5 times longer

than international cohorts. This is noteworthy as *DMD* genetic testing has been available in South Africa via the NHLS since 2000 (Kerr et al., 2013). This prolonged diagnostic delay despite locally available testing suggests barriers beyond laboratory access, potentially including delayed specialist referral and challenges accessing specialist neuromuscular care.

Overall, genetic analysis of the DMD/BMD subgroup showed that 80% had *DMD* deletions and 20% had point mutations. Although the ICGNMD study reported (Wilson et al., 2023) differences in the mutational profile in their cohort between South African (n=40) and Indian patients (n=64), our cohort has a similar mutational profile to another Indian study (n=116) wherein they found 79% had deletions and 11% had point mutations (Goyal et al., 2021). Cohort size may affect the observed distribution of variants in this case.

One BMD patient harbouring an exon 45-49 deletion, spanning the rod domain, presented with a pseudometabolic phenotype characterised by exercise-induced weakness, muscle cramps and myoglobinuria as well as hypertrophic cardiomyopathy. Although deletions in the rod domain are often associated with milder BMD (Annuar et al., 2010), exon 45-49 deletions have been linked to increased mechanical vulnerability of dystrophin and have been reported in up to 15% of rhabdomyolysis-associated dystrophinopathy (Nicolas et al., 2015; Lahoria & Milone, 2016), comparable to the 10% observed in this cohort.

MLPA reliably detects *DMD* deletions and duplications, but is unable to detect small sequence variants and complex rearrangements (Chu et al., 2025). Two cases in our cohort were *DMD* negative on MLPA, yet had a splice site deletion (Intron 32/Exon 33) and a single nucleotide variant in exon 7 detected by WES and WGS. A third proband diagnosed with DMD had apparent exon dropout of exons 14, 15 and 18. These findings, together with reports that up to one third of pathogenic *DMD* variants are not captured by MLPA (Malaga et al., 2021), support the use of WES for clinically suspicious dystrophinopathy that is MLPA-negative (Mathur et al., 2025).

Overall, these observations, alongside the diagnostic yield of DMD in this cohort, support a tiered strategy in which *DMD* MLPA remains an essential first-line test for typical childhood-onset possible dystrophinopathy in a male child, but MLPA-negative patients with a persuasive phenotype should proceed to WES to avoid missed or delayed diagnoses.

4.1.2. Facioscapulohumeral muscular dystrophy (FSHD)

Of the nine cases that were clinically suspected of being FSHD, eight (89%) were molecularly confirmed to have FSHD1 by *D4Z4* contraction testing by a specialist laboratory and aligns with published sensitivity estimates of 93% for *D4Z4* contraction testing in clinically defined FSHD cohorts (Tawil et al., 2015), supporting the use of detailed phenotyping to efficiently select patients for targeted genetic testing. Conversely, the patient clinically suspected of having FSHD, but where a molecular diagnosis has not yet been determined, presented with facial weakness, symmetrical proximal weakness and scapular winging. However, FSHD testing via Leiden University Medical Centre, WES and short-read WGS were negative for FSHD in this patient.

This undiagnosed case despite multiple genetic tests demonstrates important principles in IMD diagnosis. Negative genetic testing does not exclude genetic disease as FSHD genetic testing may fail to detect somatic mosaicism in blood-based testing, requiring muscle tissue analysis (Guruju et al., 2023). Conversely, characteristic clinical features may not be pathognomonic as IMD such as calpainopathy and valosin-containing protein (VCP) related myopathy present with similar scapular weakness and have been identified as alternate diagnoses in cases with FSHD-like phenotypes with negative *D4Z4* contraction testing (Sacconi et al., 2012). Technical limitations of sequencing platforms are important as short-read WGS cannot reliably detect the *D4Z4* repeat contraction defining FSHD1, complex structural variants in the 4q35 subtelomeric region or methylation abnormalities (Giardina et al., 2024), limiting its utility in this case. Therefore, this case highlights the diagnostic uncertainty that can arise when a suggestive clinical phenotype yields negative molecular testing, where the differential diagnosis includes both false-negative FSHD and phenotypic mimicry by alternative inherited muscle diseases.

While FSHD accounted for most of the autosomal dominant muscular dystrophies diagnoses in European ancestry patients (64%, 7/11), it was notably rare in South African admixed ancestry patients with muscular dystrophy (7%, 1/15) and absent in African ancestry patients (0/13). Its absence in those of African ancestry is consistent with the historical absence, and ongoing marked scarcity, of genetically confirmed FSHD reported in African populations, with only small case series now beginning to document such cases (Cruz et al., in press). Although the small sample size in this cohort precludes definitive conclusions regarding ancestry-specific disease distribution.

Three probands of European ancestry confirmed with FSHD did not have substantial facial weakness at time of study enrolment. Facial-sparing FSHD phenotypes are increasingly recognised in European cohorts, where reported frequencies range from 6-18% of genetically confirmed cases (Butz et al., 2003; Krasnianski et al., 2003; Pastorello, Cao & Trevisan, 2012). Although our observed frequency (37.5%, 3/8) exceeds these published figures, it may be an artefact of the small sample size rather than a definitive population difference. Notably, despite the absence of facial weakness clinically, all three probands presented with asymmetric scapular winging and a positive Beevor's sign. Although Beevor's sign is not specific to FSHD and has been reported in other myopathies, it has a high specificity (>95%) among neuromuscular diseases (Eger et al., 2010), and when combined with asymmetric scapular winging, proved to be a useful clinical indicator for suspecting FSHD in cases without facial weakness.

The high diagnostic yield (89%) for *D4Z4* contraction testing in this cohort supports consideration of targeted testing for patients presenting with asymmetric shoulder girdle and facial weakness. Although FSHD was identified predominantly in European and South African admixed ancestry individuals, the positive predictive value of this clinical phenotype suggests *D4Z4* testing may be warranted for patients with these features, regardless of ancestry.

4.1.3. Myotonic dystrophy (DM1)

There were five cases who underwent locally available *DMPK* CTG repeat expansion testing and were confirmed to have DM1 in our cohort. Three were of South African admixed ancestry and two were of European ancestry, but none had African ancestry. While there have been reports of DM1 in patients from Angola and South Africa, the genetic ancestry of these cases were not characterised in the original reports (Oliveira et al., 2022)(Goldman et al., 1996).

The restriction of DM1 to European and South African admixed ancestry individuals in this cohort is consistent with population genetic data showing that DM1 is exceedingly rare in African populations, likely reflecting the evolutionary history of the *DMPK* CTG repeat expansion as detailed previously (See Background section 1.2.1.3) (Goldman, Ramsay & Jenkins, 1994; Krause, Seymour & Ramsay, 2018). These findings inform pre-test probability, but do not preclude testing in individuals of African ancestry with clinically suggestive features.

4.1.4. Oculopharyngeal muscular dystrophy (OPMD)

Five probands from unrelated affected families presented with typical features of OPMD. At time of study enrolment, all had late-onset ptosis with three having proximal muscle weakness and all with dysphagia. Four cases were confirmed to have *PABPN1* $(GCN)_{14}$ repeat expansions with the last case awaiting confirmatory testing, but her affected sister was found to have the repeat expansion on testing in the UK. The age at symptom onset and clinical features in the OPMD subgroup are consistent with the late-onset autosomal dominant OPMD phenotype described in international cohorts (Brisson et al., 2020).

While OPMD has been reported in over 35 countries, only one South African family has previously been described, a two-generation Afrikaner family (European ancestry) carrying a $(GCN)_{15}$ repeat expansion originally reported as a Uruguayan founder mutation (Schutte et al., 2015). All four probands in this cohort harboured $(GCN)_{14}$ repeat expansions which differs from the $(GCN)_{15}$ repeat expansion identified in the previously reported Afrikaner family.

The identification of two OPMD cases in our cohort through research-based reanalysis of existing WGS data using specialised short tandem repeat (STR) genotyping algorithms (Mngadi et al., unpublished) demonstrates the underdiagnosis of repeat expansion disorders when targeted testing is unavailable. STRipy leverages flanking read patterns, fully repeated reads, mate-pair relationships and pathogenic motif counting to infer repeat read length, thereby circumventing some limitations of WES variant calling in repeat-rich regions (Halman, Dolzhenko & Oshlack, 2022). While such reanalysis represents a pragmatic interim strategy in a resource-limited setting, it is not an ideal primary diagnostic approach as it depends on prior WGS availability and requires specialised bioinformatics infrastructure.

These four unrelated patients of South African admixed ancestry indicate that OPMD, possibly introduced via European admixture, is a cause of late-onset IMD in this population. These findings, alongside the 63% diagnostic yield in the OPMD subgroup, suggest that *PABPN1* repeat expansion screening may warrant consideration in the diagnostic approach for patients presenting with characteristic late-onset ptosis, dysphagia and proximal weakness in South African populations. If local *PABPN1* testing capacity can be established, targeted repeat screening could provide diagnostic clarity and enable appropriate genetic counselling for affected families.

4.2. Inherited muscle disease phenotypes diagnosed by NGS

The majority of patients in this cohort were diagnosed via NGS after presenting with phenotypes that, while often clinically recognisable, e.g. limb-girdle, distal, extraocular muscle or metabolic phenotypes, were genetically heterogeneous and lacked corresponding locally available single-gene assays to investigate the suspected genes. In our setting, targeted molecular tests are effectively limited to *DMD* MLPA, *DMPK* repeat expansion and mtDNA deletion analysis. For all other conditions, including LGMD such as dysferlinopathy or calpainopathy and other recognisable subtypes, phenotype-guided gene-specific testing is simply not accessible. Consequently, even when a particular gene was clinically suspected, phenotype-first single-gene testing was not possible, and more advanced genetic testing (WES/WGS) was required for resolution in this subgroup.

4.2.1. RYR1-related myopathy

A male of South African admixed ancestry was diagnosed with RYR1-related myopathy due to a heterozygous likely pathogenic *RYR1* p.Ile4898Thr variant. Although this variant typically follows an autosomal dominant pattern of inheritance in families (Leão et al., 2020), this patient's presentation appears sporadic, with no family history of IMD and parental genetic testing unavailable. This case highlights a gap in local *RYR1* genetic testing in that existing local testing is restricted to two founder variants, *RYR1* c.8342_8343del and c.14524G>A, developed for confirmation of autosomal recessive centronuclear myopathy and used in specialised contexts, rather than as a broadly available state sector diagnostic service (Raga et al., 2022). As a result, these targeted tests capture only a small subset of pathogenic *RYR1* variants described in international cohorts. Consequently, most South African patients with dominant or non-founder recessive *RYR1* variants would receive a false negative result. Genetic confirmation in our proband has management implications, mandating lifelong malignant hyperthermia precautions for anaesthesia and regular respiratory monitoring.

4.2.2. Calpainopathy

Both calpainopathy cases presented with the commonly seen Leiden-Möbius pelvifemoral phenotype which was characterised by onset with predominantly symmetrical pelvic girdle muscle weakness. Their condition progressed to affect their scapular muscles thereby causing winged scapulae and hyperlordosis which correlates with international cohorts (Angelini et al., 2010). Both probands progressed to loss of independent ambulation with one being

wheelchair-bound 16 years after initial onset. One patient of African ancestry was noted to have putative compound heterozygous *CAPN3* variants, comprising a pathogenic p.Arg489Gln and p.Cys414Gly VUS novel to ClinVar. While its absence from population controls supports rarity consistent with a disease-causing allele, the lack of functional or segregation data means pathogenicity cannot be confirmed and reclassification of this novel VUS will require additional evidence to clarify its clinical significance.

We compared the prevalence of calpainopathy in our cohort, 2.2% of IMD patients (2/89), to two studies of heterogenous IMD patients conducted in Greece (n=24) and India (n=105). All three cohorts utilised WES for genetic testing. The Greek and Indian studies identified *CAPN3* variants in 4.2% (1/24) and 6.7% (7/105) of cases respectively (Zaganas et al., 2020)(Khadilkar et al., 2022). The lower *CAPN3* frequency in our South African cohort compared to the Indian cohort may be attributed to differences in consanguinity rates. However, the variation in *CAPN3* prevalence across these small cohorts may reflect sampling variability and ascertainment bias rather than true population differences, precluding definitive conclusions regarding relative *CAPN3* burden.

4.2.3. Dysferlinopathy

Two patients genetically confirmed to have autosomal recessive dysferlinopathy presented with predominant limb-girdle phenotype. There was a prolonged diagnostic delay of 24 and 43 years for these two probands respectively, in contrast to the mean 3.1 year delay reported in the multinational Clinical Outcome Study for dysferlinopathy (COS) following wider access to genetic testing (Harris et al., 2016), underscoring how lack of local testing perpetuates prolonged periods of diagnostic uncertainty in our setting.

4.2.4. SELENON-related myopathy

One proband with possible early childhood neck flexor weakness, rigid spine, scoliosis, proximal muscle weakness and respiratory dysfunction complicated by pulmonary hypertension was diagnosed as an adult with autosomal recessive SELENON-related myopathy, similar to the phenotype described in a large cohort of 132 patients in whom early-onset proximal and axial weakness, rigid spine with scoliosis and severe respiratory insufficiency were present (Villar-Quiles et al., 2020).

She was found to have biallelic pathogenic *SELENON* variants including a well-described p.Gly315Ser variant (Silwal et al., 2020) and a canonical splice donor variant, c.301+2T>C,

that is novel to ClinVar. This represents the third molecularly confirmed case of SELENON-related myopathy in South Africa with two published in a MPhil thesis (Oshi, 2021).

4.2.5. Emery-Dreifuss muscular dystrophy (EDMD)

Two probands of South African admixed ancestry presented with the classic EDMD triad of early-onset contractures, progressive humeroperoneal weakness and cardiac dysfunction, despite different genetic aetiologies and inheritance patterns, namely EMD-related EDMD1 and LMNA-related EDMD2. Both experienced substantial diagnostic delays of over 20 years prior to genetic confirmation. The EDMD1 patient progressed rapidly to wheelchair dependency with atrioventricular block, whereas the EDMD2 proband remained ambulant, but had paroxysmal atrial fibrillation, illustrating the spectrum of skeletal muscle and cardiac involvement across genotypes. This distinction is clinically important, as LMNA-related EDMD2, in particular, carries substantial long-term risk of dilated cardiomyopathy and arrhythmias, mandating lifelong cardiac surveillance and timely device therapy in both subtypes (Heller et al., 2020; Marchel et al., 2021).

4.2.6. Desminopathy

Desminopathy, caused by *DES* variants, is a progressive myofibrillar myopathy combining skeletal muscle weakness with cardiac involvement, typically inherited in an autosomal dominant pattern, but with rare autosomal recessive forms and characterised by marked phenotypic variability (Van Spaendonck-Zwarts et al., 2011). Desminopathy in this cohort demonstrated both autosomal dominant and recessive inheritance, with all three patients showing limb-girdle weakness and significant cardiomyopathy. Two South African admixed probands harboured autosomal dominant *DES* variants and presented with proximal lower limb weakness and severe cardiac involvement.

The autosomal recessive form of desminopathy is rare and published cases typically report severe early-onset muscle disease and cardiomyopathy with high adolescent mortality (Polavarapu et al., 2024). However, the third desminopathy patient reported cardiac disease at 29 years and survived until 52 years illustrating that autosomal recessive desminopathy may also exhibit a broad phenotypic spectrum. This milder phenotype has been reported in recent unrelated autosomal recessive cases, particularly in cases with splice-site or missense variants that retain partial desmin function. While p.Arg406Trp is a well described pathogenic *DES* variant (Geng et al., 2024), p.Arg454Trp and p.Gly456Arg are rarer variants reported to cause severe cardiac dysfunction that is consistent with the phenotype in our desminopathy

subgroup (Oomen et al., 2018; Liu et al., 2022). These cases emphasize the need for systematic cardiac surveillance in any patient with suspected desminopathy, regardless of inheritance pattern.

4.2.7. Myotonia congenita

Four probands of European ancestry were diagnosed with myotonia congenita. They all presented with classic childhood-onset muscle stiffness, warm-up phenomenon and percussion myotonia with skeletal muscle hypertrophy observed in three cases. Putative compound heterozygous *CLCN1* variants were identified in four probands, including the frequently reported p.Arg894* allele (Skálová et al., 2013), consistent with autosomal recessive Becker disease. The clinical heterogeneity observed, ranging from Becker subtype with later onset and myotonia with warm-up phenomenon to more generalised phenotypes with additional systemic features reflects the well-documented phenotypic variability in myotonia congenita (Gowda, Sajjan & Srinivasan, 2025). Although these probands showed classic features of myotonia congenita, there is not a locally available single-gene assay for *CLCN1* and diagnosis in this cohort was achieved via WES accessed via research collaboration. However, in South Africa where neither targeted *CLCN1* assays nor routine NGS access are readily available outside the research setting, the diagnosis of myotonia congenita remains challenging and likely underdiagnosed in affected individuals presenting with classic phenotypes.

4.2.8. Collagen VI-related myopathy

Collagen VI–related myopathy in this cohort spanned the expected severity spectrum from intermediate childhood-onset disease with contractures and respiratory compromise to milder adult-onset Bethlem myopathy (Bönnemann, 2011). Two probands had typical proximal-predominant weakness consistent with Bethlem myopathy, whereas a third presented with unusually prominent distal lower limb weakness and falls. This third patient who presented with adult-onset frequent falls and distal lower limb weakness had a *COL6A2* c.1179+2T>C VUS novel to ClinVar detected at the canonical splice donor site in intron 13.

While this specific change has not been described previously, the likely pathogenic c.1179+1G>A variant at the adjacent first nucleotide position is a known cause of Bethlem myopathy (National Center for Biotechnology Information, n.d.). Given the critical function of the splice site and the proximity to the established disease-causing variant, c.1179+2T>C is likely to disrupt splicing and cause exon 13 skipping which supports that it may be likely

pathogenic despite its novelty. Splice variants may allow for partial function that may explain the mild, distal phenotype present in this case (Bönnemann, 2011).

4.2.9. Mitochondrial and metabolic myopathies

Mitochondrial myopathies accounted for 9% of the cohort and exemplify the phenotypic and genetic heterogeneity characteristic of mitochondrial myopathies. The majority (7/8) presented with extraocular muscle weakness as a prominent phenotypic feature consistent with the preferential affinity of mitochondrial dysfunction for energetically demanding ocular muscles (Chen, Harvey, et al., 2023). Confirmed molecular diagnoses encompassed the genetic and technical spectrum of mitochondrial myopathy as the characteristic CPEO phenotype associated with single large mtDNA deletions required muscle tissue analysis for detection, whereas the *MT-ATP6* VUS and nuclear DNA mutations of genes affecting mitochondrial function such as *OPA1, TWNK* and *RRM2B* genes were identified through blood DNA sequencing. Although several patients had classic CPEO phenotypes with large mtDNA deletions amenable to targeted mtDNA analysis, many exhibited overlapping features with OPMD or other myopathies. For those with mtDNA deletions that were negative or those with atypical presentations, WES facilitated molecular diagnosis across this genetically heterogeneous group.

An interesting example was a patient with adult-onset bilateral ptosis, progressive external ophthalmoplegia, dysarthria, dysphagia, facial weakness and mild proximal upper-limb weakness with scapular winging, in whom WES identified two *TWNK* missense variants, p.Leu373Val and p.Glu375Val, in *cis* in the linker region of the Twinkle helicase. These two variants of uncertain significance, novel to ClinVar, flank the known pathogenic p.Arg374Gln variant and likely disrupt the same structural linker function required for helicase to form its ring (Bermejo-Guerrero et al., 2022). The clinical presentation of PEO with additional bulbar and limb involvement, combined with identification of both variants in his affected son, is consistent with autosomal dominant TWNK-related disease. However, as both variants occur in *cis*, determining which variant is pathogenic requires functional studies which are not readily available in resource-limited settings. Nevertheless, the co-segregation with disease in his affected son and the proximity to known pathogenic p.Arg374Gln variant suggest pathogenicity of at least one variant in this *cis* configuration.

Three patients with bilateral ptosis and ophthalmoplegia were found to have single large mtDNA deletions in the muscle mtDNA including the most frequently identified

m.8482_13460del and the next commonest m.12113_14421del, consistent with the mutational spectrum reported in large CPEO cohorts from Europe and Japan (Yamashita et al., 2008; Heighton et al., 2019).

The identification of a heterozygous *MT-ATP6* m.9026G>A variant in a South African admixed male, previously reported in a multinational collaborative study on mitochondrial DNA disorders in neuromuscular diseases (Gao et al., 2024), highlights a rare adult-onset presentation of Complex V deficiency. Classically, *MT-ATP6* variants are associated with severe paediatric syndromes like neuropathy, ataxia and retinitis pigmentosa (NARP) syndrome or Leigh syndrome. However, this patient presented with late adult-onset dysphagia, ptosis and diplopia and was initially suspected of having OPMD which is a differential diagnosis to consider in adults presenting with this phenotype (Chai, 2014). This same variant has been reported in both severe childhood-onset disease and milder late-onset presentations, underlining the marked phenotypic heterogeneity of mitochondrial mutations (Ganetzky et al., 2019).

Two patients were identified with *RRM2B* variants. In one female of South African admixed ancestry, a homozygous *RRM2B* p.Pro149Leu inconclusive VUS was classified as possibly solved based on the overall evidence. Her survival into her seventh decade with ptosis, ophthalmoplegia and fatigable proximal muscle weakness represents a relatively mild course compared with the typically severe, childhood-onset recessive RRM2B-related phenotypes described in the literature (Pitceathly et al., 2012). This case illustrates the limited ability of phenotype alone to predict genotype in mitochondrial disease and underscores the need for cautious, evidence-based interpretation of VUS, ideally supported by segregation data.

The ADOA-plus phenotype observed in a male of European ancestry characterised by proximal muscle weakness, optic atrophy and sensory neuropathy was associated with heterozygous exon 2-5 deletions in the *OPA1* gene. The patient presented as part of a multi-generational pedigree with nine affected relatives across four generations. The family demonstrates phenotypic heterogeneity with some individuals manifesting isolated visual impairment, while others presented with prominent muscle weakness illustrating the variable expressivity of OPA1-related disease (Atamena et al., 2023).

Two patients were identified with glycogen storage diseases. The first was a female of South African admixed ancestry with a waddling gait, limb-girdle weakness, lumbar hyperlordosis and areflexia who was diagnosed with late-onset Pompe disease (LOPD) due to compound

heterozygous pathogenic *GAA* variants; c.-32-13T>G which is the commonest European splice variant and a p.Arg854Ter nonsense variant, a proposed African founder variant (Turaça et al., 2015). The identification of LOPD in this patient represents an important diagnostic finding as this treatable condition is often misdiagnosed as a muscular dystrophy (Preisler et al., 2013). Although enzyme replacement therapy (ERT) has shown benefit in stabilising disease progression in many adults with LOPD (Harlaar et al., 2019), it remains unavailable in the South African setting due to cost constraints. Nevertheless, this case underscores that treatable metabolic myopathies are embedded within cohorts resembling dystrophies and highlights the clinical utility of broad genomic testing.

Another male of African ancestry was diagnosed with Glycogen storage disease IXd (GSD IXd) due to a hemizygous *PHKA1* p.Arg428His VUS novel to ClinVar. GSD IXd is an X-linked disorder caused by phosphorylase kinase (PhK) deficiency due to *PHKA1* variants (Huang et al., 2022). Given that GSD IXd is an ultra-rare metabolic myopathy with approximately 30 genetically confirmed cases reported globally, there are inconsistencies with phenotype-genotype correlation (Huang et al., 2022). Affected individuals present with muscle cramps, exercise intolerance, rhabdomyolysis and progressive muscle weakness manifesting from infancy to late adulthood. This patient presented with childhood-onset proximal lower limb weakness, delayed motor milestones, abnormal gait, lumbar hyperlordosis and scoliosis expanding the recognised phenotype beyond exercise intolerance and contributing African-specific data.

These presentations, encompassing both genetically heterogeneous mitochondrial phenotypes and rare metabolic myopathies, were ultimately diagnosed WES which was the only comprehensive genetic testing platform accessible in this setting given the absence of targeted mitochondrial or metabolic gene panels.

4.3. Unexpected findings

This cohort also contained a small number of cases with atypical or non-classical presentations that broaden the recognised clinical spectrum of IMD and illustrate how NGS can uncover diagnoses that are not clinically apparent.

4.3.1. GNE myopathy

A proband and her brother, born of a consanguineous union, presented with limb-girdle weakness and severe quadriceps weakness and were confirmed to have a homozygous likely pathogenic *GNE* p.Met265Thr variant. GNE myopathy is typically caused by compound

heterozygous variants in the kinase or epimerase domains leading to hyposialylation (Jampel et al., 2009). Notably, GNE myopathy classically presents with distal myopathy and sparing of the quadriceps muscles unlike these cases with severe quadriceps weakness. GNE myopathy has been described as having phenotypic variability with 3.2-5% of patients noted to have quadriceps weakness (Park et al., 2012; Baskar et al., 2024).

Although the p.Met265Thr variant has been found in several affected cohorts including European, Asian and Middle Eastern populations, the sibship in our cohort is of South African admixed ancestry. The p.Met265Thr variant lies in the allosteric region of the *GNE* epimerase domain, a site usually associated with dominant sialuria rather than recessive myopathy (No et al., 2013). This is the first illustration to date of a homozygous p.Met265Thr variant in the *GNE* allosteric region causing myopathy and the associated severe quadriceps weakness further broadens the clinical and mechanistic spectrum of GNE-related disease.

4.3.2. Distal nebulin myopathy

Three multi-generation, but independent families of South African admixed ancestry were identified with autosomal dominant distal myopathy due to large heterozygous *NEB* deletions, with disease segregation confirmed in two families. This represents a distinct clinical and molecular entity from the more commonly recognised autosomal recessive nemaline myopathy caused by biallelic *NEB* mutations. All three probands had the characteristic distal nebulin myopathy phenotype with predominantly distal lower limb weakness, foot drop and relative quadriceps sparing, with variable facial and proximal upper limb involvement. Their course resembled previously described Finnish cohorts with large in-frame *NEB* deletions, although weakness in our cohort was generally more severe (Sagath et al., 2025). Our cohort also distinctively presents with small fibre neuropathy in all cases, features not prominently reported in the Finnish studies.

The deletion sizes (63-64 exons) in the three families are similar to those previously identified in dominant *NEB* deletions ranging from 52-97 exons (Sagath et al., 2025). The first reported Finnish family had a 76-exon deletion (exons 14-89) with the index patient presenting at age 3 years, while his mother and grandmother had milder symptoms with adult onset (Kiiski et al., 2019). This intergenerational variation in age at onset and severity mirrors the pattern observed in our families, suggesting that factors beyond deletion size such as genetic modifiers or environmental influences may contribute to phenotypic variability.

Collectively, these findings support that dominant *NEB* deletions in the 52-97 exon size range produce a distal myopathy phenotype with variable age at onset and progressive proximal involvement, while the sensory neuropathy present in our cohort may represent a separate diagnosis rather than a direct consequence of the *NEB* deletion.

4.4. Unsolved subgroup

Approximately one third (30%) of our cohort remains unsolved following extensive testing. Of these, the majority are from the limb-girdle and EOM weakness group. These unsolved cases illustrate that there are biological, interpretation and technical limitations that prevent accurate variant detection and interpretation. Technical limitations of WES include challenges detecting structural variants, deep intronic mutations and complex repeat expansions. Additionally, interpretive challenges arise particularly for variants in genes with limited prior evidence in African and admixed populations, where the scarcity of ancestry-specific reference data complicates pathogenicity assessment. These unresolved cases therefore highlight both the need for additional molecular testing, namely long-read WGS (Yeow et al., 2025), and for more diverse genomic reference data.

4.5. Utility of tiered genetic testing for IMD in resource-limited settings

4.5.1. Diagnostic yield in this cohort

This combination of locally available genetic testing and NGS provided molecular diagnoses for patients who had often waited many years for a definitive explanation and identified several conditions with clear management consequences, particularly those requiring cardiac and respiratory function surveillance, while still leaving approximately one third of cases unsolved.

4.5.2. Phenotype vs genotype-first: lessons from this cohort

The experience in this cohort shows that while a small subset of IMD remains amenable to classic phenotype-first single-gene testing, most patients fell into clinically recognisable but genetically heterogeneous categories including limb-girdle, distal, EOM weakness, myasthenic-like syndromes with fatigable weakness and myotonia-related presentations. Although these patterns often raised clinical suspicion of specific genes, corresponding locally available single-gene assays were lacking, and these patients were therefore investigated via WES or WGS, accessed through research partnerships, despite phenotypic clues that in better-resourced settings would prompt targeted single-gene or multi-gene panel testing. More broadly, classifying cases by predominant pattern of weakness, e.g. classic

DMD versus limb-girdle or distal patterns, remains clinically useful, not because it reliably predicts a specific gene, but because it helps decide whether targeted testing is justified or whether early WES is more appropriate.

The phenotypic heterogeneity observed across most diagnostic categories, highlights the limitations of phenotype-based prediction alone. In routine practice, phenotype-first strategies can bias clinicians toward familiar, but inaccurate diagnostic categories, contributing to prolonged diagnostic odysseys in which patients undergo multiple specialist consultations, sequential diagnostic investigations and extended periods of diagnostic uncertainty before a molecular diagnosis is reached (Manjunath et al., 2025). This may be accentuated in adult-onset IMD, where patients may present with atypical clinical findings or attenuated features, increasing the likelihood of misclassification when clinicians rely solely on typical phenotype patterns (Schofer, Patzer & Quante, 2008). For most cases, particularly those with non-specific or overlapping features, early access to NGS followed by careful reverse phenotyping may prove more diagnostically efficient than prolonged attempts at clinical sub-classification. Our data therefore support a hybrid diagnostic approach in which phenotype guides targeted testing for a minority of recognisable conditions and frames variant interpretation, while the majority of patients proceed to broad, largely agnostic NGS.

4.5.3. Role of WES vs targeted panel testing in our context

In high-income countries (HIC), studies have shown that early NGS, either WES or large neuromuscular panels, is often more cost-effective than sequential single-gene testing, particularly for genetically heterogeneous IMD presentations. An Australian analysis demonstrated that large neuromuscular panels and WES reduced both time to diagnosis and overall testing costs compared with traditional diagnostic pathways, with targeted gene panels offering superior cost-effectiveness for defined clinical phenotypes (Schofield et al., 2017). Notably, intermediate targeted IMD panels are not available in South Africa. Consequently, in our tertiary neuromuscular clinic setting, diagnostic pathways moved from a small set of locally available single-gene tests directly to repeat expansion testing or WES.

In the absence of intermediate disease-specific gene panels, WES accessed via the South African Medical Research Council (SAMRC) provided the only comprehensive testing option beyond limited single-gene tests in our setting. The clinical presentations observed in this cohort, including many phenotypes that raised suspicion for specific gene groups, suggest that phenotype-directed gene panels could potentially improve diagnostic efficiency.

Targeted gene panels covering clinically defined presentations may potentially offer a more scalable and cost-effective option (Ng et al., 2022) for routine diagnostics if they could be implemented locally, while WES would remain valuable for enabling periodic re-analysis as new disease genes are discovered.

4.6. Implications for IMD diagnostics in South Africa

There are clear gaps in diagnosis and care for patients living with IMD in South Africa. The high diagnostic yield achieved through NGS in this cohort, alongside efficient targeted testing for a small number of classic phenotypes, is consistent with international recommendations that broad genomic testing should be available for most IMD presentations and other genetically heterogeneous phenotypes (Haskell et al., 2018). However, several findings from this study highlight challenges specific to South Africa that cannot be addressed through adoption of international guidelines alone.

First, this cohort identified eight probands with variants novel to ClinVar, four occurring in individuals of African or South African admixed ancestry. The high proportion of variants novel to ClinVar and variants with limited population or clinical data, particularly in African and South African admixed individuals, illustrates how reliance on predominantly European reference datasets can lead to under-recognition or misclassification of pathogenic alleles (Wilson et al., 2023). Expanding African genomic reference data and ensuring systematic sharing of curated variants from local laboratories will be crucial for improving diagnostic accuracy and reducing the burden of VUS. Secondly, extended diagnostic delay for dystrophinopathy in this cohort, despite locally available testing suggest that diagnostic barriers extend beyond laboratory capacity to include gaps in awareness of neuromuscular red flags, systemic challenges in specialist referral pathways and access to neuromuscular expertise.

To build sustainable genomic medicine capacity for IMD in South Africa, targeted investment is required across several domains. First, expansion of local sequencing and bioinformatics capability is needed for variant calling, annotation and complex variant detection including repeat expansions and structural variants. Second, clinical phenotyping capacity should be strengthened through broader access to specialist neuromuscular services and multidisciplinary teams, to ensure appropriate test selection and reduce diagnostic delays. Third, establishing African-specific genomic reference datasets and promoting data sharing from local laboratories will be essential to improve variant pathogenicity assessment and

reduce the burden of VUS that disproportionately affects under-represented populations. These priorities are broadly aligned with global initiatives to embed genomic medicine in public health systems (Wilson et al., 2023), but will require careful adaptation to our resource-constrained context.

International best-practice guidelines increasingly endorse WES or large multi-gene panels as first-tier tests for heterogeneous IMD presentations. Our findings are broadly consistent with this approach, but also emphasize that ancestry-specific disease patterns, existing single-gene assays and health system constraints should inform how such recommendations are implemented in South Africa. Phenotype-directed gene panels, where feasible, may offer a more scalable and cost-effective option for defined clinical presentations such as limb-girdle phenotypes, although WES remains valuable for presentations where clinical features do not sufficiently narrow the differential diagnosis. Consequently, there is a need for implementation studies that evaluate how advanced genomic testing, including targeted gene panels, WES and WGS, can be feasibly and equitably integrated into IMD care within the South African healthcare sector.

The high diagnostic yields for FSHD and OPMD in this cohort, despite both tests being unavailable locally and requiring international laboratory access, suggest that establishing local *D4Z4* repeat contraction and *PABPN1* repeat expansion testing capacity could be considered as part of future diagnostic capacity building. However, implementation decisions should account for the modest cohort size, restriction to European and South African admixed ancestry and require cost-effectiveness analysis comparing targeted single-gene testing to NGS approaches.

Limitations

The findings of this study should be interpreted while taking into consideration several limitations relating to sample size, cohort composition and diagnostic work-up. First, subgroup sample sizes were small, with only a few patients in each IMD subgroup, limiting the precision of estimates and precluding firm conclusions for individual conditions. Therefore, we are unable to make definitive conclusions for each condition. While the study was conducted in South Africa, 40% of probands self-assigned to South African admixed ancestry and only 15% to black African ancestry. These proportions diverge substantially from national demographics where South African admixed and African individuals comprise 8% and 81% of the population respectively (Stats SA, 2023). Based on population demographics in South Africa, the proportion of cases of European ancestry (42%) was greater than expected representing a nearly six-fold overrepresentation compared to their 7% population share. The overrepresentation of individuals of European ancestry in this cohort may reflect selection bias in tertiary neuromuscular clinic referral patterns or differential access to specialist care during the recruitment period, which coincided with the SARS-CoV-2 pandemic. Patients with private transport were able to more readily travel to Groote Schuur Hospital for consultations during this time. This limits the generalisability of ancestry-specific findings to other South African populations.

VUS are an additional challenge as they should not be used for major clinical decision-making and require regular review for reclassification (Richards et al., 2015). There are several cases of VUS and variants novel to ClinVar in our cohort. While segregation analysis can provide supporting evidence for pathogenicity, we were unable to perform this for all these cases. This reflects not only resource constraints, but also the inherent difficulty of obtaining parental samples in an adult cohort, where trios are often incomplete or unavailable.

This is a particularly relevant problem in the African setting due to the marked under-representation of African and South African populations in global genomic reference databases such as gnomAD and ClinVar, which results in a disproportionately high burden of novel variants and VUS in patients of African ancestry. Because African-specific allelic frequencies are absent or poorly characterised in these predominantly European-derived reference datasets, variants identified in African ancestry patients are more frequently classified as VUS, complicating the distinction between plausible disease-causing variants

and benign population-specific variation (Sharo et al., 2023; Dennis & Lee, 2024). Consequently, the 'probably solved' and 'possibly solved' designations used in this study should be interpreted as provisional, with the expectation that reclassification may occur as African-specific genomic reference datasets expand.

Given the small numbers within each diagnostic subgroup and the overrepresentation of European and South African admixed ancestry in this cohort, our findings should be interpreted as hypothesis-generating and should not be generalised beyond this population without validation in larger South African cohorts.

A further methodological limitation relates to the handling of missing clinical data. In this retrospective dataset, blank data fields were recoded as 'Absent' following cross-checking against physical clinical folders, based on the clinical convention of documenting abnormal findings while omitting negative ones. Although this approach was systematically applied and validated through folder review, the possibility of residual misclassification bias cannot be entirely excluded as retrospective data capture does not permit certainty that all absent fields reflect genuinely negative findings rather than documentation omissions.

Additionally, access to advanced genetic testing was contingent on research collaborations, rather than routine clinical pathways. This may have introduced selection bias regarding which patients received advanced genetic testing. The pragmatic diagnostic approach reflected real-world constraints in our setting, but means that testing decisions were influenced by factors beyond clinical presentation alone, including logistical feasibility and capacity to pursue international testing collaborations. This pragmatic and opportunistic approach is a recognised limitation of retrospective cohort studies conducted within research consortium frameworks in resource-limited settings, and the reported diagnostic yields and inter-tier comparisons should be interpreted with this context in mind.

Recommendations for future research

Future studies should prioritise the application of advanced genomic technologies such as long-read WGS and updated bioinformatic pipelines to the undiagnosed cases to better capture structural, deep intronic and repeat variants that are poorly detected by WES. Second, there is a need for ongoing re-evaluation of possibly solved and undiagnosed cases. Periodic re-analysis of existing sequencing data and, where possible, retrospective or prospective family-based follow-up may allow some variants currently classified as uncertain to be

reclassified. In line with the implications for IMD care in South Africa, future research should actively contribute to African-specific reference datasets by systematically sharing well-annotated variants and phenotypes from local IMD cohorts. Given the limited local workforce for comprehensive NGS analysis and variant curation, it will also be important to explore whether well-designed, phenotype-directed gene panels, e.g. limb-girdle gene panels, could provide a more scalable option for routine diagnostics in certain clinical scenarios in South Africa. Future research should therefore include implementation studies that compare the diagnostic yield, costs and practical feasibility of different testing pathways, such as single-gene assays, targeted gene panels and WES, in the South African healthcare setting.

Concluding remarks

To our knowledge, this study represents the largest adult single-centre heterogeneous IMD cohort reported in a diverse South African population. Despite limited access to genetic testing, scarce specialist expertise and prolonged diagnostic delays, this study achieved an overall diagnostic yield of 62% in a South African IMD cohort, a figure broadly comparable to yields reported from tertiary neuromuscular centres in high-income settings. This research characterises a distinctive local mutational landscape, including ancestry-dependent disease distribution in which conditions such as FSHD1 and DM1 were concentrated in European and, to a lesser extent, South African admixed ancestry probands, but were absent in those of African ancestry, as well as atypical NEB- and GNE-related clinical presentations and eight probands with variants novel to ClinVar. This demonstrates that both classic and atypical phenotypes can be resolved through a combination of targeted genetic testing and next generation sequencing.

This study therefore makes three main contributions for IMD care in South Africa. First, by systematically reviewing and integrating previously collected phenotypic data with curated genetic findings, this study provides initial local reference information that can inform future variant interpretation in South African IMD cases. Second, it demonstrates that a hybrid approach, combining existing targeted tests with NGS for unresolved or heterogeneous cases, is diagnostically effective in this context, while also highlighting technical and interpretive limitations that leave a subset of patients without a diagnosis and underscores the inadequacy of current IMD genetic testing outside of research-driven testing in South Africa. Third, it exposes system-level gaps that extend beyond laboratory availability, exemplified by a 10-year median delay to DMD diagnosis despite longstanding access to *DMD* MLPA, and

underscores the importance of strengthened specialist neuromuscular services, establishing African-specific genomic reference datasets and sustainable genomic testing capacity. Although derived from a cohort of modest size, these findings provide a foundation for future clinical, genomic and implementation studies aimed at improving IMD diagnosis and care in South Africa.

In summary, this work supports a hybrid diagnostic approach in our setting, in which a small minority of patients with classic phenotypes benefit from targeted single-gene or repeat expansion tests, while the majority with heterogeneous presentations require broad NGS. In this context, detailed phenotypic characterisation remains beneficial in that it helps identify the few conditions for which gene-specific tests are justified and provides the clinical context needed to interpret NGS findings, refine diagnoses and move some variants towards more confident classification. Moving forward, targeted gene panel testing for well-defined clinical groups may offer a pragmatic intermediate option between single-gene assays and WES/WGS if such panels can be implemented locally.

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Appendices

Supplementary Table 1. Variants novel to ClinVar and their associated clinical phenotypes in a South African inherited muscle diseases cohort.

Ancestry	Phenotype	Gene	Inheritance	Variant	ACMG Classification
European	Distal weakness	*COL6A2*	AD	c.1179+2T>C	VUS
African	LG weakness	*CAPN3*	AD	c.1240T>G (p.Cys414Gly)	VUS
SA Admixed	LG weakness	*EMD*	XL	chrX: 154380693-154381279del	P
European	EOM weakness	*TWNK*	AD	c.1117C>G (p.Leu373Val) c.1124A>T (p.Glu375Val)	VUS VUS
European	LG weakness	*SELENON*	AR	c.301+2T>C	P
SA Admixed	LG weakness	*DMD*	XL	c.503del (p.Ala168Valfs)	LP
African	LG weakness	*PHKA1*	XL	c.1283G>A (p.Arg428His)	VUS
SA Admixed	LG weakness	*RYR1*	AD	c.14693T>A (p.Ile4898Thr)	LP

Supplementary table 1. summarises probands in whom variants were novel to ClinVar in association with inherited muscle diseases in a South African cohort. For each case ancestry, predominant phenotype, gene, inheritance pattern, variant and ACMG classification is shown. AD: autosomal dominant, EOM: Extraocular muscle weakness, LG: limb-girdle, SA: South African, VUS: variant of uncertain significance, XL: X-linked recessive.