Title

Diagnosis and aetiology of congenital muscular dystrophy – we are halfway there

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Abstract <250 words (249)

Objectives: To evaluate the efficacy of next generation sequencing for diagnosing congenital muscular dystrophies (CMD).

Methods: A cohort of 124 CMD patients was investigated using histological and immunohistochemical staining of muscle biopsies, candidate gene sequencing and next generation sequencing (NGS).

Results: Traditional diagnostic methods identified a deficiency of merosin, α-dystroglycan or collagen VI in 51% of our CMD cohort. Candidate gene sequencing led to a genetic diagnosis in 34%. During 2013-15, investigation of 33 undiagnosed CMD patients with NGS yielded a diagnosis in 67% (22/33 patients). The diagnoses within the cohort were heterogeneous: five patients had variants in novel (*PIGY* and *GMPPB*) or recently published genes (*GFPT1* and *MICU1*); seven had variants in *TTN* or *RYR1*, large genes technically difficult to Sanger sequence; 43 patients (73%) had variants in genes known to cause CMD, but 11 patients (17%) had variants in genes associated with congenital myopathies, reflecting the overlapping clinical and histological features of these conditions. Together, a NGS neuromuscular gene panel and chromosomal microarray (CMA) could diagnose 95% of the patients in the cohort.

Conclusions: This study supports using targeted NGS as a first line tool to diagnose CMD, avoiding a muscle biopsy and associated delay to genetic diagnosis. Muscle biopsy should be reserved as a second tier investigation. The next phase of diagnostic testing for undiagnosed patients will include whole genome sequencing and RNA sequencing, and will depend on

expanding international collaborations and data sharing to increase recognition and confirmation of possible pathogenic variants in new disease genes.

Introduction <250 words (212 words)

The congenital muscular dystrophies (CMDs) are inherited disorders of skeletal muscle characterized by hypotonia and weakness within the first 2 years of life, delayed gross motor milestones and dystrophic features on skeletal muscle biopsy (1,2). These disorders are phenotypically diverse and genetically heterogeneous. The boundaries between CMD, congenital myopathies and limb girdle muscular dystrophies are blurred, with overlap in disease genes, clinical presentation and histopathological features.

In 2008, Peat and colleagues published the protein and molecular diagnostic workup of a Australasian CMD cohort (2). The diagnostic methods, muscle immunohistochemistry and Sanger sequencing of candidate genes, were described as "state-of-the-art" in an accompanying editorial (3). An immunohistochemical classification was achieved in 45% (45/101) of the cohort and a genetic diagnosis in 24% (24/101). The advent of 'next generation sequencing' (NGS) has contributed to a rapid increase in the number of known CMD genes from 12 in 2008, to 28 in 2015 (4). However, the diagnostic yield of next generation sequencing by panel testing of known disease genes, whole exome, or whole genome approach remains uncertain.

This study evaluates the diagnostic outcomes in 124 CMD patients ascertained over 35 years. NGS approaches in 33 unsolved patients provided a genetic diagnosis for 22/33 (67%) patients, double the diagnostic efficacy achieved using traditional approaches of immunostaining and Western blot that were considered state-of-the-art in 2008.

Methods (539 words)

Patient Ascertainment

CMD patients were identified retrospectively and prospectively through clinical records and the Institute for Neuroscience and Muscle Research (INRM) biospecimen bank. Clinical examination, review of medical records, muscle histology and complementary investigations such as brain MRI and muscle imaging were used to define the clinical phenotype of affected individuals.

Inclusion Criteria: Evidence of muscle weakness and hypotonia within the first 2 years of life and clinical features consistent with congenital muscular dystrophy, such as delayed gross motor milestones, congenital/early contractures or scoliosis, brain MRI consistent with laminin-α2 deficiency or α-dystroglycanopathy, or a raised CK (>200 IU/L). Only the proband was included when more than one sibling was affected. Patients with a muscle biopsy performed between 1979 and 2014 were included if it showed dystrophic changes, or non-specific myopathic findings provided the clinical criteria were met. A small number of patients were not investigated with a muscle biopsy. Deliberately broad inclusion criteria were chosen to reflect the variable pathology which can occur early in the course of disease, secondary to selective muscle involvement and in specific subtypes such as collagen VI myopathies (5,6).

Inclusion in NGS studies was based on phenotype with preference given to families with multiple affected siblings and those with DNA available from the proband and parents. Inclusion of retrospective members of the cohort was limited by the need for additional consent and the availability of DNA samples. Given the elapsed time, it was considered insensitive to re-contact some families and some were no longer contactable.

Exclusion criteria: Structural changes in skeletal muscle diagnostic of a congenital myopathy, for example rods or cores. Eleven fetuses and neonatal deaths ascertained in the Peat cohort (2) were excluded because they had not been investigated further and were no longer contactable.

Standard Protocol Approvals and Patient Consents

Ethics approval for all aspects of this study was obtained from the Human Research Ethics Committee of the Sydney Children's Hospitals Network (Approval No: 10.CHW.45). Written informed consent was obtained from all participants and inclusion in NGS studies was dependent on completion of an additional specific consent, reflecting the complexities of NGS analysis.

Immunohistochemical analysis

Immunohistochemical staining of the muscle biopsy for laminin-α2, glycosylated α-dystroglycan and collagen VI, was performed using previously reported methods (2). Probands were classified as α-dystroglycanopathy, collagen VI-related myopathy or laminin-α deficient if their biopsy showed moderate to severely reduced or absent staining.

Candidate gene sequencing

Candidate gene sequencing was guided by phenotype and immunohistochemical analysis. Methods have been previously reported for *FKRP*(2), *LARGE*, *POMT1*, *POMT2*, *FKTN* and *POMGNT1*(7), the three collagen VI genes (8), *LAMA2* (9), *SEPN1* (10), *LMNA* (11) and *DNM2* (12).

Next generation sequencing (NGS)

Targeted NGS was performed with either a research-based 45 gene panel (Panel A) (13), or a commercial 345 gene panel (Panel B) offered by PathWest Laboratory, Australia (Table e2). WES was performed by the Broad Institute using previously published methods (11). WGS was performed on 3 probands who did not have a diagnosis following WES.

Comment [GO1]: Is there a better reference, or include as supplemental methods.

Whole exome sequencing analysis pipeline.

Variant filtering was performed using the xBrowse web browser (https://atgu.mgh.harvard.edu/xbrowse). Variants were identified as outlined in Figure 1. Likely pathogenic variants were confirmed by Sanger sequencing in the proband and family members.

Results (1157)

Traditional approaches of immunostaining, Western blot and candidate gene sequencing provided a genetic diagnosis in 34%.

A cohort of 124 CMD patients was ascertained; 90 were part of the 1979-2006 cohort published by Peat and colleagues (2), and a further 34 probands were ascertained between 2006 and 2014 (Figure 2); 61 probands were female and 63 were male; 101 came from non-consanguineous families, 15 from consanguineous families and for 8 probands this information was not available. Eight probands had affected siblings.

Muscle histology was available for 118 probands. The median duration from onset of symptoms to muscle biopsy was 18 months. Immunohistochemical analysis was performed on 114 muscle biopsy specimens; 58 probands (51%) could be classified on the basis of a moderate or severe reduction in collagen VI, laminin-α2 or α-dystroglycan (Figure 3

(online)). Candidate gene sequencing was performed on the basis of clinical phenotype and immunohistochemical classification in 91 probands (Figure 3 (online)). The mean number of genes sequenced was 4 (range 1-16).

Using muscle biopsy and histological examination, immunohistochemical analysis, candidate gene sequencing and chromosomal microarray (CMA) a genetic diagnosis was achieved for 41 of 122 probands (33.6%) (Table e1 (online)). Two patients had clinical and immunohistochemical findings consistent with the genetic diagnosis (Patient 33 with abnormal αDG and Patient 71 with abnormal laminin-α2); however; only a heterozygous variant was identified on sequencing. In two patients the diagnosis was made by CMA.

Next Generation Sequencing produces a genetic diagnosis in 22/33 (66.6%) CMD patients unsolved using traditional diagnostic approaches

Of the 80 probands who remained undiagnosed after conventional investigation, 31 were available for inclusion in NGS studies (Figure 2). Two families declined participation and the remaining families were not contactable. Two recently ascertained probands (Patient 117 and 118) were investigated by NGS without a muscle biopsy or candidate gene sequencing.

Eleven patients were investigated with a NGS neuromuscular gene panel (Panel A – 6, Panel B – 4, both - 1) and 22 with WES. DNA from the parents was included in WES studies in 19 cases and in three cases affected or unaffected siblings were included. Diagnoses were made in 22/33 (66.6%) (18 confirmed, 1 possible and 3 unpublished novel findings) (Table e1 (online)). Ten were diagnosed by NGS panel and 12 by WES.

The subgroup investigated with NGS had remained undiagnosed despite extensive research-based candidate gene sequencing and was thus enriched for gene discovery. Two probands had variants in *GMPPB*, contributing to identification of this gene as a cause of α-dystroglycanopathy (14,15). Patient 44, and her affected sister, had homozygous recessive variants in *PIGY*, and a novel multisystem disease secondary to a deficiency of GPI anchor biosynthesis (16). A homozygous recessive variant in *ACTA1* was identified as a cause of rigid spine muscular dystrophy, a new phenotype of *ACTA1*-related disease (13). Three strong candidate genes are not yet published. Two patients had causative variants in genes published after enrolment in this study (*GFPT1* and *MICU1*) (17,18).

NGS also facilitated diagnosis of *RYR1* or *TTN*-related disease in 5 patients. Both genes are large and have previously been technically difficult to Sanger sequence.

In Patient 99 a heterozygous missense variant in *LARGE* was identified by WES. His phenotype was consistent with α-dystroglycanopathy. CMA detected a ..kb intragenic deletion of *LARGE* (22q12.3(33,774,511-34,221,251)) inherited in trans, highlighting the complementary nature of these investigations.

Data from WGS was available for 3 probands undiagnosed despite WES. To date this has not yielded a genetic diagnosis for these patients; however, optimization of bioinformatics is continuing. Patient 107 had a retrogene insertion in *MTMR2* of uncertain significance.

For the 63 probands who obtained a genetic diagnosis, the median age at diagnosis was 10 years (range 18 months – 42 years). The two probands investigated by NGS without muscle biopsy (Patient 117 and 118) had a genetic diagnosis 18 and 30 months after initial

presentation. The inheritance was *de novo* dominant in 25 probands, recessive in 37 probands, and X-linked in 1.

Genetic diagnoses within the cohort are heterogeneous

73% (46/63) diagnosed probands had variants in a recognized CMD gene (Figure 4). In 41/46 the gene was well known prior to this study (*FKRP*, *FKTN*, *LARGE (*2), *POMGNT1*, *POMT1* (2), *POMT2* (2), *LAMA2* (8); COL6 (16), *LMNA* (6), and *SEPN1* (2)). In the remaining 5, the gene was identified as causing CMD during, or as a result of this study (*GMPPB (2), MICU1, ACTA1* and *PIGY*).

Eleven probands (17%) had variants in genes known to be associated with congenital myopathies (*DNM2* (2), *RYR1* (4), *SIL1* (1), *ACTA1* (1), and *TTN* (3)). Patient 60 had variants in *GFPT1*, a recently published cause of a limb girdle myasthenia (17).

Biochemical and pathological features predict likelihood of a genetic diagnosis

Creatine kinase (CK) measurements were available for 114 probands. Forty probands had CK levels >1000 IU/L on at least one occasion, 24 had mild elevation (200-1000 IU/L) and 50 had normal levels. A genetic diagnosis was more likely to be obtained in those with a CK >200 IU/L (42/64, 65.6%) compared with those with a normal CK (20/50, 40%) (p=0.003). A diagnosis was also more likely, in those with a CK 200-1000 IU/L, than >1000 IU/L (19/24 compared with 23/40; p=0.039). Of the 16 patients with collagen VI-related CMD, 13 had a CK measuring ~200-1000 IU/L, none had a level >1000 IU/L, and 3 had normal levels.

Histological data was available for 119 probands. In 81 the muscle biopsy was classified as dystrophic, in 34 it was non-specific and in 3 it was normal. Probands with a dystrophic

muscle biopsy were more likely to achieve a genetic diagnosis than patients with a non-dystrophic or normal biopsy (49/81 (61%) compared with 10/37 (27%); p=0.0004).

Both factors are considered in Table 2. Elevated CK predicted a gene traditionally associated with CMD. All patients with *LAMA2*-related CMD, α-dystroglycanopathies, *LMNA*-related CMD, 1/2 with *SEPN1*-related CMD and 12/15 with collagen VI myopathies were within this group. Interestingly, the group of patients with dystrophic biopsies but normal CK included patients with mutations in *TTN*, *RYR1*, and *DNM2*. These genes are known to be associated with histological findings that mimic dystrophic findings, such as centralized nuclei and fibre size variation.

Immunohistochemical analysis accurately predicts CMD subtype

A classification could be made on the basis of immunohistochemical analysis for 58/114 probands (51%) (Figure 3 (online)). Causative variatnts were identified in *COL6A1*, *COL6A2* or *COL6A3* in 14/21 probands (67%) classified as having a collagen VI-related disorder. Only one proband (Patient 58) was missed by this classification, because the collagen VI reduction was classified as mild, rather than moderate or severe. Recessive variants in *LAMA2* were found in 7/11 (64%) probands classified as having laminin-α2 deficiency. Patient 71 had a heterozygous variant only identified by Sanger sequencing and did not have WES. No *LAMA2* patients were missed by this classification.

A genetic diagnosis was confirmed for 16/26 probands classified as having an α-dystroglycanopathy. Ten (38%) had variants in genes known to cause α-dystroglycanopathy (*FKRP*-1; *FKTN*-1; *LARGE* -2; *POMGNT1*-1; *POMT1*-2, *POMT2*-2 ; *GMPPB*-1) and 6 (23%) had variants in other genes (*DNM2*-2; *GFPT*-1; *RYR1*-3). One patient with a *GMPPB*

mutation was missed by this classification because of mild rather than moderate or severe reduction of α-dystroglycan.

Overall, a genetic diagnosis was more likely in the group with an immunohistochemical classification (39/58; 67%) compared with the group who could not be classified (19/56; 34%) (p=0.0002).

DISCUSSION 1123

This study describes a cohort of 124 potential CMD patients referred to a specialist neuromuscular diagnostic service. Patients were investigated traditionally as outlined in a recent Neurology review article on the evaluation, diagnosis and management of congenital muscular dystrophy (19). Using this approach, a genetic diagnosis was achieved in only 34%, despite this cohort being investigated in an expert research centre with access to immunohistochemical and Western blot analysis and research-based Sanger sequencing of known disease genes. Of the undiagnosed patients, 33 were investigated by either a NGS neuromuscular gene panel or WES and a diagnosis was achieved for 67%. Overall, a diagnosis was achieved for 51% (63/124) of the total cohort; however, 49 undiagnosed patients were not available for NGS studies. If these patients are excluded, a diagnosis was achieved for 63/75 (84%) patients investigated by candidate gene sequencing followed by WES if negative.

This cohort proves the value of immunohistochemical staining in correctly identifying the CMD subtype. A genetic diagnosis was significantly more likely in patients who could be classified in this way. In our cohort, antibodies to laminin-α2 and collagen VI were the most sensitive and specific. Moderate or severe reduction in α-dystroglycan was less specific,

reflecting the technical difficulties of working with antibodies to glycosylated α-dystroglycan (20). However, it is important to consider that in this large cohort an immunohistochemical classification, which is then used to guide candidate gene sequencing, could only be made in 51% (58/114) of patients.

Understanding of the clinical phenotype and natural history of different CMD subtypes is improving, such that the more common subtypes (collagen VI, laminin α2, α-dystroglycanopathies, *SEPN1*- and *LMNA*-related muscular dystrophy) should be recognized in a specialist neuromuscular clinic. However, only 35% (43/124) of our cohort had a genetic diagnosis confirming one of these subtypes. This figure is comparable with a UK cohort in which these diagnoses comprised 46% of the population (1).

Greater than 50% of the time, the neuromuscular physician is presented with a patient who does not fit easily into a CMD subtype on the basis of clinical evaluation and traditional diagnostic work up. As evidenced by the diagnoses in this cohort, there is considerable overlap between the clinical and histological features of congenital muscular dystrophy and congenital myopathies. Most patients (73%) with confirmed genetic diagnoses had variants in genes recognized to cause congenital muscular dystrophy, but 27% had alternative diagnoses. These included variants in genes better known as causes of congenital myopathy and in one case a congenital myasthenic syndrome.

A recently published Neurology review article found Level C evidence for candidate gene testing for specific congenital muscular dystrophy subtypes, and recommended considering WES as this technology becomes more accessible and affordable (19). Candidate gene sequencing is expensive, time consuming, and yields a diagnosis in less than 50% of patients.

The diversity of genetic diagnoses in our cohort, and the presence of a recognizable phenotype or immunohistochemical classification to guide candidate gene sequencing in less than 50% of the cohort, argues for using NGS as a first line investigation (Figure 5). This is now common practice in our centre. The cost of next generation sequencing is falling rapidly and is commercially available in many centres. Significantly, a neuromuscular gene panel would identify 58 of the 63 genetic diagnoses in our cohort. Two patients with micro-deletions would not have been detected, reinforcing the importance of CMA in detecting large-scale deletions not detected by NGS technology.

The diagnostic yield of prospective investigation with NGS is uncertain. The results of this study suggest it is between 50 and 85%. The largest previously published neuromuscular cohort (incorporating a broader range of disorders with onset of neuromuscular weakness or hypotonia less than 5 years of age) found a definitive genetic diagnosis for 21 of 43 (49%) using a NGS panel of 579 myopathy genes (21). In less selective cohorts the diagnosis rates are lower; 25% for 2000 consecutive patients referred to Baylor Genetics for WES for Mendelian disease (22).

Given the size of our cohort and bias in selecting probands for investigation by a gene panel versus WES, this study cannot draw conclusions about the relative efficacy of these different approaches. A previously published neuromuscular cohort study found a higher diagnosis rate for a 41 gene panel compared with clinician-requested single gene testing (46% vs 15-19%) (23). Coverage of the panel approach, with targeted capture of neuromuscular disease genes and Sanger fill-in of low-coverage exons, was better than WES, with 11 to 18% of pathogenic variants potentially missed by WES (23).

Although a targeted panel approach requires ongoing update to the panel as new genetic causes of neuromuscular disease are identified, better coverage makes this approach attractive, and we propose that it should be currently considered as the first line investigation. Patients who remain without a genetic diagnosis despite investigation with a neuromuscular panel should be considered for research-based whole exome or whole genome sequencing, where confirming candidate gene pathogenicity will require functional studies, international collaboration and identification of further affected patients (Figure 5).

Increasingly, the role of the muscle biopsy and its position in the diagnostic algorithm is being questioned. In our cohort, a genetic diagnosis was more likely in patients with an elevated CK, or dystrophic biopsy findings, however, neither was sensitive or specific. The muscle biopsy is expensive, invasive, can be challenging in infants and children with severe weakness and impaired respiratory function, and has a risk of a malignant hyperthermia reaction for some patients. In our cohort it was also associated with delay to genetic diagnosis. The muscle biopsy will not become obsolete, but should be considered after genetic testing, to help confirm the pathogenicity of novel variants by demonstrating reduced protein levels or for RNA sequencing or cDNA analysis to prove splice site disruption.

Despite the enormous advances seen in genetic diagnosis over the last 10 years, results from our cohort and the current literature, suggests that up to 50% of patients with CMD remain without a genetic diagnosis following NGS. The challenge of neuromuscular genetic research now lies with these unsolved families, who may bear variants in genes missed by bioinformatics filtering or sequencing coverage, or who have more complex genetic abnormalities that may be revealed via whole genome and RNA sequencing. As the more common causes of neuromuscular disease are identified, recognition of rarer cases, of which

there may only be one patient in any given cohort, will depend on the strength of international collaboration, open access databases and powerful bioinformatics.

Our success rate in diagnosing CMD has doubled over the past 10 years. As NGS enters routine clinical practice, it is transforming the traditional approach to diagnosis and our data supports the efficacy, time- and cost- effectiveness of this approach. Timely diagnosis has many benefits including the end of what is often a long diagnostic odyssey and can help change the focus from diagnosis to management of the child's difficulties (24). Health care and medical surveillance for complications can be individualized (11) and families are provided with information which can restore reproductive confidence and be used in prenatal diagnosis. The challenge for health care service providers is to now incorporate and streamline access to NGS panels for referring clinicians as a first-line diagnostic enquiry.

References

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1. Clement EM, Feng L, Mein R, Sewry CA, Robb SA, Manzur AY, et al. Relative frequency of congenital muscular dystrophy subtypes: Analysis of the UK diagnostic service 2001-2008. Neuromuscular Disorders. Elsevier B.V; 2012 Jun 1;22(6):522–7.
2. Peat RA, Smith JM, Compton AG, Baker NL, Pace RA, Burkin DJ, et al. Diagnosis and etiology of congenital muscular dystrophy. Neurology. 2008 Jul 29;71(5):312–21.
3. Day JW. Congenital muscular dystrophy in a new age. Neurology. 2008 Jul 29;71(5):308–9.
4. x. 14.6 Mentor Feature MH NEW.indd. 2007 Jun 9;:1–8.
5. Jimenez-Mallebrera C, Brown SC, Sewry CA, Muntoni F. Congenital muscular dystrophy: molecular and cellular aspects. CMLS, Cell Mol Life Sci. 2005 Apr;62(7-8):809–23.
6. Schessl J, Goemans NM, Magold AI, Zou Y, Hu Y, Kirschner J, et al. Predominant fiber atrophy and fiber type disproportion in early ullrich disease. Muscle Nerve. 2008 Sep;38(3):1184–91.

7. Godfrey C, Clement E, Mein R, Brockington M, Smith J, Talim B, et al. Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain. 2007 Oct 1;130(10):2725–35.

8. Baker NL. Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. Human Molecular Genetics. 2004 Nov 17;14(2):279–93.

9. Guicheney P, Vignier N, Zhang X, He Y, Cruaud C, Frey V, et al. PCR based mutation screening of the laminin alpha2 chain gene (LAMA2): application to prenatal diagnosis and search for founder effects in congenital muscular dystrophy. J Med Genet. 1998 Mar;35(3):211–7.

10. Clarke NF, Kidson W, Quijano-Roy S, Estournet B, Ferreiro A, Guicheney P, et al. SEPN1: Associated with congenital fiber-type disproportion and insulin resistance. Ann Neurol. 2006 Mar;59(3):546–52.

11. Menezes MP, Waddell LB, Evesson FJ, Cooper S, Webster R, Jones K, et al. Importance and challenge of making an early diagnosis in LMNA-related muscular dystrophy. Neurology. 2012 Apr 17;78(16):1258–63.

12. Bitoun M, Maugenre S, Jeannet P-Y, Lacène E, Ferrer X, Laforet P, et al. Mutations in dynamin 2 cause dominant centronuclear myopathy. Nature Genetics. 2005 Oct 16;37(11):1207–9.

13. O'Grady GL, Best HA, Oates EC, Kaur S, Charlton A, Brammah S, et al. Recessive ACTA1 variant causes congenital muscular dystrophy with rigid spine. European Journal of Human Genetics. Nature Publishing Group; 2014 Sep 3;:1–4.

14. Carss KJ, Stevens E, Foley AR, Cirak S, Riemersma M, Torelli S, et al. Mutations in GDP-Mannose Pyrophosphorylase B Cause Congenital and Limb-Girdle Muscular Dystrophies Associated with Hypoglycosylation of α-Dystroglycan. The American Journal of Human Genetics. The American Society of Human Genetics; 2013 Jul 11;93(1):29–41.

15. Cabrera-Serrano M, Ghaoui R, Ravenscroft G, Johnsen RD, Davis MR, Corbett A, et al. Expanding the phenotype of GMPPB mutations. Brain. 2015 Mar 23;138(4):836–44.

16. Ilkovski B, Pagnamenta AT, O'Grady GL, Kinoshita T, Howard MF, Lek M, et al. Mutations in PIGY: expanding the phenotype of inherited glycosylphosphatidylinositol (GPI) deficiencies. Human Molecular Genetics. 2015 Aug 20.

17. Huh S-Y, Kim H-S, Jang H-J, Park Y-E, Kim D-S. Limb-girdle myasthenia with tubular aggregates associated with novel GFPT1 mutations. Muscle Nerve. 2012 Sep 13;46(4):600–4.

18. Logan CV, Szabadkai G, Sharpe JA, Parry DA, Torelli S, Childs A-M, et al. Loss-of-function mutations in MICU1 cause a brain and muscle disorder linked to primary alterations in mitochondrial calcium signaling. Nature Publishing Group. Nature Publishing Group; 2013 Dec 15;46(2):188–93.

19. Kang PB, Morrison L, Iannaccone ST, Graham RJ, Bönnemann CG, Rutkowski A, et al. Evidence-based guideline summary: evaluation, diagnosis, and management of congenital muscular dystrophy: Report of the Guideline Development Subcommittee of the American Academy of Neurology and the Practice Issues Review Panel of the American Association of Neuromuscular & Electrodiagnostic Medicine. Neurology. 2015 Mar 31;84(13):1369–78.

20. Bönnemann CG, Wang CH, Quijano-Roy S, Deconinck N, Bertini E, Ferreiro A, et al. Diagnostic approach to the congenital muscular dystrophies. Neuromuscular Disorders. Elsevier B.V; 2014 Apr 1;24(4):289–311.

21. Chae J-H, Vasta V, Cho A, Lim BC, Zhang Q, Eun SH, et al. Utility of next generation sequencing in genetic diagnosis of early onset neuromuscular disorders. J Med Genet. 2015 Mar;52(3):208–16.

22. Yang Y, Muzny DM, Reid JG, Bainbridge MN, Willis A, Ward PA, et al. Clinical Whole-Exome Sequencing for the Diagnosis of Mendelian Disorders. N Engl J Med. 2013 Oct 17;369(16):1502–11.

23. Ankala A, da Silva C, Gualandi F, Ferlini A, Bean LJH, Collins C, et al. A comprehensive genomic approach for neuromuscular diseases gives a high diagnostic yield. Ann Neurol. 2014 Dec 17;77(2):206–14.

24. Zurynski Y, Frith K, Leonard H, Elliott E. Rare childhood diseases: how should we respond? Archives of Disease in Childhood. 2008 Dec 1;93(12):1071–4.

Figure 1: Whole exome sequencing analysis pipeline

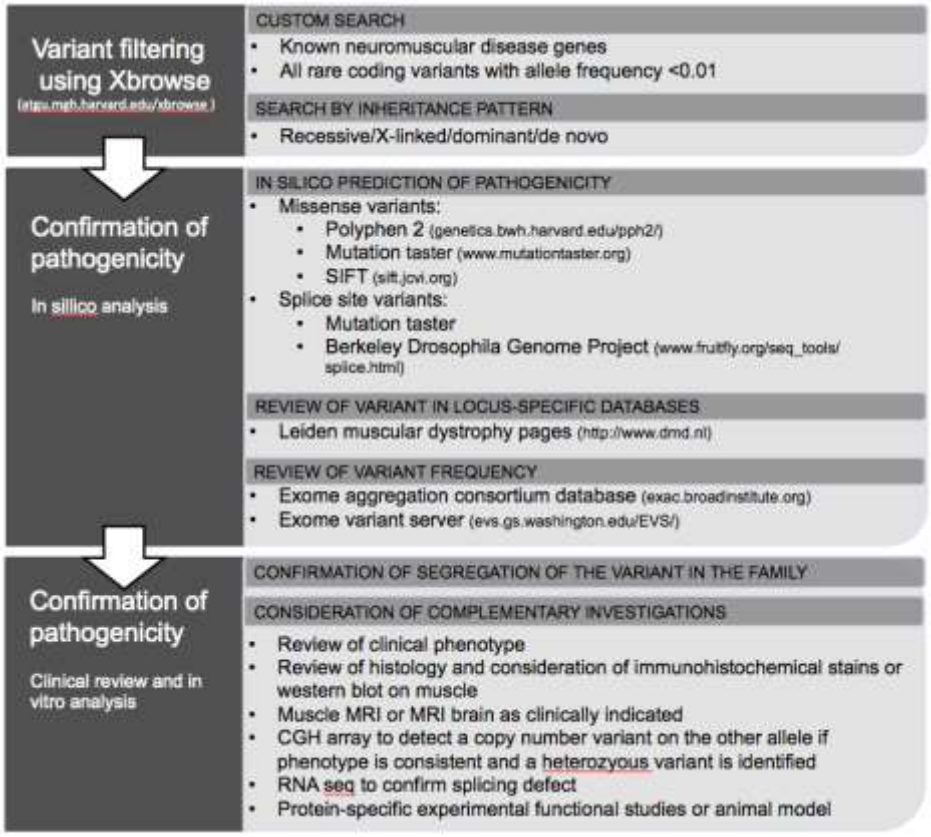
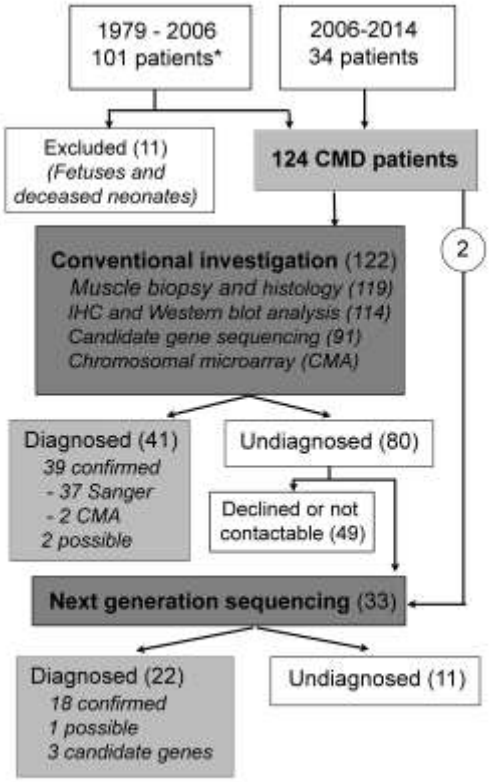

Variant filtering using Xbrowse
(atgu.mgh.harvard.edu/xbrowse/)
CUSTOM SEARCH
Known neuromuscular disease genes
All rare coding variants with allele frequency <0.01
SEARCH BY INHERITANCE PATTERN
Recessive/X-linked/dominant/de novo
Confirmation of pathogenicity
In silico analysis
IN SILICO PREDICTION OF PATHOGENICITY
Missense variants:
Polyphen 2 (genetics.bwh.harvard.edu/pph2/)
Mutation taster (www.mutationtaster.org)
SIFT (sift.jcvi.org)
Splice site variants:
Mutation taster
Berkeley Drosophila Genome Project (www.fruitfly.org/seq_tools/splice.html)
REVIEW OF VARIANT IN LOCUS-SPECIFIC DATABASES
Leiden muscular dystrophy pages (http://www.dmd.nl)
REVIEW OF VARIANT FREQUENCY
Exome aggregation consortium database (exac.broadinstitute.org)
Exome variant server (evs.gs.washington.edu/EVS/)
Confirmation of pathogenicity
Clinical review and in vitro analysis
CONFIRMATION OF SEGREGATION OF THE VARIANT IN THE FAMILY
CONSIDERATION OF COMPLEMENTARY INVESTIGATIONS
Review of clinical phenotype
Review of histology and consideration of immunohistochemical stains or western blot on muscle
Muscle MRI or MRI brain as clinically indicated
CGH array to detect a copy number variant on the other allele if phenotype is consistent and a heterozyous variant is identified
RNA seq to confirm splicing defect
Protein-specific experimental functional studies or animal model

Figure 2: Cohort ascertainment and investigation



124 CMD patients were ascertained. Conventional investigation was with protein-based screening of muscle biopsy specimens and candidate gene sequencing. Undiagnosed patients were investigated with Next Generation Sequencing technologies. 11 fetuses and deceased neonates were excluded because they had not been the subject of further investigations, and significant time had elapsed since ascertainment.

* These patients were included in the cohort published by Peat et al, Diagnosis and etiology of congenital muscular dystrophy, Neurology. 2008; 71:312-321

Figure 3 (online): Immunohistochemical analysis, candidate gene sequencing and genetic diagnoses

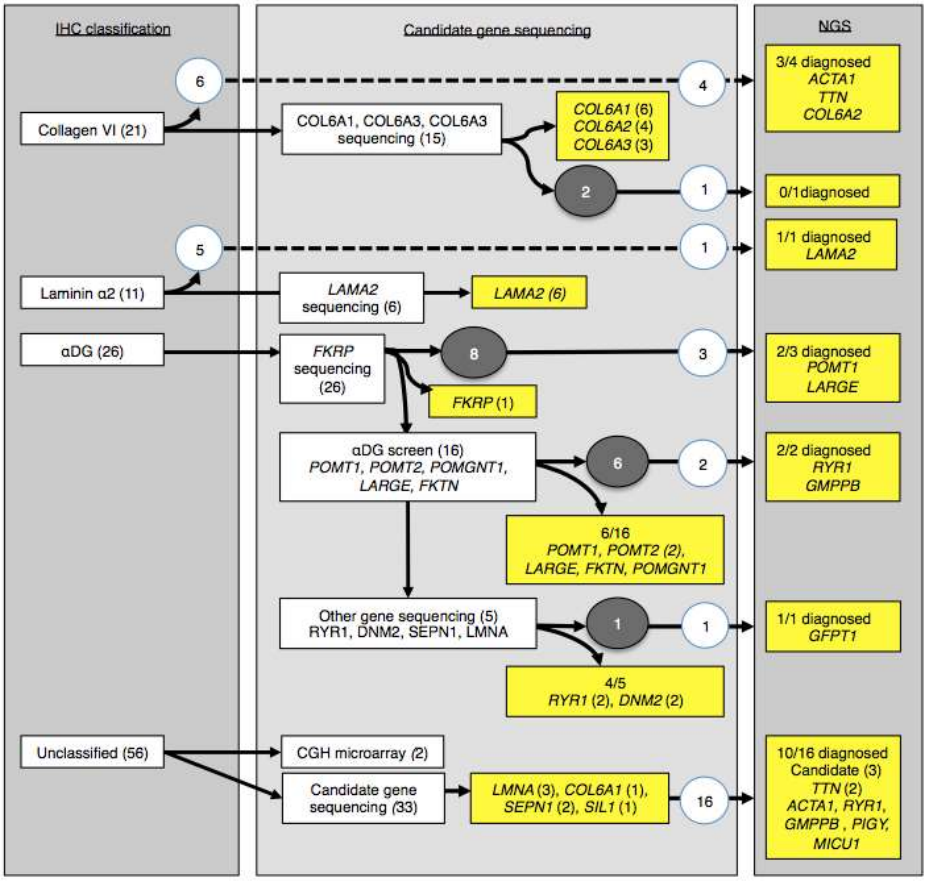
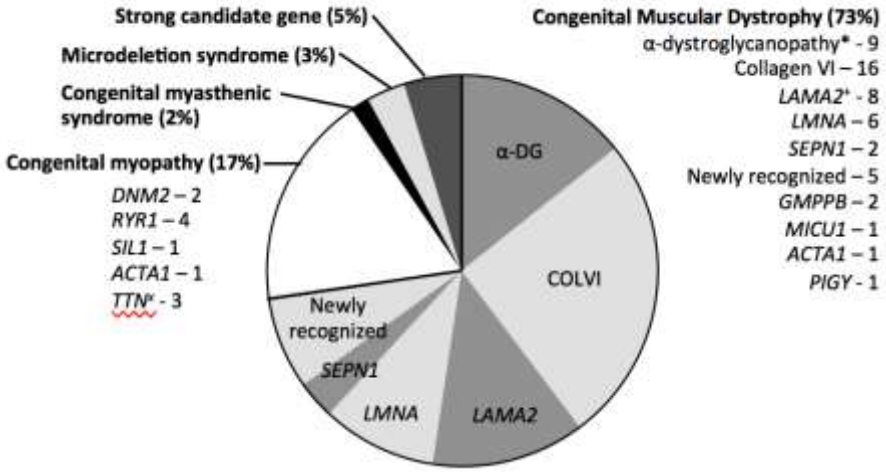


Figure 3 illustrates the classification of patients by immunohistochemical (IHC) analysis and diagnoses made by candidate gene sequencing and next generation sequencing (NGS). Left panel (IHC classification): 114 probands had immunohistochemical (IHC) analysis performed on muscle biopsy specimens. 58 probands were able to be classified on the basis of a moderate or severe reduction in collagen VI, laminin-α2 or glycosylated α-dystroglycan. 56 patients could not be classified by IHC analysis. Middle panel (Candidate gene sequencing): Candidate gene sequencing was performed on the basis of IHC classification, and when unclassified, on the basis of clinical phenotype. The gene sequenced is indicated in

a white box, and the confirmed genetic diagnoses are shown by yellow boxes. The number of patients undiagnosed after candidate gene sequencing is shown in a grey circle.

Right panel (NGS): NGS was performed on the number of patients indicated with a white circle. The confirmed diagnoses are shown in yellow.

Figure 4: Heterogeneity of genetic diagnoses in a congenital muscular dystrophy cohort



73% of the cohort had variants in genes previously recognized, or recently described as causes of congenital muscular dystrophy. 17% of patients had variants in genes better recognized as causes of congenital myopathy. One patient had a congenital myasthenic syndrome with compound heterozygous variants in *GFPT1*.

* Includes one patient with probable *LARGE*-CMD, but with only a heterozygous variant detected in *LARGE.*

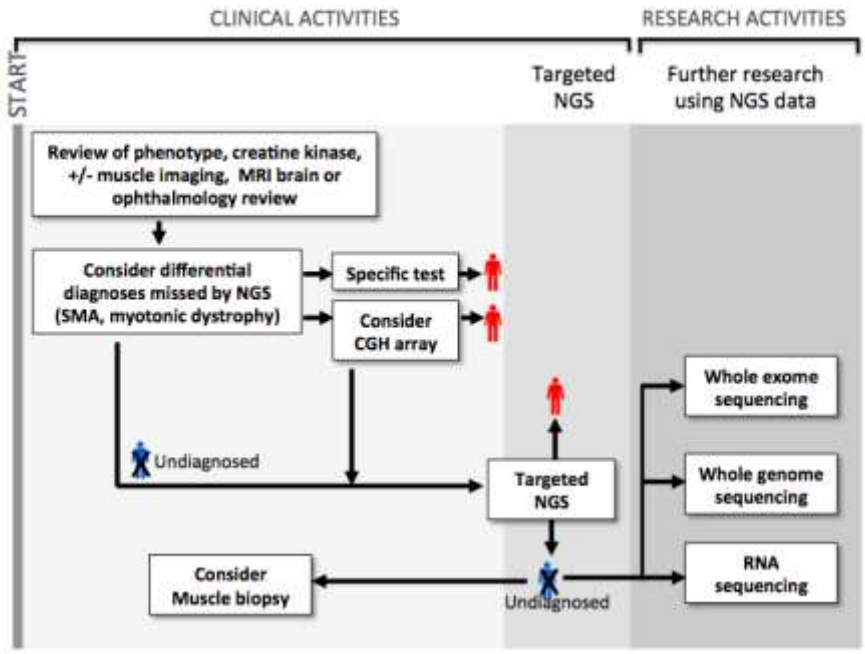
+ Includes one patient with probable *LAMA2*-CMD, but with only a heterozygous variant in *LAMA2*.

[x] Includes one patient with probable *TTN* myopathy, with a frameshift variant, and a missense variant of uncertain pathogenicity.

Table 2: Review of genetic diagnoses by muscle biopsy findings and creatine kinase levels.

	Dystrophic	Non-dystrophic
Elevated CK	**36/54 (67%)** Elevated CK (>1000IU/L): *FKRP*, *FKTN*, *POMT1* (2), *POMT2* (2), *POMGNT1*, *LARGE* (2) *GMPPB* (2), *LAMA2* (6), *LMNA*, *ACTA1* Mild elevation (<1000IU/L): *COL6A1* (7), *COL6A2* (3), *COL6A3* (2), *LMNA* (3), *PIGY*, *SEPN1*	**2/5 (40%)** *MICU1, SIL1*
Normal CK	**12/25 (48%)** *COL6A2, COL6A3, DNM2, LAMA2, RYR1 (2), TTN (3), ACTA1,* , GFPT1, Candidate	**8/24 (35%)** Microdeletion (2), *COL6A2*, *DNM2*, *RYR1*, *SEPN1*, candidate (2)
Excluded are 16 patients who either did not have a muscle biopsy, or for whom, the CK result had not been recorded.		

Figure 5: Proposed diagnostic algorithm for Congenital Muscular Dystrophy



Proposed diagnosis of suspected congenital muscular dystrophy patients using a targeted next generation sequencing neuromuscular gene panel after exclusion of diagnoses missed by this technology. Muscle biopsy should be considered in patients undiagnosed by NGS.

CMA, chromosomal microarray; SMA, spinal muscular atrophy; NGS, next generation sequencing; RNA, RNA sequencing