Genotype-guided diagnostic reassessment after exome sequencing in neuromuscular disorders: experiences with a two-step approach

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Background and purpose: Next-generation sequencing has greatly improved the diagnostic success rates for genetic neuromuscular disorders (NMDs). Nevertheless, most patients still remain undiagnosed, and there is a need to maximize the diagnostic yield.

Methods: A retrospective study was conducted on 72 patients with NMDs who underwent exome sequencing (ES), partly followed by genotype-guided diagnostic reassessment and secondary investigations. The diagnostic yields that would have been achieved by appropriately chosen narrow and comprehensive gene panels were also analysed.

Results: The initial diagnostic yield of ES was 30.6% (n = 22/72 patients). In an additional 15.3% of patients (n = 11/72) ES results were of unknown clinical significance. After genotype-guided diagnostic reassessment and complementary investigations, the yield was increased to 37.5% (n = 27/72). Compared to ES, targeted gene panels (<25 kilobases) reached a diagnostic yield of 22.2% (n = 16/72), whereas comprehensive gene panels achieved 34.7% (n = 25/72).

Conclusion: Exome sequencing allows the detection of pathogenic variants missed by (narrowly) targeted gene panel approaches. Diagnostic reassessment after genetic testing further enhances the diagnostic outcomes for NMDs.

Introduction

Neuromuscular disorders (NMDs) represent a clinically and genetically heterogeneous group of diseases affecting motor neurons, peripheral nerves, the neuromuscular junction or muscle tissue, often with an overlapping range of symptoms. A considerable proportion of NMDs are known or suspected to have a monogenic aetiology. However, due to the marked phenotypic overlap and the contribution of as yet unidentified disease genes, single gene testing has been widely unsuccessful.

With the advent of next-generation sequencing (NGS) approaches such as gene panels, exome sequencing (ES) or genome sequencing, a growing number of causative variants can be identified [1–3]. Even so, the majority of patients with NMDs still remain undiagnosed with variable success rates, mainly depending on the selected patient population and the applied method [4–12]. It is therefore a major challenge facing clinicians and geneticists to further enhance the application of NGS techniques.

For example, it is a subject of ongoing debate which exact NGS approach is optimal from a diagnostic and cost-point perspective [13]. ES has the inherent potential to identify novel disease genes and allows a diagnostic re-evaluation at a later time, whereas gene panels are postulated to secure a higher coverage. The diagnostic utility of comprehensive panels and ES has been considered to be comparable in practice [14,15]. In contrast, it is still unclear whether the widely used small-scale panels – as often
mandated by national health care providers – achieve similar results.

Another issue requiring refinement is the correct identification of causative variants against the abundance of irrelevant background variation. The widely used guidelines of the American College of Medical Genetics and Genomics (ACMG) consider various strands of genetic and clinical evidence for variant classification [16]. Whilst some variants can reliably be classified as benign or pathogenic right away, the causative effect often remains uncertain after genetic testing (variants of unknown significance, VUS) [17]. It has already been shown that uncertain findings can be successfully reclassified using clinical reconsideration, complementary family genotyping or supporting functional data [18–20]. Such approaches have the ability to reveal minor and initially overlooked clinical features, bringing to light specific phenotypic fits potentially underpinning the pathogenic relevance of variants.

In this retrospective analysis of routine ES in patients with NMDs, an evaluation was made of the degree to which a critical reassessment after ES may enhance the diagnostic outcomes in a real-world setting. Secondly, diagnostic ES was virtually compared to frequently used NGS gene panels.

### Methods

#### Patients

All patients with neuromuscular phenotypes seen at the Department of Neurology (Medical University of Vienna, Austria) who underwent diagnostic ES between July 2015 and December 2018 were retrospectively selected. The indication was determined after reviewing and complementing prior diagnostic procedures. A genetic aetiology was considered by NMD specialists, if no acquired cause could be established after an extensive diagnostic work-up.

Informed consent (also regarding actionable findings) was obtained from included patients. The study was approved by the Ethics Committee of the Medical University of Vienna.

#### Exome sequencing and data analysis

Exomes were enriched in solution with SureSelect Human All Exon Kits 50 Mb V5 and 60 Mb V6 (Agilent, Santa Clara, CA, USA). DNA fragments were sequenced as 100 bp paired-end runs on an Illumina HiSeq2500 or HiSeq4000 system (Illumina, San Diego, CA, USA). The mean average coverage in our exome dataset was 146.8×.

Variants were filtered based on the minor allele frequency (MAF), which was estimated using our in-house database (>15 000 exomes) and confirmed by ExAC, (https://exac.broadinstitute.org) or gnomAD, (https://gnomAD.broadinstitute.org). Variant prioritization was based on autosomal recessive (MAF < 0.1%) and autosomal dominant (MAF < 0.01%) filters. Copy number variation analysis was done using ExomeDepth [21] and Pindel [22]. A detailed description of the sequencing and data analysis pipeline is provided as supplementary file (Data S1).

#### Variant interpretation by genetic laboratory

Using ACMG criteria, variants were classified as (i) pathogenic, (ii) likely pathogenic or (iii) VUS [16]. VUS that were not related to the phenotype in question and (likely) benign variants were not reported. (Likely) pathogenic variants were considered sufficient for establishing a genetic diagnosis for dominant disorders. For recessive disorders, two (likely) pathogenic variants were required. Otherwise, for example in the case of one pathogenic variant and one VUS, the laboratory conclusion was considered of ‘unknown clinical significance’. Exomes were screened for actionable variants as recommended by ACMG [23]. At the time of initial analysis, basic clinical information was available for geneticists.

#### Diagnostic reassessment and variant reclassification

After ES, all (likely) pathogenic variants were considered causative, if compatible with the inheritance pattern and phenotype (definite/likely diagnoses). VUS in genes related to the NMD phenotype guided diagnostic reassessment with the aim of clarifying their clinical relevance. Investigations such as family genotyping, histology or biochemical analyses were initiated. Existing literature on previously reported families with mutations in the same gene was specifically screened to compare the phenotypes with our index cases. After reassessment, VUS were re-evaluated and partly reclassified according to ACMG [16]. Since ACMG only provides categories for variants, the following categories were additionally defined to provide patients with a firm diagnostic conclusion (as suggested by Shashi et al. [19]): (i) definite diagnosis (one pathogenic variant for dominant and two pathogenic variants for recessive disorders), (ii) probable diagnosis (one likely pathogenic variant for dominant and at least two likely pathogenic variants for recessive disorders), (iii) possible diagnosis (one VUS for dominant and either one VUS and one (likely) pathogenic variant or two VUS for recessive disorders) and (iv) no diagnosis. Final decisions were made after an
interdisciplinary discussion involving NMD specialists and a geneticist.

Comparison of exome sequencing to gene panels

To virtually compare the diagnostic yields between gene panels and ES, one commercially available targeted panel comprising less than 25 kilobases (kb) that seemed most appropriate for each individual phenotype (4–17 genes) and one comprehensive NGS panel (up to 344 genes) were retrospectively selected. The diagnostic yields of both selected panels were compared to the outcome of ES (Table S1).

Results

Patient characteristics

In all, 72 patients with neuromuscular phenotypes underwent diagnostic ES between July 2015 and December 2018 and were selected for analysis.

The median age at the time of ES was 47 years (range 19–78 years). 54.2% of all patients (n = 39) were male; 45.8% (n = 33) were female. In 30.6% (n = 22) a positive family history for the disease or a similar disease phenotype was reported. The median age at disease onset was 30 years (range 0–74 years). In 41.7% (n = 30) either the muscle or the neuromuscular junction was the predominant lesion site; 40.3% (n = 29) exhibited a more complex phenotype involving anterior horn cells or motor neurons, and 18.1% (n = 13) displayed a peripheral nerve disorder.

Molecular diagnoses

The initial diagnostic yield according to the laboratory reports was 30.6% (n = 22/72 patients). In addition, for 11 patients (15.3% of the cohort), 12 VUS in 11 different genes were reported to be potentially associated with the phenotype. After genotype-guided diagnostic reassessment and additional investigations, the final diagnostic yield was increased to 37.5% (n = 27/72 patients). In 39 individuals (54.2%) no relevant variants were identified. The main characteristics of patients with the reported variants are summarized in Table 1.

Eighteen of 27 patients (66.7%) with a genetic diagnosis after reassessment had an autosomal recessive disorder and eight (29.6%) an autosomal dominant disorder. In one patient (3.7%) a dual pathology involving DMD (hemizygous two exon deletion) and SCN4A (heterozygous missense variant) was diagnosed.

Overall, a total of 24 different OMIM (Online Mendelian Inheritance in Man) diagnoses could be established. SPG7 (MIM#607259) was represented three times and SPG4 (MIM#182601) and CMS4C (MIM#608931) were each represented twice in this cohort. Each of the remaining 21 diagnoses was represented once.

Genotype-guided diagnostic reassessment and variant reclassification

After diagnostic reassessment, the results of unknown clinical significance were reconsidered to be (probably) disease-related in five of 11 patients (Fig. 1, Table S2). In three of these five patients, this was due to specific phenotype features revealed in a second diagnostic step, e.g. muscle histology (ACMG criterion PP4), segregation analysis (ACMG criteria PM3 + PP1) and biochemical (functional) confirmation of pathogenicity (ACMG criterion PS3). One VUS in DNM2 was not considered disease-related due to the carriership of an unaffected parent, and another patient carrying a VUS in SMCHD1 showed normal D4Z4 methylation (no diagnosis). In the remaining four patients with reported VUS, pathogenicity remained uncertain after diagnostic reassessment (possible diagnosis).

As an example, a VUS in BICD2 (patient 17) led to an extensive review of the literature by the treating clinicians. Although family genotyping could not be done, previously reported families with missense variants in BICD2 were strikingly reminiscent of the patient’s specific clinical presentation (lower motor neuron disease, areflexia and marked predominance of lower limbs), and so the variant was upgraded to ‘likely pathogenic’ (likely diagnosis). Similarly, a VUS in TRPV4 (affecting a functional protein domain) was also upgraded to ‘likely pathogenic’ due to a highly specific phenotypic fit in patient 38 (lower motor neuron disease, vocal cord palsy and early respiratory involvement). One VUS in the RYR1 gene (which coexisted with one likely pathogenic variant in the same gene) in patient 39 could be changed to ‘likely pathogenic’ based on the specific features of a secondarily performed muscle biopsy, supporting RYR1-related myopathy. Patient 50 with spastic paraparesis carried one VUS (along with one pathogenic variant) in KIF1A. After ES, these variants were shown to segregate with the phenotype in two affected out of four siblings, leading to the (likely) diagnosis of SPG30. A heterozygous carriership was confirmed in both unaffected parents. Another patient with spastic paraparesis (patient 56) had one VUS in CYP7B1 (along with a pathogenic variant). A biochemical analysis of serum 27-hydroxycholesterol levels led to an upgrade to ‘likely pathogenic’, confirming SPG5A.
| Patient ID | Sex | Clinical diagnosis                                      | Age at ES (years) | Gene(s)/variant(s)                                      | Inheritance pattern | OMIM diagnosis (#MIM) | Laboratory ACMG variant classification | Diagnostic reassessment | Final ACMG variant classification | Final diagnostic conclusion |
|-----------|-----|--------------------------------------------------------|-------------------|-------------------------------------------------------|---------------------|----------------------|----------------------------------------|-------------------------|-----------------------------------|-------------------------------|
| 3         | Female | Myasthenic syndrome                                   | 26                | **CHRNE** (hom): NM_000080.3:c.1327del, p.E443Kfs*64   | AR                  | **CMS4C** (#608931) | Pathogenic              | N.A.                                   | Pathogenic                | Pathogenic                        | Definite diagnosis             |
| 4         | Female | Limb girdle muscular dystrophy                        | 45                | **SGCA** (comp het): NM_000023.2:c.229C>T, p.R77C, c.796G>A, p.V247M | AR                  | **LGMD2D** (#608099) | Pathogenic              | N.A.                                   | Pathogenic                | Pathogenic                        | Definite diagnosis             |
| 6         | Female | Cardiomyopathy, skeletal myopathy                     | 32                | **RBCK1** (hom): NM_031229.2:c.896_899del, p.E299Vfs*46 | AR                  | **PGBM1** (#615895) | Pathogenic              | Literature search, immune phenotyping | Pathogenic                | Pathogenic                        | Definite diagnosis             |
| 8         | Female | Spastic paraparesis                                   | 55                | **SPG7** (hom): NM_003119.2:c.336G>A, p.R1129Q          | AR                  | **SPG7** (#607259)   | Pathogenic              | N.A.                                   | Pathogenic                | Pathogenic                        | Definite diagnosis             |
| 9         | Male   | Limb girdle muscular dystrophy and myotonia            | 49                | **DMD** (hem): NM_000109.3:deletion of exons 48 and 49   | DP                  | **BMD** (#300376)   | Pathogenic              | N.A.                                   | Pathogenic                | Likely diagnosis (dual pathology) |
| 10        | Male   | Upper and lower motor neuron disease                  | 47                | **DNM2** (het): NM_001005361.2:c.1493A>C, p.N498T      | AD                  | **CMTDIB** (#606482) | VUS                     | Family genotyping (including trio ES) | VUS                                    | No diagnosis                      |
| 11        | Male   | Spastic paraparesis, neuropathy                       | 19                | **MFN2** (het): NM_014874.3:c.1252C>T, p.R418*          | AD                  | **CMT2A2A** (#609260) | Pathogenic              | Re-phenotyping (rare association between MFN2 and spasticity described), NCS compatible | Pathogenic                | Definite diagnosis                |
| 12        | Female | Spastic paraparesis                                   | 55                | **SPAST** (het): NM_014946.3:c.1493+2_1493+5del, p.(?)   | AD                  | **SPG4** (#182601) | Pathogenic              | N.A.                                   | Pathogenic                | Definite diagnosis                | |

(continued)
| Patient ID | Sex | Clinical diagnosis | Age at ES (years) | Gene(s)/variant(s) | Inheritance pattern | OMIM diagnosis (#MIM) | Laboratory ACMG variant classification | Diagnostic reassessment | Final ACMG variant classification | Final diagnostic conclusion |
|-----------|-----|-------------------|------------------|--------------------|---------------------|----------------------|-------------------------------|-----------------------|-------------------------------|------------------------|
| 13        | Female | External ophthalmoplegia, ptosis | 25 | CHRNAE (hom) NM_000080.3: c.1327del, p.E443K*64 | AR | CMS4C (#608931) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 17        | Female | Lower limb-predominant muscular atrophy | 33 | BICD2 (het) NM_015250: c.1673G>C, p.R558P | AD | SMALED2 (#615290) | VUS | Re-phenotyping (specific phenotype fit, predominant affection of lower limbs) | Likely pathogenic | Likely pathogenic | Likely diagnosis |
| 19        | Female | Limb girdle muscular dystrophy | 36 | CAPN3 (com het) NM_000070.2: c.1342C>T, p.R448C c.1722del, p.S575Lfs*20 | AR | LGMD2A (#253600) | Likely pathogenic | N.A. | Likely pathogenic | Likely diagnosis |
| 22        | Male | Spastic paraparesis | 63 | SPG7 (hom) NM_003119.2: c.1552 + 1G>T, p.(?) | AR | SPG7 (#607259) | Pathogenic | Family genotyping (affected brother with same homozygous variant) | Pathogenic | Definite diagnosis |
| 25        | Male | Spastic paraparesis | 62 | SPG7 (hom) NM_003119.2: c.1552 + 1G>T, p.(?) | AR | SPG7 (#607259) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 27        | Male | Lower limb predominant myopathy, dysarthria, dysphagia | 62 | PABPN1 NM_004643.3: c.19_2(4), p.A7(4) | AD | OPMD (#164300) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 30        | Female | Polyneuropathy | 33 | MARS (het) NM_004990.3: c.181_183del, p.S61del HARS (het) NM_002109.4: c.1485G>T, p.E496D | AD | AD | CMT2U (#616280) | VUS | VUS | Possible diagnosis |
| 32        | Female | Lower limb predominant myopathy | 28 | TTN (comp het) NM_001267550.1: c.96697C>T, p.R32233* c.107578C>T, p.Q35860* | AR | LGMD2J (#608807) | Pathogenic | Family genotyping (confirming biallelic location) | Pathogenic | Definite diagnosis |
| Patient ID | Sex | Clinical diagnosis                                      | Age at ES (years) | Gene(s)/variant(s)                                                                 | Inheritance pattern | OMIM diagnosis (#MIM) | Laboratory ACMG variant classification | Diagnostic reassessment | Final ACMG variant classification | Final diagnostic conclusion |
|------------|-----|--------------------------------------------------------|-------------------|-----------------------------------------------------------------------------------|---------------------|-----------------------|----------------------------------------|------------------------|-----------------------------------|------------------------------|
| 35         | Male | Spastic paraparesis, mild cerebellar atrophy          | 38                | FA2H (comp het) NM_024306.4: c.968C>T, p.P323L c.1119A>T, p.3733C>ext*48        | AR                  | SPG35 (#612319)       | Likely pathogenic Likely pathogenic       | N.A.                   | Likely pathogenic                  | Likely diagnosis              |
| 38         | Male | Lower motor neuron disease                            | 52                | TRPV4 (het) NM_021625.4: c.1119A>T, p.A312V                                      | AD                  | SPMSA (#181405)       | VUS | Literature search (fitting phenotype, early respiratory involvement) | Likely pathogenic      | Likely pathogenic                  | Likely diagnosis              |
| 39         | Female | Proximal myopathy, vertical gaze palsy                | 48                | RYR1 (comp het) NM_000540.2: c.14647-3_14647del, p.? c.4405C>T, p.R1469W        | AR                  | Minicore myopathy (#255320) | Likely pathogenic Likely pathogenic        | Muscle biopsy (specificity of phenotype) | Likely pathogenic      | Likely diagnosis |
| 40         | Male | Inclusion body myopathy                               | 70                | MYOT (het) NM_006790.2: c.179C>T, p.S60F                                         | AD                  | MFM3 (#609200)       | Likely pathogenic N.A.                   | Likely pathogenic                  | Likely diagnosis              |
| 41         | Male | Proximal myopathy                                     | 52                | MYH2 (het) NM_017534.5: c.1267G>A, p.V423M                                         | AD/AR               | MYPOP (#605637)      | VUS | Muscle biopsy/ histology                      | VUS                      | Possible diagnosis |
| 44         | Female | Facioscapulohumeral muscular dystrophy                | 29                | SMCHD1 (het) NM_015295.2: c.2510T>C, p.V837A                                      | Digenic             | FSHD2 (#158901)       | VUS | D4Z4 methylation status (normal)            | VUS | No diagnosis |
| 50         | Female | Spastic paraparesis, polyneuropathy                   | 38                | KIF1A (comp het) NM_001244008: c.2909G>A, p.R970H c.1214_1215dup, p.N405C*1    | AR                  | SPG30 (#610357)      | VUS | Segregation analysis, specific phenotype    | Likely pathogenic Pathogenic | Likely diagnosis |
| 52         | Male | Polyneuropathy                                        | 72                | PMP22 (het) 15 Mb deletion Chr17:14,075,320-15,472,674                             | AD                  | HNPP (#162500)       | Pathogenic                  | Duo ES analysis (including affected daughter) | Pathogenic | Definite diagnosis |
| 53         | Male | Spastic paraparesis                                   | 57                | SPAST (het) NM_014946.3: c.1553T>C, p.L518P                                      | AD                  | SPG4 (#182601)      | Likely pathogenic N.A.                   | Likely pathogenic      | Likely diagnosis              |

(continued)
| Patient ID | Sex | Clinical diagnosis | Age at ES (years) | Gene(s)/variant(s) | Inheritance pattern | OMIM diagnosis (#MIM) | Laboratory ACMG variant classification | Diagnostic reassessment | Diagnostic conclusion |
|------------|-----|-------------------|------------------|-------------------|--------------------|----------------------|----------------------------------------|------------------------|----------------------|
| 54         | Female | Intermittent rhabdomyolysis | 35 | CPT2 (hom) NM_000098.2: c.338C>T, p.S113L | AR | CPT II deficiency, myopathic (#255110) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 56         | Female | Spastic paraparesis | 50 | CYP7B1 (comp het) NM_004820.3: c.825T>A, p.Y275*, c.1091C>T, p.S364L | AR | SPG5A (#270800) | Pathogenic | Biochemical analysis (elevated plasma 27-hydroxycholesterol) | Pathogenic | Likely diagnosis |
| 57         | Male | Motor neuron disease | 71 | TRPV4 (het) NM_021625.4: c.134del, p.N45Mfs*54 | AD | SPMSA (#181405) | VUS | N.A. | VUS | Possible diagnosis |
| 59         | Female | Polineuropathy, action-induced myoclonus | 23 | SCARB2 (hom) NM_001204255:1 c.134del, p.N45Mfs*54 | AR | EPM4 (#254900) | Pathogenic | Epilepsy monitoring, assessment of renal function (mild proteinuria) | Pathogenic | Definite diagnosis |
| 67         | Male | Distal myopathy | 65 | HNRNP.A1 (het) NM_031157.2: c.1064-12_1086del | AD | IBMPFD3 (#615424) | VUS | N.A. | VUS | Possible diagnosis |
| 68         | Female | Spastic paraparesis, ataxia | 63 | SPG11 (hom) NM_025137.3: c.5381T>C, p.L1794P | AR | SPG11 (#604360) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 69         | Female | Sensorimotor polyneuropathy | 26 | GDAP1 (hom) NM_018972.2: c.348dup, p.Y117fs*13 | AR | CMTRIA (#608340) | Pathogenic | N.A. | Pathogenic | Definite diagnosis |
| 70         | Female | Spinal muscular atrophy | 45 | DYSF (hom) NM_0034943: c.3502C>T, p.R1768W | AR | LGMDR2 (#253601) | Pathogenic | Muscle biopsy due | Pathogenic | Definite diagnosis |

ACMG, American College of Medical Genetics and Genomics; AD, autosomal dominant; AR, autosomal recessive; comp het, compound heterozygous; DP, dual pathology; ES, exome sequencing; hem, hemizygous; het, heterozygous; hom, homozygous; N.A., not applicable; NCS, nerve conduction studies; OMIM, Online Mendelian Inheritance of Man; VUS, variant of unknown significance.
Comparison of ES to gene panels

Simulated targeted gene panels (<25 kb) included the underlying gene in 16/27 patients diagnosed by ES, leading to a diagnostic yield of 22.2%. In contrast, comprehensive gene panels would have covered the causative gene in 25/27 cases resolved by ES, resulting in a yield of 34.7%. Two patients would not have
been diagnosed with either gene panel due to atypical disease manifestations which would have led to the selection of a wrong panel. Patient 11 carrying a mutation in the polyneuropathy gene MFN2 could only be diagnosed with ES because of spastic paraparesis being the leading phenotype. Another patient (patient 59) with a predominant polyneuropathy phenotype and action-induced myoclonus was eventually diagnosed with progressive myoclonic epilepsy due to biallelic pathogenic variants in SCARB2 (Table 2).

### Actionable variants

In our cohort of 72 individuals, an actionable variant was reported in one male patient aged 52 years (1.4%). The mutation in BRCA2 (NM_000059.3: c.5073dup) was considered pathogenic according to ClinVar, (https://www.ncbi.nlm.nih.gov/clinvar).

### Discussion

Several studies have stressed the importance of a critical reconsideration of initial genetic laboratory results from a clinical perspective [19,20]. Diagnostic reassessment approaches after NGS testing are increasingly entering medical practice, since ACMG recommends not using VUS for clinical decision-making [16].

In our study, data are provided that argue in favour of such an approach. The diagnostic yield of ES in our cohort of 72 patients with NMDs was 30.6% based on the initial laboratory reports. This number could be increased to 37.5% after genotype-guided diagnostic reassessment and conducting further investigations. Evidence that led to an upgrading of VUS was either derived from additional histological, biochemical or segregation analysis or by reassessing phenotypes in comparison with families from the literature. This was the case for two of our patients (with variants in BICD2 and TRPV4), whose phenotypic overlap with previously reported patients was so specific that the reported VUS were eventually considered likely pathogenic. As exemplified by these two patients, genotype-guided secondary phenotyping makes sense, as it might reveal highly specific but initially overlooked clinical features. However, one has to be aware that this approach harbours the danger of a biased reassessment, especially if done by the treating clinician alone. Any decisions regarding

| Patient ID | Gene          | Selected targeted panel (<25 kb)     | Selected comprehensive panel | Conclusion               |
|------------|---------------|--------------------------------------|-----------------------------|--------------------------|
| 3          | CHRNE         | CMS (13 genes)                       | NMD (344 genes)             | Targeted and comprehensive |
| 4          | SGCA          | LGMD (14 genes)                      | NMD (344 genes)             | Targeted and comprehensive |
| 6          | RBCK1         | LGMD (14 genes)                      | NMD (344 genes)             | Targeted and comprehensive |
| 8          | SPG7          | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 9          | DMD, SCN4A    | LGMD (14 genes)                      | NMD (344 genes)             | Targeted and comprehensive |
| 11         | MFN2          | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 12         | SPAST         | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 13         | CHRNA         | CPEO (17 genes)                      | NMD (344 genes)             | Targeted and comprehensive |
| 17         | BICD2         | Infantile SMA (10 genes)             | NMD (344 genes)             | Targeted and comprehensive |
| 19         | CAPN3         | LGMD (14 genes)                      | NMD (344 genes)             | Targeted and comprehensive |
| 22         | SPG7          | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 25         | SPG7          | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 27         | PABPN1        | Adult SMA (14 genes)                 | NMD (344 genes)             | Targeted and comprehensive |
| 32         | TTN           | Distal myopathies (10 genes)         | NMD (344 genes)             | Targeted and comprehensive |
| 35         | FA2H          | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 38         | TRPV4         | Adult SMA (14 genes)                 | NMD (344 genes)             | Targeted and comprehensive |
| 39         | RYR1          | Congenital myopathies (7 genes)      | NMD (344 genes)             | Targeted and comprehensive |
| 40         | MYOT          | IBM (4 genes)                        | NMD (344 genes)             | Targeted and comprehensive |
| 50         | KIF1A         | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 52         | PMP22         | Inherited neuropathies (14 genes)    | NMD (344 genes)             | Targeted and comprehensive |
| 53         | SPAST         | HSP (9 genes)                        | NMD (344 genes)             | Targeted and comprehensive |
| 54         | CPT2          | Metabolic myopathies (17 genes)      | NMD (344 genes)             | Targeted and comprehensive |
| 56         | CYP7B1        | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 59         | SCARB2        | Inherited neuropathies (14 genes)    | NMD (344 genes)             | Targeted and comprehensive |
| 68         | SPG11         | HSP (9 genes)                        | HSP (56 genes)              | Targeted and comprehensive |
| 69         | GDAP1         | Inherited neuropathies (14 genes)    | NMD (344 genes)             | Targeted and comprehensive |
| 70         | DYSF          | Adult SMA (14 genes)                 | NMD (344 genes)             | Targeted and comprehensive |

CMS, congenital myasthenic syndrome; CPEO, chronic progressive external ophthalmoplegia; HSP, hereditary spastic paraplegia; IBM, inclusion body myopathy; LGMD, limb girdle muscular dystrophy; NMD, neuromuscular disorder; SMA, spinal muscular atrophy.
variant reclassification should therefore be discussed by a multidisciplinary team to minimize this risk.

Our study also adds data for the discussion whether a targeted or an exome-based NGS approach is most appropriate for routine diagnostics. Whilst comprehensive gene panels seem to offer yields similar to ES, it is questionable how well narrow gene panels perform in clinical practice (some health insurance companies, e.g. in Germany, set a limit of 25 kb) [5]. This point is particularly relevant for ambiguous phenotypes as often observed in NMDs, easily leading to a wrong panel selection.

In our cohort, a considerable proportion of patients exhibited such complex phenotypes with overlapping symptoms between various neuromuscular disease subgroups (and thus panels). For instance, in patient 11, the clinically leading feature was spastic paraparesis. ES revealed a pathogenic variant in the ‘polyneuropathy gene’ MFN2, a gene which has been associated with an additional spasticity in rare cases [24]. The usually prominent polyneuropathy phenotype was clinically not noticeable and only in retrospect evident in nerve conduction studies. Another patient (patient 59) clinically presented with a demyelinating polyneuropathy and action-induced myoclonus. ES was performed due to the complex, syndromic phenotype and surprisingly revealed a clearly pathogenic homozygous mutation in SCARB2, a gene that is usually associated with progressive myoclonic epilepsy. Subsequently, the association between SCARB2 and a polyneuropathy phenotype is rare but has already been described as part of the clinical spectrum [25].

Our analysis demonstrated that appropriately chosen simulated gene panels <25 kb would have covered only 59.3% of the responsible disease genes detected by ES. More comprehensive panels expectedly achieved a higher diagnostic yield, covering 92.6% of the detected genes. However, the two aforementioned cases resolved by ES would have been missed even by the comprehensive gene panel.

In conclusion, our analysis supports a systematic genotype-guided diagnostic reassessment after NGS in a multidisciplinary setting involving referring clinicians and geneticists. Our data further argue against the use of narrowly targeted gene panels in NMDs due to ambiguously overlapping phenotypes.

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Disclosure of conflicts of interest

The authors have no conflicts of interest related to this article.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes included in targeted and comprehensive panels.

Table S2. Basis on which VUS were upgraded after diagnostic reassessment according to ACMG.

Data S1. Supplementary methods. Detailed description of sequencing and data analysis pipeline.

References

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