ORIGINAL ARTICLE

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

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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.

Table 1 Demographic, clinical and genetic characteristics of all patients with reported variants after ES

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	Definite diagnosis
4	Female	Limb girdle muscular dystrophy	45	*SGCA* (comp het) NM_000023.2: c.229C>T, p.R77C c.739G>A, p.V247M	AR	LGMD2D (#608099)	Pathogenic 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	Definite diagnosis
8	Female	Spastic paraparesis	55	*SPG7* (hom) NM_003119.2: c.233T>A, p.L78*	AR	SPG7 (#607259)	Pathogenic	N.A.	Pathogenic	Definite diagnosis
9	Male	Limb girdle muscular dystrophy and myotonia	49	*DMD* (hem) NM_000109.3: deletion of exons 48 and 49 *SCN4A* (het) NM_000334.4: c.3386G>A, p.R1129Q	DP	BMD (#300376) HOKPP2 (#613345)	Pathogenic Likely pathogenic	N.A.	Pathogenic Likely 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)

Table 1 *(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	*CHRNE* (hom) NM_000080.3: c.1327del, p.E443Kfs*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 diagnosis
19	Female	Limb girdle muscular dystrophy	36	*CAPN3* (comp het) NM_000070.2: c.1342C>T, p.R448C c.1722del, p.S575Lfs*20	AR	LGMD2A (#253600)	Likely pathogenic Pathogenic	N.A.	Likely pathogenic 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.233T>A, p.L78*	AR	SPG7 (#607259)	Pathogenic	N.A.	Pathogenic	Definite diagnosis
27	Male	Lower limb predominant myopathy, dysarthria, dysphagia	62	*PABPN1* (het) 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.1488G>T, E496D	AD AD	CMT2U (#616280) CMT2W (#616625)	VUS VUS	Family genotyping (unaffected mother and sister)	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 Pathogenic	Family genotyping (confirming biallelic location)	Pathogenic Pathogenic	Definite diagnosis

(continued)

Table 1 *(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
35	Male	Spastic paraparesis, mild cerebellar atrophy	38	*FA2H* (comp het) NM_024306.4: c.968C>T, p.P323L c.1119A>T, p.*373Cext*48	AR	SPG35 (#612319)	Likely pathogenic Likely pathogenic	N.A.	Likely pathogenic Pathogenic	Likely diagnosis
38	Male	Lower motor neuron disease	52	*TRPV4* (het) NM_021625.4: c.935C>T, p.A312V	AD	SPSMA (#181405) HMN8 (#600175)	VUS	Literature search (fitting phenotype, early respiratory involvement)	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 VUS	Muscle biopsy (specificity of phenotype)	Likely pathogenic 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.N405fs*1	AR	SPG30 (#610357)	VUS Pathogenic	Segregation analysis, specific phenotype	Likely pathogenic Pathogenic	Likely diagnosis
52	Male	Polyneuropathy	72	*PMP22* (het) 1.5 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)

Table 1 *(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
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 VUS	Biochemical analysis (elevated plasma 27-hydroxycholesterol)	Pathogenic Likely pathogenic	Likely diagnosis
57	Male	Motor neuron disease	71	*TRPV4* (het) NM_021625.4: c.664A>G, p.N222D	AD	SPSMA (#181405) HMN8 (#600175)	VUS	N.A.	VUS	Possible diagnosis
59	Female	Polyneuropathy, 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	*HNRNPA1* (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.349dup, p.Y117Lfs*13	AR	CMTRIA (#608340)	Pathogenic	N.A.	Pathogenic	Definite diagnosis
70	Female	Spinal muscular atrophy	45	*DYSF* (hom) NM_003494.3: c.5302C>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.

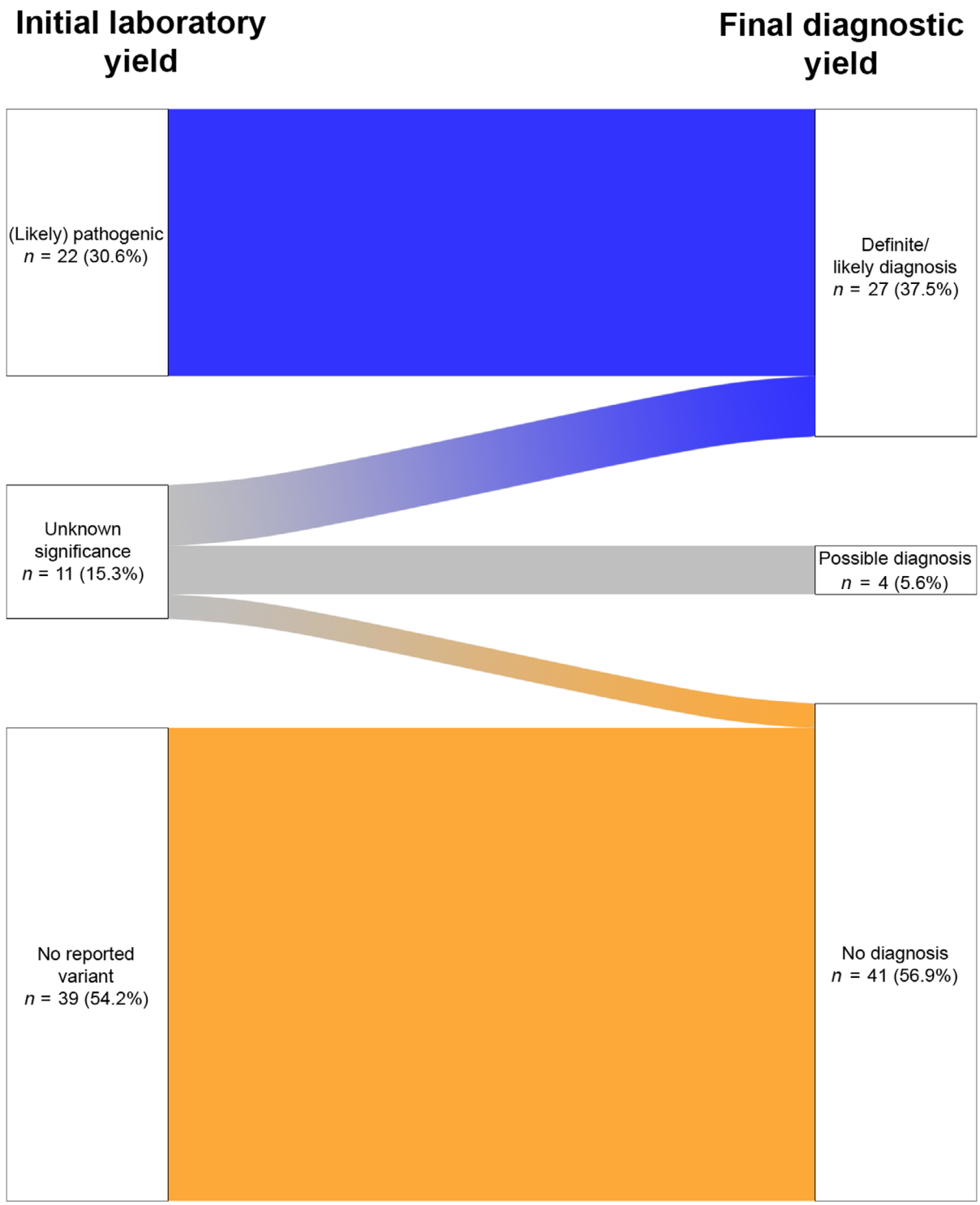

Figure 1 Comparison of diagnostic ES conclusion according to the genetic laboratory (left) with the final yield after genotype-guided diagnostic reassessment (right). [Colour figure can be viewed at wileyonlinelibrary.com]

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

Table 2 Comparison of ES to a targeted gene panel (<25 kb) and a comprehensive panel in all patients with a final diagnosis

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)	Comprehensive only
8	*SPG7*	HSP (9 genes)	HSP (56 genes)	Targeted and comprehensive
9	*DMD, SCN4A*	LGMD (14 genes)	NMD (344 genes)	Comprehensive only
11	*MFN2*	HSP (9 genes)	HSP (56 genes)	None
12	*SPAST*	HSP (9 genes)	HSP (56 genes)	Targeted and comprehensive
13	*CHRNE*	CPEO (17 genes)	NMD (344 genes)	Comprehensive only
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)	Comprehensive only
32	*TTN*	Distal myopathies (10 genes)	NMD (344 genes)	Comprehensive only
35	*FA2H*	HSP (9 genes)	HSP (56 genes)	Targeted and comprehensive
38	*TRPV4*	Adult SMA (14 genes)	NMD (344 genes)	Comprehensive only
39	*RYR1*	Congenital myopathies (7 genes)	NMD (344 genes)	Targeted and comprehensive
40	*MYOT*	IBM (4 genes)	NMD (344 genes)	Comprehensive only
50	*KIF1A*	HSP (9 genes)	HSP (56 genes)	Comprehensive only
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)	None
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)	Comprehensive only

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.

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

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 condition could be stabilized by antiepileptic treatment with levetiracetam. 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.

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