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Whole Exome Sequencing for Romanian Patients With Neurodevelopmental Disorders Through an International Collaboration

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ABSTRACT

Neurodevelopmental disorders (NDDs) are a highly diverse group of conditions that manifest through motor, cognitive, and behavioral impairments, representing the most common chronic condition encountered in children. As these often have a genetic cause or component, extensive genetic testing, particularly whole exome sequencing (WES), plays a critical role in their diagnosis, management, and prevention. Here, we present detailed clinical and genetic data on 54 Romanian patients included in the NeuroMyoDredger project, and provide an overview of the current landscape of genetic testing availability for NDDs in Romania, commenting on current barriers and the importance of integrating advanced genomic technologies into national healthcare strategies. A total of 54 undiagnosed Romanian patients with an initial clinical suspicion of unspecific NDD have benefited from singleton WES (including CNV and mitochondrial DNA analysis), with a diagnostic yield of 50%. Furthermore, a substantial proportion of cases (eight patients, 14.81%) yielded nondefinitive results involving variants of uncertain significance, with potential pathogenic relevance. Our findings align with the existing literature data, supporting the integration of singleton WES with CNV and mitochondrial variants detection as a first-line investigation in the diagnostic workflow for NDDs.

1 | Introduction

Neurodevelopmental disorders (NDDs) encompass a group of conditions that affect the development of the nervous system, typically resulting in cognitive, behavior, learning, language, or

motor difficulties. These usually manifest early in life, leading to significant functional limitations, and often persisting throughout the individual's lifespan (DSM-5—Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) [1]. Clinical presentations are highly heterogeneous, even inside specific NDDs

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subcategories, given that symptoms frequently overlap and may be associated with a specific cause or comorbid medical condition [2–4].

The etiology of NDDs remains incompletely understood, with a significant proportion of cases lacking a clearly identified cause [5]. While NDDs may arise from prenatal influences (such as alcohol/substance exposure, maternal anemia, diabetes), perinatal complications (prematurity, low birth weight), or postnatally acquired factors (malnutrition, toxin exposure) [6], a substantial number of affected individuals exhibit a genetic etiology or predisposition. The important genetic background emphasizes the critical role of genetic testing, particularly genome-wide approaches, in the diagnostic evaluation of patients with NDDs [2, 7]. This is essential as an early diagnosis helps individuals manage symptoms and improve quality of life, at the same time allowing adequate prenatal genetic counseling [8].

Along with other genetic testing methods (karyotype, microarray, *FMR1* repeat expansion analysis) [9], whole exome sequencing (WES) has been established as a first-choice diagnostic tool in clinical contexts of NDDs, demonstrating significant diagnostic yields—36% in a meta-analysis of 30 studies, with an even higher rate, 53%, in patients with syndromic NDDs [10].

In Romania, the availability of genetic testing has expanded significantly over the past 10–15 years, supported by the establishment of seven regional centers for medical genetics (Bihor, București, Cluj, Dolj, Mureș, Iași, and Timiș). These centers offer a broad spectrum of diagnostic techniques for NDDs, including conventional karyotyping, multiplex ligation-dependent probe amplification (MLPA), chromosomal microarray (CMA), polymerase chain reaction (PCR), as well as both Sanger sequencing and next-generation sequencing (NGS) in the form of targeted gene panels and WES, with significant differences between centers. Most of these investigations are provided at no cost to patients through National Health Programs. Nevertheless, access to these diagnostic services remains constrained by various socioeconomic barriers, and the diagnostic turnaround time is frequently prolonged up to several months or years due to limited funding and a lack of specialized personnel.

The aim of our study is to present clinical details and genetic findings obtained by singleton WES evaluation of 54 NDD Romanian patients within the NeuroMyoDredger project [11], an initiative aiming to provide free access to patients with neurodevelopmental or neuromuscular disorders from seven countries, and to discuss the broader implications of access to advanced genetic testing for affected individuals and their families.

2 | Materials and Methods

2.1 | Patients

Overall, the NeuroMyoDredger project provided singleton WES to a total of 245 patients across seven countries (Algeria, Chile, Egypt, France, Mexico, Peru, and Romania) [11]. The largest subcohort is represented by the Romanian patients, with 64

individuals enrolled from this country. The selection criteria consisted of clinical and/or paraclinical documentation of neurodevelopmental or neuromuscular clinical features without a confirmed genetic diagnosis. Clinical evaluations were conducted in Romania, either in the Regional Center for Medical Genetics Dolj (Craiova) or the National Institute for Mother and Child Care “Alessandrescu-Rusescu” (Bucharest). Given the considerable clinical heterogeneity of the targeted disorders, patients were grouped into two main subcategories based on the initial pretesting clinical suspicion: NDD (54 patients; 84.38%) and neuromuscular disorder (10 patients, 15.62%).

The NDD subcohort represents the only focus of this paper, being comprised of patients aged 20 years or less (only two patients above 18), with a mean age of 6.8 years (and a median of six). Twenty-four participants were female (44.44%), and 30 were male (55.56%). Clinical data related to these patients, recorded using HPO (Human Phenotype Ontology) terms, are available in Table S1.

There are no patients from consanguineous marriages. Furthermore, family history is unremarkable for all patients except patient ROU21, who has a sister with a similar clinical presentation. The patients had not previously undergone WES, microarray, or *FMR1* repeat expansion testing.

2.2 | WES

Dried blood spot cards samples were used for genetic testing. Singleton WES was performed by 3billion Inc. (Republic of Korea) on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA), utilizing the xGen Exome Research Panel v2, supplemented with xGen human mtDNA panel and xGen Custom Hyb Panel v1 (Integrated DNA Technologies, Coralville, IA, USA). Raw data processing and variant interpretation were carried out using 3billion's proprietary platform, EVIDENCE v4.1 [12], which incorporates a bioinformatics pipeline for calling single nucleotide variants (SNVs), small insertions and deletions (INDELs, < 50 bp), and large copy number variants (CNVs, ≥ 3 consecutive exons) based on GATK best practices [13], Manta [14], CoNIFER v0.2.2 [15], and 3bCNV v23.0818 (internally developed tool). Also, the platform used Mutect2 [13] for calling lower level (> 10%) heteroplasmic SNVs/INDELs in the mitochondrial genome, ExpansionHunter v5.0.0 [16] for calling repeat expansion variants, MELT v2.2.2 [17] for calling mobile element insertion variants, and AutoMap v1.2 [18] for detecting regions of homozygosity (ROH). Variant Effect Predictor v104.2 [19] is used for variant annotation. CNVs are identified by normalizing the sequencing depth-of-coverage (DOC) across target regions and comparing it against a reference panel to detect statistically significant deviations in read count ratios; when these copy number changes involve an entire chromosome, the variant is reported as an aneuploidy. While the identification of repeat expansion variants is feasible, it is restricted to 17 specific genes (*AR*, *ARX*, *ATN1*, *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *CACNA1A*, *COMP*, *FOXL2*, *HOXD13*, *HTT*, *PABPN1*, *PHOX2B*, *PRDM12*, *TBP*, and *ZIC2*). Sequences were aligned to the Genome Reference Consortium Human Build 37 (GRCh37) and Revised Cambridge Reference Sequence (rCRS) of the mitochondrial genome, respectively.



FIGURE 1 | Results for patients with NDD presentation. From a total of 54 patients with NDDs, there were 19 negative results (35.19%), eight non-definitive reports (14.81%), and 27 positive results (50%). The positive results consisted of 13 SNVs (48.15%), six indels (22.22%), seven CNVs (25.93%), and one aneuploidy (3.70%). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/cge.12026)]

Variant prioritization was conducted in accordance with the guidelines established by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) [20–22], taking into consideration the patient's phenotype, pertinent family history, and any prior test results provided by the referring physician. Only variants classified as clinically significant and relevant to the patient's clinical presentation at the time of interpretation were reported.

All identified variants were considered high-quality, and no variant validation tests were performed. Unfortunately, variant segregation tests were not available free of charge for our patients and were not performed.

2.3 | Ethics

This study was approved by the institutional ethics committee and developed in accordance with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from all participants or their legal guardians.

3 | Results

Notably, 27 positive findings were identified within the NDD subcohort, which included 54 patients, resulting in a diagnostic yield of 50% for this group. Moreover, eight patients (14.81%) from this subcohort received nondefinitive results, represented by variants of uncertain significance (VUS), with potential diagnostic relevance. The remaining 19 tests (35.19%) came back negative.

The variants underlying the positive diagnostic results were predominantly SNVs (identified in 13 patients, with one compound

heterozygote); six INDELS, seven CNVs, and one aneuploidy have also been detected (Figure 1). No mitochondrial variants, mobile element insertion variants, repeat expansions, or ROH were detected in this cohort. Eleven variants (six SNVs and five INDELS) are considered novel, as they have not been reported prior to the NeuroMyoDredger project [23]. These are represented by:

- a nonsense SNV in *EEF1D*—NM_001130053.5:c.1828C>T [24];
- another nonsense SNV in *EEF1D*—NM_001130053.5:c.874C>T [25];
- an indel in *BPTF*—NM_182641.4:c.1865-12_1868delinsC [26];
- a deletion in *ZEB2*—exons 4–10;
- an intronic SNV in *KAT6A*—NM_006766.5:c.1996 + 1G>C;
- an out-of-frame deletion in *DMD*—exons 8–30;
- a frameshift deletion in *SON*—NM_138927.4:c.4897_4900del [27];
- a missense SNV in *ZBTB20*—NM_001348800.3:c.1886C>G [28];
- a nonsense SNV in *MAP1B*—NM_005909.5:c.253C>T [29];
- a nonsense insertion in *MECP2*—NM_001110792.2:c.1089_1090insT [30];
- and a missense SNV in *EEF1A2*—NM_001958.5:c.797G>A [31].

The aneuploidy result was a trisomy 8 corresponding to patient ROU43. Karyotype validation confirmed a high-level mosaicism, with 45 out of 53 examined metaphases (85%) exhibiting the anomaly.

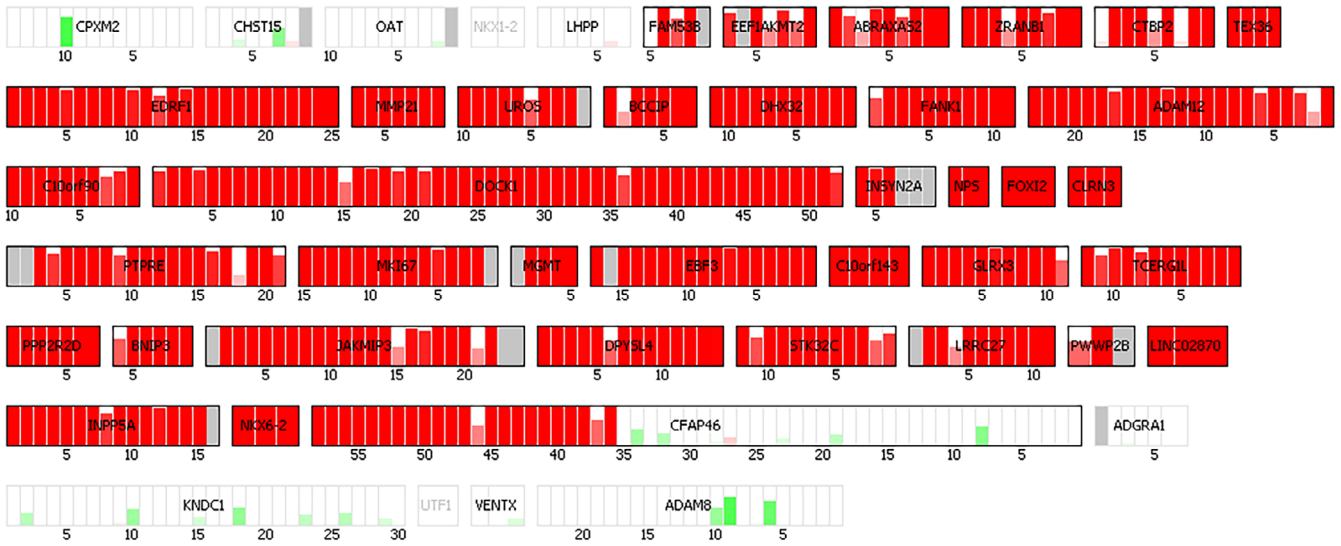


FIGURE 2 | CNV visualization using 3bCNV. This figure illustrates the standardized confidence scores for copy number variations (CNVs) generated by 3bCNV, an internally developed depth-of-coverage (DOC) based caller. Each box represents a gene, with vertical bars indicating individual exons ordered by genomic position. Red bars represent deletions (negative confidence scores) and green bars represent duplications (positive confidence scores), with the Y-axis clipped at an absolute value of 3. Noncaptured regions, such as UTRs, are shown in gray. A CNV is called when three or more consecutive exons exceed the confidence threshold (absolute score ≥ 3), denoted by a bolded outline around the gene box. The represented CNV, NC_000010.10:g.(?_126370176)_(134674486_?)del, GRCh37, was reported for patient ROU40. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

Figure 2 uses the example of the CNV identified in patient ROU40 (Table 1) to illustrate how a WES technique can detect such alterations through DOC analysis.

All variants corresponding to positive diagnoses are available in Table 1, with more detailed data (met ACMG/AMP criteria, variant frequency, variant impact, computational predictions) provided in Table S2.

Regarding the nondefinitive results found in the NDD subcohort, one patient was identified as a compound heterozygote, resulting in a total of nine detected variants (comprising eight SNVs and one INDEL). Furthermore, four of these variants are also being reported as VUS for the first time within the NeuroMyoDredger project:

- a missense SNV in *CACNA1A*—NM_001127222.2:c.4727C>T;
- a missense SNV in *NOTCH3*—NM_000435.3:c.3283C>T;
- a frameshift deletion in *KMT5B*—NM_017635.5:c.2629_2633del [50];
- and a missense SNV in *TLK2*—NM_006852.6:c.890G>T [51].

All nondefinitive, inconclusive results variants can be found in Table 2, with further variant details (met ACMG/AMP criteria, variant frequency, variant impact, computational predictions) available in Table S3.

4 | Discussion

In the extensive genomic testing and massive parallel sequencing era, important progress in elucidating the genetic etiology

of NDDs has been made, thereby shortening the diagnostic odyssey for many affected individuals [7, 10, 53]. Nevertheless, in numerous cases of exome- or genome-wide investigations, a definitive genetic cause is still to be discovered [54], with difficulties in variant identification (technical limitations) or interpretation (absence of segregation data or supporting functional evidence) contributing to this aspect.

Focusing solely on the NDD subcohort, the diagnostic yield for Romanian patients (50%, 27 positive results from 54 tests) was slightly higher compared to the one noted across all patients with NDDs in the NeuroMyoDredger project (45.12%, 37 positive results from 82 tests). One possible explanation for this difference is that most Romanian patients did not benefit from extensive investigations (genetic or otherwise) prior to their inclusion in the project. The diagnostic yield in the Romanian cohort is consistent with rates reported in other syndromic NDDs studies [10].

With regard to the novel variants corresponding to positive results, eight are linked to ultra-rare disorders, for which only a limited number of cases have been reported in the literature. For instance, the heterozygous variant identified in patient ROU39 (*BPTF*, NM_182641.4:c.1865-12_1868delinsC) is responsible for a NDD with dysmorphic facies and distal limb anomalies (OMIM 617755), a rare autosomal dominant condition described in less than 50 patients [55, 56]. Similarly, patient ROU48 is harboring a heterozygous intronic variant (NM_006766.5:c.1996+1G>C) in *KAT6A*; pathogenic variants in this gene cause another rare NDD (Arboleda-Tham syndrome, OMIM 616268, less than 100 documented cases [57]). Other Romanian patients with extremely rare NDDs diagnosed through this project are ROU55 (NM_138927.4:c.4897_4900del frameshift variant in *SON*, ZTTK syndrome, OMIM 617140, less than 100 reported

TABLE 1 | Positive results.

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU38	<i>EEF1D</i>	Genomic position: 8-144662259-G-A (GRCh37) DNA: NM_001130053.5:c.1828C>T Protein: NP_001123525.3:p.(Gln610Ter) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV; nonsense	Neurodevelopmental disorder with thin corpus callosum, hypotonia, and absent language (OMIM: 621150), autosomal recessive
ROU39	<i>BPTF</i>	Genomic position: 8-144671378-G-A (GRCh37) DNA: NM_001130053.5:c.874C>T Protein: NP_001123525.3:p.(Arg292Ter) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV; nonsense	Neurodevelopmental disorder with dysmorphic facies and distal limb anomalies (OMIM: 617755), autosomal dominant
ROU40	<i>FAM53B, EEF1AKMT2 + 35 more genes</i>	Genomic position: NC_000010.10:g(?_126370176)_ (134674486_?)del (GRCh37) Cytogenetic band: 10q26.13-q26.3 (minimum size: 8.3 Mb) Type: Deletion Zygosity: Heterozygous	Pathogenic	Known region [32, 33]; CNV	Chromosome 10q26 deletion syndrome (OMIM: 609625), autosomal dominant

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU41	SCN8A	Genomic position: 12-52099295-T-C (GRCh37) DNA: NM_001330260.2:c.1229T>C Protein: NP_001317189.1:p. (Val410Ala) Zygoty: Heterozygous	Likely pathogenic	Known variant; SNV; missense	SCN8A-related disorder (OMIM: 600702), autosomal dominant
ROU42	NR4A2, GPPD2 + 29 more genes	Genomic position: NC_000002.11:g.(?_157182256)_ (163393590_?)del (GRCh37) Cytogenetic band: 2q24.1-q24.2 (minimum size: 6.21 Mb) Type: Deletion Zygoty: Heterozygous	Pathogenic	Known region [34]; CNV	2q24.1q-24.2 deletion syndrome, autosomal dominant
ROU43	47, XY + 8	Trisomy 8 Type: Gain	Pathogenic	Known aneuploidy	Trisomy 8 (ORPHA:96061), chromosomal
ROU44	TBL1XR1, KCNMB2 + 144 more genes	Genomic position: NC_000003.11:g.(?_176743286)_ (197765538_?)dup (GRCh37) Cytogenetic band: 3q26.32-q29 (minimum size: 21 Mb) Type: Duplication Zygoty: Heterozygous	Pathogenic	Known region [35]; CNV	3q26.32-q29 duplication syndrome, autosomal dominant
ROU45	SPN, QPRT + 26 more genes	Genomic position: NC_000016.9:g. (?_29675050_(30199897_?) del (GRCh37) Cytogenetic band: 16p11.2 (minimum size: 524.8 Kb) Type: Deletion Zygoty: Heterozygous	Pathogenic	Known region [36–38]; CNV	Chromosome 16p11.2 deletion syndrome, 593 kb (OMIM: 611913), autosomal dominant

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU46	ZEB2	Genomic position: NC_000002.11:g(?_145147018)_ (145182434_?)del (GRCh37) Cytogenetic band: 2q22.3 (minimum size: 35.4 Kb) Type: Deletion	Pathogenic	New variant [11]; deletion, exons 4–10	Mowat–Wilson syndrome (OMIM: 235730), autosomal dominant
ROU47	BBS7	Zygosity: Heterozygous Genomic position: 4-122775861- CCTCT-C (GRCh37) DNA: NM_176824.3:c.712_715del Protein: NP_789794.1:p. (Arg238GlufsTer59) Zygosity: Homozygous	Pathogenic	Known variant [39]; deletion; frameshift	Bardet–Biedl syndrome 7 (OMIM: 615984), autosomal recessive
ROU48	KAT6A	Genomic position: 8-41804108- C-G (GRCh37) DNA: NM_006766.5:c.1996+1G>C Protein: NP_006757.2:p.? Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV	Arboleda–Tham syndrome (OMIM: 616268), autosomal dominant
ROU49	GALT	Genomic position: 9-34649029- G-T (GRCh37) DNA: NM_000155.4:c.855G>T Protein: NP_000146.2:p. (Lys285Asn) Zygosity: Homozygous	Pathogenic	Known variant [40, 41]; SNV; missense	Galactosemia (OMIM: 230400), autosomal recessive

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU50	<i>DMD</i>	Genomic position: NC_000023.10:g.(?_32429869)_ (32717410_?)del (GRCh37) Cytogenetic band: Xp21.1 (minimum size: 287.5 Kb) Type: Deletion	Pathogenic	New variant [11]; deletion; exons 8–30; out-of-frame	Duchenne muscular dystrophy (OMIM: 310200), X-linked recessive
ROU51	<i>TRAPPC11</i>	Zygosity: Hemizygous Genomic position: 4-184605212- G-A (GRCh37) DNA: NM_021942.6:c.1287 + 5G>A Protein: NP_068761.4:p.?	Pathogenic	Known variant; SNV; intronic	Muscular dystrophy, limb-girdle, autosomal recessive 18/LGMD R18 (OMIM: 615356), autosomal recessive
ROU52	<i>PHF6</i>	Zygosity: Homozygous Genomic position: X- 133527636-C-T (GRCh37) DNA: NM_001015877.2:c.346C>T Protein: NP_001015877.1:p. (Arg116Ter)	Pathogenic	Known variant [42]; SNV; nonsense	Borjeson–Forsman–Lehmann syndrome (OMIM: 301900), X-linked recessive
ROU53	<i>CPT1A</i>	Zygosity: Heterozygous Genomic position: 11-68566685-C-T (GRCh37) DNA: NM_001876.4:c.693+1G>A Protein: NP_001867.2:p.? Zygosity: Homozygous	Pathogenic	Known variant [43]; SNV; intronic	CPT deficiency, hepatic, type IA (OMIM: 255120), autosomal recessive

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU54	<i>DPP6</i> , <i>PAXIP1</i> + 21 more genes	Genomic position: NC_000007.13:g(?_152055672)_ (158937463_?)del (GRCh37) Cytogenetic band: 7q35-q36.3 (minimum size: 6.9 Mb) Type: Deletion Zygosity: Heterozygous	Pathogenic	Known region [44, 45]; CNV	Distal monosomy 7q36 (ORPHA: 1636), autosomal dominant
ROU55	<i>SON</i>	Genomic position: 21-34926431-TTAAAC-T (GRCh37) DNA: NM_138927.4:c.4897_4900del Protein: NP_620305.3:p.(Thr1633LeufsTer9) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; deletion; frameshift	ZTTK syndrome (OMIM: 617140), autosomal dominant
ROU56	<i>C42</i>	Genomic position: 8-86385964-A-C (GRCh37) DNA: NM_000067.3:c.275A>C Protein: NP_000058.1:p.(Gln92Pro) Zygosity: Homozygous	Likely pathogenic	Known variant [46]; SNV; missense	Osteopetrosis, autosomal recessive 3, with renal tubular acidosis (OMIM: 259730), autosomal recessive
ROU57	<i>ZBTB20</i>	Genomic position: 3-114058192-G-C (GRCh37) DNA: NM_001348800.3:c.1886C>G Protein: NP_001335729.1:p.(Thr629Arg) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV; missense	Primrose syndrome (OMIM: 259050), autosomal dominant

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU58	<i>MAP1B</i>	Genomic position: 5-71411593-C-T (GRCh37) DNA: NM_005909.5:c.253C>T Protein: NP_005900.2:p.(Arg85Ter) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV; nonsense	Periventricular nodular heterotopia 9 (OMIM: 618918), autosomal dominant
ROU59	<i>MECP2</i>	Genomic position: X-153296225-T-TA (GRCh37) DNA: NM_001110792.2:c.1089_1090insT Protein: NP_001104262.1:p.(Lys364Ter) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; insertion; nonsense	Rett syndrome (OMIM: 312750), X-linked dominant
ROU60	<i>EEF1A2</i>	Genomic position: 20-62122064-C-T (GRCh37) DNA: NM_001958.5:c.797G>A Protein: NP_001949.1:p.(Arg266Gln) Zygosity: Heterozygous	Likely pathogenic	New variant [11]; SNV; missense	Intellectual developmental disorder, autosomal dominant 38 (OMIM: 616393), autosomal dominant
ROU61	<i>ARID2</i>	Genomic position: 12-46123837-A-T (GRCh37) DNA: NM_152641.4:c.103A>T Protein: NP_689854.2:p.(Lys35Ter) Zygosity: Heterozygous	Likely pathogenic	Known variant; SNV; nonsense	Coffin–Siris syndrome 6 (OMIM: 617808), autosomal dominant
ROU62	<i>BBS12</i>	Genomic position: 4-123664110-C-T (GRCh37) DNA: NM_152618.3:c.1063C>T Protein: NP_689831.2:p.(Arg355Ter) Zygosity: Homozygous	Pathogenic	Known variant [47]; SNV; nonsense	Bardet–Biedl syndrome 12 (OMIM: 615989), autosomal recessive

(Continues)

TABLE 1 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU63	<i>GAP43</i> <i>LSAMP</i> + 56 more genes	Genomic position: NC_000003.11:g(?_115342537)_ (124215260_?)del (GRCh37) Cytogenetic band: 3q13.31-q21.2 (minimum size: 8.87 Mb) Type: Deletion	Pathogenic	Known region [48]; CNV	Chromosome 3q13.31 deletion syndrome (OMIM: 615433), autosomal dominant
ROU64	<i>GOLGA8S</i> , <i>GOLGA6L2</i> + 13 more genes	Zygoty: Heterozygous Genomic position: NC_000015.9:g. (?_23609490)_ (28566579_?) del (GRCh37) Cytogenetic band: 15q11.2-q13.1 (minimum size: 4.95 Mb) Type: Deletion	Pathogenic	Known region [49]; CNV	Chromosome 15q11.2-q13.1 Deletion (OMIM: 105830 and OMIM: 176270), autosomal dominant

Note: A list of variants associated with positive results in the NDD cohort. There is one patient with compound heterozygous status and therefore a total of 28 variants (14 SNVs, six indels, seven CNVs, and one aneuploidy). Eleven variants are reported for the first time in the NeuroMyoDredger project.

TABLE 2 | Nondefinitive results.

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU25	<i>SUCLG1</i>	Genomic position: 2-84676837-G-A (GRCh37) cDNA: NM_0038849.4:c.137C>T Protein: NP_003840.2:p. (Ser46Phe) Zygosity: Heterozygous	Variation of uncertain significance	Known variant [52]; SNV; missense	Mitochondrial DNA depletion syndrome 9 (encephalomyopathic type with methylmalonic aciduria) (OMIM: 245400), autosomal recessive
ROU28	<i>CACNA1A</i>	Genomic position: 2-84676885-T-C (GRCh37) cDNA: NM_003849.4:c.98-9A>G Protein: NP_003840.2:p.? Zygosity: Heterozygous	Variation of uncertain significance	Known variant; SNV; intronic	<i>CACNA1A</i> -related disorder (OMIM: 601011), autosomal dominant
ROU29	<i>NOTCH3</i>	Genomic position: 19-15290927-G-A (GRCh37) DNA: NM_001127222.2:c.4727C>T Protein: NP_001120694.1.p. (Ala1576Val) Zygosity: Heterozygous	Variation of uncertain significance	New variant [11]; SNV; missense	Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy 1 (OMIM: 125310), autosomal dominant

(Continues)

TABLE 2 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU33	<i>KMT5B</i>	Genomic position: 11-67925179-ATCTTC-A (GRCh37) DNA: NM_017635.5:c.2629_2633del Protein: NP_060105.3:p.(Glu877SerfsTer6) Zygosity: Heterozygous	Variant of uncertain significance	New variant [11]; deletion; frameshift	Intellectual developmental disorder, autosomal dominant 51 (OMIM: 617788), autosomal dominant
ROU34	<i>NEDD4L</i>	Genomic position: 18-56033460-C-G (GRCh37) DNA: NM_001144967.3:c.2063C>G Protein: NP_001138439.1:p.(Thr688Arg) Zygosity: Heterozygous	Variant of uncertain significance	Known variant; SNV; missense	Periventricular nodular heterotopia 7 (OMIM: 617201), autosomal dominant
ROU35	<i>TRAF7</i>	Genomic position: 16-2223813-C-T (GRCh37) DNA: NM_032271.3:c.1111C>T Protein: NP_115647.2:p.(Arg371Trp) Zygosity: Heterozygous	Variant of uncertain significance	Known variant; SNV; missense	Cardiac, facial, and digital anomalies with developmental delay (OMIM: 618164), autosomal dominant
ROU36	<i>TLK2</i>	Genomic position: 17-60642420-G-T (GRCh37) DNA: NM_006852.6:c.890G>T Protein: NP_006843.2:p.(Gly297Val) Zygosity: Heterozygous	Variant of uncertain significance	New variant [11]; SNV; missense	Intellectual developmental disorder, autosomal dominant 57 (OMIM: 618050), autosomal dominant

(Continues)

TABLE 2 | (Continued)

Patient	Gene	Variant	ACMG/AMP classification	Variant type	Disease association
ROU37	<i>EZH2</i>	Genomic position: 7-148506443-C-T (GRCh37) DNA: NM_004456.5:c.2069G>A Protein: NP_004447.2:p. (Arg690His) Zygosity: Heterozygous	Variant of uncertain significance	Known variant; SNV; missense	Weaver syndrome (OMIM: 277590), autosomal dominant

Note: A list of variants associated with nondefinitive, inconclusive results in the NDD cohort. There is one patient with compound heterozygous status and, therefore, a total of nine identified variants (eight SNVs and one indel). Four variants are reported for the first time in the NeuroMyoDredger project.

cases [58]), ROU57 (NM_001348800.3:c.1886C>G missense variant in *ZBTB20*, Primrose syndrome, OMIM 259050, less than 50 known patients [59]), ROU58 (NM_005909.5:c.253C>T nonsense variant in *MAP1B*, Periventricular nodular heterotopia 9, OMIM 618918, less than 20 patients [60]), and ROU60 (NM_001958.5:c.797G>A missense variant in *EEF1A2*, Intellectual developmental disorder, autosomal dominant 38, OMIM 616393, less than 50 patients [61]). Interestingly, two compound heterozygous likely pathogenic *EEF1D* variants (NM_001130053.5:c.1828C>T; NM_001130053.5:c.874C>T) were identified in a patient (ROU38); although initially this result was considered inconclusive [62], recent publications [63, 64] support the diagnosis of NDD with thin corpus callosum, hypotonia, and absent language (OMIM 621150) for this patient. Unfortunately, segregation studies were not available for these variants.

Given the marked clinical heterogeneity of these rare disorders, the continuous description of patients with new variants is still useful, as it can contribute to the refinement of genotype–phenotype correlations, as well as to creating or improving management guidelines. Moreover, although the remaining three novel variants are found in genes related to more extensively studied conditions (*DMD*—Duchenne/Becker muscular dystrophies [65], *ZEB2*—Mowat–Wilson syndrome [66], and *MECP2*—Rett syndrome [67]), their reporting could also prove to be important, as these findings may help address existing gaps in understanding phenotypic variability and enhance diagnostic accuracy in these well-characterized, yet clinically diverse disorders.

Regarding the nondefinitive variants in the NDD category ($N=9$), these are predicted to have an impact, but additional evidence is needed to confirm their involvement in the phenotype. Nevertheless, the reporting of 4 novel (ROU28—NM_001127222.2:c.4727C>T missense variant, *CACNA1A*; ROU29—NM_000435.3:c.3283C>T missense variant, *NOTCH3*; ROU33—NM_017635.5:c.2629_2633del frameshift variant, *KMT5B*; and ROU36—NM_006852.6:c.890G>T missense variant, *TLK2*) and five known variants of uncertain significance could contribute to future reclassifications.

As for the negative results, raw data are currently undergoing reanalysis to identify potentially overlooked variants during the initial evaluation, as recent gene–disease associations or VUS reclassifications could improve the diagnostic yield [68].

Beyond the diagnostic implications, the considerable genetic heterogeneity of NDDs is also translating into a nomenclature issue, as more and more genes are linked with such entities. Therefore, we advocate for the use of gene symbols in NDDs names, as it has already been suggested for several disorders, such as *GNAII*-related NDD [69], *HNRNP2*-related NDD [70], or *CTCF*-related disorder [71].

The main limitations of this study arise from the relatively small number of included patients, significant clinical heterogeneity, and lack of extensive investigations prior to the genetic testing. Another aspect worth mentioning is the lack of parental testing in the form of trio WES; this is due to the requirements of the grant, but also to the social/logistical difficulties in obtaining parental samples. A trio WES analysis would have significantly

increased costs, which would contribute to a more demanding implementation. Lastly, to date, functional studies for inconclusive results were not available.

4.1 | NDDs Genetic Testing in Romania as a Public Health Concept

Since NDDs are the most prevalent chronic conditions in the pediatric population and frequently have an underlying genetic component [53], genetic testing availability and accessibility represent a public health issue for which current guidelines and recommendations do not reflect the advanced knowledge level [72].

The availability of genetic testing in Romania has increased substantially in recent years [73], with a growing range of diagnostic options now accessible to patients. Both public and private healthcare providers offer genetic testing services, although the range of tests and their accessibility may vary considerably. The seven public medical genetics centers have evolved independently rather than as part of a coordinated national strategy; thus, there are important differences in the testing options each center provides. While most can perform cytogenetics, PCR, or MLPA analyses, the availability of more comprehensive or advanced tests (microarray, capillary sequencing, NGS panels, WES) is limited to a small number of laboratories; this limitation frequently results in prolonged turnaround times of up to several months or even years. Moreover, these seven centers are only located in major cities, an aspect which further hinders general access to genetic testing, particularly for individuals from rural or socioeconomically disadvantaged backgrounds, even though the majority of these tests are covered through the National Health Program PN XIII.2.3. In parallel, several private laboratories and clinics, at a national level or abroad, also offer a wide array of genetic tests; due to substantial costs decrease in the past years, an important number of patients opt to have a test in a private lab and pay out-of-pocket in order to obtain a faster result.

Although challenges in accessibility remain, first-tier testing methods for NDD patients (karyotype, Fragile X testing, CMA, WES without CNV analysis) [9, 10, 74] are available in Romania without patient costs. Within a potential future national genetic strategy for NDDs, the already existing centers could serve as a foundation for an extended, more comprehensive network that could address key issues in genetic testing services, including testing capacity, interpretation, reporting, and reanalysis options [75]. Also, although generally used for different purposes, one option worth considering is represented by pooled-WES [76], a strategy that could reduce testing costs if applied, for instance, as a screening method for large cohorts combined with follow-up Sanger sequencing variant confirmation.

A higher diagnostic yield can be obtained by combining multiple complementary techniques [75, 77] when necessary, as well as by adding other methods, such as WGS (whole genome sequencing) [78], RNAseq (RNA sequencing), or OGM (optical genome mapping) [79]. This issue is particularly relevant in the Romanian context, where WES provided through the public healthcare system does not include CNV detection; therefore, in our cohort, eight patients (seven with CNVs and one with an

aneuploidy) would not have been diagnosed if tested in Romania through WES, but they might have reached a diagnosis through a microarray or cytogenetic investigation, since five of the seven identified CNVs have a size above 5 Mb. However, the costs of integrating such comprehensive techniques in a general strategy can become overwhelming; in this regard, the diagnostic yield observed in our NDD cohort (50%) supports the utility of a first-tier, singleton WES test able to detect SNVs, INDELS, CNVs, mobile element insertion variants, several repeat expansion variants, and mitochondrial DNA SNVs and INDELS with higher heteroplasmy levels. Furthermore, this diagnostic yield is superior to microarray diagnostic rates [10, 74, 80], and similar to other WES-tested [10, 81, 82] or WGS-evaluated cohorts [83]. To our knowledge, data on Romanian patients is scarce in this area, with no published papers on WES testing in NDDs; the diagnostic yield reported here surpasses those observed in Romanian NDD cohorts evaluated by MLPA [84] or microarray [85, 86].

5 | Conclusion

To conclude, within the framework of the NeuroMyoDredger project, 54 Romanian patients with NDDs benefited from a singleton WES evaluation which resulted in a 50% diagnostic yield. These outcomes are in keeping with other literature data and support the utility of singleton WES with SNVs, INDELS, CNVs, mobile element insertion variants, specific repeat expansions, and mitochondrial variants identification as a first-tier diagnostic approach for NDD patients in Romania and other regions in need of designing genetic testing guidelines and strategies. The diagnosis rate for such patients can be improved in the next steps by using other comprehensive techniques (WGS, OGM, RNAseq). Further extensive research on NDDs' etiology is necessary to clarify which genetic methods workflows can most effectively increase diagnostic yields.

Author Contributions

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the [Supporting Information](#) of this article.

Peer Review

For transparency, the peer review documents associated with this article are available at <https://doi.org/10.1111/cge.70163>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Clinical data (HPO terms) of patients in the NDD cohort (54 patients). **Table S2:** Positive results. A comprehensive description of the variants associated with positive results in the NDD cohort (involved gene/genes, genomic position, DNA and protein effect, variant impact, ACMG/AMP criteria and classification, populational frequency, computational predictions, and associated diseases in the literature). There is one patient with compound heterozygous status, and, therefore, a total of 28 variants (14 SNVs, six INDELS, seven CNVs, and one aneuploidy). Eleven variants are reported for the first time in the NeuroMyoDredger project. **Table S3:** Nondefinitive results. A comprehensive description of the variants associated with non-definitive, inconclusive results in the NDD cohort (involved gene/genes, genomic position, DNA and protein effect, variant impact, ACMG/AMP criteria and classification, populational frequency, computational predictions, and associated diseases in the literature). There is one patient with compound heterozygous status, and, therefore, a total of nine identified variants (eight SNVs and one INDEL). Four variants are reported for the first time in the NeuroMyoDredger project.