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De novo variants in neurodevelopmental disorders—experiences from a tertiary care center

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Abstract

Up to 40% of neurodevelopmental disorders (NDDs) such as intellectual disability, developmental delay, autism spectrum disorder, and developmental motor abnormalities have a documented underlying monogenic defect, primarily due to *de novo* variants. Still, the overall burden of *de novo* variants as well as novel disease genes in NDDs await discovery. We performed parent-offspring trio exome sequencing in 231 individuals with NDDs. Phenotypes were compiled using human phenotype ontology terms. The overall diagnostic yield was 49.8% (n = 115/231) with *de novo* variants contributing to more than 80% (n = 93/115) of all solved cases. *De novo* variants affected 72 different—mostly constrained—genes. In addition, we identified putative pathogenic variants in 16 genes not linked to NDDs to date. Reanalysis performed in 80 initially unsolved cases revealed a definitive diagnosis in two additional cases. Our study consolidates the contribution and genetic heterogeneity of *de novo* variants in NDDs highlighting trio exome sequencing as effective diagnostic tool for NDDs. Besides, we illustrate the potential of a trio-approach for candidate gene discovery and the power of systematic reanalysis of unsolved cases.

KEYWORDS
autism, candidate gene, de novo variant, exome sequencing, intellectual disability, neurodevelopmental disorder, reanalysis

1 | INTRODUCTION

Neurodevelopmental disorders (NDDs) comprise a heterogeneous group of conditions affecting brain development and function and can manifest in impaired cognition, behavior, language, and motor functioning.[1] In accordance to "Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition"[2] (DSM-5), NDD encompasses intellectual developmental disorders, communication disorders, autism spectrum disorders, attention-deficit/hyperactivity disorders, specific learning disorders, and motor disorders.[2] Furthermore, patients with NDDs often demonstrate additional, (non-) neurological comorbidities.[3]

While NDDs can have numerous causes such as fetal exposure to toxicants, perinatal asphyxia and environmental contaminants, monogenic conditions make an essential contribution to the etiology of NDD.[1] The genetic etiology underlying NDD is extremely heterogeneous extending from large chromosomal aberration to single-nucleotide variants (SNVs) in >1000 of genes.[4] Nevertheless, theoretical calculations indicate that over 500 novel NDD genes remain to be discovered.[5] It has been widely acknowledged in large-scale sequencing studies that variants in protein-coding genes that have arisen *de novo* are enriched in individuals with NDDs and constitute the major cause of NDDs in outbred populations.[6-14] 42%–48% of individuals with a NDD are thought to harbor a causative *de novo* variant in known as well as yet-undiscovered disease genes.[13] However, the burden of *de novo* variants in NDD has not yet been fully illuminated.[14]

With the aim to better elucidate the genetic spectrum of (*de novo*) variants underlying rare NDDs, we describe detailed clinical and genetic findings in 231 individuals with NDDs who underwent trio exome sequencing in a single tertiary care genetic center.

2 | MATERIALS AND METHODS

2.1 | Study design

We retrospectively analyzed 231 individuals with NDDs in whom trio exome sequencing was performed in our institute. The families were recruited over a period of 3 years (August 2017 until July 2020) from different centers for human genetics, neuropediatrics, and neurology in Germany, Switzerland, Slovakia and Czech Republic. 177 (76.6%) of these 231 trios have not been published previously. Individuals were found eligible for this study if they had (1) a symptom or a constellation of symptoms consistent with a NDD (in accordance with the diagnostic criteria of "Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition"[2]) and (2) no prior genetic diagnosis. We obtained and thoroughly reviewed clinical records of all individuals and applied the human phenotype ontology (HPO) to systematically characterize the individuals' phenotype.[15] As previously published, individuals were categorized to one of two categories based on their clinical presentation: (1) isolated NDD or (2) NDD plus associated conditions defined as any additional neurological, systemic, syndromic, or other clinical

characteristic, for example, microcephaly or neutropenia.[16] Family history was collected by the referring clinician where applicable and a family history was considered as positive when a first-degree relative had a NDD.

All participants or their guardians gave written informed consent for exome sequencing and the publication of relevant findings. The study was performed in agreement with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki, and was approved by the respective local ethics committees.

2.2 | Trio exome sequencing

Exome sequencing was performed for all affected individuals and their parents using a SureSelect Human All Exon Kit 60 Mb, V6 (Agilent, Santa Clara, California) for enrichment and a Illumina NovaSeq6000 or Illumina HiSeq4000 system (Illumina, San Diego, California). Reads were aligned to the UCSC human reference assembly (hg19) with BWA v.0.7.8. SNVs and small insertions and deletions were detected using SAMtools v.0.1.19.[17] Copy number variations (CNVs) were detected with ExomeDepth and Pindel.[18,19] Mitochondrial DNA (mtDNA) variants were assessed using off-target reads as previously described.[20] Variants were analyzed in the in-house exome variant analysis database (EVAdb) using I) a recessive filter for homozygous and compound heterozygous variants with a minor allele frequency (MAF, according to in-house database with over 20 000 exomes) < 1%, II a filter for X chromosomal variants with a MAF < 0.1% and III) a filter for de novo variants with a MAF < 0.01%. IV) A phenotype-based search was conducted by performing an OMIM full term search using the three most characteristic phenotypic traits to establish a gene list. The filter queries variants with a MAF < 0.1%. In addition, CNVs with a MAF < 0.01 and mtDNA variants with a MAF < 1% were assessed. Identified variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines.[21-23]

Only cases with likely pathogenic or pathogenic variants as per ACMG (in the following designated "disease-causing") in established disease genes for NDDs were considered as solved and were reflected in the overall diagnostic yield. All genes with "strong" or "definitive" evidence for gene-disease relationship as defined by the Clinical Genome Resource (ClinGen) were considered as established disease genes.[24] Individuals with variants in candidate genes subsequently established as disease genes, were also categorized as solved and assigned to the overall yield. Individuals with (1) negative results (i.e., no variant[s] prioritized), (2) variants of uncertain significance (VUS) in NDDs associated genes or (3) variants in candidate genes for NDDs (as of November 2020) were summarized as unsolved cases. Reanalysis using updated variant annotation and newly discovered gene disease associations was performed for all cases with negative results older than ≥1 year (August 2017–September 2019).

For all established disease genes containing causative *de novo* variants, constraint metrics (pLIs and Z-scores) were extracted from Genome Aggregation Database (gnomAD) v2.1.1 to evaluate gene tolerance to loss-of-function or missense variants.[25] As recommended by gnomAD, we used pLI > 0.9 for loss-of-function variants and Z-score > 3.09 for missense variants as constraint threshold values.[26]

3 | RESULTS

3.1 | Demographic features and clinical findings

We performed parent-offspring trios in 231 individuals (117 females and 114 males) with NDDs over a period of 3 years. Age range was from 1 months to 46 years (median: 5.3 years) with 90% of individuals falling between 0 and 18 years. Parental consanguinity was reported in three cases. Information on the family history was available in 86/231 (37.2%) individuals. 9/86 (10.5%) cases had a positive family history with an affected first-degree relative. A monogenic disorder could genetically be established in a single cases with a positive family history, a *de novo PTPN11* was identified by trio analysis whereas the autism spectrum disorder remained without a monogenic explanation in the brother.

Clinical characteristics were captured using HPO terms (Table S1).[15] Among all 231 individuals, a total of n = 1291 HPO terms (median pro sample: 5, [interquartile range: 4–7]) were assigned. In summary, NDD phenotypes comprised global developmental delay (n = 175, 75.8%), intellectual disability (n = 46, 19.9%), speech delay (n = 28, 12.1%), motor delay (n = 26, 11.3%) and autistic behavior/autism (n = 26, 11.3%). Common additional features included seizures (n = 70, 30.3%), dystonia (n = 59, 25.5%), muscular hypotonia (n = 42, 18.2%), microcephaly (n = 32, 13.9%), cerebral palsy (n = 24, 10.4%), ataxia (n = 23, 10.0%), abnormal facial shape (n = 23, 10.0%), spasticity (n = 20, 8.7%) and hearing impairment (n = 13, 5.6%). Figure 1(A) gives a summary of the most frequent clinical features encountered in our cohort. The majority of individuals had NDDs plus associated conditions (n = 213/231, 92.2%), while only n = 18/231 (7.8%) individuals had isolated NDD without any additional features. The proportion of cases with NDDs plus associated conditions was higher in the subgroup with autosomal recessive inheritance (n = 19/19, 100%) in comparison with those with *de novo* variants (n = 89/93, 95.7%).

3.2 | Diagnostic yield

Overall, trio exome sequencing identified disease-causing variants in developmental disorder associated genes in 115/231 individuals reflecting an overall yield of 49.8%. The diagnostic yield was significantly higher in individuals with NDD plus associated conditions (n = 111/213, 52.1%) in comparison to individuals with isolated NDD (n = 4/18, 22.2%, p = 0.0247, Fisher's exact test).[27] 59/117 female individuals (50.4%) and 56/114 male individuals (49.1%) received a genetic diagnosis. In the group of individuals ≥18 years (n = 24/231, 10.4%), the overall yield was 50.0%. In the group of individuals <18 years (n = 206/231), the overall yield was 49.5%.

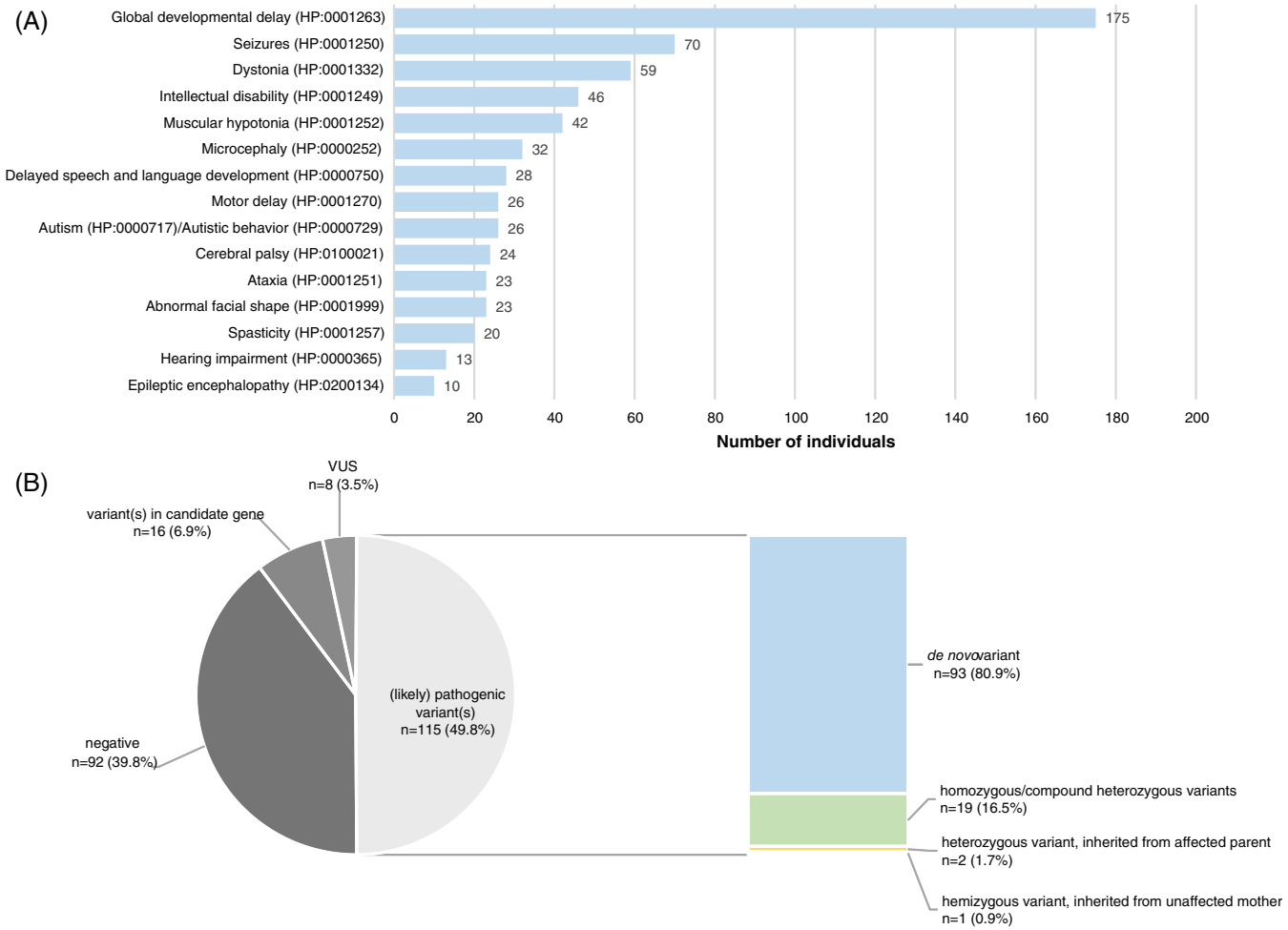

FIGURE 1 Phenotypic characterization and results of trio exome sequencing in n = 231 individuals with NDDs. (A) Distribution of the most frequent HPO categories among individuals undergoing trio exome sequencing. (B) The pie chart illustrates the results of the trio exome sequencing study (231 individuals, 426 parents) with an overall diagnostic yield of 49.8%. Solved cases were defined by the presence of disease-causing variants in established NDD-associated genes. The bar chart on the right visualizes the distribution of all disease-causing variants based on the inheritance mode with *de novo* variants representing the most frequent inheritance. 50.2% of all cases remained unsolved which included cases with negative results (i.e., no variant[s] prioritized) as well as cases with VUSs and cases with variants in candidate genes. HPO, human phenotype ontology; NDDs, neurodevelopmental disorders; VUSs, variants of uncertain significance [Colour figure can be viewed at wileyonlinelibrary.com]

In the majority of individuals (n = 93/115, 80.9%), the molecular diagnosis based on *de novo* variants in genes either associated with autosomal dominant disorders (n = 82/115, 71.3%) or with X-linked disorders (n = 11/115, 9.6%). In two cases, variants in genes/chromosomal locations linked to autosomal dominant disorders (*KMT2D*, Chromosome 16q23.2–23.3 deletion) were inherited from an affected parent (n = 2/115, 1.7%) and in one case, a variant in a gene associated with a X-linked disorder (*MECP2* duplication) was inherited from the unaffected mother (n = 1/115, 0.9%). 19/115 individuals (16.5%) harbored homozygous (n = 7/115, 6.1%) or compound heterozygous (n = 12/115, 10.4%) variants in genes related to autosomal recessive disorders. 3/7 patients with homozygous variants had a consanguineous background. A disease causing CNV (deletions >500 kb, duplications >2 Mb) was found in seven individuals leading to an overall burden of CNVs of 3.0% (n = 7/231).

116/231 individuals (50.2%) remained unsolved after trio exome sequencing. The unsolved group subsumed individuals with negative results (n = 92/231, 39.8%), individuals with VUS in DD/ID associated genes (n = 8/231, 3.5%) and individuals with variants in novel or known candidate genes for DD/ID (n = 16/231, 6.9%). These overall results are summarized in Figure 1(B).

3.3 | Characteristics of *de novo* variants

40.3% (n = 93/231) of all individuals or 80.9% of all solved cases (n = 93/115), respectively, harbored *de novo* variants in protein-coding disease genes, either in autosomal (n = 81/93, 87.1%) or X-linked genes (n = 12/93, 12.9%). Individuals with *de novo* variants in autosomal genes (n = 81) subdivided into 43 females and 38 males. Among

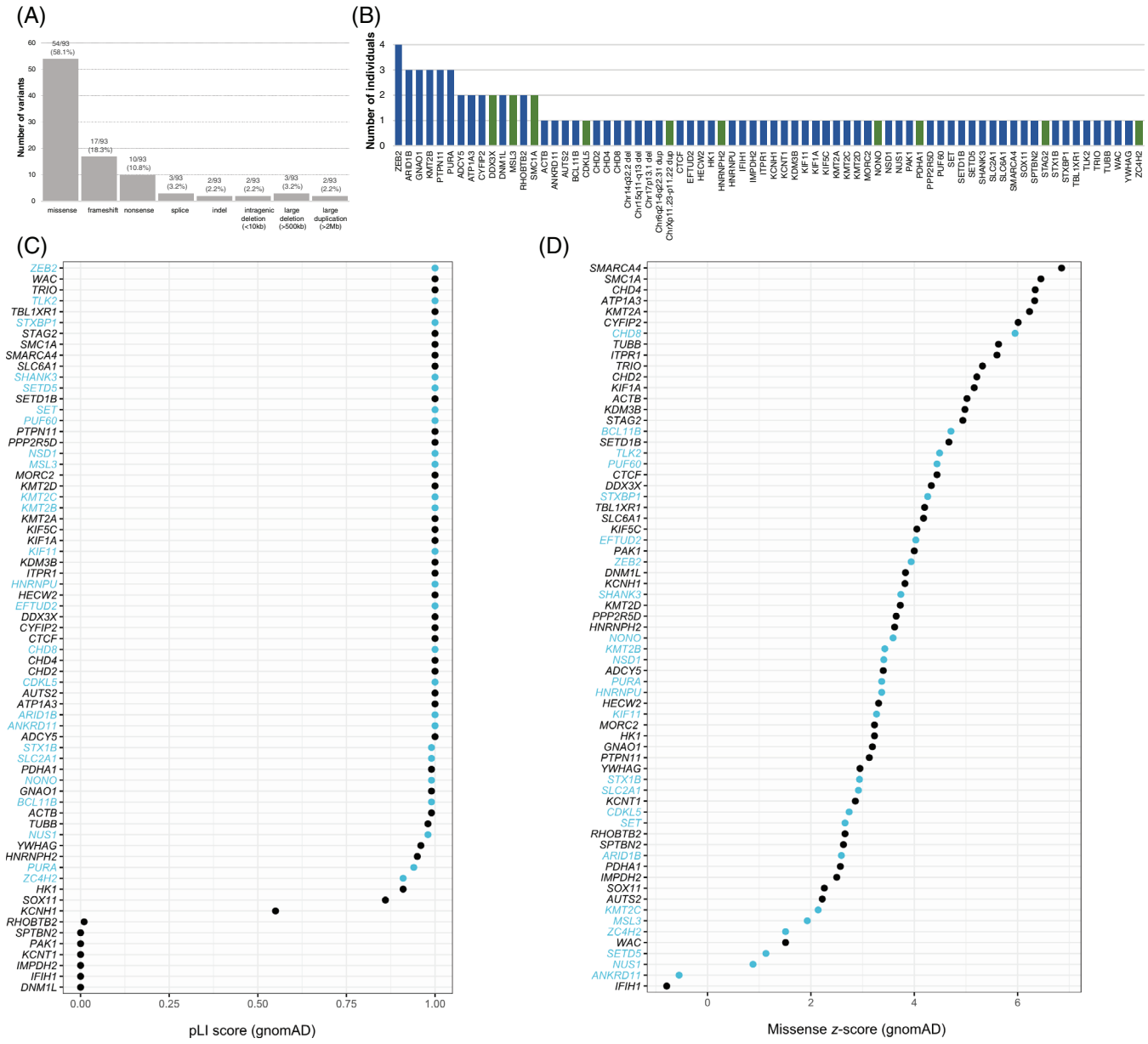

FIGURE 2 Characteristics of *de novo* variants in known disease genes identified in 93 individuals. (A) Spectrum and number of identified variant types with missense variants representing the most frequent variant type. (B) Spectrum of genes/chromosomal regions containing disease-causing *de novo* variants. The counts of individuals harboring a variant per gene/chromosomal region are shown. Genes implicated in autosomal dominant disorders are marked in blue, while genes associated with X-linked disorders are colored in green. By using trio exome sequencing, a total of 72 distinct disease entities were established. With four individuals carrying a variant in *ZEB2*, Mowat-Wilson syndrome was the most frequent molecular diagnosis. (C) Probability of being loss-of-function intolerant (pLI) score distribution of genes with *de novo* variants in autosomal and X-linked according to constraint metrics of gnomAD. Genes harboring loss-of-function (nonsense, frameshift, canonical splice site variants) variants or CNVs are highlighted in blue. (D) Missense Z-score distribution of genes with *de novo* variants in autosomal and X-linked according to constraint metrics of gnomAD. Genes harboring missense variants and in-frame deletions are highlighted in black. CNVs, Copy number variations [Colour figure can be viewed at wileyonlinelibrary.com]

individuals with *de novo* variants in X-linked genes (n = 12) were four males and eight females. We identified a variety of variant types with missense variants being the predominant type (n = 54/93, 58.1%) followed by frameshift variants (n = 17/93, 18.3%), nonsense variants (n = 10/93, 10.8%), canonical splice site variants (n = 3/93, 3.2%), indels (n = 2/93, 2.2%), intragenic deletions (<10 kb) (n = 2/93, 2.2%), large deletions >500 kb (n = 3/93, 3.2%) and large duplications >2 Mb (n = 2/03, 2.2%) (Figure 2(A)). Parental mosaicism was identified in one family (individual 47), in which the frameshift variant in *KMT2B* was identified as low-level mosaicism (in 1/216 reads) in the healthy mother. We did not encounter any cases of postzygotic mosaicism in the index patients.

A wide spectrum of diagnoses was established based on the molecular findings. In total, 72 distinct diagnoses were made with the

TABLE 1 List of all (likely) pathogenic *de novo* variants identified in this cohort (n = 93)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
1	*ACTB*	NM_001101.3	c.4G>T, p.(Asp2Tyr)	Heterozygous	Missense	25.4	Likely pathogenic	Baraitser-Winter syndrome 1	# 243310
2	*ADCY5*	NM_183357.2	c.1322C>T, p.(Ala441Val)	Heterozygous	Missense	33	Likely pathogenic	Dyskinesia, familial, with facial myokymia	# 606703
3	*ADCY5*	NM_183357.2	c.2071A>G, p.(Lys691Glu)	Heterozygous	Missense	29.1	Likely pathogenic	Dyskinesia, familial, with facial myokymia	# 606703
4	*ANKRD11*	NM_001256182.1	c.2704G>T, p.(Glu902*)	Heterozygous	Nonsense	36	Pathogenic	KBG syndrome	# 148050
5	*ARID1B*	NM_020732.3	c.2191_2192dup, p.(Pro732Serfs*14)	Heterozygous	Frameshift		Pathogenic	Coffin-Siris syndrome 1	# 135900
6	*ARID1B*	NM_020732.3	c.4009C>T, p.(Arg1337*)	Heterozygous	Nonsense	47	Pathogenic	Coffin-Siris syndrome 1	# 135900
7	*ARID1B*	NM_020732.3	c.6382C>T, p.(Arg2128*)	Heterozygous	Nonsense	52	Pathogenic	Coffin-Siris syndrome 1	# 135900
8	*ATP1A3*	NM_152296.4	c.2443G>A, p.(Glu815Lys)	Heterozygous	Missense	34	Pathogenic	CAPOS syndrome	# 601338
9	*ATP1A3*	NM_152296.5	c.266G>C, p.(Gly89Ala)	Heterozygous	Missense	24.2	Likely pathogenic	CAPOS syndrome	# 601338
10	*AUTS2*	NM_015570.4	c.1604A>C, p.(His535Pro)	Heterozygous	Missense	28.3	Likely pathogenic	Mental retardation, autosomal dominant 26	# 615834
11	*BCL11B*	NM_022898.1	c.1835del, p.(Ser612Thrfs*40)	Heterozygous	Frameshift		Pathogenic	Intellectual developmental disorder with dysmorphic facies, speech delay, and T-cell abnormalities	# 618092
12	*CDKL5*	NM_001323289.2	Deletion exon 16–18	Heterozygous	Intragenic deletion		Pathogenic	Epileptic encephalopathy, early infantile, 2	# 300672
13	*CHD2*	NM_001271.3	c.3454C>G, p.(Arg1152Gly)	Heterozygous	Missense	24.6	Likely pathogenic	Epileptic encephalopathy, childhood-onset	# 615369
14	*CHD4*	NM_001273.2	c.637A>G, p.(Ser213Gly)	Heterozygous	Missense	24.4	Likely pathogenic	Sifrim-Hitz-Weiss syndrome	# 617159
15	*CHD8*	NM_001170629.1	c.4378C>T, p.(Arg1460*)	Heterozygous	Nonsense	43	Pathogenic	*CHD8*-associated disorder	# 615032
16	Chromosome 14q32.2 deletion (~1 Mb)		chr14:100317190_101351124del	Heterozygous	CNV		Pathogenic	Chromosome 14q32.2 deletion	Not listed
17	Chromosome 17p13.1 deletion (~500 kb)		chr17:7554837_8093457del	Heterozygous	CNV		Pathogenic	Chromosome 17p13.1 deletion syndrome	# 613776

(Continues)

TABLE 1 (Continued)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
18	Chromosome 6q21-6q22.31 duplication (~16 Mb)		chr6:106960217_123957919dup	Heterozygous	CNV		Pathogenic	Chromosome 6q21-6q22.31 duplication	Not listed
19	Chromosome Xp11.23-p11.22 duplication (~2 Mb)		chrX:46736940_48693933dup	Heterozygous	CNV		Pathogenic	Chromosome Xp11.23-p11.22 duplication syndrome	# 300801
20	Chromosome 15q11-q13 deletion (~5 Mb)		chr15:23572076_28600151del	Heterozygous	CNV		Pathogenic	Prader-Willi syndrome	# 176270
21	*CTCF*	NM_006565.3	c.958C>G, p.(Arg320Gly)	Heterozygous	Missense	32	Likely pathogenic	Mental retardation, autosomal dominant 21	# 615502
22	*CYFIP2*	NM_014376.2	c.1363G>C, p.(Ala455Pro)	Heterozygous	Missense	29.5	Pathogenic	Epileptic encephalopathy, early infantile, 65	# 618008
23	*CYFIP2*	NM_001037332.2	c.2095G>C, p.(Asp699His)	Heterozygous	Missense	33	Pathogenic	Epileptic encephalopathy, early infantile, 65	# 618008
24	*DDX3X*	NM_001356.3	c.1148C>G, p.(Ala383Gly)	Heterozygous	Missense	26.6	Pathogenic	Mental retardation, X-linked 102	# 300958
25	*DDX3X*	NM_001356.3	c.977G>A, p.(Arg326His)	Heterozygous	Missense	32	Pathogenic	Intellectual developmental disorder, X-linked, syndrome, Snijders Blok type	# 300958
26	*DNM1L*	NM_005690.4	c.428C>G, p.(Thr143Arg)	Heterozygous	Missense	29.4	Pathogenic	Encephalopathy due to defective mitochondrial and peroxisomal fission-1	# 614388
27	*DNM1L*	NM_005690.4	c.1207C>T, p.(Arg403Cys)	Heterozygous	Missense	35	Pathogenic	Encephalopathy, lethal, due to defective mitochondrial peroxisomal fission 1	# 614388
28	*EFTUD2*	NM_004247.4	Deletion Exon 10	Heterozygous	Intragenic deletion		Likely pathogenic	Mandibulofacial dysostosis, Guion-Almeida type	# 610536
29	*GNAO1*	NM_138736.2	c.626G>A, p.(Arg209His)	Heterozygous	Missense	35	Pathogenic	Neurodevelopmental disorder with involuntary movements	# 617493

TABLE 1 (Continued)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
30	*GNAO1*	NM_020988.3	c.625C>T, p.(Arg209Cys)	Heterozygous	Missense	35	Pathogenic	Neurodevelopmental disorder with involuntary movements	# 617493
31	*GNAO1*	NM_020988.3	c.625C>T, p.(Arg209Cys)	Heterozygous	Missense	35	Pathogenic	Neurodevelopmental disorder with involuntary movements	# 617493
32	*HECW2*	NM_020760.1	c.3829 T>C, p.(Tyr1277His)	Heterozygous	Missense	29.2	Likely pathogenic	Neurodevelopmental disorder with hypotonia, seizures, and absent language	# 617268
33	*HK1*	NM_033498.2	c.1382C>T, p.(Thr461Met)	Heterozygous	Missense	34	Pathogenic	Neurodevelopmental disorder with visual defects and brain anomalies	# 618547
34	*HNRNPH2*	NM_019597.4	c.85C>T, p.(Arg29Cys)	Hemizygous	Missense	25	Likely pathogenic	Mental retardation, X-linked, syndromic, Bain type	# 300986
35	*HNRNPU*	NM_004501.3	c.575C>A, p.(Ser192*)	Heterozygous	Nonsense	37	Pathogenic	Epileptic encephalopathy, early infantile, 54	# 617391
36	*IFIH1*	NM_022168.3	c.2159G>A, p.(Arg720Gln)	Heterozygous	Missense	34	Pathogenic	Aicardi-Goutieres syndrome 7	# 615846
37	*IMPDH2*	NM_000884.2	c.338G>A, p.(Gly113Glu)	Heterozygous	Missense	33	Pathogenic	*IMPDH2*-associated disorder	Not listed
38	*ITPR1*	NM_002222.5	c.805C>T, p.(Arg269Trp)	Heterozygous	Missense	34	Pathogenic	Gillespie syndrome	# 206700
39	*KCNH1*	NM_002238.3	c.1405G>A, p.(Gly469Arg)	Heterozygous	Missense	34	Pathogenic	Zimmermann-Laband syndrome 1	# 135500
40	*KCNT1*	NM_020822.2	c.1283G>A, p.(Arg428Gln)	Heterozygous	Missense	34	Pathogenic	Epileptic encephalopathy, early infantile, 14	# 614959
41	*KDM3B*	NM_016604.3	c.2828G>A, p.(Arg943Gln)	Heterozygous	Missense	34	Pathogenic	Diets-Jongmans syndrome	# 618846
42	*KIF11*	NM_004523.3	c.2922G>A, p.(?)	Heterozygous	Splice	8.012	Pathogenic	Microcephaly with or without chorioretinopathy, lymphedema, or mental retardation	# 152950
43	*KIF1A*	NM_004321.6	c.760C>T, p.(Arg254Trp)	Heterozygous	Missense	34	Pathogenic	NESCAV syndrome	# 614255

(Continues)

TABLE 1 (Continued)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
44	*KIF5C*	NM_004522.2	c.420G>A, p.(Arg141Gln)	Heterozygous	Missense	34	Pathogenic	Cortical dysplasia, complex, with other brain malformations 2	# 615282
45	*KMT2A*	NM_001197104.1	c.6463C>G, p.(Pro2155Ala)	Heterozygous	Missense	22.9	Likely pathogenic	Wiedemann-Steiner syndrome	# 605130
46	*KMT2B*	NM_014727.1	c.1633C>T, p.(Arg545*)	Heterozygous	Nonsense	36	Pathogenic	Dystonia 28, childhood-onset	# 617284
47	*KMT2B*	NM_014727.1	c.521dup, p.(Thr176Aspfs*8)[a]	Heterozygous	Frameshift		Pathogenic	Dystonia 28, childhood-onset	# 617284
48	*KMT2B*	NM_014727.1	c.4847C>T, p.(Ala1616Val)	Heterozygous	Missense	32	Pathogenic	Dystonia 28, childhood-onset	# 617284
49	*KMT2C*	NM_170606.2	c.1951_1952del, p.(Glu651Lysfs*3)	Heterozygous	Frameshift		Pathogenic	Kleefstra syndrome 2	# 617768
50	*KMT2D*	NM_003482.3	c.15163_15168dup, p.(Asp5055_Leu5056dup)	Heterozygous	Indel		Pathogenic	Kabuki syndrome 1	# 147920
51	*MORC2*	NM_014941.3	c.995A>G, p.(Tyr332Cys)	Heterozygous	Missense	28.6	Likely pathogenic	Charcot–Marie–Tooth disease, axonal, type 2Z	# 616688
52	*MSL3*	NM_078629.3	c.1466+1G>A>A, p.?	Heterozygous	Splice	33	Pathogenic	Basilicata-Akhtar syndrome	# 301032
53	*MSL3*	NM_078629.3	c.1314C>A, p.(Tyr438*)	Hemizygous	Nonsense	32	Pathogenic	Basilicata-Akhtar syndrome	# 301032
54	*NONO*	NM_007363.4	c.90_114del, p.(Gln30Hisfs*18)	Hemizygous	Frameshift		Pathogenic	Mental retardation, X-linked, syndromic 34	# 300967
55	*NSD1*	NM_022455.4	c.2289_2317dup, p.(Ala773Valfs*5)	Heterozygous	Frameshift		Pathogenic	Sotos syndrome 1	# 117550
56	*NUS1*	NM_138459.3	c.238_263del, p.(Ala80Argfs*45)	Heterozygous	Frameshift		Pathogenic	Mental retardation, autosomal dominant 55, with seizures	# 617831
57	*PAK1*	NM_002576.5	c.1427 T>C, p.(Ile476Thr)	Heterozygous	Missense	29.6	Likely pathogenic	Intellectual developmental disorder with macrocephaly, seizures, and speech delay	# 618158
58	*PDHA1*	NM_000284.3	c.787C>G, p.(Arg263Gly)	Heterozygous	Missense	24.6	Pathogenic	Pyruvate dehydrogenase E1-alpha deficiency	# 312170

TABLE 1 (Continued)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
59	*PPP2R5D*	NM_006245.3	c.592G>A, p.(Glu198Lys)	Heterozygous	Missense	33	Pathogenic	Mental retardation, autosomal dominant 35	# 616355
60	*PTPN11*	NM_002834.3	c.166A>G, p.(Ile56Val)	Heterozygous	Missense	25.1	Pathogenic	Noonan syndrome 1	# 163950
61	*PTPN11*	NM_002834.3	c.417G>C, p.(Glu139Asp)	Heterozygous	Missense	27.4	Pathogenic	Noonan syndrome 1	# 163950
62	*PTPN11*	NM_002834.4	c.1510A>G, p.(Met504Val)	Heterozygous	Missense	26.4	Pathogenic	Noonan syndrome 1	# 163950
63	*PUF60*	NM_078480.2	c.1100del, p.(Leu367CysfsTer*17)	Heterozygous	Frameshift		Pathogenic	Verheij Syndrome	# 615583
64	*PURA*	NM_005859.4	c.565G>C, p.(Ala189Pro)	Heterozygous	Missense	28.6	Likely pathogenic	Mental retardation, autosomal dominant 31	# 616158
65	*PURA*	NM_005859.4	c.366_367dup, p.(Gln123Argfs*103)	Heterozygous	Frameshift		Pathogenic	Mental retardation, autosomal dominant 31	# 616158
66	*PURA*	NM_005859.4	c.640G>T, p.(Glu214*)	Heterozygous	Nonsense	39	Pathogenic	Mental retardation, autosomal dominant 31	# 616158
67	*RHOBTB2*	NM_001160036.1	c.1519C>T, p.(Arg507Cys)	Heterozygous	Missense	34	Pathogenic	Early infantile epileptic encephalopathy 64	# 618004
68	*RHOBTB2*	NM_001160036.2	c.1448G>A, p.(Arg483His)	Heterozygous	Missense	31	Pathogenic	Epileptic encephalopathy, early infantile, 64	# 618004
69	*SET*	NM_001122821.1	c.457_458del, p.(Ser153Glnfs*7)	Heterozygous	Frameshift		Pathogenic	Mental retardation, autosomal dominant 58	# 618106
70	*SETD1B*	NM_015048.1	c.5699A>G, p.(Tyr1900Cys)	Heterozygous	Missense	17.88	Likely pathogenic	Intellectual developmental disorder with seizures and language delay	# 619000
71	*SETD5*	NM_001080517.1	c.2154del, p.(Val719Leufs*18)	Heterozygous	Frameshift		Pathogenic	Mental retardation, autosomal dominant 23	# 615761
72	*SHANK3*	NM_033517.1	c.3679dup, p.(Ala1227Glyfs*69)	Heterozygous	Frameshift		Pathogenic	Phelan-McDermid syndrome	# 606232
73	*SLC2A1*	NM_006516.2	c.732del, p.(Met244Ilefs*8)	Heterozygous	Frameshift		Pathogenic	GLUT1 deficiency syndrome 1	# 606777
74	*SLC6A1*	NM_003042.3	c.149G>T, p.(Arg50Leu)	Heterozygous	Missense	24.9	Likely pathogenic	Myoclonic-atonic epilepsy	# 616421
75	*SMARCA4*	NM_001128849.1	c.1675G>A, p.(Glu559Lys)	Heterozygous	Missense	34	Likely pathogenic	Coffin-Siris syndrome 4	# 614609

(Continues)

TABLE 1 (Continued)

Individual	Gene/locus	Transcript	Variant	Zygosity	Variant type	CADD score	ACMG classification	Diagnosis	OMIM phenotype
76	*SMC1A*	NM_001281463.1	c.587G>C, p.(Arg196Pro)	Heterozygous	Missense	23.9	Likely pathogenic	Cornelia de Lange syndrome 2	# 300590
77	*SMC1A*	NM_006306.3	c.3497A>C, p.(Asn1166Thr)	Heterozygous	Missense	26.5	Pathogenic	Cornelia de Lange syndrome 2	# 300590
78	*SOX11*	NM_003108.3	c.146 T>G, p.(Ile49Ser)	Heterozygous	Missense	28.6	Likely pathogenic	Coffin-Siris syndrome 9	# 615866
79	*SPTBN2*	NM_006946.2	c.1052G>C, p.(Arg351Pro)	Heterozygous	Missense	34	Likely pathogenic	Spinocerebellar ataxia 5	# 600224
80	*STAG2*	NM_001042749	c.2860C>T, p.(Arg954Cys)	Hemizygous	Missense	32	Likely pathogenic	Mullegama-Klein-Martinez syndrome	# 301022
81	*STX1B*	NM_052874.3	c.165dup, p.(Gln56Thrfs*3)	Heterozygous	Frameshift		Pathogenic	Generalized epilepsy with febrile seizures plus, type 9	# 616172
82	*STXBP1*	NM_001032221.3	c.1261G>T, p.(Glu421*)	Heterozygous	Nonsense	47	Pathogenic	Epileptic encephalopathy, early infantile, 4	# 612164
83	*TBL1XR1*	NM_024665.4	c.799G>T, p.(Gly267Cys)	Heterozygous	Missense	34	Pathogenic	Mental retardation, autosomal dominant 41	# 616944
84	*TLK2*	NM_006852.3	c.968+1G>C, p.?	Heterozygous	Splice	25.7	Pathogenic	Mental retardation, autosomal dominant 57	# 618050
85	*TRIO*	NM_007118.2	c.3232C>T, p.(Arg1078Trp)	Heterozygous	Missense	32	Likely pathogenic	Mental retardation, autosomal dominant 44	# 617061
86	*TUBB*	NM_178014.2	c.139A>G, p.(Ile47Val)	Heterozygous	Missense	18.88	Likely pathogenic	Cortical dysplasia, complex, with other brain malformations 6	# 615771
87	*WAC*	NM_016628.4	c.1890_1892del, p.(Gln632del)	Heterozygous	Indel		Likely pathogenic	Desanto-Shinawi syndrome	# 616708
88	*YWHAG*	NM_012479.3	c.395G>A, p.(Arg132His)	Heterozygous	Missense	33	Pathogenic	Epileptic encephalopathy, early infantile, 56	# 617665
89	*ZC4H2*	NM_018684.3	c.22_23del, p.(Met8Valfs*7)	Heterozygous	Frameshift		Pathogenic	Wieacker-Wolff syndrome, female-restricted	# 301041
90	*ZEB2*	NM_001171653.1	c.353_357del; p.(Ser118Phefs*2)	Heterozygous	Frameshift		Pathogenic	Mowat-Wilson syndrome	# 235730
91	*ZEB2*	NM_014795.3	c.2761C>T, p.(Arg921*)	Heterozygous	Nonsense	39	Pathogenic	Mowat-Wilson syndrome	# 235730
92	*ZEB2*	NM_014795.3	c.770_771del, p.(Glu257Alafs*22)	Heterozygous	Frameshift		Pathogenic	Mowat-Wilson syndrome	# 235730
93	*ZEB2*	NM_014795.3	c.899A>G, p.(His300Arg)	Heterozygous	Missense	24.5	Likely pathogenic	Mowat-Wilson syndrome	# 235730

Abbreviation: CNVs, Copy number variations.

[a]The variant was identified as low-level mosaicism in the mother (in 1/216 reads, maternal DNA derived from blood).

majority of them occurring only once (n = 58/72, 79.2%). The most commonly affected gene was *ZEB2* (n = 4/72, 5.6%) associated with "Mowat-Wilson syndrome", followed by *ARID1B* (n = 3/72, 4.2%), *GNAO1* (n = 3/72, 4.2%), *KMT2B* (n = 3/72, 4.2%) and *PURA* (n = 3/72, 4.2%). Disease-causing variants in nine different X-linked genes comprising *DDX3X* (n = 2), *MSL3* (n = 2), *SMC1A* (n = 2), *CDKL5* (n = 1), *HNRNPH2* (n = 1), *NONO* (n = 1), *PDHA1* (n = 1), *STAG2* (n = 1), and *ZC4H2* (n = 1) were detected. The spectrum of genes containing disease-causing *de novo* variants is visualized in Figure 2(B). Except for one variant in *GNAO1* (NM_020988.3:c.625C>T, p.(Arg209Cys)), no recurrent variants were observed. More than half of all *de novo* variants (n = 50/93, 53.8%) were novel at the time of data interpretation and had not yet been published. All *de novo* variants were absent from the gnomAD as well as from the Database of Genomic Variants (DGV).[25] Table 1 gives an overview of all disease-causing *de novo* variants identified in this study, including the associated disorder.

We systematically evaluated constraint metrics (pLIs and Z-scores) for all genes containing (likely) pathogenic *de novo* variants (excluding CNVs spanning more than one gene). We observed that the majority of genes (n = 58/67, 86.6%) showed a pLI score > 0.9 indicating a high intolerance toward loss-of-function variants. 46/67 (68.7%) genes had a Z-score > 3.09 expressing a high constraint toward missense variants (Figure 2(C), Figure 2(D)). We further evaluated those five genes (*RHOBTB2*, *SPTBN2*, *KCNT1*, *IMPDH2*, *IFIH1*, *SOX11*) that did not show an overall constraint toward missense as well as toward loss-of-function variants (Z-scores ≤3.09 and pLIs ≤0.9). Apart from *SOX11*, whose pLI is most likely low due to the small gene size, we observed that pathogenic variants reported in those genes are all missense variants that cluster within or around a specific domain, in line with a region-specific high constraint (Table S2, Figure S2).

3.4 | Identification of novel candidate and disease genes

In cases without a definite molecular diagnosis, we sought to uncover (novel) candidate genes for NDDs. In summary, 22 different candidate genes were prioritized in 23 individuals. In the majority of individuals (n = 16), *de novo* variants in candidate genes for autosomal dominant inherited NDDs were found. Seven individuals harbored biallelic variants in candidate genes for autosomal recessive inherited NDDs. All nominated candidate genes were submitted to GeneMatcher. Six individuals were subsequently published within large collaborations connected through GeneMatcher and one individual was published as case report following two previous case descriptions, all together establishing six novel disease-associated genes for NDDs, namely *CYFIP2*, *KDM3B*, *IMPDH2*, *FITM2*, *RALGAPA1*, and *VARS*.[28-33] Those seven individuals were considered as solved and assigned to the overall yield (Supplemental Figure 1A). Furthermore, we published another three individuals from this study as single case reports proposing three novel candidate genes for NDDs (*CAMK4*, *POU3F2*, *RBL2*).[34-36] A number of the nominated candidate genes from this study is included in ongoing studies with manuscripts in process and is therefore not listed in detail.

3.5 | Systematic reanalysis of unsolved cases

We reanalyzed existing exome data from all cases with negative results older than ≥1 year (August 2017–September 2019). In summary, we performed reanalysis of 80 initially negative cases using updated variant annotation and newly discovered disease-associated genes. We achieved a diagnosis in two additional individuals increasing the overall yield from n = 113/231 (48.9%) to n = 115/231 (49.8%). Both individuals harbored variants in genes associated with autosomal recessive disorders (*SMPD4*, *UGDH*)[37,38] that had not been described as disease-associated genes at the time of data interpretation and were therefore not prioritized as potentially relevant variants. Furthermore, two previously not prioritized candidate genes were identified (Supplemental Figure 1B).

4 | DISCUSSION

In this study, we present 231 individuals with different NDDs who underwent trio exome sequencing. We further delineate the associated genetic spectrum of NDDs and corroborate the burden of *de novo* variants in NDDs.

Performing trio exome sequencing in 231 individuals with NDDs and their parents, we achieved an overall yield of 49.8%. The diagnostic yield was significantly higher in individuals with NDD plus associated conditions in comparison to individuals with isolated NDD. Our results are in accordance with a recent meta-analysis (assessing 30 articles with data on molecular diagnostic yield of exome sequencing in individuals with NDDs) that reported a diagnostic yield of 31% for isolated NDD and 53% for NDD plus associated conditions.[16] One possible reason for this difference in diagnostic yields might be that a subgroup of those cases with isolated NDD has a multifactorial basis rather than a monogenic explanation.

With regard to disease burden of CNVs in NDDs, the observed proportion of 3% in our cohort was smaller than previous estimations ranging from 10% to 15%.[24,39] This discrepancy most likely originates from a depletion of our cohort for cases with CNVs due to prior genetic work up including chromosome microarray analysis in some cases. From a phenotype perspective, the vast majority of individuals in our study displayed additional, often predominant neurological features such as dystonia or seizures further underlining the convergence in the genetics of NDDs and other neurological comorbidities.[1,30,40]

Even though it is widely recognized that *de novo* variants in protein-coding genes constitute the major genetic cause of NDDs in outbred populations, the burden as well as the genetic spectrum *de novo* variants in NDDs have not been fully elucidated yet.[14] In terms of *de novo* variants, we made several key observations in our study: First, the frequency of disease-causing *de novo* variants of 40.3% (n = 93/231) aligns with the prevalence of 42% recently presented in a large sequencing study of individuals with NDDs,[13] emphasizing the utility of trio sequencing as a first-line strategy, in particular in sporadic cases.[41,42] Second, with the identification of 72 distinct molecular diagnoses in our cohort, we replicate the enormous genetic heterogeneity underlying NDDs which

challenges diagnostic determinations based on clinical examination alone, even in disorders actually considered as highly recognizable such as Mowat Wilson syndrome.[16,43] Those findings illustrate the advantage of exome sequencing over a targeted panel sequencing approach and further support exome sequencing as first-tier for the genetic testing of unexplained NDD in clinical practice.[16,44] Third, we expand the list of disease-causing variants in NDDs-associated genes with 50 previously unreported (likely) pathogenic variants facilitating variant classification in other cases. Last, we observed that in the majority of genes containing *de novo* variants the predicted constraint metrics indicated an overall high intolerance toward loss-of-function (pLI > 0.9) and/or missense variants (Z-score > 3.09) or a region-specific constraint illustrating the importance of constraint metrics for disease gene discovery and the understanding of disease mechanism.[25]

The percentage of autosomal recessive disorders in our NDD cohort (~16%) which did not derive from a significant proportion of cases with a consanguineous background was surprisingly high in comparison to a previous study showing a low contribution (~4%) of autosomal recessive disorders to NDD in patients with European ancestry.[45] The proportion of cases with syndromal NDD was higher in the subgroup with autosomal recessive inheritance (n = 19/19, 100%) in comparison with those with *de novo* variants (n = 89/93, 95.7%) raising the question whether inclusion criteria were different in our study in comparison with previously published cohorts.

As hundreds of novel causal genes for rare NDDs still await discovery,[5] we also aimed to elucidate novel disease-associated genes for NDDs leading to the prioritization of more than 20 different candidate genes in our cohort of 231 individuals. A number of the nominated candidate genes have already resulted in publication as novel disease-associated genes,[28,29,31] once more emphasizing the potential of international data sharing and cooperation.[46,47] Most important, we illustrate that a parent-offspring trio approach is also a powerful tool for the discovery of novel disease-associated genes as it facilitates the prompt identification of *de novo* variants and assignment of zygosity for inherited variants.[42] Given the fact that our overall diagnostic yield did not include individuals with findings in new candidate genes, some of which are currently in preparation for publication, we furthermore anticipate that the actual number of molecular diagnoses in our cohort is going to increase.

The discovery of gene-disease and variant-disease associations is continually growing necessitating regular reevaluation of unsolved exomes.[48,49] In line with previous studies demonstrating an improved diagnostic yield by systematic reanalysis of existing data,[48,50] we achieved a definitive diagnosis in two additional individuals (among 80 reanalyzed individuals with initial negative results). Beyond, reanalysis in our cohort lead to the identification of two novel candidate genes for NDDs highlighting the potential of subsequent reanalysis also for disease gene discovery.[41,51]

In summary, we consolidate the contribution and genetic heterogeneity of *de novo* variants in NDDs highlighting trio exome sequencing as an excellent diagnostic tool for rare NDDs. Besides, we illustrate the potential of a trio-approach for candidate gene discovery and the power of systematic reanalysis of unsolved cases.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Additional data is available upon request from the corresponding author if in line with the consents.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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