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

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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. In accordance to “Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition” (DSM-5), NDD encompasses intellectual developmental disorders, communication disorders, autism spectrum disorders, attention-deficit/hyperactivity disorders, specific learning disorders, and motor disorders. Furthermore, patients with NDDs often demonstrate additional, (non-) neurological comorbidities.

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. The genetic etiology underlying NDD is extremely heterogeneous extending from large chromosomal aberration to single-nucleotide variants (SNVs) in >1000 of genes. Neverthe-
characteristic, for example, microcephaly or neutropenia. 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 case 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%.
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 VUSs 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
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
| 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 | 47 | Pathogenic | Coffin-Siris syndrome 1 | # 135900 |
| 6          | ARID1B     | NM_020732.3 | c.4009C>T, p.(Arg1337*) | Heterozygous | Nonsense | 52 | 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 | # 61776 |
| 17         | Chromosome 17p13.1 deletion (~500 kb) | chr17:7554837_8093457del | Heterozygous | CNV | Pathogenic | Chromosome 17p13.1 deletion syndrome | # 613776 |
| Individual | Gene/locus | Transcript | Variant | Zygosity | Variant type | CADD score | ACMG classification | Diagnosis | OMIM phenotype |
|------------|------------|------------|---------|----------|--------------|------------|---------------------|-----------|----------------|
| 18         | Chromosome 6q21-6q22.31 duplication (~16 Mb) | chr: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_0013563.3 | c.1148C>G, p.(Ala383Gly) | Heterozygous | Missense | 26.6 | Pathogenic | Mental retardation, X-linked 102 | # 300958 |
| 25         | DDX3X | NM_0013563.3 | 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 |
| 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         | HNRNU      | 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 choriorhinopathy, lymphedema, or mental retardation | # 152950 |
| 43         | KIF1A      | NM_004321.6| c.760C>T, p.(Arg254Trp) | Heterozygous | Missense | 34 | Pathogenic | NESCAV syndrome | # 614255 |
| 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 | Missense | 36 | Pathogenic | Dystonia 28, childhood-onset | # 617284 |
| 47         | KMT2B      | NM_014727.1 | c.521dup, p. (Thr176Aspfs*8) | 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, 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 |

(Continues)
| 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 | # 61583 |
| 64         | PURA       | NM_005899.4| c.565G>C, p.(Ala189Pro) | Heterozygous | Missense | 28.6 | Likely pathogenic | Mental retardation, autosomal dominant 31 | # 616158 |
| 65         | PURA       | NM_005899.4| c.366_367dup, p. (Gln123Argfs*103) | Heterozygous | Frameshift | Pathogenic | Mental retardation, autosomal dominant 31 | # 616158 |
| 66         | PURA       | NM_005899.4| 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.569A>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-atactic epilepsy | # 616421 |
| 75         | SMARCA4    | NM_001128849.1| c.1675G>A, p.(Glu559Lys) | Heterozygous | Missense | 34 | Likely pathogenic | Coffin-Siris syndrome 4 | # 614609 |
| 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 | Spineo-rebella ataxia 5 | # 600224 |
| 80        | STAG2      | NM_001042749 | c.2860G>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.

*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 VAR.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, Pou5f2, 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 of 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 cases 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.56,47 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 zygoty 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.31,51

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.

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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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