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Title: Whole Exome Sequencing in Neurodevelopmental Disorders; a Single Center Study

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Abstract

Aim: Neurodevelopmental disorders(NDD) are clinically and genetically heterogeneous group of diseases. It is difficult to diagnose the underlying origin of these diseases. We aim to evaluate whole exome sequencing(WES) results in our NDD patients and the responsible genetic variants.

Methods: In the study, WES analysis results of 25 NDD patients were evaluated retrospectively and the diagnostic yield of WES in our cases and clinical findings were examined.

Results: With WES analysis, we diagnosed 13(52%) of the patients with pathogenic(P) and likely pathogenic(LP) variants and 12(48%) patients remained unclear with variants of unsignificance(VUS). However, with phenotype consistence and following segregation analysis, we also evaluated 2 VUS as the disease causing variants and our yield rate increased to 60%. We also reported the secondary findings.

Conclusion: The diagnostic yield of WES in NDD was 60% in our study. The latest ACMG guideline recommends WES as the first-tier test in NDD. WES is time and cost effective when performed in well-selected patient, plus determining the underlying cause of NDD will provide more accurate diagnosis and clinical follow-up for the patients.

Keywords: Neurodevelopmental Disorder, Whole Exome Sequencing, Developmental Delay Disorders, Intellectual Disabilities, Congenital Abnormalities

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Introduction

Neurodevelopmental disorders (NDD) are clinically and genetically heterogeneous group of diseases. Epilepsy, intellectual disability (ID), autism spectrum disorder (ASD), and developmental delay (DD) are classified under this group (Stefanski et al., 2021).

The prevalence of DD and ID is reported to be 1-3%. Congenital anomalies (CA) may accompany these features (Xiang et al., 2021). Genetic variants such as copy number variations (CNV), small insertions/deletions (indels) and single nucleotide variations (SNV) are responsible for these manifestations. Diagnostic yield of chromosomal microarray (CMA) for DD/ID and or CA and ASD is about 12-28%. According to the 2010 American College of Medical Genetics and Genomics (ACMG) guidelines, CMA was the first-tier test for DD/ID and or CA (Manickam et al., 2021). With this test, SNV and indel detection could not be made. Over time, whole exome and genome sequencing (WES and/or WGS) became widespread and diagnosis rates of WES and/or WGS for NDD were reported as 28-68%. With the evaluation of these studies, 2020 ACMG guideline recommended WES and/or WGS for CA or DD/ID as the first-tier test. CNV analysis in whole exome sequencing is also possible thanks to technical additions (Malinowski et al., 2020).

It is difficult to diagnose this patient group, constant visits to the hospital and undiagnosed returns cause emotional problems for the family. Identifying the underlying diagnosis for CA/DD/ID may affect mortality and morbidity and reduce the burden on patients and families seeking answers (Malinowski et al., 2020). Finding the responsible gene will also be useful in understanding the pathogenesis and classifying of this large group. Due to the heterogeneous genetic background, WES analyses are more useful than single gene tests and panel tests in diagnosing (Stefanski et al., 2021). Treatment, prognosis, and follow-up processes of patients who are clearly diagnosed with

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genetic tests are also improved, and therefore a path can be followed according to the needs of the patient (Vickers and Gibson, 2019).

In this study, we aim to share the results of NDD patients who applied to our clinic and performed WES analysis and to evaluate the rates of diagnosis with WES in our clinic.

Materials and methods

Patients

This study was created by evaluating the pediatric patients admitted to our department with the diagnosis of NDD between March 2019 and May 2021 and underwent WES. In our study, the findings of 25 patients aged under 18 were evaluated retrospectively by medical genetics department records and hospital automation system. Approval was obtained from the ethics committee of our university. Informed consent was approved from the parents of our patients.

Molecular study

Genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. All coding exons and exon-intron boundaries of the genes were amplified using QIAseq Human Exome Kit (Qiagen, Hilden, Germany). Prepared library was sequenced on the Illumina NextSeq platform (Illumina Inc., San Diego, CA, USA).

Bioinformatic analysis

The data were analyzed using QIAgen Clinical Insight (QCI) Interpret software (Qiagen, Hilden, Germany). >99% base level coverage of targets at ≥20x was obtained. Sequencing data was aligned to human reference genome, hg19. QIAGEN Clinical Insight (QCI) Interpret software includes the

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following underlying databases, data reference sets and tools; QIAGEN Clinical Insight-Interpret (5.4.20190308), Ingenuity Knowledge Base (Stepford 190301.000), CADD (v1.3), Allele Frequency Community (2018-09-06), EVS (ESP6500SI-V2), Refseq Gene Model (2018-07-10), JASPAR (2013- 11), Ingenuity Knowledge Base Snapshot Timestamp (2019-03-01 11:17:42.0), Vista Enhancer hg19 (2012-07), PolyPhen-2 (v2.2.2), 1000 Genome Frequency (phase3v5b), ExAC (0.3.1), iva (Oct 4 11:04 iva-1.0.736.jar), PhyloP hg19 (2009-11), DbSNP (151), GENCODE (Release 28), CentoMD (5.0), OMIM (May 26, 2017), gnomAD (2.0.1), BSIFT (2016-02-23), TCGA (2013-09-05), Clinvar (2018-08-01), DGV (2016-05-15), HGMD (2018.3), and SIFT4G (2016-02-23).Variants were evaluated according to the ACMG (American College of Medical Genetics and Genomics) criteria, and classified as pathogenic, likely pathogenic, and variants of uncertain clinical significance were reported.

Statistics Analysis

SPSS (IBM SPSS Statistics 20) program was used for statistical analysis of the study. Descriptive statistics were used.

Results

The study consists of 14 male (56%) and 11 (44%) female patients. Mean age was 5.64 (1 to 16; SD:5.09). Developmental delay (DD) in all the 25 patients (100%), intellectual disability in 5 (20%), epilepsy in 14 (56%), congenital anomaly (CA) in 10 (40%), and other phenotypes such as autism, hypotonia, neuromotor regression in 3 (12%) of the patients were seen.

CMA tests were requested before WES analysis in 8 (32%) patients and no result was obtained describing the phenotype. With WES analysis, we diagnosed 13 (52%) of the patients with pathogenic (P) and likely pathogenic (LP) variants (Table 1) and 12 (48%) patients remained

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unclear with variants of uncertain significance (VUS). However, we reclassified the variant, as a result of segregation analysis, in 2 patients that we previously classified as VUS. These VUS variants were phenotype-associated variants. We detected that the autosomal dominant(AD) VUS variant was de novo in one of the patients. In this case, were classified the variant as LP. The other patient had also a VUS, associated with the phenotype. As a result of the segregation analysis, we determined that the homozygous variant was heterozygous in both parents, and homozygous in a sibling who had a similar phenotype. We revised the variant as LP. The diagnostic yield of WES became 60% with the revisions (Figure 1).

We included variants recommended to be reported as secondary findings in the ACMG guidelines in 4 patients. In addition, we included *MEFV* variants in the report of 2 patients due to the commonness of FMF in our country (Table 2).

Discussion

As in many clinically and genetically heterogeneous diseases, the rates of WES application are increasing in NDD. Many new responsible genes for NDD have been reported with WES analysis (Vissers et al., 2017, Xiang et al., 2021). Here, we evaluate WES results of our 25 NDD patients and the diagnostic yield of WES in this group. Our diagnostic yield was 60%. This rate is in the upper range of the literature. In a meta-analysis, the diagnostic yield of clinical exome sequencing was reported as 36% overall, 31% for isolated NDD, and 53% NDD plus associated conditions (Srivastava et al., 2019). Another review-meta-analysis study reported a diagnostic yield of WES as 23.7% in NDD patients with epilepsy, ASD, or ID (Stefanski et al., 2021). In a systematic evidence-based review, a diagnostic yield of WESand/orWGS have been reported as 28-68% in NDD patients with DD/ID/CA (Malinowski et al., 2020).

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In NDD patients with DD/ID/CA, the diagnostic yield of CMA has been reported to be 16-28% (Malinowski et al., 2020). In our study, CMA analysis was performed in 32% of the patients and none of them had a relevant result. Our WES analysis identified 14 variants of different genes that were responsible for the phenotypes. 5 of these variants were novel, and the others were previously reported variants. Of the diagnosed diseases, 7 were autosomal recessive, 4 were autosomal dominant, and 3 were X-linked.

In two siblings, P1 and P2, a p.R140H pathogenic, homozygote, missense variant in *CLP1* gene was detected; autosomal recessive (AR) pontocerebellar hypoplasia, type10 (OMIM: 615803) was diagnosed. p.R140H variant was reported as a founder mutation in Turkish families, and due to common consanguineous mating, carrier frequency was 1/1000. It has been reported that this mutation disrupts tRNA splicing and causes progressive neurodegeneration (Schaffer et al., 2014). Global DD, lack of independent sitting or walking, seizures, lack of speech, and in MRI; pontocerebellar hypoplasia, cortical atrophy, delayed myelinization were seen in the siblings consistent with the literature (Karaca et al., 2014, Schaffer et al., 2014).

In P3, *MECP2* p.R106W pathogenic variant was detected. The patient was diagnosed with Rett syndrome (OMIM: 312750). This syndrome is an X-linked dominant disease primarily seen in females with variable phenotype due to X inactivation. Clinical findings begin with 6-18 months of age. Generally, there is no clinical manifestation in early infancy. Over time, head growth slows and muscle tone decreases. Neuromotor delay and coordination disorder occur. Stereotypic hand movements, ataxic gait, scoliosis, constipation, excessive saliva, intellectual disability periodic breathing, seizures, and verbal skill deterioration may occur (Sheikh et al., 2016). Our patient was a 2-year-old female presented with neuromotor delay, hypotonia, and seizures.

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In P4, a novel p.V175A variant in *KCNQ2* gene was detected. The patient was diagnosed with developmental and epileptic encephalopathy 7, an autosomal dominant disease. The segregation analysis showed no mutations in the parents, so the de novo variant was classified as likely pathogenic. It is an autosomal dominant (AD) neurodevelopmental disorder usually characterized by resistant seizures in the neonatal period and de novo variants are responsible in most of the cases. Seizures usually resolve by 3 or 4 years of age, but neurological disorders are severe and persistent (Weckhuysen et al., 2012). The seizures of our patient started when he was a few months old and continued intermittently until he was 4-5 years old. He had spastic paraparesis and cortical atrophy in brain MRI, that is why his clinic was severe.

In P5, p.R292* pathogenic variant in *DDX3X* gene was detected. *DDX3X* is responsible for syndromic X-linked mental retardation of the Snijders Block type, which is predominantly seen in females. Intellectual disability, microcephaly, movement disorders, behavioral problems such as ASD, hyperactivity, and epilepsy are seen in the disease (Snijders Blok et al., 2015). Our patient was a female with DD/ID, epilepsy, and ASD phenotype. It was confirmed that the variant was de novo by segregation analysis.

P6 and P13 were diagnosed with Joubert syndrome 3 and 14, respectively. Both were inherited in an AR manner. Joubert syndrome is a group of diseases with genetic heterogeneity, characterized by symptoms such as neuroradiological 'molar tooth sign', hypoplasia of the cerebellar vermis, irregularity of breathing pattern, and developmental delay (Valente et al., 2005). p.L750fs*4 pathogenic, frameshift, and homozygote variant in *AHI1* gene was responsible for Joubert syndrome 3 in P6. It was confirmed that both parents were heterozygote carriers of the variant. P6 had DD and the characteristic brain MRI findings. The novel, likely pathogenic p.Q140* variant in

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TMEM237 gene was responsible for Joubert syndrome 14 in P13, who had DD, CA, hypotonia, and the characteristic brain MRI findings.

In P7, a likely pathogenic c.1516_1518delGAC variant in *SLC6A8* gene was detected that is responsible for X-linked cerebral creatine deficiency syndrome. It is characterized by DD/ID, epilepsy, ASD, and severe speech delay (Salazar et al., 2020). Our patient had DD/ID, epilepsy, lack of speech, and microcephaly.

In P8, a pathogenic p.R156* homozygote variant in *BCKDK* gene was detected that is responsible for branched-chain ketoacid dehydrogenase kinase deficiency. The same variant was reported in a Turkish family with ID, ASD, and epilepsy findings in 2012. In this study, clinical improvement has been observed in mice with branched-chain amino acid supplementation and it has been reported that the patients may benefit from branched-chain amino acid supplementation (Novarino et al., 2012).

In P9, a pathogenic p.L239R homozygote variant in *WWOX* gene was detected that is responsible for developmental and epileptic encephalopathy 28. It is an AR NDD in which resistant seizures, hypotonia, and psychomotor retardation are seen. Microcephaly, poor visual contact, and retinal degeneration may also be seen (Mignot et al., 2015). It was confirmed that both parents were heterozygote carriers of the variant. The patient had DD, epilepsy, and corpus callosum hypoplasia. His two older siblings are still living with tracheostomy, although they have not been tested yet, we suspected that they had the same diagnosis.

In P10, a pathogenic p.D106N homozygote variant in *PRUNE1* gene was detected that is responsible for neurodevelopmental disorder with microcephaly, hypotonia, and variable brain anomalies (OMIM: 617481). The same variant was reported in a Turkish family with

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microcephaly, cortical and cerebellar atrophy (Karaca et al., 2015). Our patient had severe DD, refractory seizures, hypotonia, cortical and cerebellar atrophy and died within a few months.

In P11, a pathogenic p. P65L heterozygote variant in *PCGF2* gene was detected that is responsible for Turnpenny-Fry syndrome. The segregation analysis showed that the variant was de novo; not found in the parents. It is an AD disorder with developmental delay, intellectual disability, facial dysmorphism, and skeletal abnormalities (Ercoskun et al., 2021). Our patient had DD, facial dysmorphism, and neuromotor retardation.

In P12, a likely pathogenic novel p.R308* variant in *CDH2* gene was detected. It is responsible for agenesis of corpus callosum, cardiac, ocular, and genital syndrome (OMIM:618929). The disorder characterized by DD/ID, ocular, cardiac, genital anomalies, corpus callosum hypoplasia, and craniofacial dysmorphisms (Accogli et al., 2019). Our patient had DD, corpus callosum hypoplasia, and nystagmus. The segregation analysis showed that the variant was de novo, not found in the parents.

In P14, a novel, homozygote p.G235S variant in *CACNA2D2* gene was detected. In silico tools predictions were as follows: CADD score: 27.4 (Deleterious), PolyPhen: Probably Damaging, SIFT: Damaging, PhyloP: Not Conserved, BLOSUM, MaxEntScan, B-SIFT, and QCI Inferred Activation: No Prediction. According to ACMG criteria, the variant was classified as VUS. It was confirmed that both parents were heterozygote carriers of the variant. Besides, a similarly affected older sister had the same homozygote variant. With the segregation analysis data and consistent phenotype, we evaluate the variant as likely pathogenic. *CACNA2D2* is responsible for cerebellar atrophy with seizures and variable developmental delay (OMIM:618501). It is an AR NDD characterized by cerebellar atrophy, severe refractory seizures in the first year of life, and DD

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(Butler et al., 2018). Our patient had seizures, cerebellar vermis atrophy, and DD similar to the literature.

In P15, a novel, heterozygote p.A664S variant in *IRF2BPL* gene was detected. In silico tools predictions were as follows: CADD score:17.3 (Likely Deleterious), PolyPhen: Benign, SIFT: Tolerated, Mutation Taster: Disease Causing, PhyloP: Not Conserved, BLOSUM, MaxEntScan, GeneSplicer, B-SIFT, and QCI Inferred Activation: No Prediction. According to ACMG criteria, the variant was classified as VUS. The segregation analysis showed that the variant was de novo, not found in the parents. With the segregation analysis data and consistent phenotype, we evaluate the variant as likely pathogenic. *IRF2BPL* is responsible for neurodevelopmental disorder with regression, abnormal movements, loss of speech and seizures (OMIM:618088). In this disorder, psychomotor development is normal at the beginning, followed by severe neurological regression and neurological findings (Tran Mau-Them et al., 2019). Our patient's development was normal at the beginning. After the age of 9, the phenotype of a progressive gait and speech disorder and repetitive movements emerged.

We also reported *TTN, BTD, MYH7* variants as the secondary findings for 4 patients that ACMG guidelines recommended reporting (Miller et al., 2021). Due to the commonness of FMF in our country, we also reported the *MEFV* variants of 2 patients. Since all patients were heterozygous carriers for the mutations found, segregation analysis was not performed, but families were informed about the risk of carrying this mutation.

Since consanguineous marriage is common in our country, it is more likely to see different rare diseases compared to other countries. Our study also confirmed this information. 7 of the 15 diseases were inherited in an AR manner. 6 of the parents were relatives and one was married from the same village.

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Since it is possible to screen all coding exons associated with the phenotype in a single step, the use of WES test is gradually increasing in the diagnosis of NDD diseases, considering its genetic heterogeneity. In our study, the rate of diagnosis of NDD with WES was 60%. WES test, which is still expensive in our country, seems cost effective when applied in well-selected patients compared to multiple single gene or panel tests. Determining the underlying cause of NDD will provide accurate diagnosis and clinical follow-up. It will even shed light on possible future gene therapy studies and will ensure that families are given accurate genetic counseling.

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There are no conflicts of interest.

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Authorship Contributions

Concept: C.Y.K., M.K., P. E., A.T., Design C.Y.K., M.K., P. E., A.T., Data Collection or Processing: C.Y.K., M.K., P. E., Analysis or Interpretation: C.Y.K., M.K., P. E., A.T., Literature Research: C.Y.K., M.K., P. E., Writing-Reviewing: C.Y.K., M.K., P. E., A.T

Ethics Committee Approval: This study was approved by ??? University Clinical Research Ethic Committee (decision number: B.30.2.ATA.0.01.00/129).

Informed Consent: Written informed consents were approved from the parents of our patients

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Table 1: Causal variants of NDD patients

ID/ Sex	Age (years)	Gene	Nucleotide/aa Change	RefSeq	Zygosity	ACMG criteria	impact	Ref	Inheritance	Seg.	OMIM
P1/ M	4	*CLP1*	c.419G>A (p.R140H)	NM_006831.3	Hom	P(PS3,PS4,PM2,PP3,PP5)	M	Known	AR	M/F: het	# 615803 PONTOCEREBELLAR HYPOPLASIA, TYPE 10
P2/ F	2	*CLP1*	c.419G>A (p.R140H)	NM_006831.3	Hom	P(PS3,PS4,PM2,PP3,PP5)	M	Known	AR	M/F: het	# 615803 PONTOCEREBELLAR HYPOPLASIA, TYPE 10
P3/ F	2	*MECP2*	c.316C>T p.R106W	NM_004992.4)	Het	P(PM1,PS3,PM5,PM2,PP2,PP3)	M	Known	XLD	-	# 312750 RETT SYNDROME
P4/ M	12	*KCNQ2*	c.524T>C (p.V175A)	NM_004518.6	Het	LP(PM1,PM2,PP3,PM6)	M	Novel	AD	M/F: 0	#613720 DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHY 7
P5/ F	12	*DDX3X*	c.874C>T (p.R292*)	NM_001193416.3	Het	P(PVS1,PM2,PM6)	N	Known	XLD/XLR	M/F: 0	#300958 INTELLECTUAL DEVELOPMENTAL DISORDER, X-LINKED, SYNDROMIC, SNIJDERS BLOK TYPE
P6/ M	3	*AHI1*	c.2247dupA (p.L750fs*4)	NM_017651.4	Hom	P(PVS1,PM2,PP3,PP5)	F	Known	AR	M/F: het	#608629 JOUBERT SYNDROME 3
P7/ M	11	*SLC6A8*	c.1516_1518delGAC	NM_005629.4	Hemi	LP(PM1,PM2,PM4)	I	Known	XLR	-	# 300352 CEREBRAL CREATINE DEFICIENCY SYNDROME 1
P8/ M	3	*BCKDK*	c.466C>T p.R156*	NM_001271926.2	Hom	P(PVS1,PP5,PM2,PP3)	N	Known	AR	-	#614901 BRANCHED-CHAIN ALPHA-KETO ACID DEHYDROGENASE KINASE
P9/ M	1	*WWOX*	c.716T>G p.L239R	NM_016373.4	Hom	P(PM2,PP3,BP1)	M	Known	AR	M/F: het	# 616211DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHY 28
P10 /F	1	*PRUNE1*	c.316G>A p.D106N	NM_021222.3	Hom	P(PP5,PM2,PM1,PP2,PP3)	M	Known	AR	-	#617481 NEURODEVELOPMENTAL DISORDER WITH MICROCEPHALY, HYPOTONIA, AND VARIABLE BRAIN ANOMALIES

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P11/M	2	*PCGF2*	c.194C>T p.P65L	NM_007144.3	Het	P(PS4,PM2,PP3,PM6)	M	Known	AD	M/F: 0	#618371 TURNPENNY-FRY SYNDROME
P12/	1	*CDH2*	c.922C>T p.R308*	NM_001308176.2	Het	LP(PVS1,PM2,PM6)	N	Novel	AD	M/F: 0	#618929 AGENESIS OF CORPUS CALLOSUM, CARDIAC, OCULAR, AND GENITAL SYNDROME
P13/F	4	*TMEM237*	c.418C>T p.Q140*	NM_001044385.3	Hom	LP(PVS1,PM2,PP5)	N	Novel	AR	-	#614424 JOUBERT SYNDROME 14
P14/M	2	*CACNA2D2*	c.703G>A (p.G235S)	NM_001291101.1	Hom	VUS(PP3,PM2)	M	Novel	AR	M/F: het	#618501 CEREBELLAR ATROPHY WITH SEIZURES AND VARIABLE DEVELOPMENTAL DELAY
P15/M	15	*IRF2BPL*	c.1990G>T p.A664S	NM_024496.4	het	VUS(PM2,PP2)	M	Novel	AD	M/F: 0	#618088 NEURODEVELOPMENTAL DISORDER WITH REGRESSION, ABNORMAL MOVEMENTS, LOSS OF SPEECH, AND SEIZURES

Sex; M:male, F:female, Zygosity: hom:homozygote, het:heterozygote, hemi:hemizygote impact;M:missense, N:nonsense, F:frameshift, I:in frame, Seg:segregation analysis; M/F:het(mother and father are heterozygotes) M/F:0(mother and father not carrying the variant)

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Table 2: Secondary findings of NDD patients

ID/ Sex	Age (years)	Gene	Nucleotide/aa Change	RefSeq	Zygosity	ACMG criteria	impact	Ref	Inheritance	OMIM
P21 /F	13	*TTN*	c.30928delG p.E10310fs*3	NM_001256850.1	Het	LP(PVS1,PM2)	F	novel	AR/AD	Dilated cardiomyopathy (MIM 604145)
		BTD	c.1270G>C p.D424H	NM_001370658.1	Het	P(PA2,PS4,PM1,PM3,PP3,PP5)	M	known	AR	Biotinidase deficiency (MIM 253260)
P22 /M	2	*TTN*	c.30928delG p.E10310fs*3	NM_001256850.1	Het	LP(PVS1,PM2)	F	novel	AR/AD	Dilated cardiomyopathy (MIM 604145)
		BTD	c.1270G>C p.D424H	NM_001370658.1	Het	P(PA2,PS4,PM1,PM3,PP3,PP5)	M	known	AR	Biotinidase deficiency (MIM 253260)
P24 /F	1	*BTD*	c.1270G>C p.D424H	NM_001370658.1	Het	P(PA2,PS4,PM1,PM3,PP3,PP5)	M	known	AR	Biotinidase deficiency (MIM 253260)
P12 /F	1	*MYH7*	c.2167C>T p.R723C	NM_000257.4	Het	P(PS4,PM1,PM2,PM6,PP3)	M	known	AR/AD	Familial hypertrophic cardiomyopathy 1 (MIM 192600)
P4/ M	12	*MEFV*	c.2177T>C p.V726A	NM_000243.3	Het	P(PS4,PM1,PM3,PP5,BS1,BS2,BS4,BP2,BP4)	M	known	AR	Familial Mediterranean fever, AR(MIM 249100)
P7/ M	11	*MEFV*	c.2177T>C p.V726A	NM_000243.3	het	P(PS4,PM1,PM3,PP5,BS1,BS2,BS4,BP2,BP4)	M	known	AR	Familial Mediterranean fever, AR(MIM 249100)

Zygosity: het:heterozygote, impact: F: frameshift, M:missense

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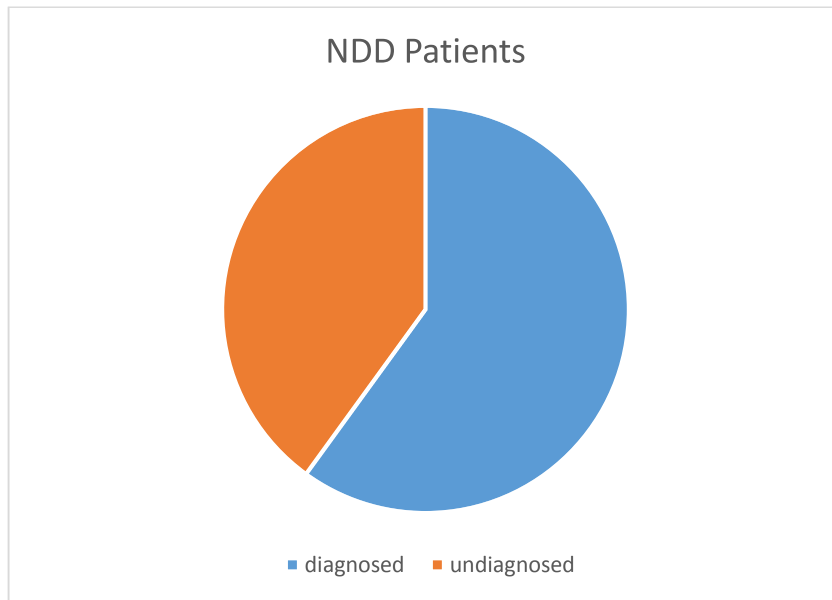
Figure 1: The diagnostostic yield of WES in our NDD patients (60%)



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