श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेन्द्रम
तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
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From,

08/11/2023

Dr. Ramshekhar N. Menon,

Professor

Department of Neurology,

Sree Chitra Tirunal Institute for Medical Sciences and Technology

Trivandrum

To,

Dr. Anju Sinha

Scientist F (Deputy Director General, Senior Grade)

Division of Reproductive, Maternal and Child Health

Indian Council of Medical Research,Ansari Nagar,New Delhi- 110029

Sub: Resubmission of Final Project Report and Statement of Expenditure and Utilisation Certificate for the project titled 'Genetics of complex Paediatric epilepsy syndromes: electroclinicoimaging based genotype - Phenotype correlations in an Indian cohort'

Respected Madam,

I am resubmitting the Project completion Report, Statement of Expenditure and Utilisation Certificate for the financial year 2022-2023 of ICMR funded project (Ref. No. 5I7II658IcHIAdhoc/2019-RBMCH dated 13t0812019) as recommended by the Project review group. The whole exome sequencing of 489 samples (including 175 probands & 155 trios) and further bioinformatics analysis were completed.

We would like to seek the permission of release of the remaining grant allocation of Rs 4,50,3351/- (as per letter 7/7/1658/CH/ Adhhoc/ 2019-RBMCH dated 19-05-2022 and 5/7/1815/CH/Adhoc/2023-RCN dated 16-03-2023). We request the adjustment of the above mentioned amount of 4,50,351 under man power (Rs. 4,22,279/-) and contingency (Rs. 28,072/-) as detailed in the utilization certificate attached with the report, at the earliest for the pending salary disbursal.

Thanking You

Yours Sincerely

Dr. Ramshekhar N Menon

Project Completion report

1.	**Project Title**	Genetics of complex pediatric epilepsy syndromes: electro-clinico-imaging based genotype- phenotype correlations in an Indian cohort
2.	**PI (name & address)**	Dr. Ramshekhar N. Menon Professor, Department of Neurology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram- 695011, Kerala, India
3.	**Co-PI (name & address)**	Dr Moinak Banerjee (Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram) Dr Ashalatha Radhakrishnan (SCTIMST, Thiruvananthapuram)
4.	**Date of start**	5th February 2020
5.	**Duration**	3 years
6.	**Objectives of the proposal**	1) To ascertain the frequency, type, inheritance patterns of pathogenic or likely pathogenic variants in complex pediatric epilepsies & epileptic encephalopathies (EE) of unknown etiology using targeted and whole exome sequencing. 2) To streamline therapeutic intervention-prognostication by correlating phenotype to genotype and pharmacogenotype. 3) To establish a registry to enable future diagnostic and precision medicine protocols in EE of infancy and childhood
7.	**Methodology**	**Inclusion criteria:** We plan to recruit trios of the probands presenting with different phenotypes that fall under the inclusion criteria. All patients with complex pediatric epilepsy (<12 years age) associated with/without developmental delay, refractory epilepsy of unknown cause or EE who will be attended to

		between 2019-2022 at the pediatric epilepsy clinic conducted at SCTIMST, Trivandrum and SAT Hospital, Trivandrum, will be included in this prospective cohort study as the core study arm. Two broad groups will be as defined below: A) Epileptic Encephalopathies (EE): This group will comprise of children in whom the interictal or ictal EEG abnormalities may result in clinically significant moderate to severe psychomotor retardation or regression which is not commensurate to the underlying epileptogenic substrate, eg. malformation of cortical development (MCD). These children may demonstrate a tendency for progression over time, with considerably dependency for daily functioning. Thus the probands along with their parents and siblings will be a part of our sample collection strategy. (N=200) B) Complex pediatric epilepsy of unknown cause/presumed genetic etiology (<12 years age): These will involve a diagnosis of symptomatic localisation related epilepsy (LRE) of focal or multifocal origin with or without developmental delay without any overt structural or metabolic cause to implicate without any overt features of EE; refractory to two or more trials of conventional antiepileptic drugs; absence of any overt neuro-cutaneous markers or recognised chromosomal aberrations. Children with drug-resistant generalized epilepsy syndromes including myoclonic atonic epilepsy, early onset childhood absences, photosensitive occipital lobe epilepsies will also be included (N=100).

		In order to overcome the issues of clinical heterogeneity, limitations of clinical exome, and to resolve the issues of denovo variants in each of these subtypes, we are using whole exome sequencing (WES) in trios. This gives better information and overcome the issues of power in clinically heterogenous group of disorders. Understanding population genetic parameters are important to resolve the issues of genetic heterogeneity in a clinically diverse group of disorders. **Trios based study:** The study is based on family trios. Variant identification will be performed on all sequenced first-degree relatives. This allows for more accurate predictive calls in all three family members, especially the proband, whose variants are inherited from parents (with the exception of de novo mutations). This method enhances the ability to determine significance of variants in low coverage regions. With this method it is also possible to call variants at suboptimal coverage levels, thus improving sensitivity with bare negative impact on specificity.EE are often caused by rare or de novo mutations. Therefore, our trio based study will be sufficient to ascertain significance of rare and de novo mutations in the study group. **Exclusion criteria:** Children with known antecedent perinatal insult or culpable epileptogenic substrates on MRI such as malformations of cortical development, gliotic scars or known neurocutaneous markers will be excluded. Progressive symptomatic causes such as storage disorders, progressive myoclonus epilepsies, poliodystrophies will be excluded by appropriate tests. Evident metabolic epilepsies will be excluded using specific tests that will be done as a blanket screening in all children with EE and refractory epilepsy with developmental delay and these will include: plasma amino acids, serum homocysteine, lactate,

		ammonia, acylcarnitine profile, total and free carnitine, and urine for organic acids and mucopolysaccharides. Recognised chromosomal aberrations with dysmorphism will undergo karyotyping with Fluorescent Insitu Hybridization (FISH) probes for specific deletions/duplications based on the phenotype and if proven, will not be included among the probands. **Phenotyping:** All study participants who are identified as probands will undergo overnight or 24-hour video EEG along with multimodal imaging which will include structural MRI, DTI, arterial spin labelling and MR-Spectroscopy. EE phenotypes will be classified as per current International League Against Epilepsy (ILAE) criteria asearly infantile EEwith/without suppression-burst, early myoclonic encephalopathy, migrating partial epilepsy of infancy, West syndrome, Mark and-Blume-Ohtahara syndrome with multiple independent spike-foci, DS, Lennox-Gastaut syndrome (LGS), Landau-Kleffner syndrome, EE with electrical status epilepticus in sleep (ESES), refractory focal/generalized epilepsies of unknown cause such as myoclonic-atonic epilepsy and atypical/atonic variant of Rolandic epilepsy of childhood. Developmental assessment will be done by the neuropsychologist, speech therapist and occupation therapist. Cognitive disabilities will be classified in accordance with the Diagnostic & Statistical Manual for mental disorders (DSM- version V) as well as with development and intelligence quotient scales. The data of initial metabolic evaluation done on these subjects as mandated as per the standard of care guidelines to exclude metabolic disorders include blood tandem mass spectroscopy and urine evaluation for organic aciduria which are done routinely in these children. When suspected to have disorders like neurotransmitter

		disorders or glucose transporter defects (GLUT-1) deficiency, the results of fasting CSF study for glucose, neurotransmitter (pterine levels) and aminoacid levels if done during the evaluation process will be documented as part of the endophenotyping. CSF analysis will not be separately done for the purpose of this genetic study. **Demographic details**, family history, detailed clinical history, any comorbid condition will be recorded and appropriate blood samples will be collected. Blood samples will be shipped to RGCB for NGS based resequencing of critical candidate genes and exome sequencing of epilepsy panel genes of trios. Endophenotyping using clinical data including dysmorphism, developmental assessments, electrophysiological (EEG) and multi-modal MRI analysis will be collected. The data of initial metabolic evaluation done on these subjects as mandated as per the standard of care guidelines to exclude metabolic disorders include blood tandem mass spectroscopy and urine evaluation for organic aciduria which are done routinely in these children. When suspected to have disorders like neurotransmitter disorders or glucose transporter defects (GLUT-1) deficiency, the results of fasting CSF study for glucose, lactate, neurotransmitter (pterine levels) and aminoacid levels if done during the evaluation process will be documented as part of the endophenotyping. CSF analysis will not be separately done for the purpose of this genetic study. All protocols and methodology will be carried out with written informed consent of the parents of all proband children as per the established guidelines laid down by ICMR for resolving ethical concerns. 10 ml peripheral blood sample will be collected in EDTA vials, for Deoxyribo nucleic acid (DNA) isolation, from the study participants which would include probands and their parents and siblings. Genomic

		DNA will be isolated from peripheral lymphocytes using conventional Phenol Chloroform method. The strategy for identifying genetic risk for EE will be as follows: **Resequencing of Critical candidate genes:** The most commonly screened genes and their mutations for a given EE phenotype will be screened as per the **table 1** shown below. This has been established as per the SCTIMST experience gained so far and also after extracting information from published literature and thereby limiting only to one or two genes. However, the most common gene will be used for complete resequencing in all available trios and probands and all proband negative for this common gene will be screened for whole exome**(Figure 1).**Complete resequencing of the critical candidate genes shown in the table will be carried out for the trios using Sanger based sequencing in ABI3130XL. **Exome sequencing** Candidate gene negative subjects will undergo WES. Identified novel variants will also be evaluated in comparison to the candidate gene positive EE subtype. Genomic DNA extracted from peripheral blood cells which will be quantified using TaqMan™ RNase P Detection Reagents Kit. From this 50 ng of DNA will be used to amplify using 294,000 primer pairs across 12 primer pools with 10 cycle of amplification, to collect the protein coding regions of human genome DNA using Ion AmpliSeq™ Exome RDY Kit and the Ion AmpliSeq™ HiFi Mix. The amplicons will be treated with FuPa Reagent to partially digest the primers and phosphorylate the amplicons for ligating the adapters.The generated libraries will be normalized using the Ion Library Equalizer™ Kit. The exome libraries thus

		generated will be run on a single Ion PI™ Chip which will be determined to generate ~ 90 million with average read depth ~ 500×, 99% alignment to reference sequence. The AmpliSeq™ Exome RDY Kit has the capability of identifying novel copy number variants, single-nucleotide polymorphisms (SNPs) and small insertion/deletions (indels) in a single kit. The exon-enriched DNA libraries will be sequenced in Ion Proton™ System and read using its Ion Reporter™ Software. Since our study is trio-based therefore, Ion AmpliSeq™ exome trio workflow in the Ion Reporter™ Software will be used which uses WES data from the trio to characterize variant alleles in the proband that may be responsible for the phenotype. The Ion Proton™ System will be used as a sequencing platform due to its availability in RGCB. Sequences will be aligned against the reference genome (GRCh37/hg19) using TMAP Alignment and various file formats will be generated using the BAM, BAI, FASTQ, VCF and TSV files that contain a comprehensive list of all of the variants identified via the Ion Reporter software. **Variant Filteration and Variant prioritization (SOP figure to be attached of analysis plan and also functional structural analysis))**: The raw FastQ datawas processed as per the recommendations of Genome Analysis Toolkit (GATK) "Best Practices for Germline SNP &Indel Discovery". Basic QC checking of the raw data obtained was performed using FastQC tool and Adapter and low quality sequences (phred score<15) will be removed using cutadapt. The QC passed sequences will be aligned to the reference human genome (hg19) using BWA-MEM algorithm. Aligned data in SAM format will be sorted, converted in to BAM file and PCR duplicates will be removed

		using Picard Tools (broadinstitute.github.io/picard/). Subsequently, realignment around indels and base recalibration will be performed using GATK and cleaned BAM file will be generated. Alignment QC and target region coverage in both depth and breadth will be calculated from the BAM file generated from the preceding step using Qualimap. Variants in VCF format will be created from the cleaned BAM file using samtools itself, and also GATK and Dindel. GATK and samtools both call SNPs and indels, while Dindel only identifies indels. The variations will be annotated using ANNOVAR and GEMINI and will be filtered as follows: i.) Variations in the coding regions will be selected as they would contribute to alterations in the functionality of the protein formed. ii.) Classification of sequence variants will be defined as per the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines. As per the ACMG/AMP guidelines the classification of variants relies on a weighted, evidence-based process that first evaluates all the available information for each variant identified in a patient extracted from public databases such as 1000 genomes, Exome variant server, ExAC, and GnomAD, including sub-populations and then "buckets" the variants into categories that appear as a report. The guidelines detail four levels of evidence (very strong, strong, moderate and supporting), which apply to two broad categories (pathogenic/likely pathogenic and benign/ likely benign). iii.) Each of these levels of evidence further breaks down into combinations which provide overall weight for pathogenicity. Variations predicted to be functionally deleterious will be selected. The functional predictions will be done using scores of algorithms in ljb26 databases (prediction on the basis of evolutionary conservation and protein structure).

		The algorithms include SIFT, PolyPhen2 HDIV/ HVAR, LRT, Mutation Taster, Mutation Assessor and FATHMM scores. Each trio will be individually assessed. The depth of the coverage of each of these variants will be assessed. These variants will further be prioritized on the basis of the presence of variations in the segregating (linkage) regions, differential expression of the genes harboring variations in publically available expression datasets relevant to epilepsy per se, their presence in the known pathways or association with known genes and various scores associated with variations. In silico analysis of these variants will be checked if the variants are predicted to be damaging using SIFT, Polyphen2_HDIV, Polyphen2_HVAR, LRT, MutationTaster, LR, FATHOM, Mutation Assessor, MetaLR, PROVEAN, MetaSVM, RadialSVM, Variant Effect Scoring Tool3 (VEST3) and Combined Annotation Dependent Depletion (CADD) score. Evolutionary conservation score of the variant positions will be calculated using phastCons7way_vertebrate, GERP++_RS, SiPhy_29way_logOdds and GERP++_NR and gene-based pathogenicity estimation will be calculated using Residual Variation Intolerance Score (RVIS). **New risk variants** New risk variants will be identified by filtering the raw variant using two criteria: (1) The variant must be a missense variant (2) The variant should not be present in known public databases such as 1000 genomes, ExAC, Exome variant server and GnomAD, including sub-populations.

		For all analyses, the hg19 genome will be used as a reference and ACMG criteria will be applied for variant stratification. **De Novo mutations**: The identification of de novo mutations will be done on comparisons between the WES data of probands and parents. *De novo* and rare inherited mutations (including SNVs and small indels) in each trio will be detected by the mirTrios program based on the VCF files produced by GATK. Alternatively, for De novo variants we will also use DeNovoGear, an algorithm frequently used by EuroEPINOMICS trios. It takes the advantage of Bayesian analysis. After alignments are organized for each individual using BWA, potentially significant de novo variants will be highlighted with the GATK Unified Genotyper for all family members in a trio. Gene loci bearing potentially pathogenic de novo mutations should meet the following criteria:[9] (1) the read depth in both parents should be ≥ to 10; (2) the depth of coverage in the child should be at least 10% of the sum of the coverage in both parents; (3) for de novo variants, less than 5% of the reads in either parent should carry the alternate allele; (4) at least 25% of the reads in the child should carry the alternate allele; (5) the normalized, phred-scaled likelihood (PL) scores for the offspring genotypes AA, AB and BB, where A is the reference allele and B is the alternate allele, should be >20, 0 and >0, respectively; (6) the PL scores for both parents should be 0, >20 and >20; (7) at least three variant alleles must be observed in the proband; and (8) the de novo variant has to be located in a CCDS exon targeted by the exome enrichment kit. De novo mutations will be screened based on following five classes. The Class 1 mutations will be de novo mutations in known and established epilepsy genes including SCN1A, SCN2A, CDKL5 or STXBP1. Sufficient evidence exists where

		in these mutations are involved in pathogenesis of the epilepsy phenotype. Also mutations in novel genes where recurrent de novo variants have been to identical phenotypes in literature should be Class 1 mutations. Class 2, are mutations in genes that have been identified in other neurodevelopmental disorders such as intellectual disability or autism. However, these genes will be approached with a certain degree of caution similar to the de novo copy number variants which are known to have a broad phenotypic range (eg.CACNA1H identified by us in our DS series). Class 3 mutations are de novo mutations in "potential" genes including previously undescribed ion channel genes expressed in the CNS. Everything else in the probandsare defined as Class 4 and of uncertain significance. Class 5 is constitute by non-pathogenic variants, that are presented as de novo mutations in genes that were previously identified in control populations. All Variant(s) thus identified will be confirmed by Sanger sequencing. **Pathway and Network Analyses**. The candidate genes segregating with the disease and having rare deleterious variations will be analyzed for the enrichment in the biological pathways using Enrichr and ClueGo. Kyoto encyclopedia of genes and genomes (KEGG), GeneOntology (GO), Wikipathways enrichment analysis will be performed using ClueGO and CluePediawih applications of Cytoscape. In ClueGo settings; the GO biological processes, GO cellular components, KEGG, REACTOME and WikiPathways will be selected to identify the pathways enriched by the candidate genes in the families. The p-value will be set to 0.05. Interaction map of candidate genes will be plotted. Protein interaction relationship data will be obtained from the BioGrid (V33)

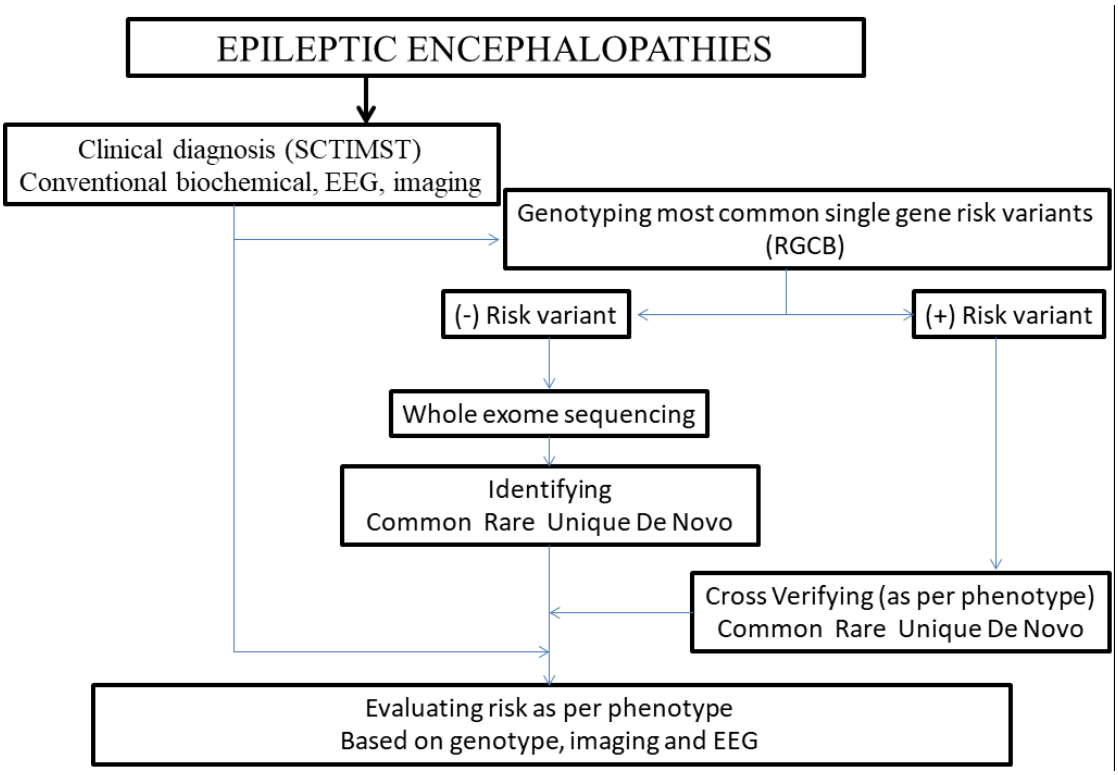
		(thebiogrid.org/) and STRING (V34) (string-db.org/) databases. Protein–protein interactions (PPIs) and gene coexpression networks for the HM-relevant genes will be constructed using the Cytoscape software tool (V3.1 36) (cytoscape.org/).
8.	**Interim modification of objectives/methodology (with justifications)**	NIL

Table 1: Critical candidate genes listed based on literature and SCTIMST experience

(EIEE- Early-onset infantile epileptic encephalopathy; IGE- Idiopathic generalized epilepsy; LGS- Lennox Gastatut syndrome; EE- Epileptic Encephalopathy)

Epileptic encephalopathy phenotypes	**Prospective sample size**	**Resequencing of Candidate genes**	**Exome sequencing**
Dravet syndrome/Dravet borderline phenotype	50	SCN1A, SCN2A, PCDH19 in females	SCN1A (-), SCN2 A (-), PCDH 19 (-)
Ohtahara syndrome/EIEE with suppression burst	25	STXBP1 in males, CDKL5 in females, SCN2A	STXBP1(-) in males, CDKL5 (-)in females, SCN2A (-)
West syndrome/LGS/MarkandBlume-Ohtahara syndrome	50	STXBP1 in males, CDKL5 in females if phenotype typical	STXBP1(+/-) in males, CDKL5(+/-) in females
Myoclonic atonic epilepsy and refractory IGE of childhood	25	SCN1A, SCN2A, SLC2A1	SCN1A,(-) SCN2A,(-), SLC2A1 (-)
Migrating partial epilepsy of infancy	15	KCNT1, SCN 2A, SCN1A	KCNT1, SCN2A, SCN1A (-)
Neonatal onset EIEE with refractory focal epilepsy	15	KCNQ2	KCNQ2(-)
EE with Continuous spike and wave discharges in sleep & Landau Kleffner Syndrome	20	GRIN2A	GRIN2A (-)

Figure 1



9. Summary on progress (during the period of report)

SRF recruited in the project has been undergoing PhD program at SCTIMST and has completed five seminars on 'Basics of Molecular Genetics', 'Genetics of Epilepsy', 'Next Generation Sequencing techniques', 'ACMG classification criteria and databases' and 'Whole exome sequencing analysis' and course work modules of 8 credits as part of the PhD curriculum and submitted semi annual report during the period January 2022 to August 2022, August 2022 to December 2022, and January 2023 to July 2023 and has passed the Comprehensive Written and Oral exam conducted on 28th September 2023.

RA and SRF had presented case discussions of 64 probands in the third year of the study. Subsequently, in the six months extension period, they had presented the WES analysis of remaining 20 cases. The discussions were held as per the schedule below:

Presenter	Topic	Date
RA & SRF	Whole exome sequencing analysis: Case presentation	08/02/2023
RA & SRF	Whole exome sequencing analysis: Case presentation	15/02/2023
RA & SRF	Whole exome sequencing analysis: Case presentation	22/02/2023
RA & SRF	Whole exome sequencing analysis: Case presentation	01/03/23
RA & SRF	Whole exome sequencing analysis: Case presentation	15/03/23
RA & SRF	Whole exome sequencing analysis: Case presentation	22/03/2023
RA & SRF	Whole exome sequencing analysis: Case presentation	29/03/23
RA & SRF	Whole exome sequencing analysis: Case presentation	19/04/23
RA & SRF	Whole exome sequencing analysis: Case presentation	26/04/23
RA & SRF	Whole exome sequencing analysis: Case presentation	03/05/23
RA & SRF	Whole exome sequencing analysis: Case presentation	10/05/23
RA & SRF	Whole exome sequencing analysis: Case presentation	17/05/23
RA & SRF	Whole exome sequencing analysis: Case presentation	24/05/23
RA & SRF	Whole exome sequencing analysis: Case presentation	31/05/23
RA & SRF	Whole exome sequencing analysis: Case presentation	07/06/23

RA & SRF	Whole exome sequencing analysis: Case presentation	17/06/23
RA & SRF	Whole exome sequencing analysis: Case presentation	21/06/23
RA & SRF	Whole exome sequencing analysis: Case presentation	28/06/23
RA & SRF	Whole exome sequencing analysis: Case presentation	05/07/23
RA & SRF	Whole exome sequencing analysis: Case presentation	12/07/23
RA & SRF	Whole exome sequencing analysis: Case presentation	15/07/23

An Institutional Ethics Committee clearance was obtained prior to the start of the project. (IEC/1473/2019)

The RA has attended the Epilepsy OPD clinics (SCTIMST) thrice a week and carried out daily visits to the Epilepsy wards (RMNCEC, SCTIMST) to recruit patients according to the sample inclusion criteria. Pre-test genetic counseling is offered to all patients, wherein details regarding the purpose and nature of the tests are explained. A detailed three-generation pedigree chart is maintained after interviewing family members of each patient. A signed informed consent form is obtained and documented for each patient after detailed pre-test counselling carried out by the PI. Photographs of children with facial dysmorphisms are taken and maintained as records. We have collected 5ml blood samples from a total of 175 patients and their parents (trios). The first 91 patients' samples were collected in the first year, 66 patients' samples were collected in the second year and 18 patients' samples in the third year. The characteristics of the patients' whose samples have been collected are described in Table 2 below.

Table 2- Diagnoses of patient cohort

Diagnosis	No.
DEE unclassified	63
Focal epilepsy	41
West Syndrome	22
Dravet Syndrome	24
Lennox Gastaut Syndrome	12
Generalised epilepsy	8
Landau-Kleffner Syndrome	5

The samples are transported to RGCB for DNA isolation, resequencing of candidate genes and whole exome sequencing which will be carried out by SRF.

Isolation of genomic DNA from Whole Blood

Peripheral blood (5 ml) samples of probands and their parents were collected in EDTA vials (total number of samples collected- 434). The genomic DNA was isolated from lymphocytes obtained from anticoagulated blood. Modified salting out method was used for DNA extraction (Miller et al., 1988). Ten times volume 1X RBC Lysis buffer [155mM NH_4Cl, 12mM $NaHCO_3$, 0.1mM EDTA, pH-7.3] was added to 4 ml blood sample and kept for 10 minutes rotation at room temperature. Afterwards, it was centrifuged at 10,000 rpm for 10 minutes and the supernatant containing red blood cells was discarded. The WBC pellet was resuspended in equal volume of Tris-EDTA buffer (1M Tris, 0.5M EDTA, pH-8.0), to which 32 µl proteinase K (20mg/ml) and 100 µl of SDS (20 %) were added. This solution was then incubated at 55°C for 60 minutes followed by 15 minutes incubation at 65°C. During this step, the SDS ruptures the WBCs and the proteins in the cells is digested by the action of proteinase K.

1.52 ml of 4M NaCl was then added followed by equal volume of Chloroform: Isoamyl alcohol (24:1). The samples were rotated for 10 minutes and then centrifuged at 6000 rpm for 15 minutes at room temperature. The aqueous layer alone was collected and double the volume of absolute alcohol was added and mixed gently. The lump of DNA was collected into 1.5 ml fresh centrifuge

tube. The DNA was washed twice in 70% alcohol. The pellet was then dried and dissolved in TE buffer and stored at -20°C. DNA isolation of 414 samples including 155 probands was completed. The quantity and quality of DNA was analyzed using Nanodrop. The ratio of absorbance at 260 nm and 280 nm was used to estimate the purity of the DNA. A ratio between 1.7-1.9 was considered as good quality DNA without protein contamination. The concentration of 1µl DNA was quantified and samples were further diluted (1µg/10µl) for exome sequencing.

Whole exome sequencing (WES) was performed using Exome Agilent SureSelect all exon V6 method. The data were analysed using various Bioinformatics tools and customised in house pipelines and annotated with publicly available databases to identify the risk variants associated with epilepsy and DEE. BWA (Burrows-Wheeler Aligner) was used to align raw fastq data to the human reference genome (hg19/GRCh37). The SNPs and INDELs were identified following the best practise standards provided by the Genome Analysis Toolkit (GATK). ANNOVAR was used to annotate the variants detected. The variants which passed the depth and quality filter were classified based on inheritance pattern including homozygous recessive, Autosomal dominant, Compound heterozygous, X-linked and De novo variants. In the primary analysis, variant reporting was restricted to 2812 genes associated with Epilepsy, intellectual disability, developmental delay and autism spectrum disorder. The in silico prediction tools CADD, SIFT, PolyPhen2, MutationTaster, and REVEL score were used to analyse the possible harmful impacts of the variations. Variants having a CADD score of less than 20 and an ExAC allele frequency of greater than 1% were eliminated. The variants were ranked based on the phenotypes of the proband by manually reviewing the evidence from several databases such as ClinVar, OMIM, HPO, and literature searches. The variants were classified based on American College of Medical Genetics and Genomics (ACMG) classification criteria. Sanger sequencing validation of identified pathogenic variants was done.

Results

A total of 489 blood samples were collected which included 175 probands and 314 parents. Due to lack parental consent issues for 3 probands, no parental samples were obtained. For 13 probands, consent was obtained but only a single parent sample was obtained. Whole exome sequencing analysis of 480 samples including 175 probands and 150 trios has been completed and genetic test reports have been issued to these patients. The diagnostic yield (75/175=**42.8%)** was investigated based on the number pathogenic or likely (P/LP) pathogenic variants identified. Sanger sequencing validation of P/LP variants were conducted. No potential disease causing variants were identified

in 28.5% (50/175) of the patients and variants of uncertain significance (n=114) were identified in the remaining 28.5% of the patients. The most common type of disease-causing variant identified was missense variants (76%), and the remaining included frameshift variants (10%), non-sense variants (9%), splice site variants (3%) and CNVs (2%) (**Figure 2a**). 44% of the disease causing variants were identified in ion channel genes and most of them were *SCN1A* variants associated with Dravet syndrome (**Figure 2b**). In neurometabolic genes, 20% of pathogenic/likely pathogenic variants were identified and the remaining variants (36%) were identified in genes *DCX, GNAO1, TUBA3E, NOVA2, TSC2, TUBB3, CDKL5, PCDH19, TSC1, AKT3, C12ORF57, SZT2, NR2F1, UFSP2, GNAO1, and KMT2C.* The protein network analysis of pathogenic or likely pathogenic variants identified from study showed that most of the disease causing variants associated with developmental and epileptic encephalopathy are enriched in ion channel genes (**Figure 4**).

60% of the P/LP variants were de novo variants, and 12% of the variants followed a homozygous recessive pattern of inheritance (**Figure 3**). The diagnostic yield of Dravet syndrome and West syndrome was more than 50% (**Table 4**). Patients with age of onset of epilepsy less than 1 year achieved a diagnostic yield of 52.3% (**Table 5**).

Sample Size

Total number of samples collected (N=489)	489
Total number of probands	175
Total number of trios	141 single child affected 9 siblings affected
Parent sample was not collected for	3
Single parent sample was collected for	13
Whole exome sequencing done	480 samples

Table 3- Pathogenic or Likely pathogenic variants identified

De novo	*SCN1A (n=12), KCNT1 (n=4), CHRNA2 (n=2), SCN2A (n=2), GNAO1 (n=2), CHRNA4, SLC2A1, DCX, NDUFS2, TUBA3E, TSC2, NOVA2, CDKL5, GABRB2, IDH2, TUBB3, SETD1B, , PCDH19, IDH2, SCN8A, TSC1, SETD1A, SCN3A, STXBP1, NR2F1, WDR45, KMT2C, GABRA1, GABRG2, CHD8, CDK8*
Compound heterozygous	*C12ORF57, ALG3, SZT2*
Autosomal Dominant	*SCN1A(n=3), SCN8A, KCNT1*
Homozygous recessive	*ARG1, CLN5, NHLRC1 (n=2), BTD, ITPA, ALDH18A1, UFSP2 (n=2)*
X-linked	*DCX, USP9X, POLA1*
Likely Pathogenic/Pathogenic	75
Variant of Uncertain Significance	113

Table 4: P/LP variants and VUS identified in DEE using WES

DEE classification	Yield (P/LP)	VUS
West syndrome (*n*=22)	50%(11/22)	27.7% (6/22)
Dravet Syndrome (*n*=24)	54.16%(13/24)	29.16%(7/24)
Lennox-Gastaut syndrome (LGS) (*n*=12)	16.6%(2/12)	41.6%(5/12)
Landau-Kleffner syndrome (LKS) (*n*=5)	No P/LP variants	5/5
Focal Epilepsy (*n*=41)	26.8%(11/41)	41.46%(17/41)
Generalized Epilepsy(*n*=8)	25%(2/8)	37.5%(3/8)
DEE Unclassified (*n*=63)	53.9%(34/63)	15.8%(10/63)

Table 5: Yield of WES based of age of onset of epilepsy

Age of onset of epilepsy	Yield (P/LP variants identified)
Patients with age of onset of epilepsy < 6 months (n=44)	54.5% (24/44)
Patients with age of onset of epilepsy between 6 to 12 months (n=40)	52.5% (21/40)
Patients with age at onset <12 months (N=84)	52.3% (45/84)
Patients with age of onset of epilepsy between 12 to 36 months (n=50)	40% (20/50)
Patients with age of onset < 3years (N=134)	48.5% (65/134)
Patients with age of onset of epilepsy > 3 years(n=41)	24.3% (10/41)

Figure 1: Variant distribution of whole exome sequencing

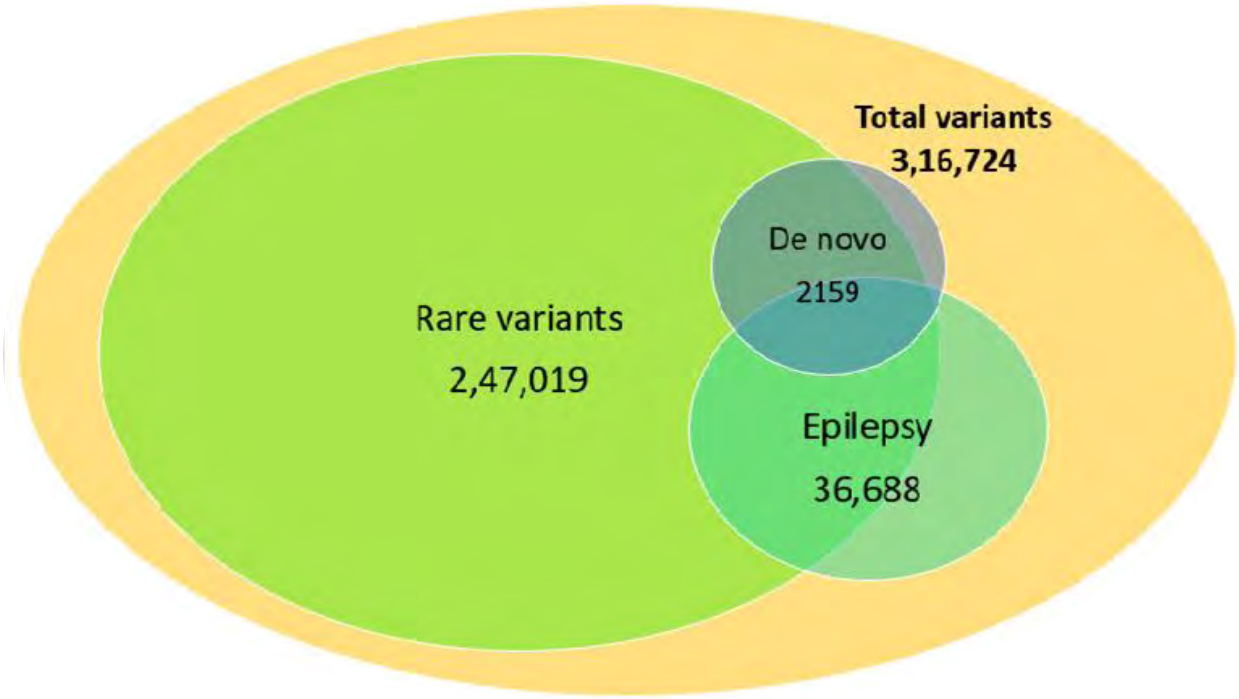
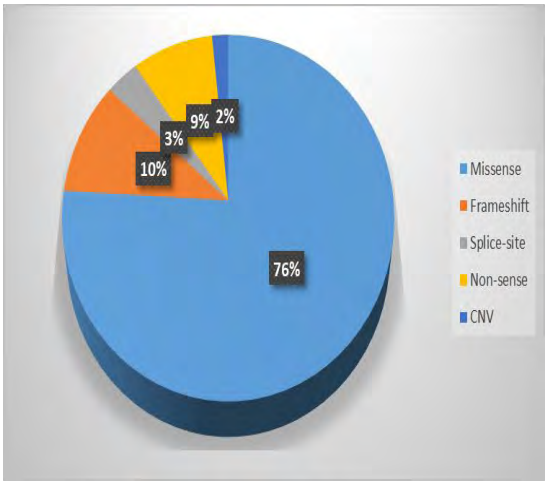


Figure 2:

a) Type of disease causing variants



b) Type of monogenic causes

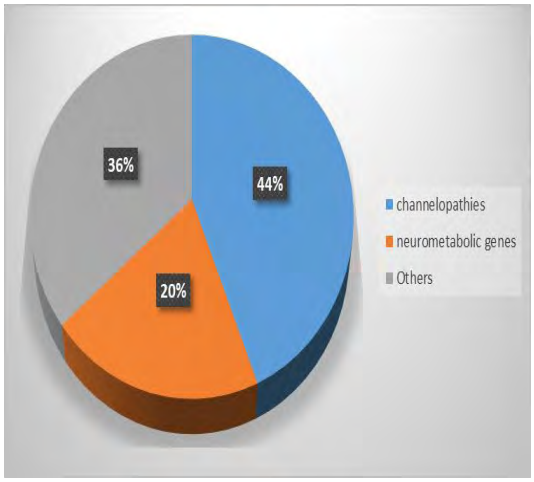


Figure 4: Protein network analysis of pathogenic or likely pathogenic variants identified from the study

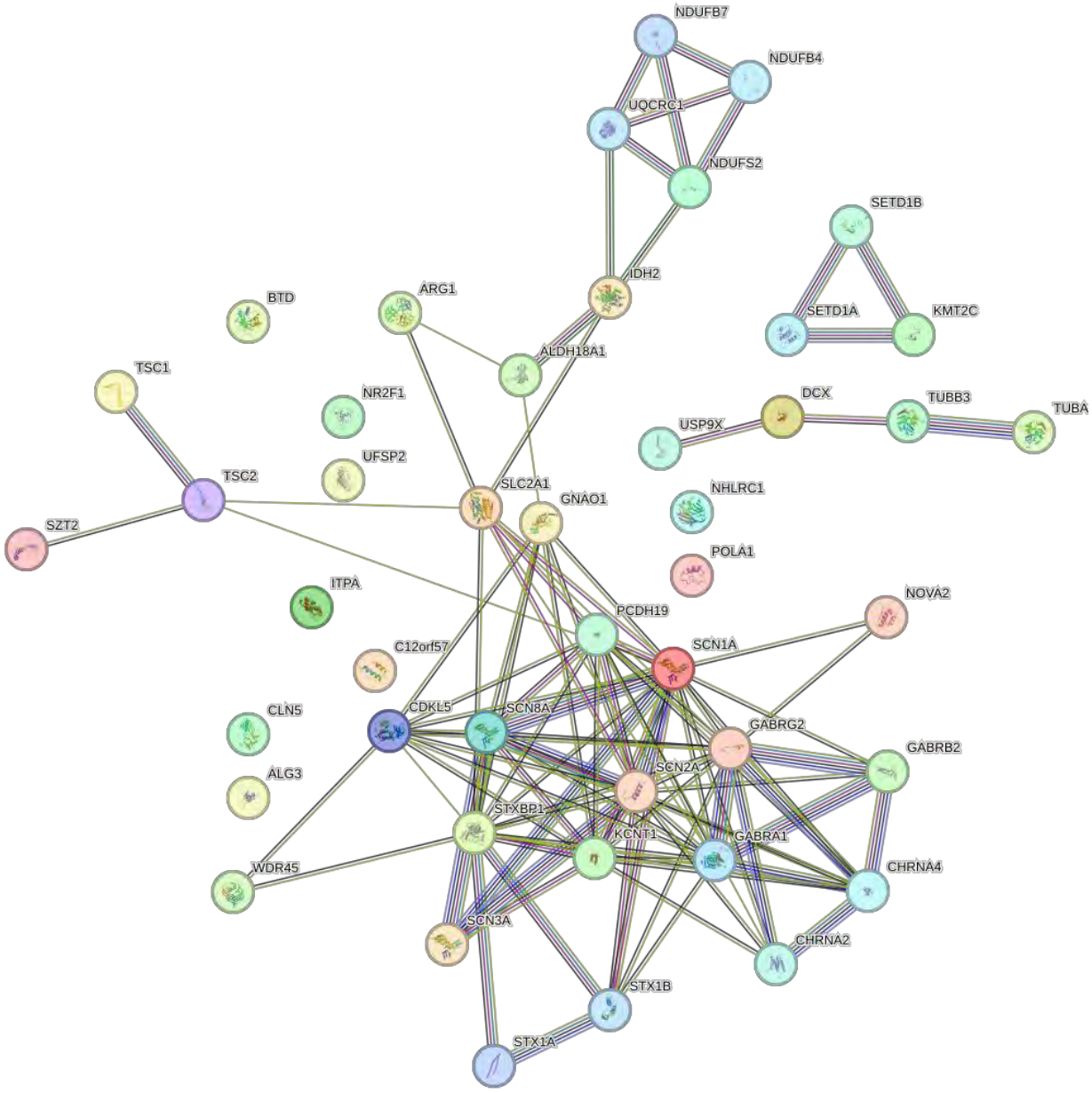


Figure 5: Inheritance pattern of disease causing variants identified

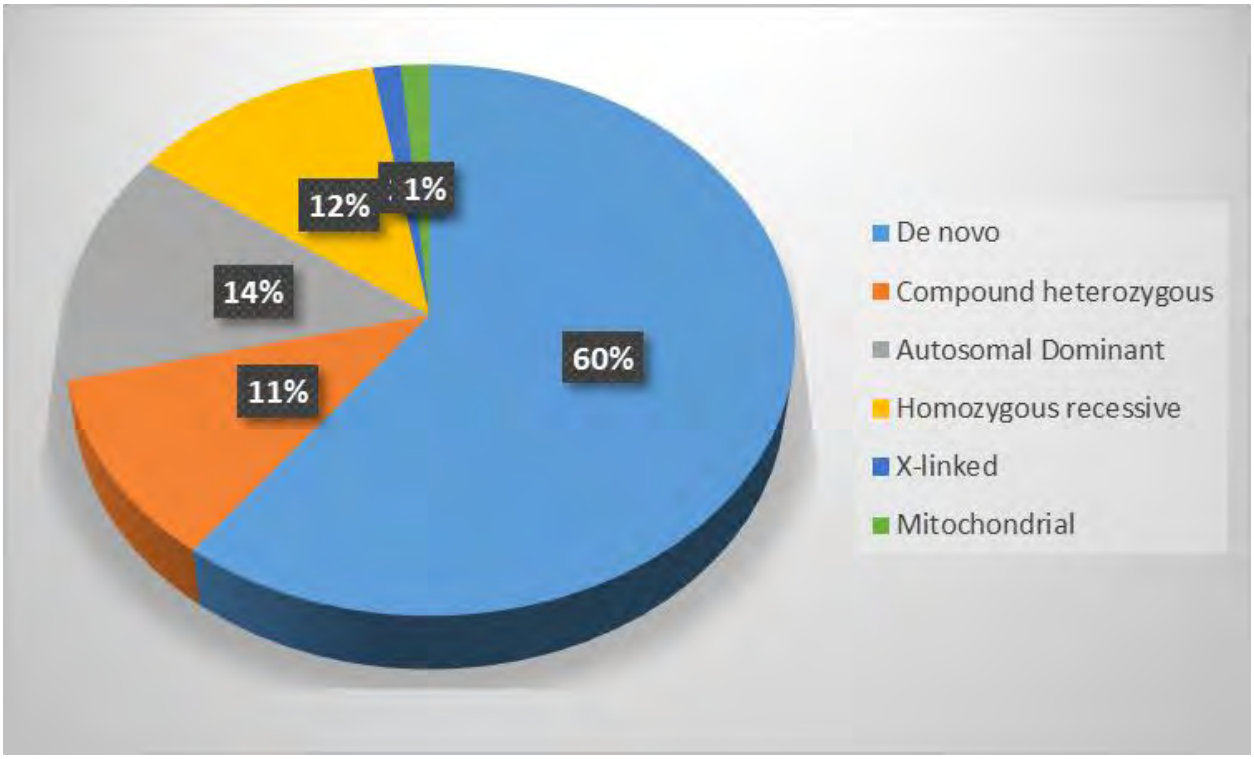


Table 5: List of Novel P/LP variants identified in our cohort

Sl.No	Gene	Variant	ACMG Classification	Inheritance	SIFT_prediction	PolyPhen_Prediction	CADD score
1	TUBA3E	c.G392A	Pathogenic	De novo	D	D	25.4
2	NOVA2	c.G134C; p.G45A	Pathogenic	De novo	D	D	25.7
3	TUBB3	c.T2G;p.M1?	Pathogenic	De novo	D	D	29.1

4	GABRG2	c.C917T;p.S306F	Pathogenic	De novo	D	D	33
5	CDKL5	c.G517C;p.A173P	Pathogenic	De novo	D	D	28.8
6	GABRB2	c.T949G;p.F317V	Pathogenic	De novo	D	D	26.7
7	STXBP1	c.A434C:p.Y145S	Pathogenic	De novo	D	D	25.7
8	ITPA	c.G511T:p.E171X;	Pathogenic	Homozygous recessive	D	-	39
9	NHLRC1	c.C138G:p.C46W	Pathogenic	Homozygous recessive	D	D	25.3
10	SCN1A	c.1724delT:p.F575Sfs*48	Pathogenic	De novo	-	-	32
11	SCN1A	**c.5339T>G | p.Met1780Arg**	Pathogenic	De novo	D	D	28
12	SCN2A	**c.T585A:p.D195E**	Pathogenic	De novo	D	D	24.2

Table 6 – List of promising VUS (VUS-D) identified

Sl No	Gene	Variant	Inheritance	Classification
1	SCN3A	c.A2410G:p.T804A	Autosomal dominat	VUS-D
2	GSTT2B, DDTL	Chromosome 22q11.23 duplication	De novo	VUS-D
3	BSN	c.A10658G:p.Y3553C; c.G9563T:p.G31	Compound heterozygous	VUS-D

		88V		
4	GABBR2	c.A2503C:p.N835H	De novo	VUS-D
5	TCF20	c.C2483T:p.A828V	Heterozygous	VUS-D
6	RNF112	c.G59T:p.R20I	De novo	VUS-D
7	SCN3A	c.A2263G:p.T755A	De novo	VUS-D
8	CAMK2A	c.1463-2A>T	De novo	VUS-D
9	PDK2	c.C512T:p.P171L	Homozygous Recessive	VUS-D
10	SHQ1	c.T850C:p.Y284H	Homozygous Recessive	VUS-D

Table 7- List of Copy number variants (CNVs) identified

Sl.No.	CNV	Inheritance	ACMG classification
1.	Chr 22q13.1 duplication	De novo	VUS
2.	Chr 15q duplication	De novo	VUS
3.	Chr 22q duplication	De novo	VUS
4.	Chr 2q duplication	De novo	VUS
5.	Chr 4p deletion	De novo	VUS
6.	Chr 2q11.2 duplication	De novo	VUS
7.	Chr 2p duplication	De novo	VUS
8.	Chr 17q duplication	De novo	VUS
9.	Chr 13q14.3 deletion	-	VUS
10.	Chromosome 13q32.3 deletion	De novo	Likely pathogenic

Case study of a novel homozygous recessive variant identified in ALDH18A1 gene associated with De Barsy Syndrome

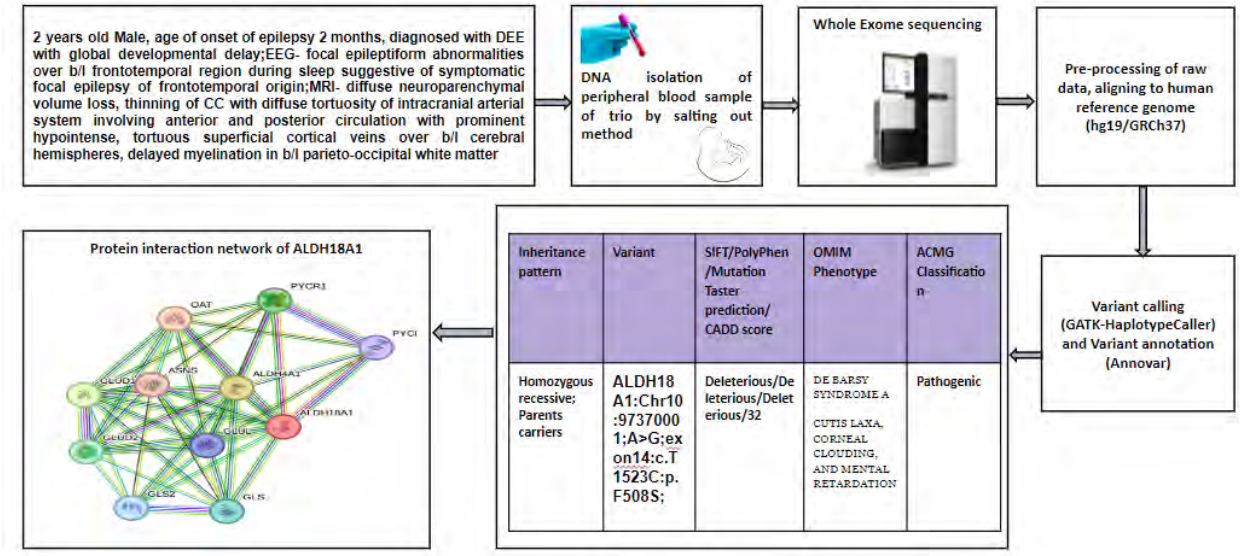
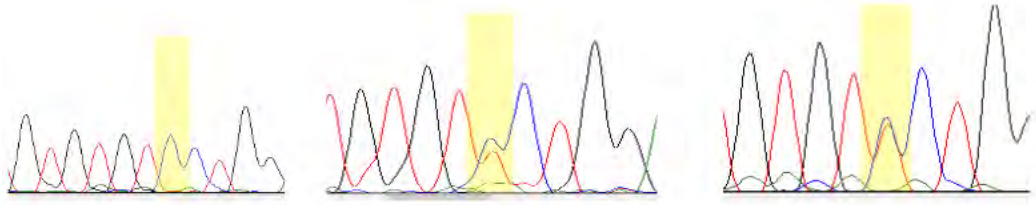


Figure 6: Sanger sequencing image of c.T1523C:p.F508S variant identified in ALDH18A1 gene



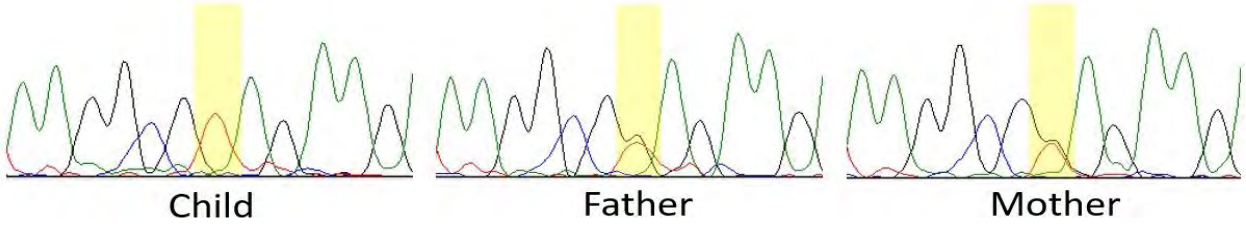
Case study of an infant with novel variant in *ITPA* gene

1.5 year old male, born of 3rd degree consanguineous parentage. Diagnosed with focal epilepsy left temporal with global developmental delay (GDD) and movement disorder. Expired in early infancy due to aspiration pneumonia. His biochemical investigations which included urine organic acid estimation, tandem mass spectroscopy analysis of amino acids and biotinidase and lactate levels were normal. He had focal seizures since 4 months of age, associated with fever. MRI: prominent subdural spaces with mild diffuse volume loss. Video EEG: showed temporal slowing (left>right) with left temporal spike and wave discharges (mid and posterior) and left posterior head region.

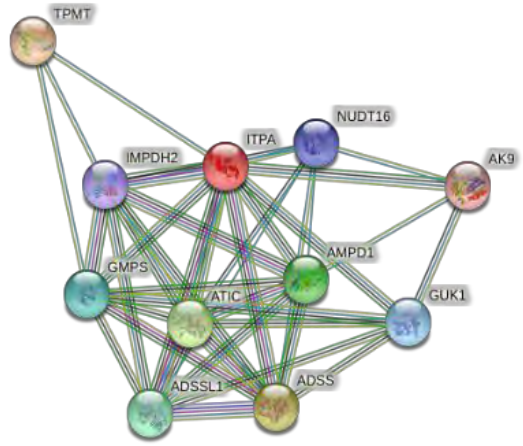
Variant/ACMG Classification	**ITPA: Chr20:3204034:c.G511T:p.E171X; stop gain/Pathogenic**
Inheritance	**Homozygous recessive (Parents are carriers)**

Figure 7

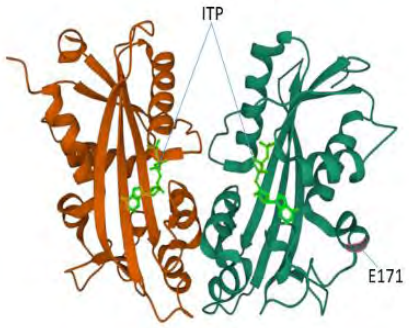
a) Sanger sequencing result of novel variant identified in ITPA gene



b) Protein interaction network of ITPA



c) PDB structure of ITPA



Case study of a child with a pathogenic variant in *ARG1* gene

Proband: 5 year 11 month old female born of third degree consanguineous parentage, presented with seizures since 2.5 years age followed by regression of milestones since 4 years

Seizure semiology: Brief spasms in sleep with no atonic drops, has these spells on slowly awakening from sleep

VEEG: LGS with diffuse background slowing maximal over the frontal regions, multifocal and generalized spikes along with sleep activation of generalized slow spike wave discharges and poorly formed sleep architecture

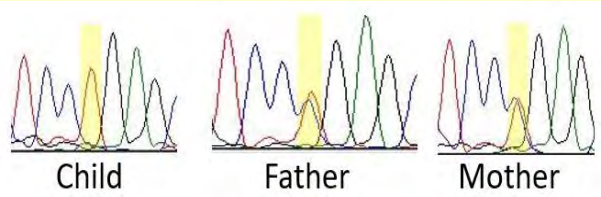
MRI: Bilateral diffuse and periventricular hyperintensities with arterial spin labeling hypoperfusion over bilateral parietooccipital regions

Developmental assessment: Global developmental delay

Plasma arginase level: Low (21.5nmol/l)

WES analysis in trio: Homozygous recessive variant in ARG1 gene (Chr6:131905002;c.G923A;p.R308Q). Parents are carriers of the mutant allele.

Sanger sequencing Image:



In silico structural and functional validation of a novel variant of uncertain significance identified in STXBP1 gene

A 6.5 year old female, diagnosed with myoclonic seizures, Rett like disorder with myoclonus ataxia. Video EEG:multifocal and generalised spikes and polyspikes. She had regression of motor and language milestones after events. Methylation profile of MeCP2 was negative. Her clinical exome sequencing identified a VUS in STXBP1 gene. After WES of trio, a likely pathogenic variant in STXBP1 gene was identified and further in silico functional/structural prediction analysis, the variant is re-classified as pathogenic.

Inheritance pattern	Variant	SIFT Prediction/MutationTaster prediction. CADD score	Protein structure prediction (Missense3D)	OMIM Phenotype	ACMG Classification
De novo	STXBP1: Chr9:130425488 exon7:c.A434C:p.Y145S	Deleterious/Deleterious/25.7	Structural Damage detected	Developmental and epileptic encephalopathy-4	VUS Re-classified into a Pathogenic variant after parental segregation and *in silico* structural/functional analysis

To assess the pathogenicity of VUSs on protein function and structure stability, in silico analysis tools were used based on protein stability, protein interaction pattern and protein 3D structural analysis (**Figure 8**). To identify the interactions of protein with other relevant proteins, STRING (Search Tool for the Retrieval of Interacting Genes) (https://string-db.org/) was used. (**Figure 9**)

Protein stability is a primary factor affecting the function and activity of biological molecules. Protein unfolding free energy is dependent on protein stability. So, it is important to precisely quantify the influence of mutation on protein stability by analysing the effect of mutation on free energy. IMutant (http://gpcr2.biocomp.unibo.it/cgi/predi_ctors/I-Mutant3.0/I-Mutant3.0.cgi), an online support vector machine based tools to predict protein stability changes based on single site mutations, was used to predict the stability of protein.

A mutation in a specific site, such as a conserved residue, can cause a change in the protein's structure and function, so, it is crucial to figure out how mutations affect protein structure to predict

the pathogenicity of a variant. Missense3D, a web-based tool to predict the structural changes introduced by an amino acid substitution, was used to predict the effect of mutation on protein strutcure. SWISS-MODEL servers were also used for modelling three dimensional protein structures.

Variants of Uncertain significance were grouped into Promising VUS/Incidental VUS according to data- Frequency data of variants, *In silico* functional predictions, *de novo* data, clinical data and protein structure/stability data analysed with computational prediction tools (iMutant, Missense3D, etc). Molecular dynamic simulation was used to re-classify the promising VUS.

Molecular Dynamics simulation

The structure of wild type (**Figure 10b**) and mutant protein (**Figure 10c**) was modeled using SWISSMODEL and quality assessment was done using Ramachandran plot. Molecular dynamics simulations were performed using GROMACS 5.1.2 software. All systems were soaked in a cubic box of water molecules using the gmx editconf module and gmx solvate module for solvation. All systems were equilibrated a constant temperature (300K), by utilizing the NVT and NPT process for 100 ps. The final simulations were performed for 100ns. Radius of gyration (Rg) (**Figure 10e**), Root mean square deviation (RMSD) (**Figure 10f**), and Root mean square fluctuation (RMSF) (**Figure 10g**), were analysed using GROMACS. MDS of wild type STXBP1 and its mutant form revealed that the mutation affect protein stability, residual fluctuation and function.

Figure 8: *In silico* analysis of VUS based on protein stability, protein interaction pattern and protein 3D structural analysis

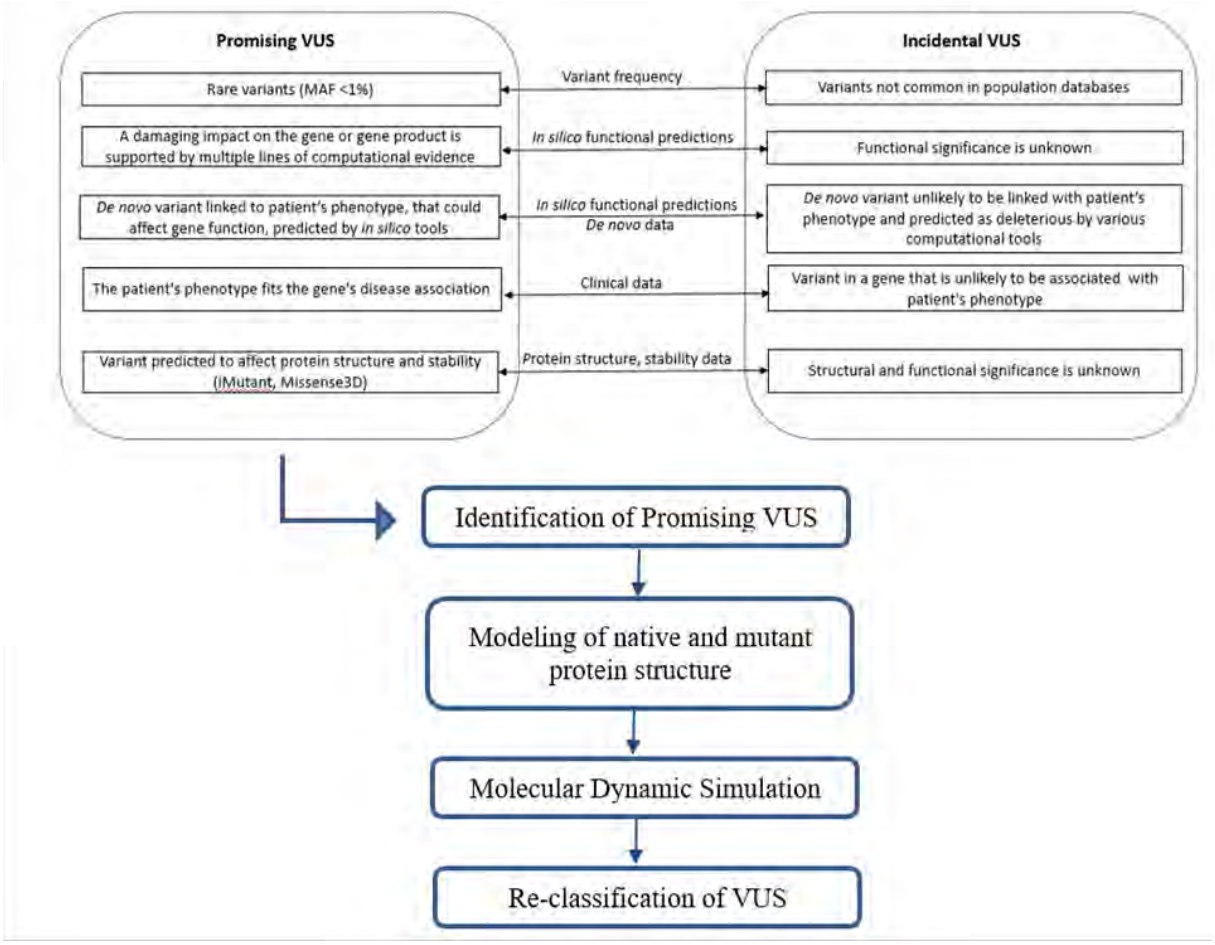


Figure 9: Protein interaction network of STXBP1 gene

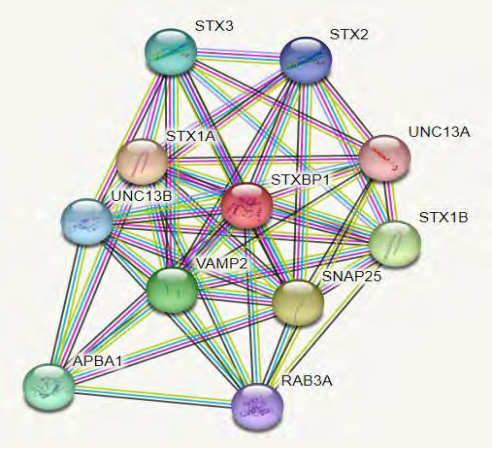
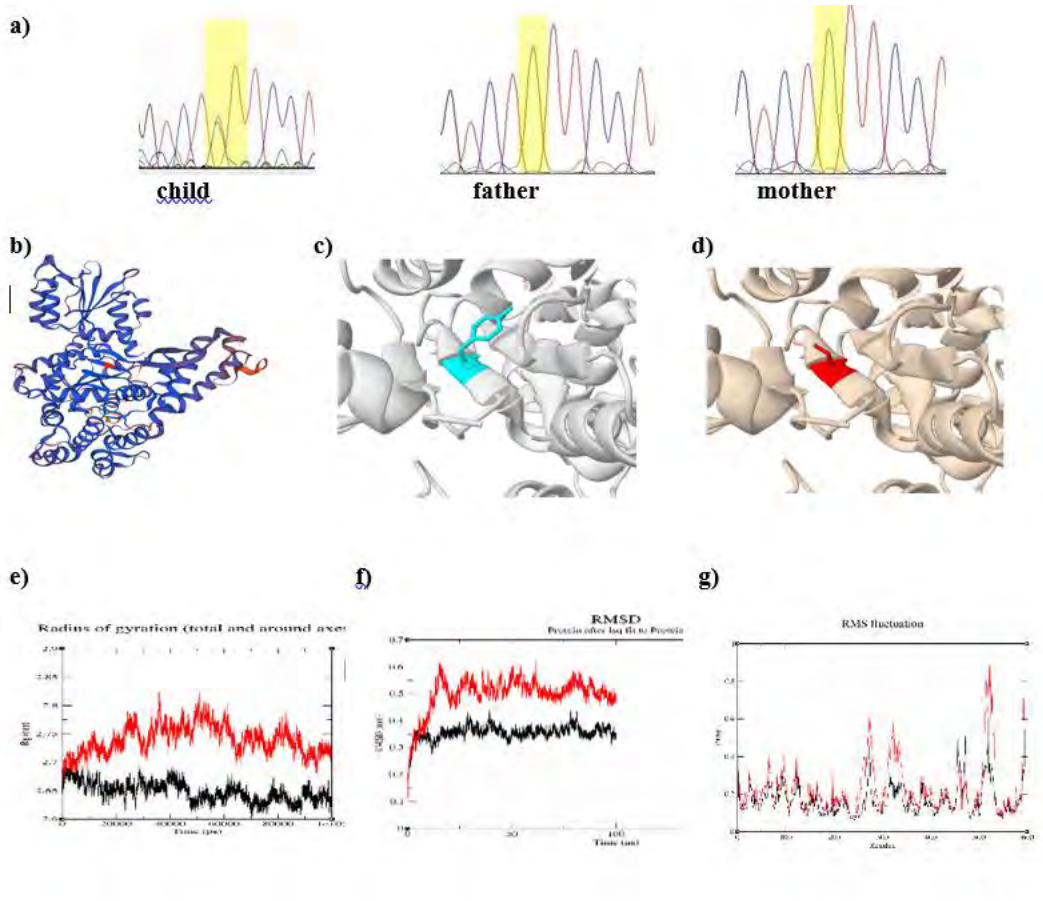


Figure 10: a) Sanger Sequencing image of probands with de novo STXBP1 variant and parents with homozygous wild type alleles. b) structure of mutant protein predicted by SWISSMODEL c) Wild type protein structure d) Mutant protein structure e) MDS analysis- Radius of Gyration f) RMSD g) RMSF



10. **Applied value of the project**

The study is unique in several ways.

1. The novelty in this study is that this is the first comprehensive genetic-electro-clinical-imaging based study on EE in India.
2. The study tries to identify genotype phenotype correlates based on clinico-EEG-imaging based evaluation. The results thus derived will offer a wealth of clinical data that can be utilised to develop treatment protocols for India.

3. It also tries to follow up the patients for their medication profile and subsequent response to medication.
4. The response to medication will further be addressed by their pharmacogenetics and immunogenetic traits.

11. **Research work that remains to be done under the project**

WES analysis has predicted certain variants as VUS, since they have distinct phenotype match. The validation of these VUS variants by in silico functional/structural predictions can help to reclassify them as variants of potential significance. These confirmations can aid in an accurate disease diagnosis and streamline the treatment strategies for the pediatric patient group. Post-test counseling along with genotype phenotype correlations are rendered to the families. Cost effectiveness and post genetic test counseling surveys are also being administered.

12. **Publications**

1. Nandini Mitta, Ramshekhar N Menon, Amy McTague, Ashalatha Radhakrishnan, Soumya Sundaram, Ajith Cherian, G.K. Madhavilatha, Ashraf U Mannan, Sheela Nampoothiri, Sanjeev V. Thomas. Genotype-phenotype correlates of infantile-onset developmental & epileptic encephalopathy syndromes in South India: A single centre experience. Epilepsy Res 2020 Oct;166:106398. (Attached in Appendix 2)

2. Manna Jose, Prashanth Paulose, Soumya Sundaram, Ashalatha Radhakrishnan, Sheela Nampoothiri, Ramshekhar N. Menon. Utility of clinical exome sequencing in progressive myoclonus epilepsy syndromes- An exploratory analysis. Clinical Genetics 2021, Feb;101(2):270-271. (Attached in Appendix 2)

3. Alfiya F.*, Manna Jose*, Soumya V. Chandrasekharan, Soumya Sundaram, Madhusoodanan Urulangodi, Bejoy Thomas, Ashalatha Radhakrishnan, Moinak Banerjee and Ramshekhar N. Menon. C12orf57 pathogenic variants: a unique cause of developmental encephalopathy in a South Indian child. Journal of Genetics (2022) 101:30. (Attached in Appendix 2)

4. Alfiya, F.*, Manna Jose*, Chandrasekharan, S. V, Rudrabhatla, P. V., Sundaram, S., Radhakrishnan, A., Banerjee, M., & Menon, R. N. (2022). Visual sensitive epilepsy in GLUT-1

deficiency syndrome– expanding the phenotype. Epileptic Disorders (2023); 25(2):265-268.(Attached in Appendix 2)

5. Harini Pavuluri*, Manna Jose*, Alfiya F*, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee, Ramshekhar N Menon (2023). Arginase deficiency- an unheralded cause of developmental epileptic encephalopathy. Epileptic Disorders (2023) doi: 10.1002/epd2.20081. (Attached in Appendix 2)

6. Jukkarwala A, **Menon RN**, Sunesh ER, Radhakrishnan A. Electroclinical Phenotype-Genotype Homogeneity in Drug-Resistant "Generalized" Tonic-Clonic Seizures of Early Childhood. *Clin EEG Neurosci*. 2021;52(5):371-375. doi:10.1177/1550059420953735

7. Chandrasekharan SV, **Menon RN**, Nanda S, et al. Does Etiology and Hypsarrhythmia Subtype Influence Outcome in West Syndrome? Challenges Encountered from a Referral Center Perspective. *Neurol India*. 2022;70(1):188-196. doi:10.4103/0028-3886.336325

8. Manna Jose, Alfiya F., Harini Pavuluri, Pavan Kumar Rudrabhatla, Soumya V. Chandrasekharan, Jithu Jose, Moinak Banerjee, Soumya Sundaram, Ashalatha Radhakrishnan, Ramshekhar N. Menon (2023). Metabolic causes of pediatric developmental & epileptic encephalopathies (DEE)- genetic variant analysis in a south Indian cohort. (Under review)

9. Krishna Nikhil*, Manna Jose*, Alfiya Fasaludeen*, Moinak Banerjee, Soumya Sundaram, Ashalatha Radhakrishnan, Ramshekhar Menon. Impact of *SCN1A* variant subtype on electro-clinical phenotype in Dravet syndrome. (Under review)

10. Alfiya F., Manna Jose, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee and Ramshekhar N. Menon. Yield of WES in trios in drug resistant childhood epilepsy syndromes of uncertain etiology (Under manuscript preparation)

11. Akash J*, Alfiya F*, Manna Jose*, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee and Ramshekhar N. Menon. GNAO1 encephalopathy spectrum in South Indian male probands (Under manuscript preparation)

12. Alfiya F*, Manna Jose*, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee and Ramshekhar N. Menon. Spectrum of ion channelopathies in DEE phenotypes. (Under manuscript preparation)

13. Kiren George Koshy, Alfiya F, Manna Jose, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee and Ramshekhar N. Menon. WDR45 variants-an unrecognized cause of DEE in males. (Under manuscript preparation)

14. Alfiya F, Manna Jose, Ashalatha Radhakrishnan, Moinak Banerjee, Ramshekhar N. Menon and Soumya Sundaram Genotype- phenotype correlations in CDKL5 mediated encephalopathy (Under manuscript preparation)

16. Saranya B Gomathy, Alfiya F, Karthika Ajit, Ashalatha Radhakrishnan, Bejoy Thomas, Ramshekhar N. Menon and Soumya Sundaram, De barsy syndrome due to aldh18a1 mutation – expanding the spectrum of a rare neurocutaneous syndrome (Under review)

17. Krishna Mohan, Alfiya F, Ashalatha Radhakrishnan, Soumya Sundaram, and Ramshekhar N. Menon, GABA receptor variants in Epileptic Encephalopathy (Under Manuscript preparation)

18. Bharani, Alfiya F, Ashalatha Radhakrishnan, Soumya Sundaram, and Ramshekhar N. Menon , Potassium channel variants in Developmental Epileptic encephalopathy (Under manuscript preparation)

19. Alfiya F; Amy McTague; Manna Jose; Soumya Sundaram; Moinak Banerjee; Madhusoodanan UK; Ashalatha Radhakrishnan, Genetic variant interpretation for the neurologist- a pragmatic approach in the era of next-generation sequencing in childhood epilepsy (Under review)

Paper presentations

1. Dr Ramshekhar N Menon- invited talk- The spectrum of genetic developmental epileptic encephalopathies- Brain Hour- McMaster Pediatric Neurology Rounds- 14th May 2021- McMaster University, Canada (Virtual)
2. Dr Ramshekhar Menon- Invited faculty for Digital CME- The Genetic Landscape of Epilepsies: Implications for Diagnostic Evaluation- Virtual recording (Indian Academy of Neurology organized by Omnicuris)
3. Dr Ramshekhar N Menon- Invited faculty and Treasurer of ECON 2022, Thiruvananthapuram, Aug 2022- Presented a lecture on ION CHANNELS IN REFRACTORY CHILDHOOD EPILEPSY-WHERE ARE WE?

4. Dr Ramshekhar N Menon - Invited faculty of IANCON 2021, NIMHANS, Bangalore- Debate on Older vs Newer Antiseizure medicines (Virtual)
5. Dr Ramshekhar N Menon - Invited faculty- Webinar on "Occipital Transients"- Indian Epilepsy Society- March 2023
6. Dr Ramshekhar N Menon, Invited faculty- Webinar on " Idiopathic focal epilepsies:- Indian Epilepsy Society- June 2022
7. Invited facuty- Society of Neurocritical Care Webinar, Pharmacotherapy in Status Epilepticus (SE), February 2023
8. Invited faculty- Webinar, Pre-surgical and candidature for epilepsy surgery, NeurosciencesSociety of India, February 2022
9. Dr Ramshekhar N Menon, Invited faculty- Kerala Association of Neurology annual meeting- Poovar - April 2022- Genetic testing is helpful in all pediatric patients with epilepsy (resistant/unknown etiology)
10. Dr Ramshekhar N Menon, Invited faculty- Neuropedicon Nov 2021- Refractory seizures, EEG progression pattern and presurgical evaluation oriented special EEGs
11. Research Associate presented a paper in the Award paper category titled 'Metabolic causes of pediatric developmental and epileptic encephalopathies (DEE)- mutation analysis in a South Indian cohort' at ECON, Joint conference of Indian Epilepsy Association and Indian Epilepsy Society, Thiruvananthapuram, August 5-7, 2022; Won Third Prize for the presentation. (Appendix 2 abstract).
12. Research Associate presented a poster titled 'GLUT-1 deficiency syndrome with unique features of visual sensitive epilepsy- a case study' at ECON, Joint conference of Indian Epilepsy Association and Indian Epilepsy Society, Thiruvananthapuram, August 5-7, 2022. (Appendix 2 abstract)
13. SRF presented a paper titled 'Denovo mutations in Developmental and Epileptic Encephalopathies (DEE) in an Indian cohort' at ECON, Joint conference of Indian Epilepsy Association and Indian Epilepsy Society, Thiruvananthapuram, August 5-7, 2022. (Appendix 2 abstract)
14. SRF presented a poster titled 'A novel ITPA variant associated with Developmental and Epileptic Encephalopathy (DEE)' at ECON, Joint conference of Indian Epilepsy Association and Indian Epilepsy Society, Thiruvananthapuram, August 5-7, 2022. (Appendix 2 abstract)

15. Research Associate presented a paper titled ‘Do potential variants of uncertain significance (VUS) contribute to diagnosis in progressive myoclonus epilepsies?’ at the Silver Jubilee Conference of R. Madhavan Nayar Centre for Comprehensive Epilepsy Care, SCTIMST, Thiruvananthapuram, August 3-4, 2022. (Appendix 2 abstract)

16. SRF presented a paper titled ‘Genetic Diagnostic Yield of Childhood Developmental Epileptic Encephalopathies: A Comparative Study of Whole Exome Sequencing and Global Screening Array’ at the Silver Jubilee Conference of R. Madhavan Nayar Centre for Comprehensive Epilepsy Care, SCTIMST, Thiruvananthapuram, August 3-4, 2022. (Appendix 2 abstract)

7. SRF presented a paper titled ‘Potential variants identified in metabolic genes linked to Developmental and epileptic encephalopathy’ at 47th Annual Conference of ISHG, Andhra University, 23rd to 25th January 2023.

8. SRF presented a paper titled “Pathogenic Metabolic gene variations in Developmental and epileptic encephalopathy” at the National Seminar: Epigenetics meets Metabolomics, Institute for Communicative and Cognitive Neurosciences, Shoranur, Kerala, June 2-3, 2023.

9. SRF presented a paper titled “Diagnostic yield of Trio-Whole exome sequencing and Clinical exome sequencing in children with Developmental and epileptic encephalopathy” at Epilepsy conference, ECON23, Marriot hotel, Jaipur, Rajasthan, 21st to 23rd July, 2023.

10. SRF selected for presenting a digital poster titled “Functional and structural characterization of variants of uncertain significance identified from whole exome sequencing of developmental and epileptic encephalopathies in an Indian cohort: an *In Silico* approach” at the 35th International Epilepsy Congress, 2-6 September, 2023, Dublin, Ireland

11. RA selected for presenting a digital poster titled “Metabolic basis of pediatric developmental and epileptic encephalopathies (DEE)- genetic variant analysis in a South Indian cohort” at the 35th International Epilepsy Congress, 2-6 September, 2023, Dublin, Ireland

12. Sambha Murthy Krihsna Mohan Mavuru, Alfiya F, Manna Jose, Soumya Sundaram, Ashalatha Radhakrishnan, and Ramshekhar N. Menon. "CNVS IN REFRACTORY EPILEPSY – DOES IT REALLY MATTER..!?", Award paper presentation at Epilepsy conference, ECON23, Marriot hotel, Jaipur, Rajasthan, 21st to 23rd July, 2023.

13. Sambha Murthy Krihsna Mohan Mavuru, Alfiya F, Manna Jose, Soumya Sundaram, Ashalatha Radhakrishnan, and Ramshekhar N. Menon. "CNVs in refractory epilepsy; A diagnostic Odyssey" Selected for Digital poster presentation at the 35th International Epilepsy Congress, 2-6 September, 2023, Dublin, Ireland.

13. Any patents applied for- NA

Awards

1. SRF won Second prize for the award paper presentation titled "Diagnostic yield of Trio-Whole exome sequencing and Clinical exome sequencing in children with Developmental and epileptic encephalopathy" at Epilepsy conference, ECON23, Marriot hotel, Jaipur, Rajasthan, 21st to 23rd July, 2023.
2. RA won First prize for the Poster presentation titled "Significance of pathogenic variants in CDKL5 gene in developmental and epileptic encephalopathy" at Epilepsy conference, ECON23, Marriot hotel, Jaipur, Rajasthan, 21st to 23rd July, 2023.
3. Third Prize for the award paper presentation. Research Associate presented a paper in the Award paper category titled 'Metabolic causes of pediatric developmental and epileptic encephalopathies (DEE)- mutation analysis in a South Indian cohort' at ECON, Joint conference of Indian Epilepsy Association and Indian Epilepsy Society, Thiruvananthapuram, August 5-7, 2022;(Appendix 2 abstract).

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY
TRIVANDRUM – 695011

Statement of Accounts
(Period 05.02.2020 TO 04.08.2023)

1. Sanction Letter No. : 5/7/1658/CH/Adhoc/2019-RBMCH
2. Total Project Cost (1st, 2nd & 3 Year) : Rs. 9931045/-
3. Sanction /Revised Project cost (if applicable): N A
4. Date of Commencement of Project : 05.02.2020
5. Proposed Date of Completion : 04.08.2023
6. Statement of Expenditure : From 05.02.2020 to 04.08.2023

Sl. No.	Sanctioned / Heads	Funds Allocated (1st, 2nd & 3rd Year)	Expenditure Incurred				Balance as on 04.08.2023	Remarks
			I Year	II Year	III Year	IV year		
A	Salaries	3051206	752461	1157305	1022362	314474	(-) 195396	
B	Consumables	9749649	3500000	3700000	2459589	0.00	90060	
C	Travel	50000	0.00	0.00	0.00	0.00	50000	
D	Contingency	300000	100000	100000	44664	0.00	55336	
E	Non-recurring (Equipments)	103000	103000	0.00	0.00	0.00	0.00	
F	Overhead Expenses	696216	237072	247072	212072	0.00	0.00	
	Total	13950071	4692533	5204377	3738687	314474	0.00	

Note: (1) Unspent balance in the project as on 04.02.2023 in the heads consumables, Travel and contingency, Rs 314474/- re-appropriated to Manpower head as per approval received ICMR letter dated 16.03.2023.

(2) 10 % of the 3rd year grant Rs. 4,50,351/- retained by ICMR may be released favoring SCTIMST as this amount is committed for payment to consumables.

Sr Accounts Officer
Date: 24.07.2023

VENKITA SUBRAMANIA IYER
Sr. ACCOUNTS OFFICER
Sree Chitra Tirunal Institute for
Medical Sciences and Technology
Thiruvananthapuram

Principal Investigator
Proj # 5389

DR. RAMSHEKHAR MENON
MD; ONB; DM (BOMBAY), EPILEPSY FELLOWSHIP
Consultant Neurologist
Sree Chitra Tirunal Institute
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Trivandrum-11; Reg No: TCMC 3095

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY
TRIVANDRUM – 695011

Check list for covering note to accompany Utilization Certificate of grant for the project for the period from 05.02.2023 to 04.08.2023

1.	Title of the project	Genetics of complex Pediatric epilepsy syndromes:electro-clinico imaging based genotype-phenotype correlations in an Indian cohort
2	Name of the Institution	Sree chitra Tirunal Institute for medical Sciences and Technology, Trivandrum
3	Principal Investigator	Dr Ramshekhar Menon Additional Professor, Neurology Department, SCTIMST
4	ICMR letter No. and date sanctioning the project.	5/7/1658/CH/Adhoc/2019-RBMCH dated 13.08.2019
5	Head of account as given in the original sanction letter	
6	Amount received during the year (Please give No. & Date of ICMR's sanction letter for the amount and period)	Rs. 0.00
7	Total amount that was available for expenditure (excluding commitments) during the year (Sl.No.6+7)	Rs. 314474/-
8	Actual expenditure (excluding commitments) incurred during the year.	Rs. 314474/-
9	Balance amount available at the end of the year, ie as on 04.08.2023	Rs. 0.00
10	Amount already committed, if any.	0.00
11	Amount to be carried forward to the next year (if applicable). Indicate the amount already committed with supporting documents.	Rs. 0.00

Utilization Certificate

Certified that out of Rs. 0.00 (Rupees **Zero only)** of grants-in-aid sanctioned during the year 2023-24 in favour of Director, SCTIMST under ICMR Letter No. Nil dated Nil and **Rs. 314474.00 (Rupees Three** lakh Fourteen Thousand **Four Hundred and Seventy Four only)** on account of unspent balance of the previous year, a sum of **Rs. 314474/- (Rupees Three lakh Fourteen Thousand Four Hundred and Seventy Four only)** has been utilized for the purpose of "Genetics of complex Pediatric epilepsy syndromes:electro-clinico imaging based genotype-phenotype correlations in an Indian cohort" for which it was sanctioned and that the balance of **Rs. 0.00 (Rupees** Zero only) remaining unutilized at the end of 04.08.2023 ~~has been surrendered to ICMR (vide cheque No .....NIL..... Dated....NIL...../ will be adjusted towards the grants-in-aid payable during the next year i.e. 2023-24.~~

The 10% retained by ICMR Rs. 450351/- may be released early favoring Director, SCTIMST.

Sr. Accounts Officer

24.07.2023

N. VENKITA SUBRAMANIA IYER
Sr. ACCOUNTS OFFICER
Sree Chitra Tirunal Institute for
Medical Sciences and Technology
Thiruvananthapuram

Principal Investigator
Proj # 5389

DR. RAMSHEKHAR MENON
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for DIRECTOR
SCTIMST 29/7/2023.

निदेशक / DIRECTOR
श्री चित्र तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान
Sree Chitra Tirunal Institute for
Medical Sciences and Technology
तिरुवनंतपुरम / Trivandrum-695011

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DOI: 10.1002/epd2.20008

Epileptic Disorders

CASE VIGNETTE

Visual-sensitive epilepsy in GLUT-1 deficiency syndrome: Expanding the phenotype

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Indian Council of Medical Research, Grant/Award Number: 2018-2670

Keywords: GLUT-1 deficiency syndrome, hypoglycorrhachia, next generation sequencing, reflex photosensitive epilepsy

Glucose, the primary fuel for brain energy metabolism, is transported by glucose transporter type 1 (GLUT-1) across the blood–brain barrier. Mutations in solute carrier family 2 member 1 (*SLC2A1*) gene result in defective transfer of glucose and a spectrum of neurological manifestations together called GLUT-1 deficiency syndrome (GLUT-1DS). The association between GLUT-1DS and intractable infantile epilepsy responsive to ketogenic diet (KD) has been clearly elucidated.[1–3] Low fasting cerebrospinal fluid (CSF) glucose levels are demonstrated in most patients and provide a diagnostic clue, thereby driving early genetic confirmation and initiation of KD. Often, patients with GLUT-1DS are treated with antiseizure medication (ASM) in the early stages which fails to address the underlying metabolic disturbance during early brain development. Studies show that low brain glucose supply early in life can lead to profound diminution of cerebral microvasculature.[4] However, reflex phenotypes, such as photosensitive epilepsy or eyelid myoclonia with/without absence among the genetic generalized epilepsies (GGE) that have thalamo-cortical generators, have not been reported. Time delay to genetic confirmation and initiation of the ameliorative KD has not been addressed critically. We give an account of the clinical and genetic odyssey of a child with a novel visual-sensitive epilepsy phenotype associated with GLUT-1DS who was evaluated using whole-exome sequencing (WES) trio analysis, by virtue of variants of uncertain significance (VUS), detected using a limited epilepsy gene panel. We also compared this phenotype with those from other patients with GLUT-1DS in our prospectively maintained database.

A 5-year-old girl, born of a nonconsanguineous parentage, presented with recurrent seizures and developmental delay with no prior antecedents or perinatal insults. From 6 months of age, the child was noted to have a tendency to look at bright objects, resulting in multiple brief episodes of eye blinks, after which she would cry holding her head on a daily to weekly basis. She was also noted to have moderate developmental delay, especially in language, and was prone to temper tantrums, reduced eye contact, and interest in rotating objects which worsened from 3 years of age. These paroxysmal events were refractory to ASMs including sodium valproate, ethosuximide, and clobazam. From 4 years of age, the seizure burden (severity, frequency, and self-induction) and behavioral issues worsened. On examination, she had no facial dysmorphism, microcephaly, neurocutaneous markers, or focal neurological deficits.

The developmental assessment was performed using the Revised Denver Developmental Scale (Denver II Technical Manual. 1990; Denver Developmental Materials, Inc.), Vineland Social Maturity Scale (VSMS), and Childhood Autism Rating Scale (CARS). She was found to have global delay; developmental quotients for the different domains were social—27, fine motor—42, language—30, and gross motor—21. Receptive Expressive Emergent Language Scale

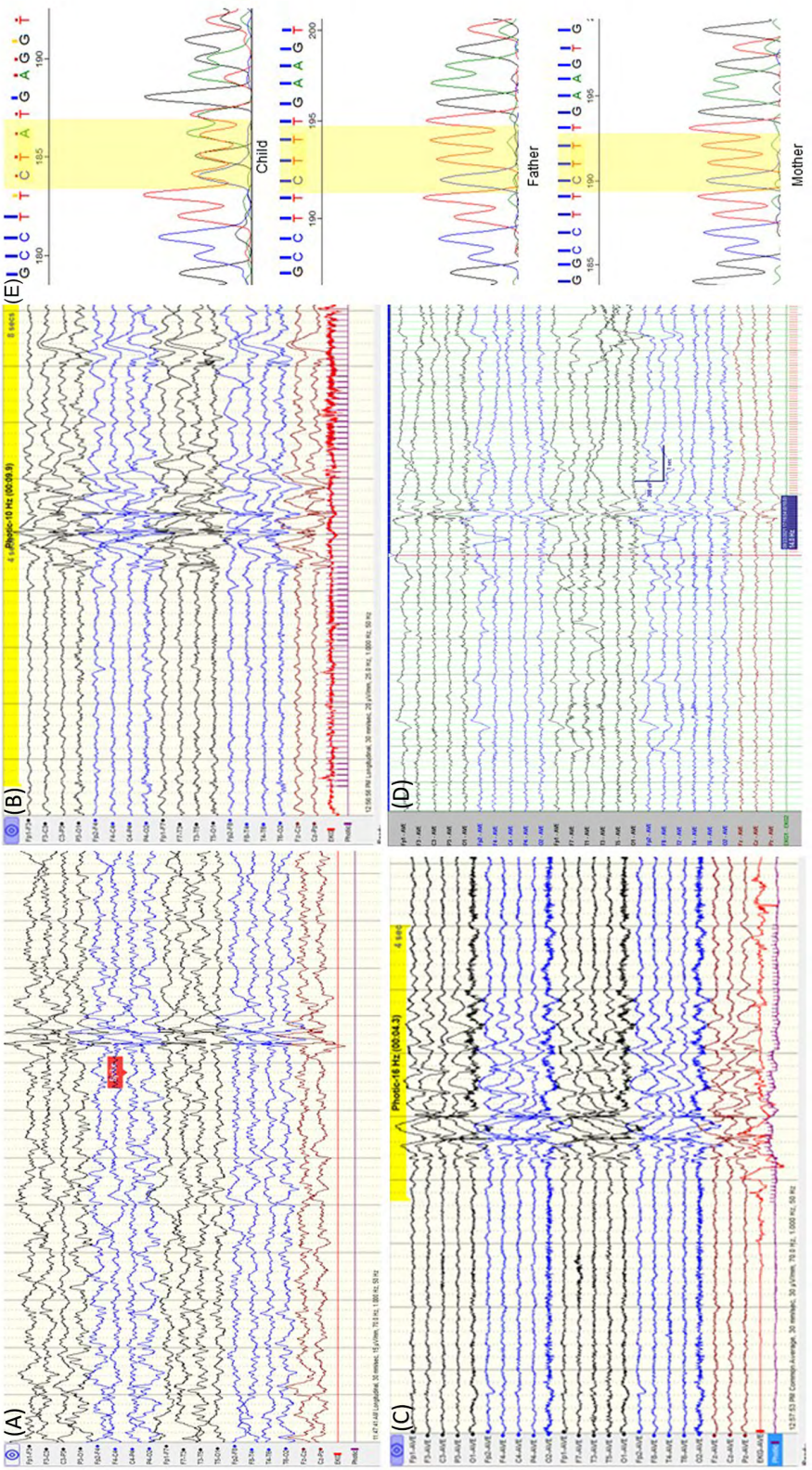

FIGURE 1 (A) EEG images of the patient, showing generalized epileptiform discharges. (B) EEG showing a photoparoxysmal response at 10 Hz. (C) photoparoxysmal response at 16 Hz. (D) Follow-up EEG revealing abortive generalized discharges on photic stimulation at 14 Hz which were inconsistent, following initiation of the ketogenic diet. (E) Sanger sequencing to validate the de novo deletion, c.1134_1136del, in the proband.

scores revealed a receptive language age of 12–14 months and expressive language age of 8–9 months. On the Vineland Social Maturity Scale, her social quotient was 47. There was no family history of epilepsy, movement disorders, developmental delay, or autism.

Her video-electroencephalography (EEG) showed generalized slowing and generalized and bilateral occipital spike/polyspike-and-wave discharges with eye closure sensitivity. Photic stimulation produced a Grade IV photoparoxysmal response (PPR) at 10 and 16 Hz (Figure 1) with absences associated with eyelid myoclonia. Her initial clinical exome sequencing, employing a panel of 500 genes, revealed VUS in *SLC2A1* and *CHD2*. The combination of infantile refractory absences with eyelid myoclonia, GDD, and photosensitivity led us to contemplate the possibility of developmental epileptic encephalopathies secondary to potential *SCN1A*, *SYNGAP1*, and *CHD2* variants. WES analysis revealed a heterozygous de novo in-frame deletion in the *SLC2A1* gene (ENST00000426263.3:Chr1:43393418-43393420;exon9:c.1134_1136del:p.F379del) which was classified as a pathogenic variant.[5] A heterozygous de novo variant in the *ABCC10* gene (Chr6:43414112;exon14:c.A3388C:p.M1130L) was also identified with no phenotype match. The incidental variants identified in this proband were present in both the unaffected parents: heterozygous-dominant missense variants in the *KCNMA1* (Chr10:78709061;c.G2224A;p.V742I) and *CHD2* gene (Chr15:93524599;c.T2978C;p.M993T). A fasting CSF study revealed hypoglychorrachia, confirmatory of GLUT-1DS (36 mg/dL; CSF:plasma glucose ratio of .49). The child was initiated on KD and was optimized on sodium valproate and clobazam. Within 3 months of initiation of KD, there was an improvement in her absences, and eyelid myoclonia completely stopped, and there was a steady improvement in language with the persistence of abortive generalized discharges on the EEG.

A summary of the clinical, electrographic, and genetic findings of this proband in comparison to other patients with GLUT-1DS in our cohort is summarized in Table S1.

Our series expands the phenotypic spectrum of GLUT-1DS in photosensitive epilepsy as seen in genetic generalized epilepsies (GGE), in addition to previously reported associations with resistant typical or atypical absences, myoclonic, clonic, tonic, and focal-onset seizures, nonconvulsive status epilepticus, paroxysmal movement disorders, microcephaly, ataxia, and psychomotor delay, as noted in other patients in our cohort, none of whom exhibited photosensitivity (Table S1).[2]

Adult antisense GLUT-1DS mice replicate the human phenotype of GGE (which may or may not be associated with photosensitivity) and concurrent robust thalamocortical electrical oscillations correlating to hypometabolism have been demonstrated in PET studies.[5,6] This is not surprising as the thalamus and cerebral cortex display the lowest level of GLUT-1 expression and glucose uptake, as shown in mutant mouse models.[5] Diagnostic delays can be avoided by lowering the threshold to conduct fasting CSF glucose estimations and/or genetic testing in children with refractory GGE or visual-sensitive epilepsy phenotypes with developmental impairments. Our analysis also highlights the importance of diligent WES trio analysis to establish de novo, potentially pathogenic, *SLC2A1* deletions along with tests to establish genotype–phenotype correlations. For the proband (with a coexistent VUS in *CHD2*, inherited from an unaffected parent; a gene uniquely associated with photosensitive epilepsy, especially when de novo[7,8]) the confirmatory test was CSF glucose estimation. Although a "double-hit" involving *SLC2A1* and *CHD2* can be hypothesized, the inherited nature of the latter gene from an unaffected parent makes this unlikely. The evidence that the KD improved seizures and language supports the pathogenic role of *SLC2A1* in this patient.

ACKNOWLEDGMENTS

We thank the neuro technologists, allied health staff, and all faculty of R Madhavan Nayar Centre for Comprehensive Epilepsy Care for their support. The study was funded by an extramural grant from the Indian Council of Medical Research Ref. No. 5/7/1658/CH/Adhoc/2019-RBMCH dated 13/08/2019.

FUNDING INFORMATION

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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SUPPORTING INFORMATION

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

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Test yourself

1. Which biochemical test should be performed in children with refractory generalized epilepsy with or without photosensitivity?
2. What are the classic features of GLUT1-DS?
3. What is the role of WES trio analysis in suspected GLUT-1DS?

Answers may be found in the supporting information.

Epileptic Disorders / Early View

CLINICAL COMMENTARY

Arginase deficiency—An unheralded cause of developmental epileptic encephalopathy

Harini Pavuluri, Manna Jose, Alfiya Fasaludeen, Soumya Sundaram, Ashalatha Radhakrishnan, Moinak Banerjee, Ramshekhar N. Menon

First published: 27 May 2023
https://doi.org/10.1002/epd2.20081

Harini Pavuluri, Manna Jose, and Alfiya Fasaludeen contributed equally to the study. [Correction added on 8 June 2023, after first online publication: Alfiya Fasaludeen has been added in the preceding equal contribution statement].

Abstract

Arginase deficiency, which leads to hyperargininaemia is a rare urea cycle disorder caused by a mutation in the ARG1 gene. It is an under-recognized cause of pediatric developmental epileptic encephalopathy, with the key coexistent clinical features being developmental delay or regression and spasticity. Detection of ARG1 gene mutation on genetic testing is the confirmatory diagnostic test. However, elevated levels of plasma arginine and low plasma arginase level can be considered as biochemical markers for diagnosis. We present two cases of arginase deficiency with genetically confirmed ARG1 mutation in one and biochemical confirmation in both. As the spectrum of epilepsy in arginase deficiency has been less explored, we attempted to elucidate the novel electroclinical features and syndromic presentations in these patients. Informed consent was obtained from families of patients. Electroclinical diagnosis was consistent with Lennox Gastaut syndrome (LGS) in the first patient while the second patient had refractory atonic seizures with electrophysiological features consistent with developmental and epileptic encephalopathy. Though primary hyperammonaemia is not a consistent feature, secondary hyperammonaemia in the setting of infectious triggers and drugs like valproate (valproate sensitivity) has been well described as also observed in our patient. In the absence of an overt antecedent in a child with spasticity and seizure disorder, with a progressive course consistent with a developmental epileptic encephalopathy, arginase deficiency merits consideration. Diagnosis often has important therapeutic implications with respect to dietary management and choice of the appropriate antiseizure medications.

CONFLICT OF INTEREST STATEMENT

Journal of Genetics (2022)101:30
https://doi.org/10.1007/s12041-022-01371-0

RESEARCH NOTE

C12orf57 pathogenic variants: a unique cause of developmental encephalopathy in a south Indian child

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Abstract. Open reading frame variants which lack stop codons such as *C12orf57* variants are known to cause Temtamy syndrome, an extremely rare disorder characterized by intellectual disability, seizures, facial dysmorphism and agenesis of corpus callosum. *C12orf57* was initially reported to be required for human corpus callosum development. We report the first child who is of Indian origin with developmental and epileptic encephalopathy (DEE) with a unique phenotypic evolution as focal onset reflex seizures. We performed whole exome sequencing of genomic DNA isolated from peripheral blood samples of proband and his parents. Two pathogenic compound heterozygous variants, a start loss variant (Chr12:7053285:c.1A>G) and a premature stop gain variant (Chr12:7053327:c.43C>T), involving the *C12orf57* gene were identified in the proband. Our case report which details genotyping in this rare syndromic developmental encephalopathy, with no prior cases reported from India, expands the ethnic spectrum of patients.

Keywords. developmental encephalopathy; agenesis of corpus callosum; epilepsy; *C12orf57*; compound heterozygous.

Introduction

Temtamy syndrome is a rare disorder, which is caused by mutations involving open reading frame 57 (*C12orf57*) in chromosome 12. It was first described in 1991 and is phenotypically characterized by intellectual disability (ID), epilepsy, craniofacial dysmorphism, agenesis of the corpus callosum, and optic coloboma (Temtamy *et al.* 1991, 1996). No prior reports from the Indian subcontinent exist in literature focussing on potential mutations in this syndrome that lead to epileptogenesis and callosal anomalies. Pathogenic variants were first reported in 2013 in four siblings of a Saudi Arabian family with a deleterious nucleotide change (c.1A>G; p.Met1Val) and eight pathogenic variants have been reported till date (Salih *et al.* 2013; Alrakaf *et al.* 2018; Wang *et al.* 2020) of which seven are of Middle Eastern and one of Asian (China) ethnicity. Homozygous and compound heterozygous mutations in *C12orf57* have been described (Platezer *et al.* 2014). We report the first Indian male with genetically proven Temtamy syndrome who as diagnosed using whole exome sequencing in trios to have compound heterozygous variants involving *C12orf57*. Informed consent for publication was obtained from the parents.

Case presentation

A male, born of south Indian nonconsanguineous parentage, presented to us in early childhood with global developmental delay and no history of perinatal insults. There was a history

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of seizures since 84 days of life which had a phenomenology consistent with symmetric myoclonic jerks. Overall initial electroclinical features were consistent with 'symptomatic' myoclonic epilepsy of infancy and he was initiated on levetiracetam. At 2 years follow up, the child was noted to have global developmental delay. Developmental gains were noted and he was able to speak 10–15 words and was able to walk with support with a developmental age reaching 12 months. Although his epilepsy was initially well controlled with levetiracetam, at 3 years he had two episodes of focal-onset seizures of left opercular semiology both related to drug default. He was subsequently noted to have autistic traits with hand dyspraxia. At 4 years of age, he developed a new semiology of seizures characterized by left hemispheric asymmetric tonic seizures which frequently were associated with auditory and visual reflex triggers with no overt myoclonus or ataxia. Sodium valproate followed by lamotrigine was added and ketogenic diet was initiated. He continues to have brief nondisabling reflex startle-induced spasms with no major consciousness impairing events. There was family history of pharmacoresponsive focal epilepsy of temporal plus origin with hippocampal sclerosis on MRI and hearing impairment respectively among two paternal relatives and psychiatric illness in maternal grandmother (figure 1a).

Physical examination revealed facial dysmorphism characterized by microcephaly (head circumference = 41 cm), hypertelorism, depressed nasal bridge, strabismus, full cheeks, thick lips, dental anomalies and open mouth (figure 1b). He had truncal hypotonia with no overt limb spasticity, dystonia or ataxia. He had good auditory and visual regard with normal ophthalmological examination. His routine blood investigations including thyroid profile was normal. Metabolic workup including arterial blood gas analysis, ammonia, lactate and pyruvate were within normal limits. His echocardiogram showed a 3-mm ostium secondum atrial septal defect. His initial CT head showed agenesis of corpus callosum. Developmental assessment using the revised Denver developmental scale (Denver II Technical Manual 1990; Denver developmental materials, Inc.) showed (developmental quotient being 20 on gross motor, eight on fine motor, 34 on language and 20 on personal social domains). His EEG showed occasional interictal epileptiform discharges (IED) over bilateral temporal regions along with mild to moderate degree of nonspecific disturbance in electrophysiological function intermittently over left temporal region. A follow up EEG after the onset of tonic seizures and reflex epilepsy showed multifocal IED predominantly in the form of bilateral independent frontotemporal sharp waves with sleep showing activation in a pseudo periodic fashion along with asynchronous background activity, fairly formed sleep spindles and focal paroxysmal fast activity (figure 1c). Brain MRI confirmed partial agenesis of corpus callosum and incomplete rotation of hippocampi (figure 1d).

Whole exome sequencing and bioinformatic analysis

Genomic DNA was isolated from the peripheral blood samples of proband and his parents using salting out method

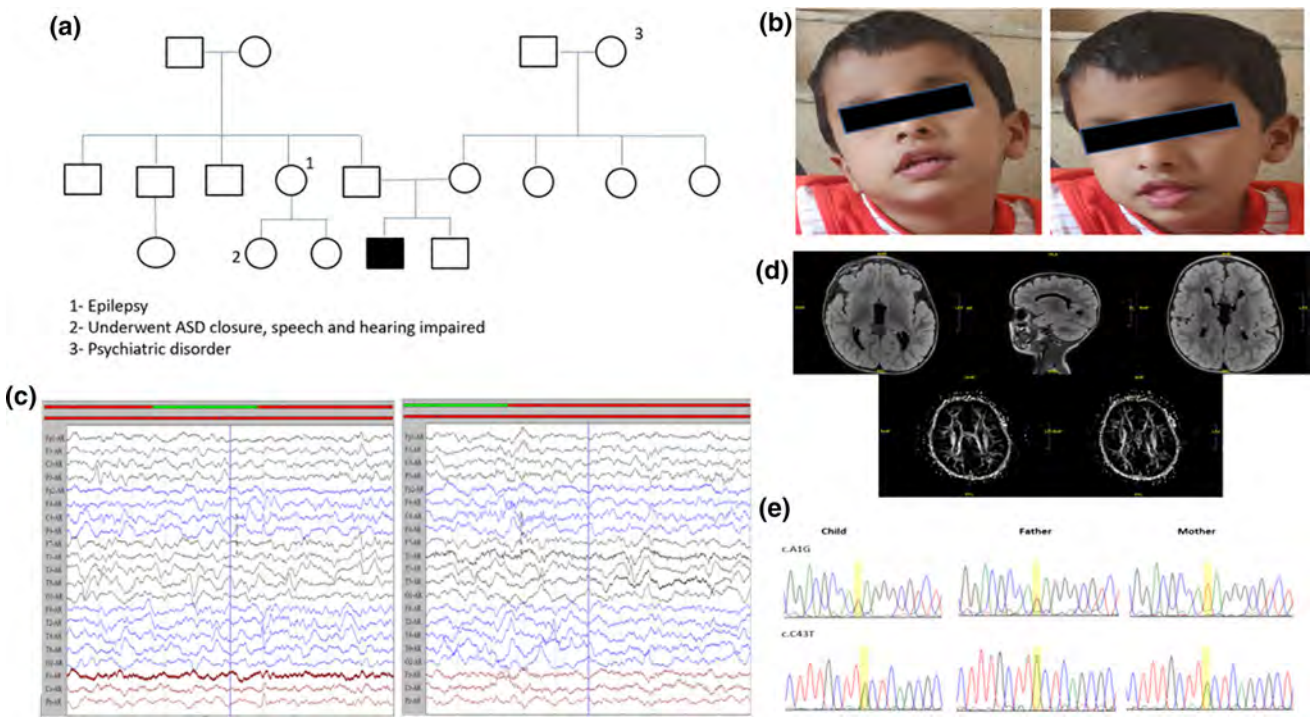

Figure 1. (a) Pedigree analysis, (b) photograph of the patient, (c) EEG images (d) MRI images and (e) Sanger sequencing electrogram of compound heterozygous variants in *C12orf57*.

Table 1. Details of compound heterozygous variants identified in the proband.

Variant	Gene	Zygosity	CADD score	SIFT prediction	PolyPhen2 prediction	Mutation-taster prediction	REVEL score	OMIM phenotype	ACMG classification
Chr12:7053285; exon1:c.A1G: p.Met1Val; (Platzer *et al.* 2014)	*C12orf57*	Het	21.9	Deleterious	Benign	Deleterious	0.492	Temtamy syndrome (MIM number: 218340)	Pathogenic (PVS1, PS3, PM2, PP5)
Ch12:7053327; exon1:c.C43T:p.Q15X	*C12orf57*	Het	60	–	–	Deleterious	–		Pathogenic (PVS1, PM2, PP5)

of DNA extraction. Sequencing libraries were generated using Agilent SureSelect Human All ExonV6 kit (Agilent Technologies) and paired end sequenced on Illumina HiSeq X Ten (Illumina) for 150 cycles according to manufacturer's instructions. Raw fastq files were aligned to human reference genome (hg19/GRCh37) using Burrows-Wheeler Aligner (Li and Durbin 2010). The SNPs and INDELs were called using Genome Analysis Toolkit's (GATK) best practice guidelines (Van der Auwera *et al.* 2013). The variants identified were annotated using ANNOVAR (Wang *et al.* 2010).

The variants which passed the depth and quality filter were considered for inheritance pattern models includes homozygous recessive, autosomal dominant, compound heterozygous, X-linked and *de novo* variants. The variant reporting was restricted to 2413 genes, extracted from literature searches and databases, which are associated with epilepsy, intellectual disability, developmental delay and autism spectrum disorder. The potential damaging effects of the variants were assessed with the *in silico* prediction tools CADD (http://cadd.gs.washington.edu/), SIFT (http://sift.jcvi.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/) and REVEL (https://sites.google.com/site/revelgenomics/) score. The variants with a CADD score of greater than 20 and ExAC (http://exac.broadinstitute.org/) allele frequency less than 1% were filtered out. Based on the phenotypes of proband, the variants were prioritized by manually assessing the evidence from various databases including ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), OMIM (https://www.omim.org/), HPO (https://hpo.jax.org) and also the literature searches. Sanger sequencing was performed to validate the genotypes of the proband and his parents.

Results

From the whole exome sequencing analysis, 188,598 variants were identified after alignment and variant calling, which were then filtered against various databases. Twenty-three thousand six hundred and nine variants were filtered out from the epilepsy associated genes which includes both SNPs and INDELs. From this list of variants, 55 variants were then picked out with CADD score $>$ 20 and MAF $\leq$ 0.01. Autosomal dominant variants identified were present in either of the unaffected parent. The proband was identified with two potentially damaging compound heterozygous variants in *C12orf57* gene; loss of start codon variant and (NM_138425.4:c.1A>G; rs587776954; Chr12:7053285,exon1:c.1A>G:p.Met1Val) premature stop gain variant (NM_138425.4:c.43C>T; rs1565574197; chr12:7053327, exon1:c.C43T:p.Q15X). His father was a heterozygous carrier of chr12:7053285, A>G variant and mother carried another variant chr12:7053327, C>T. The pathogenicity classification of variants by American College of Medical Genetics and Genomics (ACMG) guidelines suggested that

Table 2. Genotype and phenotype data of patients identified with Temtamy syndrome from literature and the present study.

	Temtamy *et al.* (1996)	Alrakaf *et al.* (2018)	Wang *et al.* (2020)	Present study
Country	North Africa	Middle East	East Asian	India
Developmental delay	GDD	GDD	GDD	GDD
Behavioural abnormalities	–	Autistic features	NIL	Autistic features
Seizures	–	GTCS, MS, FS, tonic infantile spasms	GTCS	MS, focal onset auditory reflex seizures, asymmetric tonic seizure
Congenital anomalies	Talipes equinovarus, flat feet, moderate dilatation of aorta	ASD, VSD	ASD	ASD, flat feet
Dysmorphic features	Iris coloboma, myopia, hypertelorism, frontal bossing, elongated face, arched eyebrows, antimongoloid slanting of eyes, beaked nose, low set simple lop ears, long philtrum, short upper lip, micrognathia, dental anamolies	Frontal bossing, low set, posteriorly rotated ears, depressed nasal bridge, hypertelorism, micrognathia, epicanthal folds, and upslanted palpebral fissures	Colobomatous microphthalmia, frontal bossing, low set ears, depressed nasal bridge, ocular hypertelorism, micrognathia, single transverse palmar crease	Microcephaly, frontal bossing, hypertelorism, depressed nasal bridge, strabismus, full cheeks, thick lips, open mouth, low-set ears, dental anamolies
MRI findings	Dilated cerebral ventricles, complete agenesis of CC	Abnormal CC, abnormal septum pellucidum, ventriculomegaly	Expanded lateral ventricles, agenesis of CC	Agenesis of CC
C12orf57 mutation	–	c.1A>G, p.(Met1?), c.53-2A>G, c.-3_2delinsG, c.43C>T, p.(Gln15*), c.229+2T>C	c.3G>C (p.Met1Ile)	Loss of start codon variant exon1:c.1A>G:p.Met1Val Premature stop gain variant exon1:c.C43T:p.Q15X

GDD, global developmental delay; GTCS, generalised tonic clonic seizures; MS, myoclonic seizures; CPS, complex partial seizures; FS, febrile seizures; ASD, atrial septal defect; PS, pulmonary stenosis; CC, corpus callosum.

both chr12:7053285;c.A1G and chr12:7053327;c.C43T are pathogenic variants (table 1). Both the missense variants prediction tools, SIFT and MutationTaster, predicted the first variant (c.1A>G) as a deleterious variant. It prevents the translation of *C12orf57* by demolishing the initiation codon (Salih *et al.* 2013). The second variant (c.C43T) has a high CADD score and it establishes a stop codon which ends the translation of *C12orf57* prematurely (Platzer *et al.* 2014). We confirmed the variants in the proband and his parents by Sanger sequencing (figure 1e).

Discussion

This report highlights compound heterozygous variants involving the *C12orf57* gene in a patient of south Indian ethnicity with atypical presentation of Temtamy syndrome with respect to clinical and radiological features. The diagnosis in this patient was established by whole exome sequencing performed in trios and confirmed by Sanger sequencing. Two previously reported heterozygous variants were identified in our patient with concordant phenotypic features (Platzer *et al.* 2014; Alrakaf *et al.* 2018). *C12orf57* gene is ubiquitously expressed in human tissues and is essential for the development of human corpus callosum (Akizu *et al.* 2013). A few pathogenic variants in *C12orf57* are reported in literature causing Temtamy syndrome of which the c.1A>G pathogenic variant in start codon is the most frequent causing severe reduction in protein levels, suggesting a loss of function (Salih *et al.* 2013). The c.3G>C pathogenic variant also affects the same start codon, but at different bases and affects protein expression level resulting in clinical manifestations (Wang *et al.* 2020). The Q15X variant is a pathogenic variant and predicted to cause loss of normal protein function either through protein truncation or nonsense-mediated mRNA decay (Zahrani *et al.* 2013; Platzer *et al.* 2014; Alrakaf *et al.* 2018) (https://www.ncbi.nlm.nih.gov/clinvar/variation/620193). It has been noted that the *C12orf57* is a three-exon gene that encodes a 126-residue protein (Akizu *et al.* 2013). A recent functional study reports that *C12orf57* controls synaptic scaling in excitatory neurons (Jiang 2019), however the mechanisms behind epileptogenesis are poorly understood as in Aicardi syndrome in which differential methylation patterns in several neurodevelopmental networks are hypothesized to be putative, leading to phenotypic heterogeneity (Piras *et al.* 2017). As opposed to Aicardi syndrome in which cortical abnormalities are described, our patient did not demonstrate epileptogenic lesions on MRI and reflex sensitivity could represent a consequence of neuronal hyperexcitability of the sensory or association cortices, with a synchronized discharge spreading to functionally connected cortical or subcortical structures through white matter tracts. It is also possible given the increased synaptic activity postulated with this variant, physiological responses are responsible for the induction of synchronization of larger networks or functionally connected epileptogenic cortex, although this remains to be conclusively proven (Jiang 2019).

Phylogenetic analysis indicated that the protein is highly conserved as a single copy across evolution. Other genes which have been implicated in agenesis of corpus callosum include *MCOLN1*, *HERC2*, *DCLK2*, *CACNA1A* and *KCNH3* (Meloche *et al.* 2020). This child had global developmental delay, truncal hypotonia and symptomatic epilepsy with reflex seizures which is refractory to sodium valproate and lamotrigine favouring a developmental encephalopathy. Dysmorphic features in Temtamy syndrome are not distinct from patients with intellectual disability and syndromic agenesis of corpus callosum such as Aicardi syndrome (Donnai and Barrow 1993; Aicardi 2005; Ganesh *et al.* 2005). The current patient's phenotype was similar to the literature reports of craniofacial anomalies consisting of arched eyebrows, antimongoloid slant of the eyes, beaked nose, low-set and simple lop ears, full cheeks, long philtrum, short upper lip, and micrognathia (Temtamy *et al.* 1991). Previously reported ocular abnormalities such as coloboma and microphthalmia were not found in this child. The most frequent congenital heart defect, i.e. ASD was also observed in the present case. The phenotype and genotype of previously reported cases of Temtamy syndrome in comparison to our poband is detailed in table 2. The epilepsy phenotype with focal-onset seizures, startle-induced tonic seizures, spasms and occasional secondary generalized seizures suggests a diffuse network centered on cortical hyperexcitability with multifocal IEDs on EEG. This phenotype has however been reported with other callosal-agenesis syndromes such as Aicardi syndrome (Grosso *et al.* 2007) suggesting the role of the pathogenic variant in leading to dysfunction in epileptogenic pathways centred on reflex seizures. This case report expands the ethnic spectrum of patients with this rare syndrome and highlights the utility of WES in trios in rare DEE phenotypes.

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Does Etiology and Hypsarrhythmia Subtype Influence Outcome in West Syndrome? Challenges Encountered from a Referral Center Perspective

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Free article

Abstract

Background: Prediction of outcome of West syndrome (WS) in relation to etiology and electrophysiology remain pertinent challenges.

Objective: This study aimed to compare electro-clinical and imaging characteristics between WS of "unknown-etiology"; "symptomatic"WS; to gauge the evolution and impact of electroencephalographic (EEG) patterns on seizure outcomes.

Materials and methods: Electro-clinico-radiological data of 76 children with WS who were followed up for atleast 1 year was collected for reviewing clinical, therapeutic and EEG profiles (sub-typed as typical and modified hypsarrhythmia [HA]). Quantified seizure scores were assessed.

Results: Among 76 children included in this retrospective analysis, 31 (40.8%) were of unknown-etiology and 45 (59.2%) were "symptomatic" (structural cause/developmental-encephalopathy). Children with symptomatic WS (p = 0.037), specifically with gliosis on imaging (p = 0.05) and typical HA (including the multifocal subtype; P = 0.023) were more likely to have other seizure types before onset of spasms and exhibit prior delay or regression in milestones (p = 0.017). There was negative correlation between time to diagnosis and reduction in seizure scores (r = -0.32; p = 0.005).Significant reduction was noted in seizure scores with pharmacotherapy, irrespective of etiology (P < 0.001 in unknown-etiology and symptomatic subgroups). Seizure freedom rates did not differ between typical and modified HA groups (p = 0.215) with a higher proportion of children with meaningful reduction in seizure scores in the former sub-group (p = 0.030). Children who failed to achieve seizure remission were more likely to exhibit developmental impairment (p = 0.019).

Conclusions: Early diagnosis and initiation of optimal therapy is crucial towards improving outcome, irrespective of etiology (which impacts pre-spasm development) and HA subtypes.

Keywords: Epileptic spasms; etiology; hypsarrhythmia; outcome; west syndrome.

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Clin EEG Neurosci. 2021 Sep;52(5):371-375. doi: 10.1177/1550059420953735. Epub 2020 Sep 3.

Electroclinical Phenotype-Genotype Homogeneity in Drug-Resistant "Generalized" Tonic-Clonic Seizures of Early Childhood

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Abstract

Purpose: Children with refractory focal to bilateral tonic-clonic seizures, despite normal high-resolution imaging, are often not subjected to genetic tests due to the costs involved and instead undergo multimodality presurgical evaluation targeted at delineating a focal onset. The objective of this study was to ascertain genotype-phenotype correlations in this group of patients.

Method: An online hospital database search was conducted for children who presented in 2019 with drug-resistant epilepsy dominated by nonlateralizing focal-onset/rapid generalized (bilateral) tonic-clonic seizures (GTCS), subjected to presurgical evaluation and subsequent genetic testing due to absence of a clear focus hypothesis.

Results: Phenotypic homogeneity was apparent in 3 children who had onset in infancy with drug-resistant GTCS (predominantly unprovoked and occasionally fever provoked) and subsequent delayed development. 3-Tesla magnetic resonance imaging (MRI) scans were negative and video EEG documented a homogeneous pattern of multifocal and/or generalized epileptiform discharges with phenomenology favoring probable focal-onset/generalized-onset bilateral tonic-clonic seizures. All 3 tested positive for *SCN1A* gene variants (heterozygous missense substitution variants in 2 children, one of which was novel and a novel duplication in one that led to frameshift and premature truncation of the protein), suggestive of *SCN1A*-mediated epilepsy. This electroclinical profile constituted 3 out of 25 patients with *SCN1A*-epilepsy phenotypes at our center.

Conclusions: These cases suggest that children with early-onset drug-resistant "generalized" epilepsy are likely to have a genetic basis although the presentation may not be typical of Dravet syndrome. Hence, genetic testing for *SCN1A* variants is recommended in children with drug-resistant MRI negative focal-onset/generalized-onset bilateral tonic-clonic seizures before subjecting them to exhaustive presurgical workup and to guide appropriate treatment and prognostication.

Keywords: EEG; SCN1A; drug-resistant epilepsy; generalized tonic clonic seizures; genetics.

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Genotype-phenotype correlates of infantile-onset developmental & epileptic encephalopathy syndromes in South India: A single centre experience

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Abstract

Introduction

A paucity of literature exists on genotype- phenotype correlates of 'unknown-etiology' infantile-onset developmental-epileptic encephalopathies (DEE) from India. The primary objective was to explore the yield of genetic testing in identifying potential disease causing variants in electro-clinical phenotypes of DEE

Methods

An observational hospital-based study was undertaken on children with unexplained refractory seizure-onset ≤12 months age and developmental delay, whose families consented and underwent genetic testing during a three year time period (2016–2018) by next-generation sequencing (NGS) or multiplex ligand protein amplification. Yield was considered based on demonstration of pathogenic/likely pathogenic variants only and variants of unknown significance (VUS) were documented.

Results

Pathogenic/likely pathogenic variants were identified in 26 (31.7 %) out of 82 children with DEE. These included those variants responsible for primarily DEE- 21(76.7 %); neuro-metabolic disorders- 3(18.6 %) and chromosomal deletions- 2(4.7 %). Of these patients, early-infantile epilepsy onset ≤ 6 months age was noted in 22(84.6 %). The DEE studied included Ohtahara syndrome associated with *STXBP1* and *SCN8A* variants with yield of 50 % (2/4 tested); early myoclonic encephalopathy (no yield in 2); West syndrome with *CDKL5*, yield of 13.3 % (2/15 tested); epilepsy of infancy with migrating partial seizures due to *CACNA1A* and *KCNT1* variants, yield of 67 % (2/3 tested); DEE-unclassified with *KCNQ2, AP3B2, ZEB2,* metabolic variants (*SUOX, ALDH7A1, GLDC*) and chromosome deletions (chr 1p36, chr2q24.3); yield of 32 % (8/25 tested). Patients with Dravet syndrome/Dravet-like phenotypes (N = 33) had variants in *SCN1A* (N = 10), *SCN1B*, *CHD2*; yield of 36.4 % (12/33 tested; 57.1 % from NGS). Eighteen patients with potential variants (*SCN1A, SCN2A, SCN8A, KCNQ2, ALDH7A1* which also included VUS) could be offered targeted therapy.

Conclusions

Our study confirms a good yield of genetic testing in neonatal and infantile-onset DEE provided robust phenotyping of infants is attempted with prognostic and therapeutic implications, particularly relevant to centres with resource constraints.

Introduction

Infantile epileptic encephalopathies include various phenotypes of epileptic encephalopathies (EE), which are characterized by seizure-onset before 12 months of age.(Zhang et al., 2017) The majority of EE have onset ≤ 6 months of age and are referred to as early-onset EE (EOEE) or early infantile EE (EIEE).(Hwang and Kwon, 2015; Nieh and Sherr, 2014)The syndromes include phenotypes with refractory seizures often accompanied by frequent focal, multifocal or generalized epileptiform abnormalities with age-inappropriate ontogeny on electroencephalography (EEG), and developmental delay (DD), regression or intellectual disability (ID).(Berg et al., 2010; Nieh and Sherr, 2014) EIEE include Ohtahara syndrome (OS), West syndrome (WS), early myoclonic encephalopathy (EME), epilepsy of infancy with migrating focal seizures (EIMFS), and Dravet syndrome (DS) as well as refractory focal or generalized epilepsy with developmental impairment which cannot be classified into named syndromes but may broadly come under the description of developmental and epileptic encephalopathies (DEE).(Kalser and Cross, 2018; Scheffer et al., 2017) Identifiable primary causes include structural, neurodegenerative, metabolic, pathogenic copy number variants, or chromosomal deletion/duplication syndromes. Increasingly, a number of novel and *denovo* genetic causes are being identified in childhood epilepsy, especially DEE of uncertain aetiology.(Alam and Lux, 2012; McTague et al., 2016) DEE is a genetically heterogeneous disorder: over 100 genes have been suggested to be involved in the aetiology of these syndromes.(McTague et al., 2016) Many DEE cases are sporadic occurring in patients with no family history of seizures or epilepsy.(Epi et al., 2013) Next-generation sequencing (NGS), which includes clinical exome and whole-exome sequencing (WES) allows the analysis of a variety of genes simultaneously and is very useful in large sample analysis or multi-gene analysis. In one study, 265 monogenic epilepsy-associated genes were sequenced using targeted NGS in 33 patients with various well-phenotyped epilepsy syndromes and 48 % were shown to have disease-causing variants. (Lemke et al., 2012) Employing the more expensive whole genome sequencing identified variants in four of the six patients with DEE. (Martin et al., 2014)Another study using NGS suggested a diagnostic yield of 28 % for an underlying genetic cause in patients with EE in a retrospective cohort study with aetiologies including metabolic disorders, pathogenic copy number variants (CNVs) and variants in *SCN1A, SCN2A, SCN8A, KCNQ2, STXBP1, PCDH19,* and *SLC9A6*genes.(Mercimek-Mahmutoglu et al., 2015)This information is lacking from the subcontinent wherein the advent of disease-specific panels has largely transformed diagnostics although challenges remain with regard to commercial costs, variant curation, absence of local population-based data, clinical interpretation and reluctance of unaffected family members to undergo testing.(Ganapathy et al., 2019)

With this background, we undertook this study with the following objectives:

1) To study the yield of genetic testing using commercially available epilepsy gene panels and or MLPA in the broad category of DEE in a south Indian cohort, in terms of prevalence of potentially pathogenic variants with added documentation of variants of unknown significance (VUS) which may have clinical implications.

2) To qualitatively assess variant subtypes with estimates of pathogenicity in various phenotypes of DEE

Section snippets

Study design and subjects

This hospital based retrospective observational study was conducted at a comprehensive epilepsy care referral centre in Trivandrum, Kerala, India. Patients with DEE or drug-resistant epilepsy of uncertain or unknown etiology with unprovoked seizure-onset at or under 12 months of age with/without co-existent developmental delay or regression and whose families consented and underwent genetic testing between Jan 2016 to Dec 2018 were included. Electro-clinical syndromes and DEE were defined and…

Patient profiles

A total of 82 patients with DEE underwent genetic testing during the three year time period (2016–2018) and potential variants were noted in 43(52.4 %) patients. Early infantile onset of epilepsy was noted in 36/43 [neonatal 14 (32.6 %),

1–6 months in 22 (51.2 %)] and onset > 6 months in 7 (16.2 %). The yield of pathogenic/likely pathogenic variants were identified in 26 (31.7 %), VUS-D in 8 (9.8 %) and VUS in 8(9.8 %) and a benign susceptibility variant in 1(1.2 %) while testing did not…

Discussion

Our study identified pathogenic/likely pathogenic variants in nearly one-third of patients diagnosed to have DEE over a 3 year period, with a majority presenting in early infancy. The yield exceeded 30 % in most DEE syndromes (including unclassified subtypes) with the notable exception of WS. Besides diagnostic, prognostic and predictive utility in the entire cohort, this had therapeutic implications in ion-channelopathies and neuro-metabolic disorders all of which constituted around 50 % of…

Conclusions

Our observational study which is the first of its kind from the Indian sub-continent reports a diagnostic yield of 31.7 % for an underlying genetic cause in patients DEE. The results in terms of yield are broadly similar to a Western population. These variants included *SCN1A*, *SCN8A*, *KCNT1*, *CACNA1A*, *KCNQ2, CDKL5,AP3B2, ZEB2, SCN1B, CHD2,* genes responsible for inherited metabolic disorders and chromosomal deletions. Additionally, identification of certain VUS such as *SCN2A, ALDH7A1* had…

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Declaration of Competing Interest

The authors declare that they have no known competing interest.…

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LETTER TO THE EDITOR

Utility of clinical exome sequencing in progressive myoclonus epilepsy syndromes: An exploratory analysis

Manna Jose, Prashant Poulose, Soumya Sundaram, Ashalatha Radhakrishnan, Sheela Nampoothiri, Ramshekhar N. Menon

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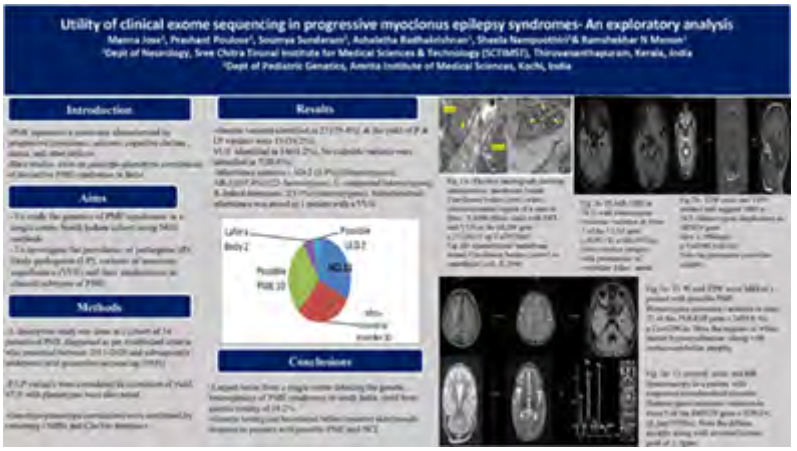
Graphical Abstract



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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/cge.14090.

DATA AVAILABILITY STATEMENT

The clinical and genetic data used in the study is stored in the repository of the corresponding author based on approval obtained from the Institute Ethics Committee and is available for review on request.

Genetic Diagnostic Yield of Childhood Developmental Epileptic Encephalopathies: A Comparative Study of Whole Exome Sequencing and Global Screening Array

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Developmental and epileptic encephalopathy (DEE) is a heterogeneous group of conditions chracterized by the co-occurrence of epilepsy and intellectual disability (ID), frequently accompanied by recurrent epileptiform activity and developmental regression. Next generation sequencing techniques such as Whole Exome Sequencing (WES), covers 1-1.5% of the human genome comprising of around 20000 genes facilitates the identification of familial or *denovo* pathogenic variants using a family trio design. Global Screening Array (GSA) consists of 6,54,027 markers including single nucleotide variants, small insertions and deletions and Copy Number Variants, rather than Single Nucleotide variants to detect rare Mendelian mutations. In this study we performed a comparative analysis of WES with GSA to assess the cost effectiveness, genetic diagnosis yield and to characterize the molecular findings and inheritance patterns in patients with well-defined DEE phenotypes analysed in trios.
We performed WES and GSA on 6 trios comprised of unaffected parents and a child diagnosed with DEE. An average of 6,39,813 variants from GSA and 1,95,357 variants from WES were identified in 4 probands with DEE phenotypes of West Syndrome (N=2), Lennox Gastaut syndrome (LGS) epilepsy (N=1), symptomatic myoclonic epilepsy (N=2) and encephalopathy with continuous spike wave in sleep (CSWS) (N=1). The diagnostic yield of pathogenic variants from WES is 50% (3/6) and from GSA analysis is 16.6% (1/6) with a denovo variant identified by both techniques. Despite GSA covers a wider range of genetic markers, the WES approach in trios appears to indicate a higher yield in children suspected of having DEE when compared to GSA in trios.

Denovo mutation identified in SCN1A gene associated with Dravet syndrome using whole exome sequencing

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(Funding source: Indian Council of Medical Research)

Dravet syndrome (DS) is a prototype developmental and epileptic encephalopathy of childhood , also known as severe myoclonic epilepsy of infancy (SMEI), affects individuals in early childhood and progresses to damage cognitive, behavioural, and motor functioning. The condition is characterised by generalised tonic, clonic, myoclonic,and tonic-clonic along with focal onset seizures seizures that occur within the first year of life and are initially triggered by fever. The genetic aetiology of DS is mutations in the neuronal voltage-gated sodium channel alpha subunit gene SCN1A. Here, we report a rare, denovo mutation (c.T5339C;p.M1780T) in SCN1A gene in a South Indian child diagnosed with DS using whole exome sequencing in trios.

Genomic DNA isolated from peripheral blood samples of proband and her parents was used for whole exome sequencing. Raw fastq files were aligned to human reference genome (hg19/GRCh37) using BWA (Burrows-Wheeler Aligner). The single nucleotide variants were called using Genome Analysis Toolkit's (GATK) best practice guidelines and VarScan is used to identify denovo variants. The variants identified were annotated using ANNOVAR. The variant is predicted as a potentially damaging variant by various protein function prediction tools including SIFT (Sorting Intolerant From Tolerant) and PolyPhen2.The pathogenicity classification of variants by American College of Medical Genetics and Genomics (ACMG) guidelines suggested that the identified denovo variant is a pathogenic variant. We validated the identified variant in proband and parents by Sanger sequencing.

Whole exome sequencing can be used to identfiy novel, denovo mutations in the evaluation of individuals with SMEI phenotypes, since the detection of a denovo mutation has diagnostic, therapeutic and genetic counselling implications.

Keywords: Dravet syndrome, SCN1A, Whole exome sequencing

Diagnostic yield of Trio-Whole exome sequencing and Clinical exome sequencing in children with Developmental and epileptic encephalopathy

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Exome sequencing is the preferred method for the molecular diagnosis of Developmental and epileptic encephalopathies (DEE) due to their distinct clinical and genetic variability. The aim of this study is to assess the clinical utility and cost-effectiveness of Whole exome sequencing (WES) over targeted or clinical exome sequencing (CES). We performed WES/CES in 492 probands (<12 years) to have well defined or unclassified phenotypes of DEE. Out of 492 probands, 114 underwent trio-WES and 26 underwent CES prior to trio-WES. Pathogenic or likely pathogenic variants (P/LP) identified in 39% (193/492) of our patients. 159 out of 492 had Variants of uncertain significance (VUS) and 140 out of 492 had no potentially disease causing variants identified. The diagnostic yield of CES was 40.1% without performing the parental segregation. Trio-WES revealed a diagnostic yield of 27.19% which includes 24.5% de novo variants and 5.26% of variants with homozygous recessive and compound heterozygous pattern of inheritance. For 26 out of 125 patients who underwent CES prior to trio-WES, trio-WES was able to identify 30.7% of the P/LP variations, whereas CES could only achieve a diagnostic yield of 7.69%. The cost comparison analysis revealed that Trio-WES (₹45000) is the preferred cost-effective method for first-tier assessment as it has the better diagnostic yield when compared to CES and trio-WES after negative CES results in an additional cost of ₹13000. This study substantiates the utility of trio-WES as a useful diagnostic and cost effective tool in determining the aetiology of patients with unknown causes of DEE.

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Potential variants identified in metabolic genes linked to Developmental and epileptic encephalopathy

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Developmental and Epileptic Encephalopathies (DEE) describes rare neurodevelopmental disorders with early infantile onset, characterized by the co-occurrence of epilepsy, intellectual disability, autism spectrum disorder and developmental delay. DEE can be caused by Inborn errors of metabolism (IEM) and which are caused by genetic variations that block a biochemical pathway essential to cellular function, affecting the brain primarily. Here, we have performed Whole exome sequencing of 11 trios with probands being diagnosed to have well defined or unclassified phenotypes. Sequencing libraries were generated using Agilent SureSelect Human All ExonV6 kit and paired end sequenced on Illumina HiSeq. Raw fastq files were aligned to human reference genome (hg19/GRCh37) using BWA (Burrows-Wheeler Aligner). The SNPs and INDELs were called using Genome Analysis Toolkit's (GATK) best practice guidelines. The variants were annotated using ANNOVAR and filtered based on inheritance pattern, *in silico* predictions and allele frequency. American College of Medical Genetics and Genomics (ACMG) criteria was used for variants classification. We have identified pathogenic variants in metabolic genes in 9 out of 11 patients. Pathogenic homozygous recessive variants identified in genes *ITPA, ARG1, CLN5, BTD,* and *NHLRC1.* De novo variants identified in 3 patients and two of them were pathogenic (*SLC2A1* and *IDH2*) and a variant of uncertain significance in *SLC1A2* gene was identified in a proband diagnosed with probable progressive myoclonic epilepsy. In patients with complex DEE phenotypes, the identification of metabolic variants can direct investigations and have therapeutic significance.

Title: Denovo mutations in Developmental and Epileptic Encephalopathies (DEE) in an Indian cohort

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Introduction: DEE are largely known to be caused by denovo genetic pathogenic variants however current next generation sequencing techniques do not incorporate testing in trios to establish denovo variants which are usually confirmed by Sanger sequencing. This study aimed to establish the prevalence and subtypes of denovo variants using WES in trios among a cohort of children with DEE.

Methods: We performed whole exome sequencing (WES) on 26 consecutive trios consist of unaffected parents and a proband diagnosed to have well defined or unclassified phenotypes of DEE. American College of Medical Genetics and Genomics criteria was used for variants classification.

Results: We found 24 denovo mutations (pathogenic/likely pathogenic, LP/P=18 and promising Variants of Uncertain Significance, VUS=6) in our cohort. Among the P/LP variants, 14 were missense point mutations and 4 were frameshift deletions. Probands with known DEE phenotypes had P/LP variants in genes *SCN1A* (Dravet syndrome), *TSC2* (West Syndrome), *KCNT1* (Ohtahara syndrome) and *IDH2* (Lennox Gastaut syndrome) and in probands with unclassified phenotypes (20/26=76.9%), we detected P/LP variants in *CHRNA4, SLC2A1, MCTP2, DCX, TUBA3E, NOVA2, TUBB3, CHRNA2, CDKL5, SCN8A* and *GABRB2* genes.

Out of 26 probands, 5 underwent clinical exome sequencing previously and reported 9 VUS and no P/LP variants were identified. WES showed an additional yield and established denovo P/LP variants in 69.2% (18/26) and potential VUS with predicted deleterious effects on functional prediction tools in 23% (6/26).

Conclusions: This is the first study from the Indian subcontinent that substantiates the role of trios based WES as opposed to singleton limited panel based testing in identifying denovo mutations in DEE. This enables early diagnosis with implications for prognostication, drug development and treatment personalization in DEE.

Funding source: Indian Council of Medical Research (ICMR); ICMR Ref. No. 5/7/1658/CH/Adhoc/2019-RBMCH

Comparative analysis of Whole Exome Sequencing and Global Screening Array yield in evaluation of childhood developmental epileptic encephalopathies

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Source of funding ICMR grant No.5/7/1658/CH/Adhoc/2019-RBMCH

Rationale

Developmental Epileptic Encephalopathy(DEE) represents a heterogeneous group of conditions wherein cognitive and behavioural deterioration occurs as a consequence of frequent seizures or interictal epileptiform abnormalities or due to the underlying etiology. Techniques for next generation sequencing such as Whole Exome Sequencing(WES), covers 1-1.5% of the human genome comprising of around 20000 genes facilitates the identification of familial or *denovo* pathogenic variants using a family trio design. Global Screening Array(GSA) consists of 6,54,027 markers including single nucleotide variants, small insertions and deletions and Copy Number Variants, rather than Single Nucleotide Polymorphisms to detect rare Mendelian mutations. In this study we performed a comparative analysis of WES with GSA to assess the cost effectiveness, genetic diagnosis yield and to characterize the molecular findings and inheritance patterns in patients with well-defined DEE phenotypes analysed in trios.

Methods

Genomic DNA extracted from peripheral blood sample of 4 probands and their biological parents were used for WES and GSA analysis. WES was done with Illumina Exome Sureselect Agilent V6 method and Infinium Global screening array-24 V3 beadchip was used to identify clinically relevant markers in trios. The Data were analysed using different bioinformatic tools and customised in house pipelines and annotated with publicly available databases to identify the risk variants associated with DEE. The variants were classified based on American College of Medical Genetics and Genomics (ACMG) classification criteria. Yield was considered based on identification of pathogenic/likely pathogenic variants and variants of unknown significance were documented.

Results

An average of 6,39,813 variants from GSA and 1,95,357 variants from WES were identified in 4 probands with DEE phenotypes of West Syndrome, Lennox Gastaut syndrome (LGS) epilepsy, symptomatic myoclonic epilepsy and encephalopathy with continuous spike wave in sleep (CSWS). Only an average of 12,112 variants were present in common for both GSA and WES which includes 4223 exonic variants, 5427 intronic variants, 27 splicing variants and remaining intergenic/3'UTR/5'UTR variants. The diagnostic yield of pathogenic variants from WES is 75% (3/4) and from GSA analysis is 25% (1/4) with a denovo variant identified by both techniques (DCX associated with Lissencephaly) (**Table 1**). The cost of GSA ($66) compared favourably to WES ($342) which is 5 times more expensive.

Conclusions

Despite a wider coverage of genetic markers with GSA, the WES technique in trios does seem to suggest a higher yield in children suspected with DEE when compared to GSA in trios. Although WES in trios is more effective in identifying point mutations, a denovo variant could be identified

by both techniques. In our study of rare DEE phenotypes of uncertain etiology, GSA has the extra advantage of reducing costs required to process a large number of samples. Larger comparative studies with segregation analyses are likely to throw further light on the preferred cost-effective approach to genetic analysis of DEE, especially from a low middle income country perspective.

Table 1- Comparison of risk variants identified from WES and GSA

Patient ID	**Diagnosis**	**Risk variants identified from WES (hg19)**				**Risk variants identified from GSA (hg19)**			
P1	4 yrs old Male, gender; Symptomatic myoclonic epilepsy, microcephaly with craniofacial dysmorphism with corpus callosal agenesis	Gene	Variant	Zygosity	ACMG class	Gene	Variant	Zygosity	ACMG class
		C12orf57 Compound heterozygous	rs587776954; Chr12:7053285; c.A1G:p.M1?	Het; Mother carrier;Father recessive;	Likely pathogenic (Temtamy syndrome)	-	-	-	-
		C12orf57 Compound heterozygous	rs156554197; Chr:12 7053327 c.43T:p.Q15X	Het; Father carrier; Mother recessive;	VUS	-	-	-	-
P2	10 years old Female; LGS, autistic traits, lissencephaly	DCX	rs587783563; Chr X: 110644366; c.G800A p.R267H	Het; X-linked *Denovo*	Likely pathogenic (Lissencephaly with double cortex)	DCX	rs587783563 Chr X: 110644366 c.G800A p.R267H	Het; X-linked Denovo	Likely pathogenic
P3	9 years old Male; Intellectual disability Encephalopathy with CSWS and craniofacial dysmorphism, congenital heart disease	MCTP2	Chr15:95013587;c.C2221G:p.L741V;	Het; Denovo	Likely pathogenic (Chrmosome 15q26-Qter Deletion Syndrome)	-	-	-	
P4	5 years old Male; West syndrome evolving into multifocal epilepsy	PHIP	rs147526156; Chr 6: 79671522; c.G3541P:p.A1181S	Het; Mother carrier; Father recessive;	VUS-Parent unaffected	-	-	-	-

		CUX2	rs537978708; Chr:12 111729306; c.G386A: p.R129Q;	Het; Mother carrier;Father recessive;	VUS-parent unaffected	-	-	-	-

Comparing Yield of Whole Exome Sequencing and Global Screening Array for Genetic Diagnosis of Childhood Developmental Epileptic Encephalopathies

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Developmental Epileptic Encephalopathy(DEE) represents a heterogeneous group of conditions wherein cognitive and behavioural deterioration occurs as a consequence of frequent seizures or interictal epileptiform abnormalities or due to the underlying etiology. Next generation sequencing techniques such as Whole Exome Sequencing(WES), covers 1-1.5% of the human genome comprising of around 20000 genes facilitates the identification of familial or *denovo* pathogenic variants using a family trio design. Global Screening Array(GSA) consists of 6,54,027 markers including single nucleotide variants, small insertions and deletions and Copy Number Variants, rather than Single Nucleotide Polymorphisms to detect rare Mendelian mutations. In this study we performed a comparative analysis of WES with GSA to assess the cost effectiveness, genetic diagnosis yield and to characterize the molecular findings and inheritance patterns in patients with well-defined DEE phenotypes analysed in trios.

We performed WES and GSA on 4 trios comprised of unaffected parents and a child diagnosed with DEE. An average of 6,39,813 variants from GSA and 1,95,357 variants from WES were identified in 4 probands with DEE phenotypes of West Syndrome, Lennox Gastaut syndrome (LGS) epilepsy, symptomatic myoclonic epilepsy and encephalopathy with continuous spike wave in sleep (CSWS). The diagnostic yield of pathogenic variants from WES is 75% (3/4) and from GSA analysis is 25% (1/4) with a denovo variant identified by both techniques. Despite a wider coverage of genetic markers with GSA, the WES technique in trios does seem to suggest a higher yield in children suspected with DEE when compared to GSA in trios.

GLUT-1 deficiency syndrome with unique features of visual sensitive epilepsy- a case study

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Rationale- A unique case of GLUT-1 deficiency syndrome (GLUT-1DS), with visual sensitive epilepsy, autism, and global developmental delay (GDD), having particular features of self-induction of seizures (predominantly eyelid myoclonia) and photo-paroxysmal response on EEG which was not described in GLUT-1DS previously.

Case report- A 5-year-old girl, born of a nonconsanguineous parentage presented with habitual seizures since 6 months of age and developmental delay. The child did not have any subsequent perinatal insult or ICU stay. From 6 months of age, the child was noted to have a tendency to look at bright objects resulting in sudden brief episodes of eye blinks, events would last few seconds with variable frequency – multiple episodes per day to once in a week. She was noted to have moderate GDD, especially for language on administration of Denver Developmental Scale (Denver II Technical Manual. 1990; Denver Developmental Materials, Inc.), Vineland Social Maturity Scale (VSMS) and Childhood Autism Raring Scale (CARS). She was also noted to have abnormal behavior with temper tantrums, reduced eye contact, interest in rotating objects which worsened from 3 years of age. Magnetic resonance imaging of the brain showed ill-defined patchy areas of altered signal intensity in bilateral peri-trigonal white matter with prominent posterior horns of bilateral lateral ventricles. Her video-electroencephalography (EEG) showed generalized slowing, generalized and bilateral occipital spike/polyspike and wave discharges with eye closure sensitivity. Photic stimulation produced grade IV photo-paroxysmal response at 10 and 16Hz with absences having associated eyelid myoclonia recorded. CSF analysis revealed hypoglycorrhachia following which whole exome sequencing was performed for the proband and her parents and identified a de novo deletion in exon 9 of the SLC2A1 gene and a familial CHD2 variant (identified in an unaffected parent).

Discussion- Our study expands the phenotypic spectrum of GLUT-1DS to photosensitive epilepsy as seen in genetic generalized epilepsies in addition to previously reported associations with resistant typical or atypical absences, myoclonic, clonic, tonic, focal onset seizures, non convulsive status epilepticus, paroxysmal movement disorders, microcephaly, ataxia and psychomotor delay. Diagnostic delays can be avoided by incorporating genetic testing and fasting CSF glucose estimations in children with refractory generalized epilepsy with developmental impairments.

Pathogenic Metabolic gene variations in Developmental and epileptic encephalopathy

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Developmental and Epileptic Encephalopathies (DEE) are a group of rare neurodevelopmental disorders with early infantile onset, characterized by the co-occurrence of epilepsy, intellectual disability, autism spectrum disorder and developmental impairment. DEE can be caused by Inborn errors of metabolism (IEM) and which are caused by genetic variations that block a biochemical pathway essential to cellular function, affecting the brain primarily. Here, we have performed Whole exome sequencing (WES) of 20 trios with probands being diagnosed to have well defined or unclassified phenotypes. Sequencing libraries were generated using Agilent SureSelect Human All ExonV6 kit and paired end sequenced on Illumina HiSeq. Raw fastq files were aligned to human reference genome (hg19/GRCh37) using BWA (Burrows-Wheeler Aligner). The SNPs and INDELs were called using Genome Analysis Toolkit's (GATK) best practice guidelines. The variants were filtered based on inheritance pattern, *in silico* predictions and allele frequency and annotated using ANNOVAR. American College of Medical Genetics and Genomics (ACMG) criteria was used for variants classification. Pathogenic/likely pathogenic variants in metabolic genes were identifed in 13 out of 20 probands. These included homozygous recessive (*ITPA, ARG1, NHLRC1, CLN5, BTD*), *de novo* (*SLC2A1, GABRA1, GABRB2, GABRG2, STXBP1, IDH2*) and compound heterozygous variants (*ALG3*). In patients with complex DEE phenotypes, the identification of metabolic variants can direct investigations and have therapeutic significance.

Metabolic basis of pediatric developmental and epileptic encephalopathies (DEE)- genetic variant analysis in a South Indian cohort

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Abstract

Purpose- Epilepsy is seen in patients with inborn errors of metabolism and metabolic dysfunction is crucial in brain disorders. These can present as developmental epileptic encephalopathy (DEE) or distinctive phenotypes- infantile spasms. The detection rates of metabolic causes of DEE using next generation sequencing has been rarely reported.

Method- A prospective hospital based study was carried out in 385 children with DEE, who underwent genetic testing (whole exome sequencing in trios- 71 and clinical exome sequencing- 314). Metabolic disorders were evaluated with biochemical assays and when required cerebrospinal fluid estimations were performed.

Results- A total number of 154 pathogenic/likely pathogenic (P/LP) variants in 385 children were identified; 90 were diagnosed with suspected metabolic disorders. P/LP variants in metabolic genes were identified in 39 out of 90 (43.3%) and promising variants of uncertain significance in 29 (32.2%). Of these patients, onset of seizures less than 6 months of age was noted in 44.4%. These included variants for progressive myoclonus epilepsies (PME) (21; 53.8%) with *ACOX1, EPM2A, CLN1/PPT1, CLN2/TPP1, CLN3, CLN5, CLN6, CLN7/MFSD8, GBA, POLR3B, GLDC, KCTD7, L2HGDH*, *MT-TL1, NHLRC1, PLP1*; DEE unclassified with focal/multifocal seizures (9; 23.1%)- *SUOX, ITPA, AP3B2, SLC35A2,* Ch16p13.2 duplication syndrome, *ABCD1, NDUFV2*; generalized epilepsy (5;12.8%)- *SLC2A1, PTS*; early myoclonic encephalopathy (2; 5.1%)- *POLG, MFF;* LGS (1; 2.6%)- *ARG1;* West syndrome (1; 2.6%)- *BTD.* Biochemical tests were confirmatory in only 15.2% and 3 out of 17 who underwent skin biopsies for PME syndromes were confirmatory (17.6%).

Conclusion- Our cohort demonstrates for the first time from the Indian subcontinent that identification of metabolic variants can guide investigations and therapeutic implications in

patients with variable DEE phenotypes. The highest yield is noted in phenotypes such as PME, DEE unclassified and mitochondrial cytopathy. A high utility is noted given the low yield of available biochemical tests indicating cost-effectiveness of this approach.

Acknowledgement- The study was funded by an extramural grant from Indian Council of Medical Research- Ref. No. 5/7/1658/CH/Adhoc/2019-RBMCH dated 13/08/2019.

Functional and Structural Characterization of Variants of Uncertain Significance identified from Whole Exome Sequencing of Developmental and Epileptic Encephalopathies in an Indian Cohort: an *In Silico* Approach

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Purpose

Developmental and epileptic encephalopathies (DEE) refers to a group of rare, sporadic neurodevelopmental disorders with early infantile onset. Whole-exome sequencing (WES) is the preferred method for the molecular diagnosis of these disorders since they are characterized by significant clinical and genetic heterogeneity. The detection of a large number of variants of Uncertain Significance (VUS) from these next generation sequencing techniques and its use in clinical contexts are still challenging. Here, we are proposing an *in silico* approach to re-classify the VUS, identified from WES, into promising VUS and incidental VUS.

Method

WES analysis was performed for 97 probands including 90 trios. The variants were classified according American College of Medical Genetics and Genomics (ACMG) classification criteria. To re-classify the VUS identified from WES, *in silico* functional and structural predictions were performed. The impact of VUS on protein stability, interactions and structure was detected using iMutant, STRING, and Missense3D. The structural effect of variant re-classifed as promising VUS was performed with Molecular Dynamic Simulation (MDS) for 100ns using GROMACS. Post simulation analysis was performed with Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF) and Radius of gyration (Rg).

Results

We obtained a diagnostic yield of 41.2% (40/97) and identified 67% (65/97) of VUS from WES analysis of probands with DEE. The *in silico* functional and structural study of a novel *de novo* VUS identified in *STXBP1* (c.A434C:p.Y145S) gene was re-calssifieid as a promising VUS. The MDS of wild type *STXBP1* and its mutant form showed that the mutation affect the protein structure on different levels.

Conclusion

The real behavior of wild type protein and its mutant form in their environment was reproduced with MDS. This study would establish a structural and functional characterization pipeline for reclassifying the VUS identified from WES of DEE.

Title: A novel ITPA variant associated with Developmental and Epileptic Encephalopathy (DEE)

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Introduction: Inosine triphosphate pyrophosphatase (ITPA) encoded by ITPA gene plays an important role in purine metabolism and biallelic loss of function variants in ITPA gene have been linked to epileptic encephalopathies. Here we report a novel homozygous recessive pathogenic variant in ITPA gene, identified in a proband diagnosed with focal epilepsy left temporal with global developmental delay (GDD) and movement disorder, who expired in early infancy due to aspiration pneumonia.

Case presentation- A 1.5 year old boy, born of third degree consanguineous parentage presented with habitual seizures since 4 months of age and GDD. The child had to undergo ICU stay for pathological jaundice. He had poor weight gain and had failure to thrive. On examination, he had microcephaly, hypotonia and involuntary movement with dyskinesias. He did not have visual or auditory regard. His biochemical investigations which included urine organic acid estimation, tandem mass spectroscopy analysis of amino acids and biotinidase and lactate levels were normal. He had focal seizures since 4 months of age, associated with fever. He was in remission from seizures since six months before he expired. Magnetic resonance imaging of the brain showed prominent subdural spaces with mild diffuse volume loss. His video-electroencephalography (EEG) showed temporal slowing (left>right) with left temporal spike and wave discharges (mid and posterior) and left posterior head region (PHR). Whole exome sequencing of proband and his parents revealed a homozygous recessive pathogenic mutation in exon 8 of the ITPA gene c.511T:p.E171X in proband and parents were heterozygous for the same variant.

Discussion- ITPA mutations are associated with Developmental and Epileptic Encephalopathy- 35 (DEE-35), which is an autosomal recessive neurodegenerative disorder characterized by onset of seizures in the first months of life associated with essentially no normal development. The assessment of ITPase activity and ITP accumulation in erythrocytes along with WES could be a potential diagnostic tool for DEE-35. Our report expands the spectrum of metabolic causes of DEE in India.

Funding source: Indian Council of Medical Research (ICMR); ICMR Ref. No. 5/7/1658/CH/Adhoc/2019-RBMCH

Significance of pathogenic variants in *CDKL5* gene in developmental and epileptic encephalopathy

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Abstract

Rationale- The human Cyclin-Dependent Kinase-Like 5 (*CDKL5*) gene encodes a serine/threonine kinase that plays major role in brain development and function. Disease-causing variants of CDKL5 cause Developmental and Epileptic Encephalopathy-2 (DEE2).

Methods- A prospective hospital-based study was carried out in 510 children with DEE, who underwent genetic testing by next generation sequencing (whole exome sequencing in trios- 120 and clinical exome sequencing- 390) after obtaining parental consent.

Results- We report twelve patients with variants in *CDKL5* gene- seven pathogenic variants, of which five are novel, one likely pathogenic variant and four variants of uncertain significance (VUS). In six female patients with pathogenic variants, we identified two frameshift deletions, one non-sense variant, one de novo missense, one splice site and one copy number variant (CNV). A pathogenic hemizygous frameshift deletion was identified in a male patient with West syndrome. One likely pathogenic CNV was identified in another male patient. Three male probands diagnosed with West syndrome, Lennox Gastaut syndrome and autistic phenotypes harboured hemizygous VUS in *CDKL5* and one VUS with potential damaging effect (VUS-D) was identified in a female proband.

Conclusion- Our study, largest in the Indian sub-continent, establishes the importance of *CDKL5* screening in patients affected with DEE, regardless of the patient gender, contrary to previous studies where CDKL5 mutations were identified predominantly in females. Genetic testing in patients with suspected CDKL5 disorders can offer confirmatory diagnoses and thus could avoid diagnostic delays and also has the potential to avoid needless interventions in some patients with refractory seizures.

Metabolic causes of pediatric developmental & epileptic encephalopathies (DEE)- mutation analysis in a south Indian cohort

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Abstract

Rationale- Epilepsy is seen in patients with inborn errors of metabolism and metabolic dysfunction is crucial in brain disorders. These can present as developmental- epileptic encephalopathy (DEE) or distinctive phenotypes such as infantile spasms. The detection rates of metabolic causes of DEE using next generation sequencing has been rarely reported.

Methods- A prospective hospital based study was carried out in 288 children with DEE, study period from 2015-2022, who underwent genetic testing by next generation sequencing (whole exome sequencing in trios- 71 and clinical exome sequencing- 217) after obtaining parental consent. Metabolic disorders were evaluated with blood and urine biochemical assays and when required CSF estimations were performed.

Results- Pathogenic/likely pathogenic variants in metabolic genes were identified in 29 out of total 121 P/LP variants in 288 children (10.1%) with DEE with promising VUS in 27 (9.40%). Of these patients, onset of seizures < 6 months was noted in 16 (55.2%). These included variants for progressive myoclonus epilepsies (14; 48.3%) with *ACOX1, EPM2A, CLN3, CLN5, CLN6, GBA, MFSD8, POLR3B, CLN1/PPT1, CLN2/TPP1*; GLUT-1 deficiency syndrome (4;13.8%)- *SLC2A1*; Myoclonus ataxia syndrome (4; 13.8%)- *GLDC, KCTD7, L2HGDH*; DEE unclassified with focal/multifocal seizures (3; 10.3%)- *SUOX, ITPA*; Mitochondrial cytopathy (2; 6.9%)- *MT-TL1, PLP1;* Lennox Gastaut syndrome- Arginase deficiency (1; 3.4%)- *ARG1;* West syndrome- Biotinidase deficiency (1; 3.4%)- *BTD.* Biochemical tests when available were confirmatory in 7 out of 29 (24.1%) and 3 out of 17 who underwent skin biopsies for PME syndromes were confirmatory (17.6%).

Conclusions- Our cohort demonstrates for the first time from the Indian subcontinent that identification of potential metabolic variants by genetic testing can guide investigations and implies therapeutic implications in patients with variable DEE phenotypes. The highest yield is noted in phenotypes such as in PME, GLUT1-DS and DEE unclassified. A high utility is noted given the low yield of available biochemical tests indicating cost-effectiveness of this approach.

Genotype-phenotype correlations of variants of uncertain significance identified in progressive myoclonic epilepsies

Abstract

Rationale- There are limited number of studies which ascertain the yield of genetic testing in progressive myoclonus epilepsy (PME) phenotypes in India.

Methods-A prospective study was carried out in a cohort of patients who met the clinical criteria for electroclinical phenotypes of PME between 2013-2020 and were subsequently investigated by clinical exome sequencing to identify the presence of variants of uncertain significance (VUS).

Results- Thirty four patients diagnosed as probable/possible PME syndromes underwent genetic testing (19 males and 15 females). Twelve patients (35.3%) had a positive family history of either epilepsy or global developmental impairment. Consanguineous parentage was noted in 14 (41.2%). Phenotypes included neuronal ceroid lipofuscinosis in 11(32.4%), Lafora body disease in 2(5.9%), Unverricht Lundborg Disease in 1(2.9%), mitochondrial disorders in 9(26.5%) and possible PME in 11(32.4%). Positive skin biopsies were obtained in only 3 out of 20 patients (15%). Genetic variants were identified in 27(79.4%), of which variants of unknown significance with potential phenotype match (VUS) were identified in 14(41.2%) and no variants were identified in 7 patients (20.6%). VUS were reported in cases where there was a strong and specific correlation between the gene and patient's phenotype.

Conclusion- Our study reports a significant number of VUS in patients with PME and reiterates the need of segregation studies in trios and in silico functional studies to resolve the classification of suspicious PME. Genotype-phenotype correlations need to be robust among VUS.

National Science Day 2022

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For the Poster presentation entitled

"Comparing yield of whole exome sequencing and global screening array for genetic diagnosis of childhood developmental epileptic encephalopathies"

This was organised by Sree Chitra Tirunal Institute for Medical Sciences and Technology on 25 February 2022.

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This certificate is awarded to

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(delegate) for successfully participating in the workshop on EEG on August 3-4,2022 as part of the Silver Jubilee Celebration of R Madhavan Nayar Centre for Comprehensive Epilepsy Care held at Thiruvananthapuram.

This Workshop has been awarded 5 hrs credit points by Kerala State Merdical Council Vide approval letter C1- 11850 / 2022 /MC-CME.

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Dr.Ashalatha Radhakrishnan (Organizing Secretary)
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for presenting the abstract

Functional and structural characterization of variants of uncertain significance identified from whole exome sequencing of developmental and epileptic encephalopathies in an Indian cohort: an *In Silico* approach

35th International Epilepsy Congress

02 - 06 September 2023, Dublin

J Helen Cross
Congress Co-chair

Francesca Sofia
Congress Co-chair

STANDARD OPERATING PROCEDURES

"Genetics of complex Pediatric epilepsy syndromes: electro clinico imaging based genotype-phenotype correlations in an Indian cohort"

Prepared by

ALFIYA F, MANNA JOSE, MOINAK BANERJEE, RAMSHEKHAR N MENON

Funded by: ICMR Ref. No. 5/7/1658/CH/Adhoc/2019-RBMCH dated 13/08/2019

Standard Operating Procedures

DNA isolation

Peripheral blood (5 ml) samples of probands and their parents were collected in EDTA vials (total number of samples collected- 489). The genomic DNA was isolated from lymphocytes obtained from anticoagulated blood. Modified salting out method was used for DNA extraction (Miller et al., 1988). Ten times volume 1X RBC Lysis buffer [155mM NH4Cl, 12mM NaHCO3, 0.1mM EDTA, pH-7.3] was added to 4 ml blood sample and kept for 10 minutes rotation at room temperature. Afterwards, it was centrifuged at 10,000 rpm for 10 minutes and the supernatant containing red blood cells was discarded. The WBC pellet was resuspended in equal volume of Tris-EDTA buffer (1M Tris, 0.5M EDTA, pH-8.0), to which 32 µl proteinase K (20mg/ml) and 100 µl of SDS (20 %) were added. This solution was then incubated at 55°C for 60 minutes followed by 15 minutes incubation at 65°C. During this step, the SDS ruptures the WBCs and the proteins in the cells is digested by the action of proteinase K. 1.52 ml of 4M NaCl was then added followed by equal volume of Chloroform: Isoamyl alcohol (24:1). The samples were rotated for 10 minutes and then centrifuged at 6000 rpm for 15 minutes at room temperature. The aqueous layer alone was collected and double the volume of absolute alcohol was added and mixed gently. The lump of DNA was collected into 1.5 ml fresh centrifuge tube. The DNA was washed twice in 70% alcohol. The pellet was then dried and dissolved in TE buffer and stored at -20°C. DNA isolation of 414 samples including 156 probands was completed. The quantity and quality of DNA was analyzed using Nanodrop. The ratio of absorbance at 260 nm and 280 nm was used to estimate the purity of the DNA. A ratio between 1.7-1.9 was considered as good quality DNA without protein contamination. The concentration of 1µl DNA was quantified and samples were further diluted (50ng/µl) for exome sequencing.

Whole exome sequencing

Whole-Exome sequencing libraries were prepared using **SureSelectXT Human All Exon V6+UTR**. Biotinylated oligonucleotide capture probes, also called as baits, that are designed for the human exons were provided with the kit and used to enrich the region of interest (whole exome) by hybridization. The workflow involves shearing of DNA, repairing ends, adenylation of 3' ends, followed by adapter ligation. At each step the products were purified. The adapter sequences were added onto the ends of DNA fragments to generate paired-end libraries. The resulting adaptor-ligated libraries were purified, qualified and hybridized with an

exomespecific biotinylated capture library. After hybridization, the targeted molecules were captured on streptavidin beads. The resulting enriched DNA libraries were multiplexed by adding index tags by amplification, followed by purification. Indexed captured library DNA were assessed to check the quality and quantity of the captured libraries. Finally, indexed captured library DNA were sequenced on Illumina HiSeq series to generate 2X150bp sequence reads at 80 - 100X sequencing on target depth. The generated sequence data were checked for necessary quality control. A minimum of 75% of the sequenced bases were of Q30 value. Sequenced data were processed to generate FASTQ files.

Bioinformatic analysis

The data were analysed using various Bioinformatics tools and customised in house pipelines and annotated with publicly available databases to identify the risk variants associated with epilepsy and DEE. The quality of raw data which is in fastq format was performed using FASTQC and the adapters were removed using cutadapt tool. BWA (Burrows-Wheeler Aligner) was used to align raw fastq data to the human reference genome (hg19/GRCh37). The SNPs and INDELs were identified following the best practise standards provided by the Genome Analysis Toolkit (GATK). ANNOVAR was used to annotate the variants detected. The variants which passed the depth and quality filter were classified based on inheritance pattern including homozygous recessive, Autosomal dominant, Compound heterozygous, X-linked and De novo variants. In the primary analysis, variant reporting was restricted to 2812 genes associated with Epilepsy, intellectual disability, developmental delay and autism spectrum disorder. The in silico prediction tools CADD, SIFT, PolyPhen2, MutationTaster, and REVEL score were used to analyse the possible harmful impacts of the variations. Variants having a CADD score of less than 20 and an ExAC allele frequency of greater than 1% were eliminated. The variants were ranked based on the phenotypes of the proband by manually reviewing the evidence from several databases such as ClinVar, OMIM, HPO, and literature searches. The variants were classified based on American College of Medical Genetics and Genomics (ACMG) classification criteria.

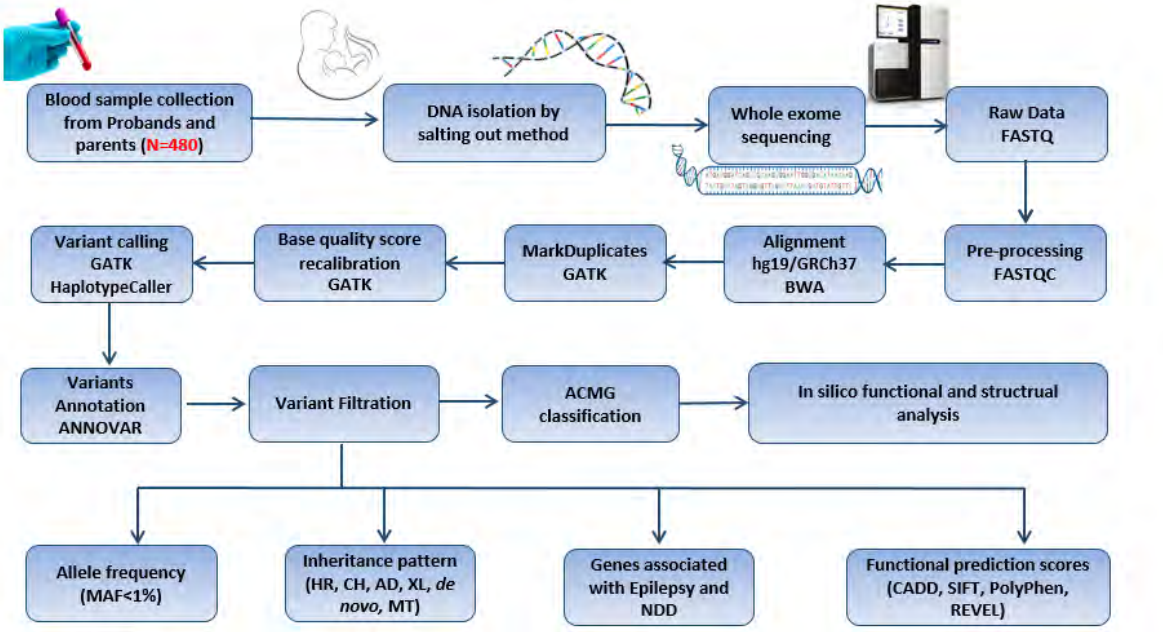


Figure 1: Workflow of Whole exome sequencing analysis

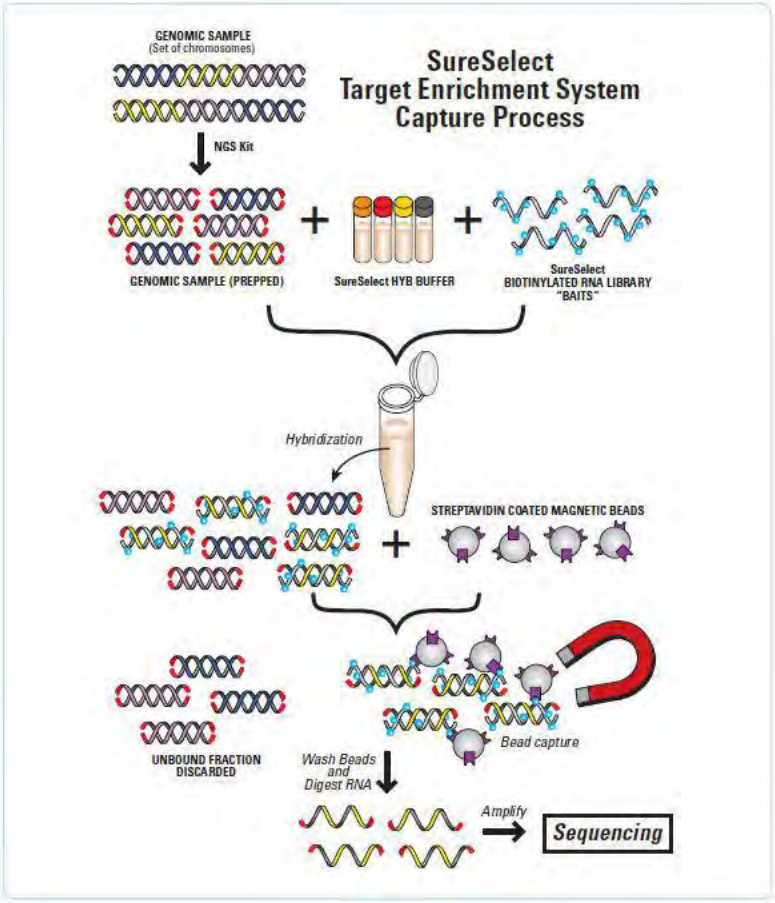


Figure 2: Capture process for whole exome sequencing

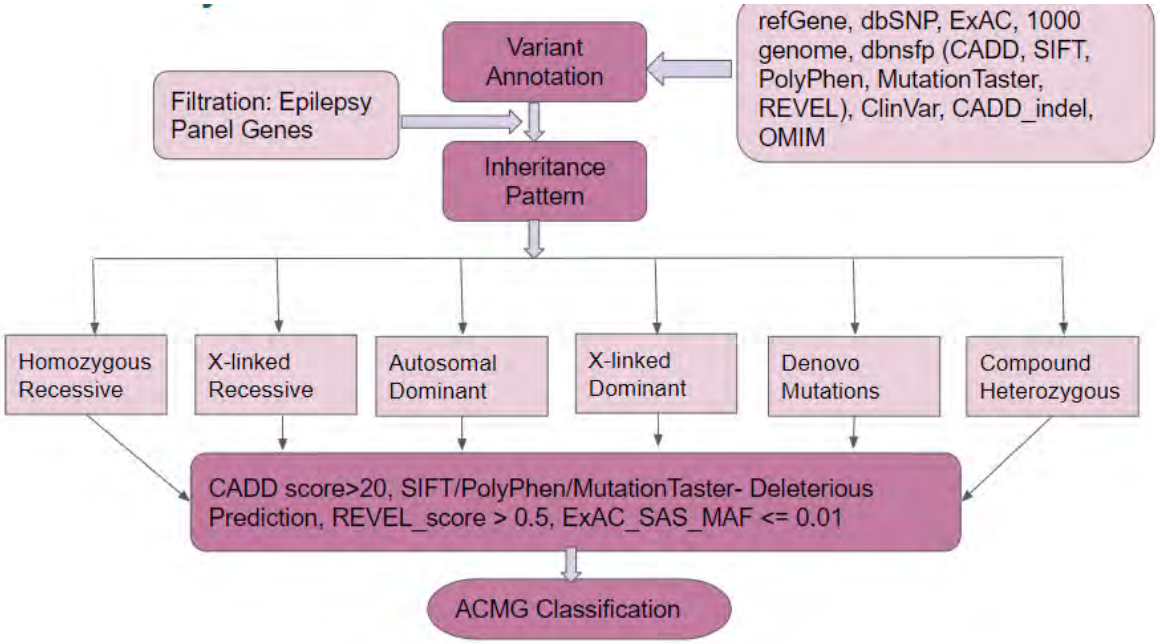


Figure 3: Workflow of variant annotation and filtration

Scripts for Bioinformatic analysis of WES raw data

bwa mem -M -R '@RG\tID:sample\tLB:sample\tPL:ILLUMINA\tpm:HISEQ\tSM:sample' hg19idx R1.fastq.gz R2.fastq.gz > aligned_reads.sam

samtools view -bS aligned_reads.sam > aligned_reads.bam

samtools sort aligned_reads.bam -o sample.sorted.bam

samtools index sample.sorted.bam

java -jar /home/hmg/Desktop/ngs/picard/picard.jar MarkDuplicates -I sample.sorted.bam --REMOVE_DUPLICATES -O dedup_reads.bam -M metrics.txt

samtools index dedup_reads.bam

#gatk HaplotypeCaller -R hg19.fa -I dedup_reads.bam -O raw_variants.vcf

gatk HaplotypeCaller -R hg19.fasta.gz -I dedup_reads.bam -O raw_variants.vcf

gatk SelectVariants -R hg19.fasta.gz -V raw_variants.vcf -select-type SNP --exclude-filtered -O raw_snps.vcf

gatk SelectVariants -R hg19.fasta.gz -V raw_variants.vcf -select-type INDEL --exclude-filtered -O raw_indels.vcf

gatk VariantFiltration -R hg19.fasta.gz -V raw_snps.vcf -filter "QD < 2.0 || FS> 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0 || SOR > 4.0" --filter-name 'basic-snp-filter' -O filtered_snps.vcf

```
gatk VariantFiltration -R  hg19.fasta.gz -V raw_indels.vcf -filter "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0 || SOR > 10.0" --filter-name 'basic_indel_filter' -O filtered_indels.vcf
gatk SelectVariants  -R  hg19.fasta.gz -V filtered_snps.vcf  -select-type SNP --exclude-filtered -O filtered_snps_1.vcf
gatk SelectVariants  -R  hg19.fasta.gz -V filtered_indels.vcf  -select-type SNP --exclude-filtered -O filtered_indels_1.vcf
gatk BaseRecalibrator -R hg19.fasta.gz -I dedup_reads.bam --known-sites filtered_snps_1.vcf --known-sites filtered_indels_1.vcf -O recal_data.table
gatk ApplyBQSR -I dedup_reads.bam -bqsr recal_data.table -O recal_reads.bam
gatk BaseRecalibrator -R hg19.fasta.gz -I recal_reads.bam --known-sites filtered_snps_1.vcf --known-sites filtered_indels_1.vcf -O post_recal_data.table
gatk AnalyzeCovariates -before recal_data.table -after post_recal_data.table -csv analyzecovariates.csv
gatk HaplotypeCaller -R hg19.fasta.gz -bamout EPbamout.bam -I recal_reads.bam -O raw_variants_recal.vcf
gatk SelectVariants -R  hg19.fasta.gz -V raw_variants_recal.vcf  -select-type SNP --exclude-filtered -O raw_snps_recal.vcf
gatk SelectVariants -R  hg19.fasta.gz -V raw_variants_recal.vcf -select-type INDEL --exclude-filtered -O raw_indels_recal.vcf
gatk VariantFiltration -R  hg19.fasta.gz -V  raw_snps_recal.vcf -filter "QD < 2.0 || FS> 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0 || SOR > 4.0" --filter-name 'basic-snp-filter' -O filtered_snps_recal.vcf
gatk VariantFiltration -R  hg19.fasta.gz -V raw_indels_recal.vcf -filter "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0 || SOR > 10.0" --filter-name 'basic_indel_filter' -O filtered_indels_recal.vcf
gatk SelectVariants -R  hg19.fasta.gz -V filtered_snps_recal.vcf  -select-type SNP --exclude-filtered -O filtered_snps_final.vcf
gatk SelectVariants -R  hg19.fasta.gz -V filtered_indels_recal.vcf -select-type INDEL --exclude-filtered -O filtered_indels_final.vcf

#denovo
#varscan
```

samtools mpileup -B -q 1 -f hg19.fasta Father.bam Mother.bam child.bam > trioEP44.mpileup

java -jar /home/hmg/Desktop/ngs/VarScan.v2.3.9.jar trio trio.mpileup trio.mpileup.output --min-coverage 5 --min-var-freq 0.20 --p-value 0.05 -adj-var-freq 0.05 -adj-p-value 0.15

#Trio snp and indels concat (combining vcf of snp and indels of a sinlge sample)

bgzip trio.mpileup.output.snp.vcf
tabix trio.mpileup.output.snp.vcf.gz

bgzip trio.mpileup.output.indel.vcf
tabix trio.mpileup.output.indel.vcf.gz

bcftools concat -a trio.mpileup.output.snp.vcf.gz trio.mpileup.output.indel.vcf.gz -Ov -o TrioVarscan.vcf
#Annotation
perl table_annovar.pl humandb/ -buildver hg19 -out EP -remove -protocol refGene,cytoBand,exac03,avsnp150,dbnsfp41a,clinvar_20200316,revel,gnomad211_genome, intervar_20180118,ALL.sites.2015_08,mcap13,cadd13gt20,caddindel -operation g,r,f,f,f,f,f,f,f,f,f,f -nastring . -vcfinput -polish
#

The compound heterozygous variants were identified using *in silico* tool FMFilter. (https://fmfilter.sourceforge.net/)

Sanger sequencing validation of Pathogenic/Likely pathogenic variants identified

Primer designing

The primers of pathogenic/likely pathogenic variants identified were designed using the NCBI primers. The DNA sequences spanning the variant region for primer designing were obtained from NCBI. The length of primers ranged from 18-22bp. GC content of the primers designed was 50%. The melting temperature of forward and reverse primer, absence of primer dimers, loop formation etc were analyzed using the online tool for Oligocalc. Finally an *in silico* PCR

was done using UCSC genome browser to assure the designed primers amplify the region containing the variant.

PCR amplification

The designed primers were obtained as lyophilized oligonucleotides from Sigma Genosys. The primer is suspended in the required amount of nuclease free water to make to 100uM. The suspended primer stock is stored at -20^{0}C, and diluted to a concentration of 20ul for use in PCR. The constituents of the PCR reactions are as follows: Nuclease free water, 1X buffer, 200uM of deoxynucloetide mixture, 2pmol of primer and 0.5 units of Taq polymerase. 20ng of target DNA is added as sample in the reaction. All the components of the PCR reaction were mixed properly by vortexing. PCR was carried in Thermal cycler.

Cycle sequencing by BIG Dye Terminator

The amplification of DNA by PCR was verified by gel electrophoresis. Sequencing was done using Applied Biosystems Big Dye terminator v3.1 cycle sequencing kit. The sequencing reaction mix consists of nuclease free water, sequencing buffer, 10 microlitre of forward or reverse primer and sequencing reaction mix. The polymerase present in Big Dye terminator kit is Amplitaq Gold polymerase. The final volume of the reaction was made to 10 microlitre.

Clean up of sequencing product

After the sequencing PCR, the products were cleaned up to purify the sequenced DNA from other excess reagents. Sequencing clean up removes the remnants of post cycle sequencing reaction – excess dNTPs, primers, enzymes, unincorporated fluorescent dyes and residual slats. 2uL of 125uM EDTA, 10 microlitre of nuclease free water and 2uL of 3M sodium acetate was added to the sequencing product and vortexed well. The samples for incubated for 15 minutes followed by which centrifugation was done at 10,000 rpm for 20 minute at 4^{0}C. The supernatant was discarded and the pellet was washed with 70% alcohol. After 2 rounds of washing, the DNA pellet was air dried.

Capillary sequencing

Formamide was added to the air-dried samples and was subjected to PCR conditions for 10 minutes at 95^{0}C for denaturation. The denatured samples were loaded into the capillary sequencer for analysis of the sequence. The ABI PRISM DNA analyzer used in the study is automated to handle 96 samples at a time. The long capillaries in the analyzer are filled in by POP7 polymer for resolving the sequences. DNA fragments move towards the end of capillary, with smaller fragments moving faster than longer fragments. The fluorophores in the attached ddNTPs, a specific color fluorescence emission occurs. Thus a four color sequencing emission spectra is generate. DNA sequences upto 1000 bases can be read accurately using automated

sequencer. The sequences obtained can be analyzed using the software sequence analyzer V.1.1.

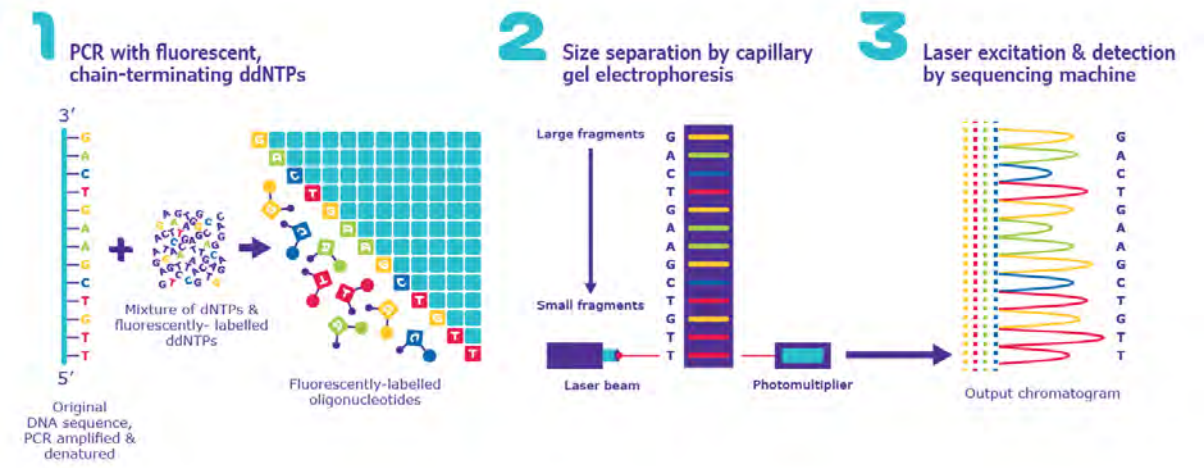
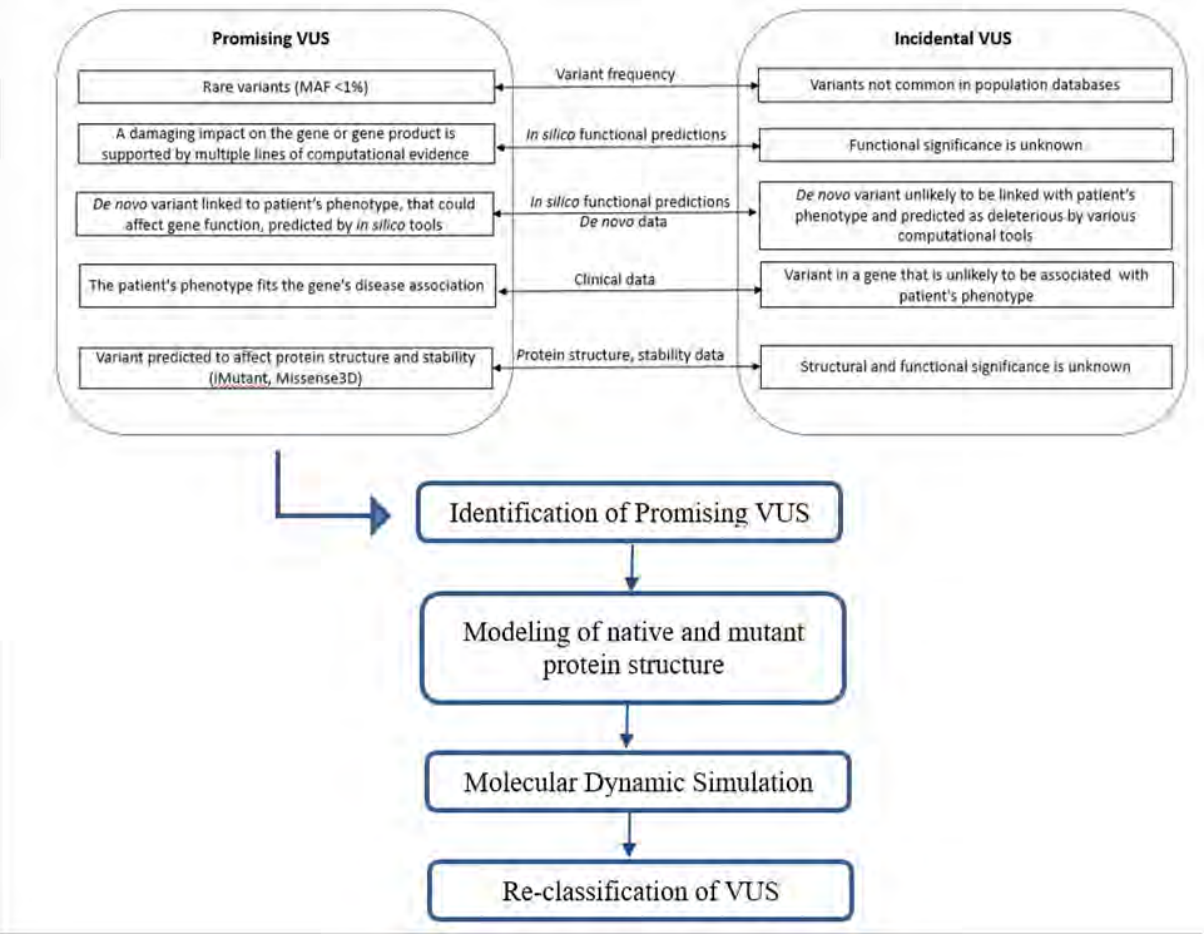


Figure 3: Basic steps in Sanger Sequencing (https://www.sigmaaldrich.com/technical documents/articles/biology/sanger-sequencing.html**)**

In silico functional and structural analysis of deleterious variants



To assess the pathogenicity of VUSs on protein function and structure stability, in silico analysis tools were used based on protein stability, protein interaction pattern and protein 3D structural analysis. To identify the interactions of protein with other relevant proteins, STRING (Search Tool for the Retrieval of Interacting Genes) (https://string-db.org/) was used.

Protein stability is a primary factor affecting the function and activity of biological molecules. Protein unfolding free energy is dependent on protein stability. So, it is important to precisely quantify the influence of mutation on protein stability by analysing the effect of mutation on free energy. IMutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi), an online support vector machine based tools to predict protein stability changes based on single site mutations, was used to predict the stability of protein.

A mutation in a specific site, such as a conserved residue, can cause a change in the protein's structure and function, so, it is crucial to figure out how mutations affect protein structure to predict the pathogenicity of a variant. Missense3D, a web-based tool to predict the structural changes introduced by an amino acid substitution, was used to predict the effect of mutation on protein strutcure. SWISS-MODEL servers were also used for modelling three dimensional protein structures.

Variants of Uncertain significance were grouped into Promising VUS/Incidental VUS according to data- Frequency data of variants, *In silico* functional predictions, *de novo* data, clinical data and protein structure/stability data analysed with computational prediction tools (iMutant, Missense3D, etc). Molecular dynamic simulation was used to re-classify the promising VUS.

Molecular Dynamics simulation

The structure of wild type and mutant protein was modeled using SWISSMODEL and quality assessment was done using Ramachandran plot. Molecular dynamics simulations were performed using GROMACS 5.1.2 software. All systems were soaked in a cubic box of water molecules using the gmx editconf module and gmx solvate module for solvation. All systems were equilibrated a constant temperature (300K), by utilizing the NVT and NPT process for 100 ps. The final simulations were performed for 100ns. Radius of gyration (Rg), Root mean square deviation (RMSD), and Root mean square fluctuation (RMSF), were analysed using GROMACS.

Copy Number variant identification

CoNIFER pipeline was used to detect copy number variants (CNVs) from the Whole exome sequencing data. CoNIFER is a tool that detects copy number variants and genotypes the copy number of duplicated genes using exome sequencing data. In order to remove persistent and significant capture biases in exome reactions across sample batches, singular value decomposition (SVD) was used to process exome data. CoNIFER removes batch biases and allows exome sequences from numerous experimental runs to be mixed. CoNIFER can reliably identify rare CNVs and estimate the copy number of duplicated genes up to about eight copies using current exome capture kits when used in conjunction with a short read aligner like mrsFAST, which can align reads to multiple sites. (**Figure 3**)

```
python conifer.py rpkm \
       --probes Exome-Agilent_V6.bed \
       --input $sample"_recal_reads.bam" \
       --output RPKM/$sample".rpkm.txt"
done
python conifer.py analyze \
       --probes Exome-Agilent_V6.bed \
       --rpkm_dir ./RPKM/ \
       --output analysis.hdf5 \
       --svd 2 \
       --write_svals singular_values.txt \
       --plot_scree screeplot.png \
       --write_sd sd_values.txt
python conifer.py call \
       --input analysis.hdf5 \
       --output calls.txt
```

Annotation and ACMG classification of CNVs

ClassifyCNV is a command-line tool that implements the 2019 ACMG guidelines to evaluate the pathogenicity of germline duplications and deletions. In accordance with the ACMG criteria, the programme computes a pathogenicity score each copy-number variant (CNV) using pre-prepared, publicly available databases. (**Figure 4**)

```
python3 ClassifyCNV.py –infile file.hg19.bed --GenomeBuild hg19 --precise
```

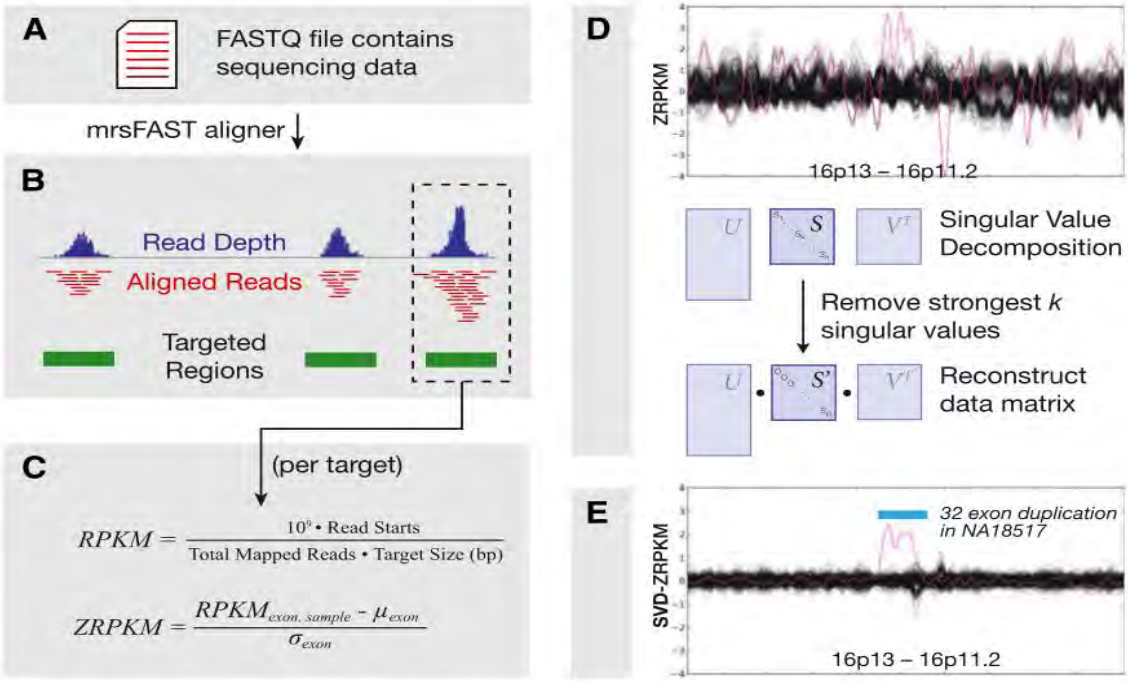


Figure 3: CoNIFER pipeline to detect CNVs (https://conifer.sourceforge.net)

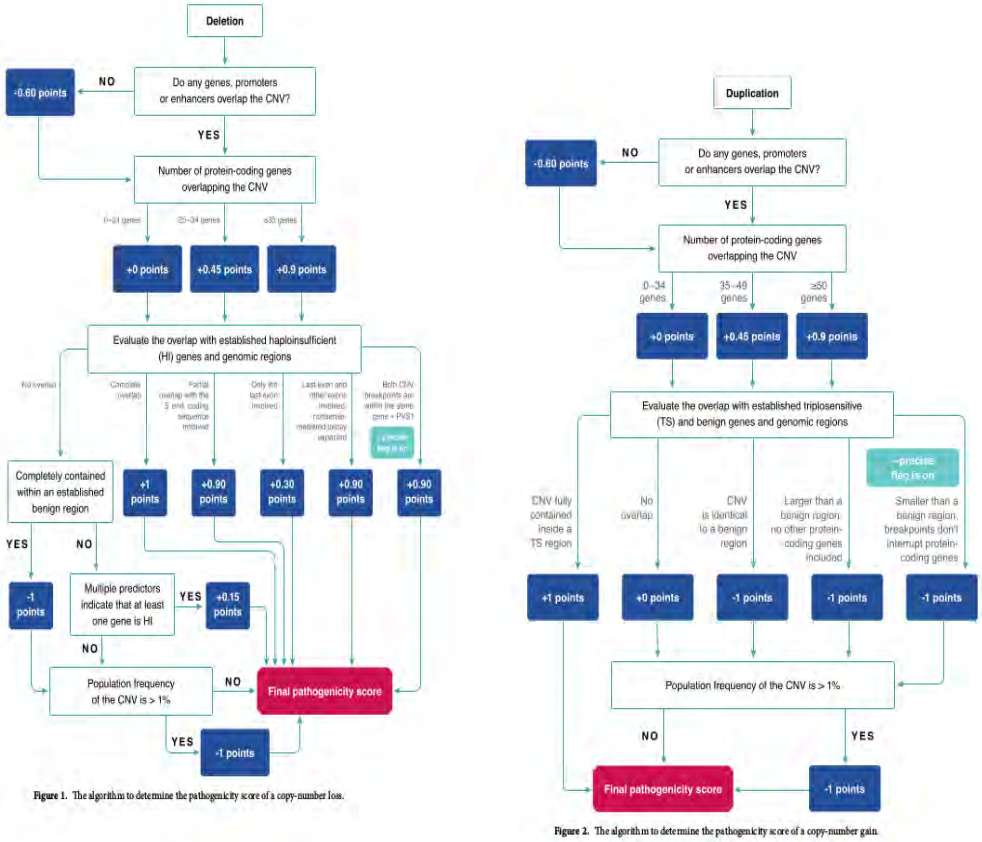


Figure 4: ACMG classification of CNVs using ClassifyCNV tool (https://github.com/Genotek/ClassifyCNV)