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CLINICAL AND GENETIC STUDY OF DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHY IN ARGENTINEAN PEDIATRIC PATIENTS

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Abstract **Introduction**: The aim of this study was to extend our knowledge of the genetic background of Argentinean pediatric patients with developmental and epileptic encephalopathy (DEE) applying a next generation sequencing (NGS) panel. **Methods**: Thirty one patients with DEE were studied, including these phenotypes: Dravet syndrome (n:7), Dravet like syndrome (n:3), West syndrome (WS) (n:6), WS that evolved to Lennox-Gastaut syndrome (LGS) (n:4), epilepsy of infancy with migrating focal seizures (n:2), continuous spikes and waves during slow sleep evolving to LGS (n:1), LGS (n:1), myoclonic status in non-progressive encephalopathy (n:1), myoclonic atonic epilepsy (n:1), epileptic encephalopathy with multifocal spikes (n:1) and unclassified epileptic encephalopathy (n:4). Fifty-two genes frequently associated with DEE were studied by NGS in genomic DNA from peripheral blood. **Results**: Relevant variants were detected in 12 cases; 6 novel pathogenic or likely pathogenic variants, 6 previously reported as pathogenic and 1 variant of unknown significance. Single-nucleotide heterozygous variants were identified in the *SCN1A* (5), *GABRG2* (1), *STXBP1* (2) genes, a mosaic variant in *SCN2A* (1) and a homozygous variant in *SCN1B* (1). Additionally, a heterozygous deletion involving the *SCN1A, SCN2A* and *SCN3A* genes (1), and the most frequent triplet repeat expansion in the *ARX* gene (1) were detected. **Discussion**: Genetic diagnosis was made in 39% of patients. We emphasize the importance of considering mosaic variants, copy number variants and hereditary forms when designing and interpreting molecular studies, to optimize diagnosis and management of patients. Approximately 42% of the detected variants were novel, expanding the knowledge of the molecular basis of DEEs in Latin-American patients.

Key words: developmental and epileptic encephalopathy, NGS panel, molecular diagnosis, novel gene variants, mosaicism, copy number variants

Resumen ***Estudio clínico y genético de encefalopatía epiléptica y del desarrollo en pacientes pediátricos argentinos***

Introducción: El objetivo del estudio fue ampliar el conocimiento de las bases moleculares de las encefalopatías epilépticas y del desarrollo (EED) en pacientes pediátricos argentinos aplicando un panel de secuenciación de nueva generación (NGS). **Métodos**: Se analizaron 31 pacientes con los fenotipos clínicos de síndrome de Dravet (n:7), síndrome símil Dravet (n:3), síndrome de West (SW) (n:6), SW que evoluciona a síndrome de Lennox Gastaut (SLG)(N:4), epilepsia de la infancia con crisis focales migratorias (n:2), actividad de punta onda continua durante el sueño que evolucionan a SLG (n:1), SLG (n:1), encefalopatía no progresiva con estatus mioclónico (n:1), epilepsia mioclónica atónica (n:1), encefalopatía epiléptica con espigas multifocales (n:1) y encefalopatía epiléptica indeterminada (n:4). Se estudiaron los 52 genes más frecuentemente asociados a EED a través de NGS, en ADN extraído de sangre periférica. **Resultados**: Se identificaron variantes relevantes en 12 casos, de las cuales 5 fueron nuevas y 6 previamente reportadas como patogénicas o posiblemente patogénicas, mientras que una variante fue clasificada como de significado incierto. Variantes heterocigotas, de nucleótido único, se identificaron en los genes *SCN1A* (5), *GABRG2* (1), *STXBP1* (2), una variante en mosaico en *SCN2A* (1) y otra homocigota en *SCN1B* (1). Además, se detectó una deleción que involucra a los genes *SCN1A, SCN2A* y *SCN3A* (1) y la expansión de repeticiones de tripletes más frecuente en el gen *ARX* (1). **Discusión**: Se alcanzó el diagnóstico molecular en el 39% de los pacientes. Remarcamos la importancia de considerar variantes en mosaico, variantes en el número de copias y formas heredadas al momento de diseñar e interpretar los estudios moleculares, de tal forma de optimizar el diagnóstico y seguimiento de los pacientes con EED. Cabe destacar, que el 42% de las variantes detectadas fueron nuevas, ampliando nuestro conocimiento sobre las bases moleculares de las EED en población latino americana.

Palabras clave: encefalopatía epiléptica y del desarrollo, panel de NGS, diagnóstico molecular, variantes genéticas nuevas, mosaicismo, variantes en el número de copias

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KEY POINTS

- The developmental and epileptic encephalopathy are a clinically and genetically heterogeneous group and it is often challenging to determine their genetic cause.
- Genetic testing is essential to prevent unnecessary and potentially harmful diagnostic procedures and management, and to determine precision medicine strategies.
- A genetic diagnosis was made in 39% of the patients and approximately 42% of the detected variants were novel.
- We emphasize the importance of considering mosaic variants, copy number variants and hereditary forms when designing and interpreting molecular studies, in order to optimize diagnosis and management of these patients.

The International League Against Epilepsy (ILAE) defines epileptic encephalopathy as a condition in which the epileptic form activity itself contributes to severe cognitive and behavioral impairments beyond what expected from the underlying pathology alone (such as a cortical malformation)[1].

In 2017, the new concept of "developmental and epileptic encephalopathy" (DEE) was introduced for those cases in which the developmental consequences arise directly from the effect of a genetic variant, in addition to the effect of the frequent epileptic activity on development [2].The recognition of DEE is critical, as there is a treatable component with the potential to improve the developmental outcome and cognition. Optimal management depends on a number of factors, such as the age of the patient, comorbidities, current medications and, most importantly, the underlying etiology, which seems to be mainly genetic[3].

The DEEs are a clinically and genetically heterogeneous group and it is often challenging to determine their genetic cause. Major advances in molecular diagnosis over the past decades have contributed to our increased knowledge on the underlying causes of DEEs and genotype-phenotype correlations and have led to a rapid increase in the discovery of genes across the human disorders[4–6]. However, most genomic data is reported from developed countries, whereas data from developing countries is underrepresented.

The search term "developmental and epileptic encephalopathy" led to more than 250 entries associations in the Online Mendelian Inheritance in Man database (OMIM) demonstrating the wide genetic heterogeneity underlying the DEEs.

Next generation sequencing (NGS) is a relatively new technique, which allows the simultaneous study of hundreds of genes. It is being applied to genetic testing of diseases or syndromes that are caused by a single or by many different genes, and has the potential to detect causal variants, including *de novo* and familial variants associated with DEEs and, due to the variable phenotypic presentations of the latter disorders, to greatly improve molecular diagnosis[7].

Genetic testing is essential to determine precision medicine strategies and to prevent unnecessary and potentially harmful diagnostic procedures and management. A genetic diagnosis can also provide useful information on the natural history and prognosis of a disease and facilitate better targeted genetic counseling. Furthermore, it allows the subject and their family to enter gene-specific networks of families with the same condition[3, 8, 9].

The aim of this study was to extend our knowledge of the genetic background of Argentine children with DEE seen at a public tertiary pediatric hospital, applying a targeted NGS panel designed to analyze 52 epilepsy candidate genes in a group of 31 DEE patients.

Materials and methods

Study population

We retrospectively analyzed 31 patients with DEE diagnosed and followed at the Department of Neurology of *Hospital de Pediatría J. P. Garrahan*, a public tertiary-care pediatric center, between 2019 and 2020. We included patients with DEE with age at onset younger than 12 months and an unknown etiology after ruling out structural abnormalities. The occurrence of metabolic disorders and/or large chromosomal abnormalities were investigated according to the epileptic syndrome. The following study variables were analyzed: sex, age at onset, family history, seizure semiology and neurodevelopment. Standard electroencephalography (EEG) and/or video EEG recordings and magnetic resonance imaging (MRI) were reviewed. Epileptic syndromes were diagnosed and classified according to the ILAE classification[2, 10].

This study was approved by our Institutional Ethics Committee. Written informed consent for the study was obtained from patients, parents and/or tutors.

Molecular studies

Genomic DNA was extracted from peripheral blood using Chemagic 360 (PerkinElmer, USA). A panel of the fifty-two genes (Table 1) most frequently associated with DEEs was designed based on bibliographic references using a customized enrichment strategy (SureSelect XT Low Input, Agilent Technologies, USA) which was run following the manufacturer's protocols. The obtained enriched libraries were sequenced for paired-end reads of 150 bp in a MiSeq sequencer using the MiSeq Reagent Kit v2 (300-cycles) (Illumina, USA) with a mean coverage depth of 250X per sample.

After sequencing, raw data were processed using an in-house bioinformatic pipeline to detect single nucleotide variants (SNV) and copy number variants (CNV) in all 31 cases according to international guidelines, as previously published by our group[11].

The high coverage-depth obtained allowed us to analyze the presence of mosaic variants. Alternate allele fraction (AAF) (mosaic variant reads/total reads) was calculated using the data generated by NGS. For autosomal variants and X-linked variants in females, a variant was considered possibly mosaic if the AAF was less than 36% or greater than 64% by NGS analysis, while AAF higher than 10% was used as a threshold to identify mosaic variants in X-linked genes in males[12].

TABLE 1.– *Gene panel related to developmental and epileptic encephalopathies tested by next generation sequencing*

AARS	*CHD2*	*GABRG2*	*KCNQ3*	*QARS*	*SLC6A8*
ADSL	*CNTNAP2*	*GAMT*	*KCNT1*	*SCN1A*	*SLC9A6*
ALDH7A1	*CTSO*	*GATM*	*MECP2*	*SCN1B*	*STXBP1*
ALG13	*DNM1*	*GRIN1*	*PCDH19*	*SCN2A*	*TBC1D24*
ARX	*FOLR1*	*GRIN2A*	*PIGA*	*SCN8A*	*UBE3A*
CACNA1A	*FOXG1*	*GRIN2B*	*PLCB1*	*SLC12A5*	*WWOX*
CACNA2D2	*GABRA1*	*HCN1*	*PNKP*	*SLC25A22*	*ZEB2*
CASK	*GABRB2*	*KCNA2*	*PNPO*	*SLC2A1*	–
CDKL5	*GABRB3*	*KCNQ2*	*POLG*	*SLC6A1*	–

The presence of the genetic variants was confirmed by Sanger sequencing and/or array comparative genomic hybridization (CGH) (SurePrint G3 8x60K, Agilent Technologies, USA), as necessary. Variant classification was performed according to the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines[13]. When pathogenic or likely pathogenic variants were found, samples from the patient's presumed biological parents were tested by Sanger sequencing to classify the case as being hereditary (present in one or both parents) or *de novo* variants (present only in the patient). All novel variants were submitted to ClinVar NCBI database.

In patients with Dravet syndrome (DS) and Dravet like syndrome (DLS) without relevant single nucleotide variants, multiplex ligation-dependent probe amplification (MLPA) for the *SCN1A* gene was performed using SALSA MLPA probemix P137 (MRC-Holland, Amsterdam) according to the manufacturers' instructions.

Results

This report includes genomic results obtained from 31 pediatric patients with DEEs with the following clinical phenotypes: DS (n:7), DLS (n:3), West syndrome (WS) (n:6), WS that evolved to Lennox-Gastaut syndrome (WS/LGS) (n:4), epilepsy of infancy with migrating focal seizures (EIMFS) (n:2), continuous spikes and waves during slow sleep (CSWSS) that evolved to LGS (n:1), LGS (n:1), myoclonic status in non-progressive encephalopathy (MSNE) (n:1), myoclonic atonic epilepsy (n:1), EE with multifocal spikes (n:1) and unclassified epileptic encephalopathy (UEE) (n:4). Eighteen of the patients were female. Median age at symptoms onset was 4.8 months (range 0-12 months).

Relevant variants were detected in 12 cases (39%) and the main clinical characteristics of these patients are shown in Table 2. Of these 12 cases, five showed novel pathogenic or likely pathogenic variants, six were previously reported as pathogenic and one was classified as variant of unknown significance (VUS) (Table 3).

Relevant single-nucleotide heterozygous variants were identified in the *SCN1A* (sodium voltage-gated channel alpha subunit 1), *GABRG2* (gamma-aminobutyric acid type A receptor subunit gamma2) and *STXBP1* (syntaxin binding protein 1) genes, a mosaic variant in *SCN2A* (sodium voltage-gated channel alpha subunit 2) and one homozygous variant in *SCN1B* (sodium voltage-gated channel beta subunit 1*)*. Additionally, a heterozygous deletion involving the *SCN1A, SCN2A* and *SCN3A* (sodium voltage-gated channel alpha subunit 3) genes was detected in one patient, and the most frequent triplet repeat expansion in the *ARX* (aristaless related homeobox) gene was observed in another one in hemizygous state (Table 3 and Fig. 1).

Dravet and Dravet like syndrome

We identified pathogenic variants in the *SCN1A* gene in four cases with DS; one was missense, one was nonsense and two were frameshift variants. One of them was novel while the remaining three had been previously reported[14-16]. All alterations were *de novo.*

Three of the four variants identified in the *SCN1A* gene generate a premature stop codon and are predicted to give rise to truncated proteins that affect normal function of the alpha subunit of the sodium channel $Na_v1.1$. The variants detected in patient #4 (p.Cys336ValfsTer16) and patient #7 (p.Arg1636Ter) have been previously reported as associated with DS while the frameshift variant identified in patient #8 (p.Phe1831fsTer6) was *novel*. This latter variant was classified as pathogenic (ACMG criteria: PVS1, PM2, PM6, PP4). The variant c.4178 A>C p.(His1393Pro) detected in patient #6 is located within the extracellular loop, connecting the fifth and sixth segment in the third domain of the sodium channel protein $Na_v1.1$, that involves the gate and voltage-sensing region of the channel. This pathogenic variant has been previously reported in a patient with DS[14-16].

Finally, in patient #1 with DS we identified a novel homozygous missense variant in the *SCN1B* gene (p.Glu56Lys) affecting a highly conserved amino acid in the extracellular domain of the beta-1 subunit of the sodium channel. The variant was classified as likely pathogenic (ACMG criteria: PM1, PM2, PP2, PP3, PP4). This

TABLE 2.– *Summary of clinical information of patients with a molecular diagnosis*

Patient ID	Diagnosis	Gender	Age of onset	Current age	Type of seizures	Additional clinical findings	Initial EEG	Current EEG	Brain MRI	Treatment
1	DS	F	6 m	3.5 y	FSE, MS	GDD	Normal	Normal	Normal	LVT, VPA, KD
2	EIMFS	F	3 m	3 y	FS, MS, Focal Status	GDD	Asymmetric bilateral rhythmic spike alternate in both hemispheres	Rhythmic sharp activity in the θ to α frequency range involving the Rolandic region	Normal	LVT, TPM, DCI, KD
3	EIMFS	F	2 d	3 y	FMS	GDD	Disorganization of background, slowing, high amplitude, spike activity, burst suppression	Diffuse slow waves	Corpus callosum dysgenesis	LVT, TPM, CBZ, KD, PHT, VPA, VGB, CBD
4	DS	F	6 m	3 y	MFSE, CS	GDD	Focal spikes	Focal spikes	Normal	LVT, TPM,
5	CSWSS/ LGS	M	1 d	4 y	MS, FS, TS, TCS	GDD, Arthrogryposis	Slow waves	Epileptic encephalopathy with continuous spike and waves during sleep	Normal	CLB, DCI
6	DS	F	5 m	7 y	FSE, TS, MS	GDD	Normal	Diffuse slow waves	Normal	LVT, DCI, TPM, KD
7	DS	M	5 m	3 y	FS, FSE	GDD	Normal	Normal	Normal	LVT, CLB
8	DS	M	10 m	13 y	FSE, FS	ID, Ataxia	Normal	Normal	Unilateral hippocampal sclerosis	LVT, CLB VPA, CLB, CBD
9	WS/LGS	M	3 m	7 y	IS, CS, MS, TS	GDD	Hypsarrhythmia	Disorganization of background, slowing, high amplitude, spike activity, burst suppression	Normal	LVT, VPA, TPM, CLB
10	WS/LGS	F	2 m	11 y	IS, TS, TCS	GDD, Ataxia	Hypsarrhythmia	Diffuse slow spike and slow waves (< 2.5 Hz) activity	Normal	CLB, TPM, VPA, KD
11	MSNE	F	6 m	7 y	MS	ID, Ataxia	Subcontinuous multifocal slow spike-waves, predominating in frontocentral regions	Multifocal slow spike-waves	Normal	VPA, ETS, TPM, KD
12	LGS	F	12 m	4 y	FOS	GDD	Spike waves	Multifocal spikes	Normal	LVT, ETS, CLB, KD

EEG: electroencephalogram; MRI: magnetic resonance imaging; DS: Dravet syndrome; EIMFS: epilepsy of infancy with migrating focal seizures; CSWSS: continuous spikes and waves during slow sleep; LGS: Lennox-Gastaut syndrome; WS: West syndrome; MSNE: myoclonic status in non-progressive encephalopathy; F: female; M: male; d: days; m: months; y: years; FSE: febrile status epilepticus; MS: myoclonic status; FS: febrile seizure; FMS: focal myoclonic seizure; MFSE: multiple febrile status epilepticus; CS: clonic seizure; TS: tonic seizure; TCS; tonic clonic seizure; IS: infantile spasms; FOS: focal seizure; GDD: global developmental delay; ID: intellectual disability; LVT: levetiracetam; VPA: valproic acid; KD: ketogenic diet; TPM: topiramate; DCI: rufinamide; CBZ: carbamazepine; PHT: phenytoin; VGB: vigabatrin; CBD: cannabidiol; CLB: clobazam; ETS: ethosuximide.

TABLE 3.– *Relevant variants identified by a multigenic next generation sequencing- panel for developmental and epileptic encephalopathies*

Patient	Gene	Zygosity	Inheritance	Transcript (Refseq)	Variant	SIFT	Poly Phen 2	Mutation taster	ACMG	Reported
1	*SCN1B*	Hom	*Both parents*	NM_199037.4	c.166G>A p.(Glu56Lys)	T	PD	DC	LP	Novel
2	*SCN1A-SCN2A-SCN3A*	Het	*De novo*	/	arr[GRCh37] 2q24.3 (165903731x2, 166011638_166918961x1, 166925143x2)	/	/	/	P	Novel
3	*SCN2A*	Mosaic (17%)	*Mosaicism (17%)*	NM_021007.2	c.4018G>A p.(Val1340Ile)	D	PD	DC	P	Novel
4	*SCN1A*	Het	*De novo*	NM_001165963.1	c.1005del p.(Cys336ValfsTer16)	/	/	/	P	PMID: 24168886
5	*SCN1A*	Het	*Presumably de novo**	NM_001165963.1	c.4036T>C p.(Ser1346Pro)	D	PD	DC	LP	Novel
6	*SCN1A*	Het	*De novo*	NM_001165963.1	c.4178A>C p.(His1393Pro)	T	Benign	Polymorphism	P	PMID: 17129991
7	*SCN1A*	Het	*De novo*	NM_001165963.1	c.4906C>T p.(Arg1636Ter)	/	/	DC	P	PMID: 21248271
8	*SCN1A*	Het	*De novo*	NM_001165963.1	c.5489dup p.(Phe1831fsTer6)	/	/	/	P	Novel
9	*ARX*	Hem	*Maternal*	NM_139058.3	c.315_335dup p.(Ala109_Ala115dup)	D	PD	DC	P	PMID: 12376946
10	*STXBP1*	Het	*De novo*	NM_003165.3	c.874C>T p.(Arg292Cys)	D	PD	DC	P	PMID: 24781210
11	*STXBP1*	Het	*Presumably de novo**	NM_003165.3	c.1060T>C p.(Cys354Arg)	D	PD	DC	P	PMID: 23708187
12	*GABRG2*	Het	*ND*	NM_198903.2	c.403C>T p.(Leu135Phe)	D	PD	DC	VUS	ClinVar ID: 850807

*Refseq: reference sequence according to NCBI; ACMG: American college of medical genetics and genomics; Hom: homozygous; Het: heterozygous; Hem: hemizygous; T: tolerated; D: damaging; PD: probably damaging; DC: disease causing; LP: likely pathogenic; P: pathogenic; VUS: variant of unknown significance; ND: not determined; *father not available.*

variant is predicted to be probably damaging and disease-causing by PolyPhen-2 and Mutation Taster, respectively, suggesting that this novel variant might affect protein activity and function. Both parents are heterozygous for the variant and declared that there is no consanguinity within the family.

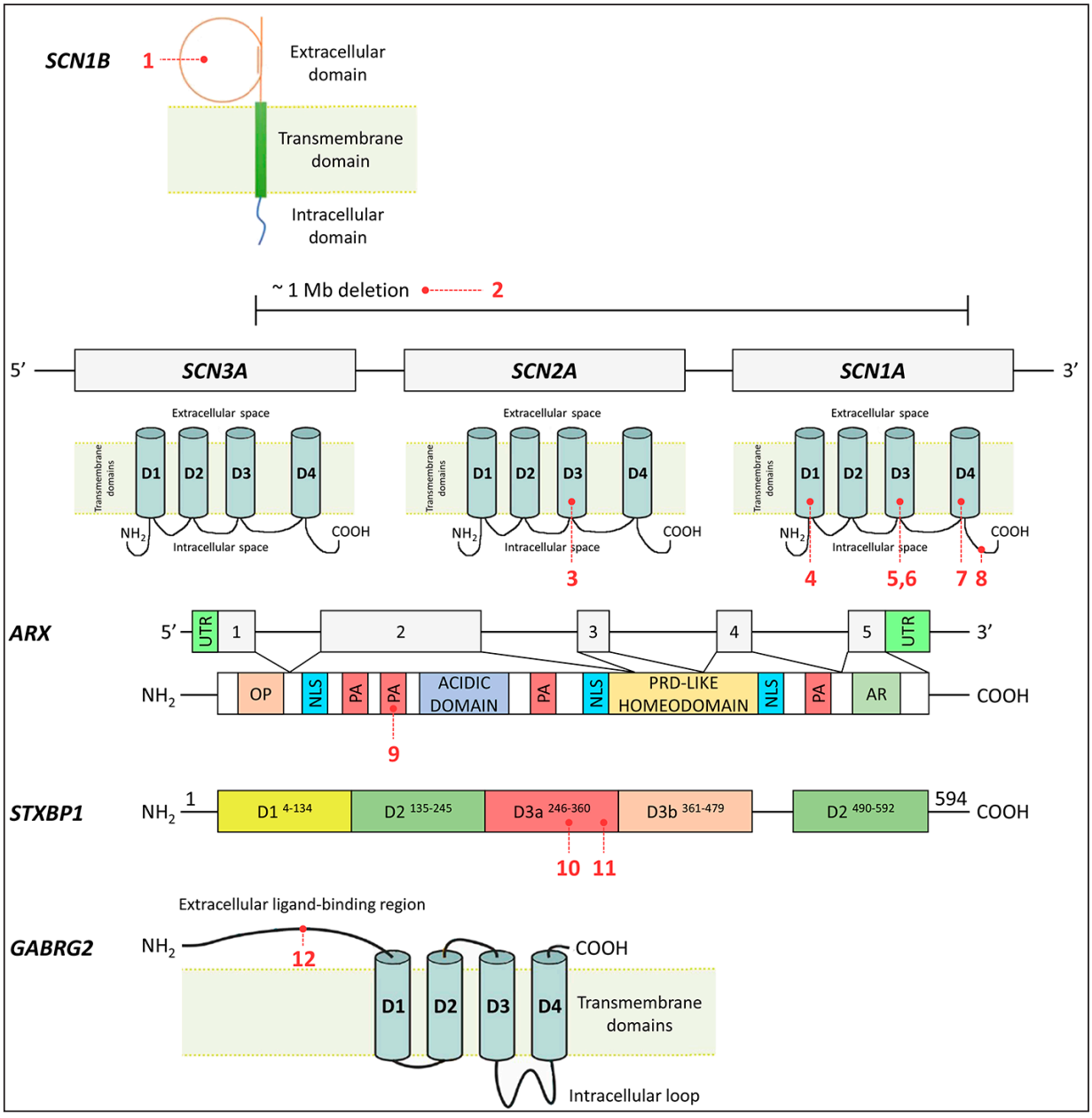
In the 3 patients with DLS no relevant variants were detected.

MLPA for CNVs in the *SCN1A* gene was performed in five patients without relevant variants and resulted negative in all of them.

Epilepsy of infancy with migrating focal seizures

Two relevant novel variants were identified associated with sodium channels. In patient #2, we found a mosaic variant in the *SCN2A* gene (p.Val1340Ile) with approximately 17% of the AAF in leucocytes. This variant affects the fifth transmembrane segment of the third domain of the sodium channel protein type 2 subunit alpha, that involves the gate and voltage-sensing region. *In silico* tools suggest that this novel variant could be deleterious for protein activity and function. Secondly, in patient #3, we identified a CNV

Fig. 1.– Schematic representation of the genes, protein domains and associated variants detected (indicated with patient numbers)



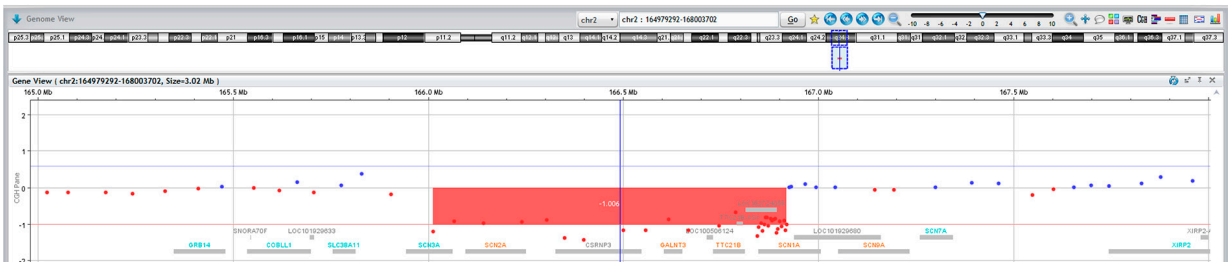
D: domain; UTR: untranslated region; OP: octapeptide domain; NLS: nuclear localization signals; PA: poly-alanine expansion repeats; AR: Aristaless domain/C-peptide; SCN1B: sodium voltage-gated channel beta subunit 1; SCN3A: sodium voltage-gated channel alpha subunit 3; SCN2A: sodium voltage-gated channel alpha subunit 2; SCN1A: sodium voltage-gated channel alpha subunit 1; ARX: aristaless related homeobox; STXBP1: syntaxin binding protein 1; GABRG2: gamma-aminobutyric acid type A receptor subunit gamma 2.

that affects the *SCN3A, SCN2A* and *SCN1A* genes. This variant was detected through a bioinformatics pipeline applied to the NGS panel and was confirmed by array-CGH (Figure 2). Both novel alterations were classified as pathogenic and their presence is in agreement with the clinical phenotype of the EIMFS patients[9].

West syndrome/Lennox-Gastaut syndrome

We identified two previously reported variants in patients with WS that evolved to LGS, in the *ARX* and *STXBP1* genes. In patient #9 we detected one of the most common polyalanine expansion mutations in the *ARX* gene

Fig. 2.– Array comparative genomic hybridization results confirming the deletion in chromosome 2



The red box indicates the size of the genomic deletion. Online Mendelian Inheritance in Man (OMIM) genes: SCN3A: sodium voltage-gated channel alpha subunit 3; SCN2A: sodium voltage-gated channel alpha subunit 2; SCN1A: sodium voltage-gated channel alpha subunit 1; GALNT3: polypeptide N-acetylgalactosaminyltransferase 3; TTC21B: tetratricopeptide repeat domain 21B

c.315_335dup; p.(Ala109_Ala115dup), which is predicted to expand the original 16 alanine residues to 27 alanine residues in the first polyalanine tract of the homeobox protein ARX, a transcription factor required for normal brain development[17]. Since NGS studies have limitations to detect triplet repeat expansions, this variant was confirmed by Sanger sequencing due to strong clinical suspicion. The variant was classified as pathogenic and was also present in the patient's mother.

In patient #10 we identified a recurrent *de novo* variant in the *STXBP1* gene (p.Arg292Cys) associated with WS[18]. The variant affects a highly conserved amino acid in the domain 3a of the syntaxin-binding protein and was classified as pathogenic.

Besides, we identified a previously reported missense variant (p.Leu135Phe) in the *GABRG2* gene in a patient with LGS (#12). *In silico* tools strongly suggest that this variant could be deleterious for protein activity and function; however, we consider that there is not enough clinical evidence to confirm the deleterious impact of the variant and therefore we have classified it as VUS (ACMG criteria: PM2, PP2, PP3). At the same time, the variant was identified in his healthy father, indicating possible incomplete penetrance, as previously reported by other authors [19,20].

Developmental and epileptic encephalopathy with a particular electroclinical pattern

We identified a novel variant in the *SCN1A* gene in patient #5 who had CSWSS that evolved to LGS. The variant (p.Ser1346Pro) detected in the *SCN1A* gene is predicted to affect a topological domain in the intracellular loop, between the fourth and fifth segment in the third domain of the $Na_v1.1$ protein. It is predicted to be deleterious, probably damaging and disease-causing by SIFT/PROVEAN, PolyPhen-2 and Mutation Taster, respectively, suggesting that this novel variant might affect normal protein activity and function. It was absent in the mother's sample while the father's sample was not available. The variant was classified as likely pathogenic (ACMG criteria: PM1, PM2, PP2, PP3, PP5).

In patient #11, who presented with a myoclonic status, a presumably *de novo* missense variant (p.Cys354Arg) in the *STXBP1* gene was identified. This variant has been previously reported as pathogenic and was associated with WS and Ohtahara syndrome[21].

Discussion

Here we present a group of 31 pediatric patients with DEE studied at a public reference center in Argentina and analyzed with a customized NGS panel of 52 epilepsy-related genes. The incorporation of NGS analysis into our diagnostic approach led to a molecular diagnosis in 39% of the cases. These results are similar to those previously reported by our group, even though, with a different molecular strategy and a different group of patients, and are in agreement with those published in the literature, reporting diagnostic yields ranging from 10% to 48.5%[3, 11, 22-26].

Approximately 66% of the patients in whom a molecular diagnosis was made showed a variant in genes that encode for ion channels, which agrees with previously reported data[3, 6, 27, 28]. It is well known that pathogenic variants in sodium channel genes, including *SCN1A, SCN1B, SCN2A, SCN3A, SCN8A* and *SCN9A*, are responsible for a large proportion of cases with pediatric genetic epilepsies[23], the three most common being *SCN1A, SCN2A* and *SCN8A*. This is especially true for DS, EIMFS and other DEE where the impact of the variants in the ion channels is relevant for selection of the most appropriate treatment.

In four of the seven patients with DS identified here, *SCN1A* variants were found. Although the functional effect of the variants is unknown, it is well established that the mechanism underlying epileptogenesis in *SCN1A*-associated DS is a loss-of-function (LoF) in sodium channels affecting inhibitory interneurons. Therefore, sodium-channel-blocking drugs should be avoided in DS[29].

In addition, elucidation of the mechanisms responsible for *SCN1A*-related DS has stimulated innovative research into precision treatments to correct the underlying molecular or functional defect.

As expected, all the DS patients with a *SCN1A* mutation presented with *de novo* variants. Nevertheless, we observed a very infrequent pattern of inheritance in a DS patient who had a homozygous variant in the *SCN1B* gene. To our knowledge, this alteration is the tenth homozygous variation in the sodium channel accessory subunit gene reported in DEE patients[30-32]. Both parents of the patient harbor the heterozygous p.(Glu56Lys) variation and have no history of seizures.

No relevant variants were identified in 28% of the patients with DS, as was to be expected based on the reports in the literature, since *SCN1A* variants or deletions are found in 70-80% of tested DS cases[14, 33]. A substantial minority of patients has no identified cause, and a smaller minority has a confirmed pathogenic variant involving a different gene, similar to our patient with the homozygous *SCN1B* variant.

In the patients with DLS no relevant variants were detected. In recent years, a large number of genes associated with DLS have been reported, but data are still insufficient to establish a genotype-phenotype relationship; therefore, in this analysis we decided to include in our panel only those genes with a proven relationship with DS or DLS (*SCN1A, SCN2A, SCN8A, SCN9A, SCN1B, PCDH19, GABRA1, GABRG2, STXBP1, HCN1, CHD2* and *KCNA2*)[19].

In the EIMFS cases we identified a mosaic *SCN2A* variant and a CNV that involved the *SCN1A*, *SCN2A* and *SCN3A* genes, in agreement with previous studies describing *SCN2A* as the second-most frequent gene after *KCNT1* involved in this syndrome[9]. In contrast to patients with *SCN1A* variants, neonatal and early infantile epilepsies related to *SCN2A* variants may show a good response to sodium-channel blockers, as did our patient with the mosaic *SCN2A* variant. This effect was explained by other authors through functional studies that showed that *SCN2A*-associated early-onset epilepsies are due to gain-of-function (GoF) variants with increased sodium channel activity and show a good response to sodium channel blockers, whereas variants found in late-onset forms have a LoF effect without a meaningful response to sodium-channel blockers[34].

The mosaic *SCN2A* variant identified here supports the increasing number of patients with DEE associated with variants with somatic mosaicism[9]. It is important to be aware that a proportion of patients with DEE may have somatic mosaic variants that can easily be missed by exome sequencing, a technical approach that usually shows a lower depth of coverage than customized gene panels. Currently, it has been widely established that NGS is able to detect germline mutations present in all of the patients' cells; however, somatic and mosaic variants can be difficult to identify. High-sequence depth, which is more commonly reached with targeted gene panels, aids in the detection of mosaic mutations with studies more commonly associated with somatic mutations relying on coverage >100X for accurate variant calling[7, 12].

Importantly, using an in house specific CNV-detection pipeline, we identified a CNV in a patient with EIMFS studied using the NGS panel, which seemed to involve the *SCN1A* and *SCN2A* genes. The application of array CGH allowed us to describe a 1Mb heterozygous deletion in patient #2 that involved not only *SCN1A* and *SCN2A*, but also the *SCN3A* gene, which was not included in our customized NGS panel (Table 1). The deleted region also includes the OMIM genes *GALTN3* and *TTC21B,* associated with recessive diseases and thus cannot be related with the patient's phenotype. NGS is increasingly being employed in the detection of CNVs using bioinformatic approaches, although a second method is necessary to confirm them and precisely determine the size of the involved region. The development of effective bioinformatics pipelines employed in the detection of CNVs has added another layer of complexity to the applications of NGS for diagnostic testing[7].

In the group of patients with WS that evolved to LGS, one had a heterozygous variant in the *GABRB2* gene. Genes related to inhibitory GABA neurotransmitters play a key role in genetic epilepsies, including DEEs and genetic generalized epilepsies[35]. Genes that encode subunits of GABA(A) receptors, including *GABRG2, GABRA1, GABRB3* and *GABRD* are part of the long list of epilepsy genes. A patient with LGS carrying a *GABRB3* mutation was successfully supplemented with vinpocetine, an alkaloid with pharmacological effects that include vasodilatation, antioxidation, anti-inflammation, synaptic modulation and antithrombotic properties, leading to reduction of epileptiform activity on EEG recordings and improvement of cognitive, behavioral and language functions[36].

The other patient with WS/LGS had several relatives with intellectual disability and epilepsy with a genealogy showing an X-linked pattern; therefore, an alteration in the *ARX* gene was suspected. The most common polyalanine expansion mutation in the *ARX* gene c.315_335dup; p.(Ala109_Ala115dup), was confirmed by Sanger sequencing.

A specific epilepsy syndrome was observed in one of the patients with a variant in the *STXBP1* gene who developed myoclonic *status epilepticus*. Pathogenic variants in this gene are most commonly associated with Ohtahara syndrome, WS and non-syndromic intellectual disability with or without epilepsy, while their association with myoclonic status had not been previously reported[37].

It should be noted that the number of negative results in patients with DEE in this cohort is in agreement with data reported by other authors[3, 6, 7]. Nevertheless, we

emphasize the need of performing additional studies in depth to these patients, for example by exome sequencing, to reach a precise diagnosis and hopefully treatment optimization.

Furthermore, the diagnostic yield in our study was similar to that reported in the literature, although only the 52 genes most frequently associated with DEE were included. This demonstrates that a small group of genes are responsible for the majority of DEE, which is relevant when searching for an efficient diagnostic approach in countries with limited access to NGS panels or exome sequencing, such as Argentina.

In conclusion, using an NGS panel, a genetic diagnosis could be made in 39% of patients, including in those with rare genetic variants, such as one CNV and one mosaic alteration, and establishing new genotype-phenotype associations that have not been previously described. We emphasize the importance of considering mosaic variants, CNV and hereditary forms when designing and interpreting molecular studies in order to optimize the diagnosis and management of patients with DEE. Approximately 42% of the detected variants were novel, expanding the knowledge of the molecular basis of DEEs in Latin-American patients. Molecular characterization is becoming the new diagnostic paradigm in DEE patients. Knowing the genetic cause of DEE will improve the treatment tailoring of some genetically determined forms of epilepsy.

Conflict of interest: None to declare

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