Official Journal of the European Paediatric Neurology Society

Original article

The most recurrent monogenic disorders that overlap with the phenotype of Rett syndrome

S. Vidal [a,d], N. Brandi [b], P. Pacheco [c], J. Maynou [c,d], G. Fernandez [c,d], C. Xiol [a,d], A. Pascual-Alonso [a,d], M. Pineda [a], Rett Working Group, J. Armstrong [c,d,e,*]

[a] Sant Joan de Déu Research Foundation, Barcelona, Spain
[b] School of Medicine, Universitat de Barcelona, Barcelona, Spain
[c] Molecular and Genetics Medicine Section, Hospital Sant Joan de Déu, Barcelona, Spain
[d] Institut de Recerca Pediàtrica Hospital Sant Joan de Déu, Barcelona, Spain
[e] CIBER-ER (Biomedical Network Research Center for Rare Diseases), Institute of Health Carlos III (ISCIII), Madrid, Spain

ARTICLE INFO

Article history:
Received 19 December 2018
Received in revised form
12 February 2019
Accepted 28 April 2019

Keywords:
Rett syndrome
Genotype-phenotype correlations
Monogenic disorders
MECP2
RTT
Rett-like

ABSTRACT

Rett syndrome (RTT) is an early-onset neurodevelopmental disorder that is caused by mutations in the *MECP2* gene; however, defects in other genes (*CDKL5* and *FOXG1*) can lead to presentations that resemble classic RTT, although they are not completely identical. Here, we attempted to identify other monogenic disorders that share features of RTT. A total of 437 patients with a clinical diagnosis of RTT-like were studied; in 242 patients, a custom panel with 17 genes related to an RTT-like phenotype was run via a HaloPlex-Target-Enrichment-System. In the remaining 195 patients, a commercial TruSight-One-Sequencing-Panel was analysed. A total of 40 patients with clinical features of RTT had variants which affect gene function in six genes associated with other monogenic disorders. Twelve patients had variants in *STXBP1*, nine in *TCF4*, six in *SCN2A*, five in *KCNQ2*, four in *MEF2C* and four in *SYNGAP1*. Genetic studies using next generation sequencing (NGS) allowed us to study a larger number of genes associated with RTT-like simultaneously, providing a genetic diagnosis for a wider group of patients. These new findings provide the clinician with more information and clues that could help in the prevention of future symptoms or in pharmacologic therapy.

* *Corresponding author*. Molecular and Genetics Medicine Section, Hospital Sant Joan de Déu, Pg. Sant Joan de Déu 2, planta 0, Esplugues de Llobregat, 08950, Barcelona, Spain. Fax: + 34 93 600 9760.
E-mail address: jarmstrong@sjdhospitalbarcelona.org (J. Armstrong).

https://doi.org/10.1016/j.ejpn.2019.04.006
1090-3798/

1. Introduction

Rett syndrome (RTT; OMIM#312750) is an early onset neurodevelopmental disorder that almost exclusively affects girls and has an incidence of 1:10,000 live female births.[1] This syndrome is first recognized in infancy with a period of apparently normal development (up to the age of 6–18 months), followed by a regression characterized by a loss or deterioration of speech and purposeful hand use and motor apraxia, which may be associated with epilepsy and dysautonomic features, including disturbed breathing and sleep as well as gastrointestinal dysmotility.[2,3] Hand stereotypies and breathing abnormalities, including hyperventilation and/or breath holding episodes, are distinct clinical features that often present and can help in the diagnosis. Facial dysmorphism is not distinct, and few have subtle dysmorphic facial features that do not enable a clinical diagnosis.[4] The type and severity of symptoms are highly different in individuals.

Although the majority of RTT patients have pathogenic variants in the gene encoding methyl-CpG binding protein 2 (*MECP2*, OMIM*300005), approximately 5% of classic RTT and 25% of atypical RTT patients are negative for *MECP2* pathogenic variants.[5,6] In this group of atypical RTT patients, some have variants in other genes that are also related to RTT, such as cyclin-dependent kinase-like 5 (*CDKL5*; OMIM*300203) and forkhead box protein G1 (*FOXG1*; OMIM*164874). Recently, as a consequence of large-scale genetic screening technologies, other genes not previously related to RTT have been associated with RTT-like phenotypes, such as transcription factor 4 (*TCF4*; OMIM*602272) and myocyte-specific enhancer factor 2C (*MEF2C*; OMIM*60066).[7]

Next generation sequencing (NGS) has emerged as a potentially powerful tool for the study of this type of genetic disease.[8–11] The aim of this study was to continue to extend and improve the diagnosis of monogenic disorders that share features with RTT. Here, we report accurately the 40 cases of a cohort of 437 patients with features of RTT, described by Vidal S. et al. 2017,[9] no *MECP2* defects and variants which affect gene function in six genes associated with other monogenic disorders. Here, we compare different disorders and causative genes to RTT features.

2. Material and methods

2.1. Subjects and ethical issues

A cohort of 437 Spanish patients who presented with clinical features associated with RTT or RTT-like phenotypes was recruited at Sant Joan de Déu Hospital in Barcelona from different Spanish Hospitals.[9] The study was approved by the ethical committees of Hospital Sant Joan de Déu, CEIC: *Comité d'Ètica d'Investigació Clínica* - Fundació Sant Joan de Déu (internal code: PIC-101-15). Patients or their parents gave signed informed consent for genetic studies, and blood samples from patients and controls were obtained according to the Helsinki Declaration of 1964, as revised in 2004.[12] Patients had been diagnosed following the usual clinical parameters[13] and according to the recently revised RTT Search International Consortium criteria and nomenclature.[6] Patients who almost completely fulfilled the criteria, including the main features, such as psychomotor delay with or without regression stereotypic hand movements and absent language or limited to only a few words, were also included.

2.2. Library preparation and bioinformatic pipeline

Libraries of the 242 patients' samples were generated using a custom-made panel with 17 genes associated with a RTT-like phenotype through HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA) and 195 using the TruSight One Sequencing Panel kit (Illumina, San Diego, CA) (Supplementary Table S1)[9]; both according to the manufacturer's sample preparation protocol, and all of them were sequenced on an Illumina NextSeq 500. The variant calling pipeline was developed at the Bioinformatics Unit from the Molecular Genetics Department at the Sant Joan de Déu Hospital. The bioinformatic analysis was divided into several steps: quality control, alignment, variant calling, variant annotation and, finally, filtering. Before and after the adaptor and low quality reads were removed (cutadapt v.1.13), read quality control was assessed using FastQC v.0.11.5.[14,15] The reads were aligned to the human reference genome sequence (hg19/GRCh37) using Burrows-Wheeler Aligned through BWA-MEM v.0.7.15.[16] The aligned reads were filtered by means of mapping quality and duplicates to ensure high quality data using BEDtools v.2.26.0[17] and Picard tools v2.9.0.[18] Once the sequences were filtered, variant calling was determined using SAMTools v.1.5,[19] FreeBayes v1.1.0,[20] VarScan v2.4.0[21] and GATK v3.7.[22] The variants were annotated using SnpEff v.4.3 and included nucleotide and amino acid annotations (dbSNP), population frequencies (gnomAD and internal database), and clinical information (Clinvar, OOMIM). The variants were filtered based on population frequencies (<0.01), coverage (>= 20), amino acid impact (High or Moderate according to SnpEff), pathogenic scores, clinical significance and inheritance patterns. To reduce the amount of variants to be analysed, another filtering layer that took into account specific lists of genes that contained putative targets (RTT-like genes, GABA and glutamate pathway and epilepsy genes) was applied (Supplementary Table S1). Copy-number variant (CNV) were detected using R-package ExomeDepth v1.1.10 based on read-depth method. Deletions and duplications identified were annotated using Database of Genomic Variants version March 2016 and internal database,[23] all CNV detected by NGS of them were confirmed by CGH array 400k.

2.3. Molecular analysis

To identify the potential causative variants, we checked the variants by matching their affected phenotypes and inheritance patterns of respective genes checked by Sanger sequencing of the index cases and their parents. Moreover, we considered the pathogenicity by *in silico* predictors:

MutationTaster (http://www.mutationtaster.org/), SIFT-PROVEAN (http://sift.jcvi.org/), Sorts Intolerant From Tolerant (SIFT), Protein Variation Effect Analyzer (PROVEAN) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), and American College of Medical Genetics and Genomics (ACMG) guidelines were used (https://www.acmg.net/docs/standards_guidelines_for_the_interpretation_of_sequence_variants.pdf). In addition, databases in the literature, such as the RettBASE: *MECP2* Variation Database, the Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD), HGMD® Professional 2018.2 and the Single Nucleotide Polymorphism Database (dbSNP), were revised. All variants detected and their subsequent segregation studies were performed by Sanger sequencing.

3. Results and discussion

A total of 40 patients with clinical features of RTT-like had variants which affect gene function in six genes associated with other monogenic disorders (Fig. 1 and Table 1). All variants were not registered in ExAC or gnomAD. A total of 25% of these variants (10/40) were considered pathogenic or likely pathogenic in ClinVar, and only in 10% of variants (4/40) did one or more programs predict that there could be possibly benign or neutral changes. The variants information is shown in Supplementary Table S2.

We obtained partial or complete clinical information from 37 patients (Table 2); therefore, the data and percentages are related to the total, with the clinical information of the specific feature available. All the patients presented with psychomotor delay and mental retardation, and these were severe in 75.7% of the patients (28/37). The most common characteristics of our cohort of RTT-like patients were autistic features (30/34) and breathing dysfunction (30/34). A total of 51.7% of the patients (15/29) showed acquired microcephaly, and 34.4% (11/32) had abnormalities of the brain revealed by magnetic resonance imaging (MRI). A total of 74.2% of patients (23/31) presented with hypotonia, and 68.4% (26/38) were able to walk, while a further ten patients (26.3%) who had a more severe phenotype never walked, and two of them (5.3%) had lost this ability. Language skills were limited to a few words in 25.8% of patients (8/31), one (3.2%) had lost the skills, and 75.7% (22/31) never acquired them. Additionally, epilepsy was present in 61.1% of the patients (22/36), 79.4% (27/34) showed typical RTT hand stereotypies, hand wringing or hand washing, and 53.3% of patients (16/30) showed mood disturbances the most common of which was disruptive agitation (14/30); only 13.3% showed sudden laughing (4/30) and 6.7% aggressiveness (2/30).

In Table 3, we summarize the most relevant clinical manifestations of the patients with typical RTT associated with the classic *MECP2*, *CDKL5* and *FOXG1* alterations and those observed in the present series of Rett-like associated genes. Many overlapping characteristics were detected; some cardinal features are also highlighted, such as the absence of developmental regression in patients with pathogenic variants at *TCF4*, *KCNQ2* and *MEF2C* or the absence of breathing dysfunction in those with molecular alterations at *STXBP1*, *SCN2A*, *KCNQ2*, *MEF2C* or *SYNGAP1*.

3.1. STXBP1 *gene*

We describe twelve RTT-like patients with different ten variants in the *STXBP1* gene (Syntaxin-binding protein 1; OMIM#602926). This protein plays an important role in presynaptic vesicle docking and fusion, a necessary mechanism for neurotransmitter release.[24] Reduced *STXBP1* expression has been shown to increase synaptic depression at both GABAergic and glutamatergic synapses, with greater impact on GABAergic interneurons,[25] a pathway that is also altered in RTT patients.[26] Mutations in *STXBP1* have been associated with EEIE7 and a series of neurodevelopmental disorders, including RTT-like syndrome. Our patients showed a combination of stereotypies, autistic features, and regression that have been already described in patients with mutations in *STXBP1*.[27]

The twelve *STXBP1* variants identified in our RTT-like patients include one in-frame deletion, one duplication, two splicing site variants and six missense changes, all within the protein domains. Eight variants were demonstrated to be *de novo*, and one was inherited from the mother; in one case, the mother was not a carrier, and no sample was available from the father; for two cases, inheritance information was not available.

Regarding missense variants, the previously described *STXBP1* variants p.R292C (x2), p.R292H (x2), p.R406C and c.1359+1G > T were *de novo* and have been related to EEIE7.[28] Within the three novel variants, p.R235Q was found to be *de novo*, p.S516R was inherited from an asymptomatic mother and, for p.K7E, the family study was not available. All pathogenicity prediction tools predicted that these variants were likely to be pathogenic. Both splicing variants can affect the correct splicing of the RNA, c.326-3C > G mutates the splice acceptor site, and c.1359+1G > T disrupts the donor splice site. Finally, if the duplication of exon 10 is in tandem, it could affect the structure of the protein and its functionality. To confirm the pathogenicity of these variants, functional studies should be done. For the inherited variants, studies should be performed to see if the carrying progenitor is a mosaic and whether the RNA of these patients is altered.

We had partial or complete clinical information about all patients, except patient 11 (c.1359+1G > T). We found that severe intellectual disability (ID) with epilepsy and absence of speech are the most common clinical features present in patients with likely pathogenic variants in *STXBP1*. Most of them had severe to profound ID, and only one, patient 7 (seizure-free), had a moderate ID. Nearly 95% of patients published to date present with epilepsy. In our cohort, 8 out of 11 (73%) presented with epilepsy.[29] There were only three patients without seizures: patient 3 c.326-3C > G, patient 9 with duplication and patient 7 with p.R292H. As in patient 8 (also carrying the p.R292H), the onset of epilepsy occurred at 7 years, and patient 7 was only 3 years old; we therefore hypothesized that epilepsy may still appear and that this feature may be related to the affected variant. In fact, when comparing the four patients with changes in arginine 292, it

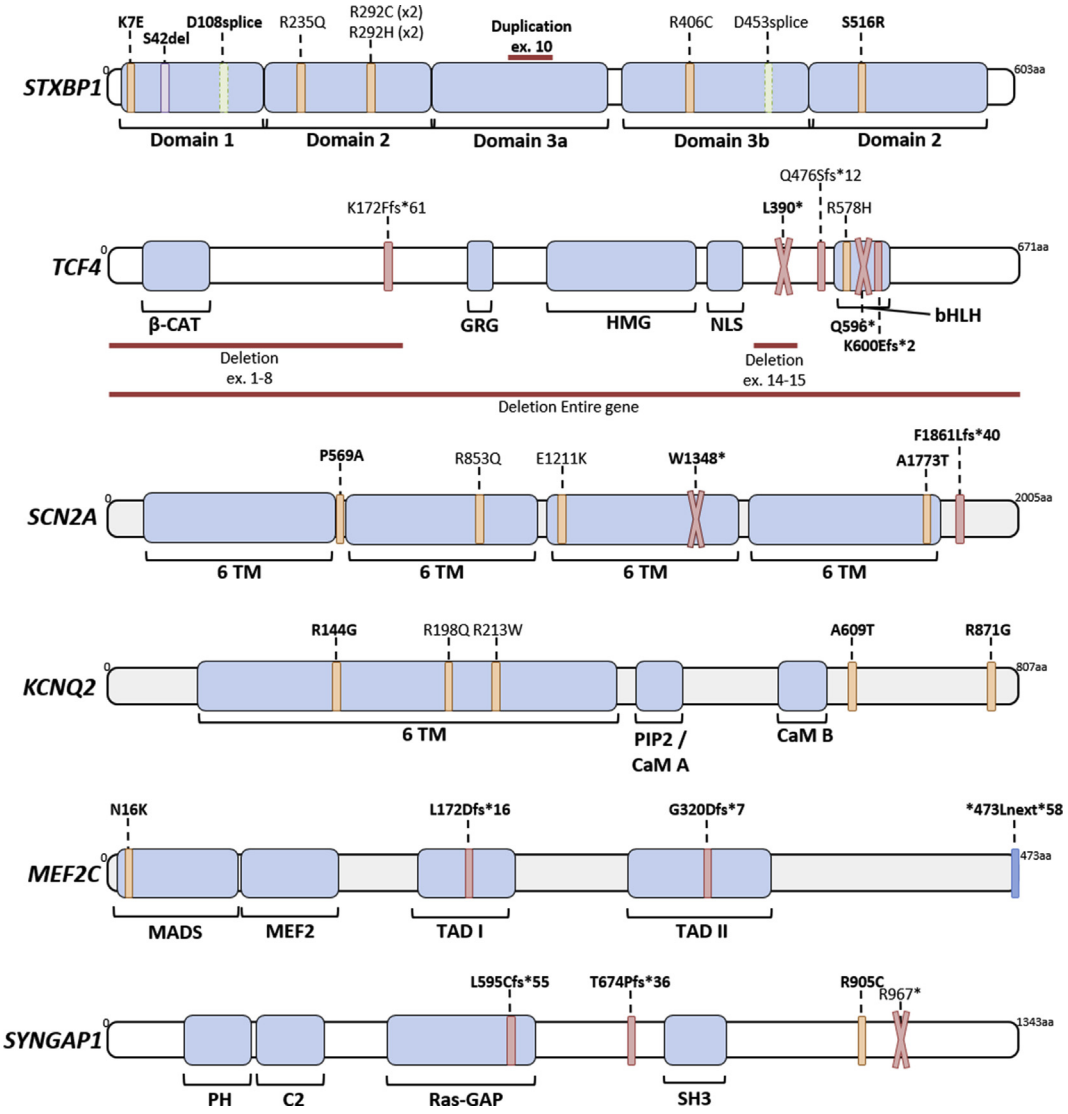

Fig. 1 – Protein structure and location of the variants identified in RTT-like patients. Novel variants in bold; orange box, missense variants; red box, frameshift deletions; red cross, nonsense variants; green box, splice site variants; blue box, stop lost variant; and purple box, in-frame deletion. Abbreviations: β -CAT = N-terminal β-catenin-binding domain; GRG = the interaction domain with the groucho/TLE transcriptional co-repressors; HMG = DNA-binding domain; NLS = the nuclear localization signal; bHLH = basic helix-loop-helix; 6TM = six transmembrane domains; PIP2 = proximal C-terminal domain that binds phosphatidylinositol 4,5-bisphosphate; CaM A = domain which binds calmodulin; CaM B = the more distal domain which binds calmodulin; MADS-box and MEF2 = N-terminal region involved in DNA binding and dimerization; TADI and TADII = activation domains; PH = Pleckstrin Homology domain; C2 = C2 domain; SH3 = SRC Homology 3 domain.

seems that the change at this residue to a cysteine is slightly more severe than the change to histidine. Although the number of patients was very small, we observed that the two patients with a change to cysteine presented with earlier onset of epilepsy, were microcephaly and had not yet acquired walking/ambulation. In contrast, the other two patients with the histidine alteration were able to walk. Regarding the other patient without epilepsy (patient 9), we must conduct further studies to determine whether the duplication of exon 10 of the gene is causative of disease. First, it is important to study the segregation to know if the variant was inherited from parents or is *de novo*. Then, it must be determined if the duplication is in tandem and whether it negatively affects the reading frame of the protein, creating a non-functional protein.

3.2. TCF4 *gene*

We found nine RTT-like patients with anomalies in the *TCF4* gene: three gross deletions, one in-frame indel, three frameshift, one nonsense and one missense change. Six of these

Table 1 – Potentially pathogenic and causative variants detected. Abbreviations: NA = Not Available; Path = Pathogenic; DC = Disease causing; Dam = Damaging; Del = Deleterious; Pol = Polymorphism; Tol = Tolerate; Neu = Neutral; ACMG = American College of Medical Genetics; PA = Potential Alteration; NE = No effect; PVS = Pathogenic Very Strong; PM = Pathogenic Moderate; PP = Pathogenic Supporting; BP = Benign Supporting.

Gene	Patient	Inheritance	Zygosity	Type of seq. Change	cDNA/Protein change	dbSNP	ClinVar	Mutation taster, PROVEAN, SIFT, Polyphen-2, HSF3.1	ACMG Classification
STXBP1	1	NA	Heter.	Missense	c.19A > G/p.(K7E)	–	–	DC, Dam, Del, Dam, PA	PM2, PP3
(NM_003165.3)	2	*de novo*	Heter.	In-frame deletion	c.128_130delTCC/p.(S42del)	–	–	DC, -, -, -, -	PM2, PM4
	3	Mother not carrier	Heter.	Splicing variant	c.326-3C > G/-	–	–	-, -, -, -, Br Acceptor	PM2, PP3
	4	*de novo*	Heter.	Missense	c.704G > A/p.(R235Q)	–	–	DC, Dam, Del, Dam, PA	PM2, PP3
	5	*de novo*	Heter.	Missense	c.874C > T/p.(R292C)	–	–	DC, Dam, Del, Dam, PA	PM2, PM5, PP3
	6	*de novo*	Heter.	Missense	c.874C > T/p.(R292C)	–	–	DC, Dam, Del, Dam, PA	PM2, PM5, PP3
	7	*de novo*	Heter.	Missense	c.875G > A/p.(R292H)	rs796053361	Likely path. Allele	DC, Dam, Del, Dam, PA	PM2, PM5, PP3, PP5
	8	*de novo*	Heter.	Missense	c.875G > A/p.(R292H)	rs796053361	Likely path. Allele	DC, Dam, Del, Dam, PA	PM2, PM5, PP3, PP5
	9	NA	Heter.	Gross duplication	129pb incl. ex. 10/-	–	–	-, -, -, -, -	–
	10	*de novo*	Heter.	Missense	c.1216C > T/p.(R406C)	rs796053367	Likely path. Allele	DC, Dam, Del, Dam, PA	PM2, PM5, PP3, PP5
	11	*de novo*	Heter.	Splicing variant	c.1359+1G > T/-	–	–	-, -, -, -, Br Donor	PVS1, PM2, PP3
	12	Maternal	Heter.	Missense	c.1548C > A/p.(S516R)	–	–	DC, Dam, Del, Dam, PA	PM2, PP3
TCF4	13	*de novo*	Heter.	Frameshift deletion	c.514_517delAAAG/p.(K172Ffs*61)	rs398123561	Path. Allele	DC, -, -, -, -	PVS1, PM2, PP5
(NM_001083962.1)	14	*de novo*	Heter.	Gross deletion	c.1069 + 118_1350 + 119del5450/-	–	–	-, -, -, -, -	–
	15	*de novo*	Heter.	In-frame indel	c.1169_1175delinsAAA/p.(L390*)	–	–	DC, -, -, -, -	PVS1, PM2
	16	*de novo*	Heter.	Frameshift deletion	c.1438delC/p.(Q476Sfs*12)			DC, -, -, -, -	PVS1, PP2, PP3
	17	*de novo*	Heter.	Missense	c.1733G > A/p.(R578H)	rs121909123	Path. Allele	DC, Dam, Del, Dam, NE	PM1, PM2, PM5, PP3, PP5
	18	*de novo*	Heter.	Nonsense	c.1786C > T/p.(Q596*)	–		DC, -, -, -, -	PVS1, PM1, PM2, PP3
	19	NA	Heter.	Frameshift deletion	c.1798_1799delAA/p.(K600Efs*2)	–	–	DC, -, -, -, -	PVS1, PM1, PM2
	20	NA	Heter.	Gross deletion	2.2mb incl. entire gene/-	–	–	-, -, -, -, -	–
	21	NA	Heter.	Gross deletion	0.42mb incl. ex.1–8/-	–	–	-, -, -, -, -	–
SCN2A	22	Paternal	Heter.	Missense	c.1705C > G/p.(P569A)	–	–	DC, Tol, Del, Dam, PA	PM2, PP2, PP3
(NM_021007.2)	23	*de novo*	Heter.	Missense	c.2558G > A/p.(R853Q)	rs794727152	Path. Allele	DC, Dam, Del, Dam, PA	PM2, PP2, PP3, PP5
	24	Mother not carrier	Heter.	Missense	c.3631G > A/p.(E1211K)	rs387906684	Path. Allele	DC, Dam, Del, Dam, PA	PM2, PP2, PP3, PP5
	25	*de novo*	Heter.	Nonsense	c.4043G > A/p.(W1348*)	–	–	DC, -, -, -, -	PVS1, PM1, PM2, PP3
	26	*de novo*	Heter.	Missense	c.5317G > A/p.(A1773T)	rs796053162	Likely path. Allele	DC, Dam, Del, Dam, NE	PM2, PM5, PP2, PP3, PP5
	27	*de novo*	Heter.	Frameshift deletion	c.5583delT/p.(F1861Lfs*40)	–	–	DC, -, -, -, -	PVS1, PM2, BP4
KCNQ2	28	NA	Heter.	Missense	c.430C > G/p.(R144G)	–	–	DC, Dam, Del, Dam, PA	PM2, PM5, PP2, PP3, PP5
(NM_172107.3)	29	*de novo*	Heter.	Missense	c.593G > A/p.(R198Q)	rs796052621	Conflicting path.	DC, Dam, Del, Dam, PA	PM1, PM2, PP2, PP3
	30	NA	Heter.	Missense	c.637C > T/p.(R213W)	rs118192203	Path. Allele	DC, Dam, Del, Dam, PA	PM1, PM2, PM5, PP2, PP3
	31	Maternal/ paternal	Hom.	Missense	c.1825G > A/p.(A609T)	–	–	Pol, Tol, Neu, Ben, NE	PM2, PP2
	32	Maternal	Heter.	Missense	c.2611A > G/p.(R871G)	–	–	DC, Dam, Neu, Ben, PA	PM2, PP2, PP3
MEF2C	33	*de novo*	Heter.	Missense	c.48C > G/p.(N16K)	–	–	DC, Dam, Del, Dam, PA	PM1, PM2, PP3
(NM_002397.4)	34	*de novo*	Heter.	Frameshift insertion	c.513_514insGA/p.(L172Dfs*16)	–	–	DC, -, -, -, -	PVS1, PM2
	35	*de novo*	Heter.	Frameshift deletion	c.959_960delGT/p.(G320Dfs*7)	–	–	DC, -, -, -, -	PVS1, PM2
	36	*de novo*	Heter.	Stop loss	c.1421G > T/p.(*473Lnext*58)	–	–	Pol, -, -, -, -	PM2, PM4

(*continued on next page*)

Table 1 – (continued)

Gene	Patient	Inheritance	Zygosity	Type of seq. Change	cDNA/Protein change	dbSNP	ClinVar	Mutation taster, PROVEAN, SIFT, Polyphen-2, HSF3.1	ACMG Classification
SYNGAP1 (NM_006772.2)	37	Mother not carrier	Heter.	Frameshift deletion	c.1783delC/p.(L595Cfs*55)	rs587780470	Likely path. Allele	DC, -, -, -, -	PVS1, PM2
	38	*de novo*	Heter.	Frameshift deletion	c.2020delA/p.(T674Pfs*36)	–	–	DC, -, -, -, -	PVS1, PM2
	39	NA	Heter.	Missense	c.2713C > T/p.(R905C)	–	–	DC, Dam, Del, Dam, -	PM2
	40	NA	Heter.	Nonsense	c.2899C > T/p.(R967*)	–	–	DC, -, -, -, -	PVS1, PM2

variants are novel: c.1069 + 118_1350 + 119del5450, c.1169_1175delinsAAA, c.1786C > T (p.Q596*), c.1798_1799delAA (p.K600Efs*2), deletion of 2.2 Mb (the entire gene) and deletion of 0.42 Mb (including exons 1–8). *TCF4* encodes a broadly expressed basic helix-loop-helix (bHLH) protein that forms a homodimer or heterodimer with other bHLH proteins. These dimers bind DNA at Ephrussi (E) box sequences. Alternative splicing produces a number of different *TCF4* isoforms with distinct N-termini that differ in their subcellular localization and transactivation capacity.[30] Seven out of the nine variants found produced a truncated protein, leading to haploinsufficiency of the transcription factor. The gross deletion encompassing the whole gene also results in haploinsufficiency of the protein. In addition, we found a missense variant in the bHLH domain located in a recurrent mutation site. This variant affects an evolutionarily highly conserved arginine residue, constituting the E-box recognition motif. It has been previously demonstrated that such an impairment of the functional bHLH domain reduces the interaction with *ASCL1* in transactivating an E-box-containing reporter construct to a similar degree as that of haploinsufficient stop mutations.[31]

Mutations in *TCF4* have been associated with Pitt-Hopkins syndrome (PTHS; OMIM*610954)that is characterized by ID, epilepsy, microcephaly, facial dysmorphisms, postnatal growth restriction, and intermittent hyperventilation.[31] Episodic hyperventilation/apnea, microcephaly, and autism spectrum disorders (ASD)-related stereotypies hand movements may steer clinicians towards a misdiagnosis of RTT-like rather than PTHS. The presence of distinct facial features is more consistent with PTHS and helps to distinguish PTHS from RTT, but patients do not always have these facial distinctions, which are often not clearly defined during the first year of life.[32]

We had partial or completed clinical information of all *TCF4* RTT-like patients. Interestingly, abnormal MRI (4/7), absence of walking (4/9) or loss of walking ability (1/9), absence of speech (7/8), seizures (3/9) and autism features (8/8) were observed in our patients, suggesting a possible RTT-like phenotype. There was no correlation between the clinical features and the variants. Patients 13 and 16, carrying the upstream frameshift (p.Lys172Phefs*61, supposedly the more aggressive variant) and the missense variant (p.R578H, supposed to be the less affected protein), respectively, had the most moderate clinical characteristics: purposely hand use, ability to walk and only moderate mental retardation. Seizures were not present in the patient with the missense variants, which contradicts what had been previously suggested.[33]

3.3. SCN2A *gene*

SCN2A (neuronal voltage-gated sodium channel NaV1.2; OMIM*182390) encodes one of the sodium channels involved in the initiation and propagation of action potentials in numerous neuron classes. *SCN2A* is expressed early in brain development; each domain of the protein contains six membrane-spanning segments S1–S6, where S1–S4 forms the voltage-sensing domains, and S5–S6 forms the pore loops and DEKA-selectivity filter. Variants in *SCN2A* are associated with three disorders: gain-of-function variants leading to

Table 2 – Summary of clinical information available for all patients with mutations in the *STXBP1*, *TCF4*, *SCN2A*, *KCNQ2*, *MEF2C* and *SYNGAP1* genes. Abbreviations: NA = Not available; y = years; m = month; AED = Antiepileptic drugs; VPA = Valproate; CZ = Carbamazepine; LEV = Levetirazetam; ETO = Etosuximide; LTG = Lamotrigine; ACTH = adrenocorticotrophin hormone; CLB = Clobazam; CLN = Clonazepam; ZNS = Zonisamide; PRM = Primidone; OXC = Oxcarbazepine; PHT = Phenytoin; RFM = Rufinamide; BRV = Brivaracetam.

Patient	Gene	Sex/Age evaluation	Acquired microcephaly	Brain MRI	Hypotonia	Psychomotor delay	Walking/Age	Apraxic gait	Speech	Mental retardation	Autistic behaviours	Breathing dysfunction	Hands use
P1	*STXBP1*	F/11y	No	Normal	No	Yes	Aided/22m	No	Loss	Profound	Yes	Yes	Yes
P2	*STXBP1*	F/9y	Yes	Normal	Yes	Yes	Aided/NA	Yes	NA	Moderate	No	No	Yes
P3	*STXBP1*	M/18y	NA	Normal	Yes	Yes	Aided with help/NA	No	No	Profound	Yes	No	NA
P4	*STXBP1*	M/13y	No	Normal	No	Yes	Aided/2y 3m	No	NA	Profound	Yes	No	Yes
P5	*STXBP1*	M/6y	Yes	Abnormal	Yes	Yes	No/-	–	No	Profound	Yes	Yes	Yes
P6	*STXBP1*	M/14y	Yes	Abnormal	Yes	Yes	No/-	–	No	Profound	Yes	No	Yes
P7	*STXBP1*	F/4	No	Abnormal	No	Yes	Aided with help/3y 9m	Yes	No	Moderate	No	No	Yes
P8	*STXBP1*	M/8y	No	Normal	Yes	Yes	Aided with help/NA	Yes	No	Profound	NA	No	No
P9	*STXBP1*	F/32y	Yes	Normal	No	Yes	Aided/2y	No	No	Profound	Yes	No	No
P10	*STXBP1*	F/5y	No	NA	Yes	Yes	Loss/NA	–	No	Profound	Yes	NA	NA
P11	*STXBP1*	M/14y	NA	NA	NA	NA	Aided/NA	Yes	NA	NA	NA	NA	NA
P12	*STXBP1*	F/9y	No	Abnormal	No	Yes	Aided/2y	Yes	No	Profound	Yes	Yes	Yes
P13	*TCF4*	F/9y	No	Normal	No	Yes	Aided/30m	Yes	No	Moderate	Yes	Yes	Yes
P14	*TCF4*	F/15y	Yes	Normal	Yes	Yes	Aided with help/NA	Yes	No	Profound	Yes	No	No
P15	*TCF4*	F/17y	Yes	NA	Yes	Yes	Loss/NA	–	NA	Profound	Yes	NA	NA
P16	*TCF4*	F/12y	NA	Abnormal	No	Yes	Aided/NA	No	No	Moderate	Yes	Yes	No
P17	*TCF4*	F/13y	Yes	Abnormal	Yes	Yes	Aided/3y	Yes	Few words	Profound	Yes	Yes	Yes
P18	*TCF4*	F/6y	Yes	NA	Yes	Yes	No/-	–	No	Profound	Yes	NA	NA
P19	*TCF4*	F/2y 4m	No	Normal	Yes	Yes	No/-	–	No	Profound	Yes	No	NA
P20	*TCF4*	F/7y	NA	Abnormal	NA	Yes	No/-	–	No	Profound	Yes	No	Yes
P21	*TCF4*	M/1y 6m	No	Abnormal	No	Yes	No/-	–	No	Profound	NA	No	Yes
P22	*SCN2A*	M/5y	Yes	NA	NA	Yes	No/-	–	No	Profound	Yes	No	NA
P23	*SCN2A*	M/6y	No	Normal	NA	Yes	No/-	–	No	Profound	Yes	No	No
P24	*SCN2A*	F/NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
P25	*SCN2A*	F/5y	NA	Normal	NA	Yes	Aided/NA	NA	Few words	Profound	Yes	No	Yes
P26	*SCN2A*	F/25y	Yes	Normal	Yes	Yes	Aided/3y	Yes	No	Profound	Yes	Yes	Yes
P27	*SCN2A*	F/25y	Yes	Normal	NA	Yes	Aided/2y	NA	Few words	Profound	Yes	NA	Yes
P28	*KCNQ2*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
P29	*KCNQ2*	F/5y	Yes	Abnormal	Yes	Yes	No/-	No	Few words	Profound	Yes	Yes	Yes
P30	*KCNQ2*	M/5y	No	Normal	Yes	Yes	Aided/6y	No	NA	Moderate	NA	No	No
P31	*KCNQ2*	F/4y	No	Normal	Yes	Yes	Aided/24m	No	No	Profound	Yes	No	Yes
P32	*KCNQ2*	M/2y	Yes	Normal	Yes	Yes	No/-	–	No	Profound	Yes	No	Yes
P33	*MEF2C*	F/24y	NA	Normal	Yes	Yes	Aided with help/6y	NA	Few words	Profound	Yes	NA	NA
P34	*MEF2C*	F/6y	NA	Abnormal	Yes	Yes	Aided/NA	NA	Few words	Profound	No	NA	NA
P35	*MEF2C*	F/8y	NA	Normal	Yes	Yes	Aided with help/1y 2m	NA	No	Profound	Yes	NA	NA
P36	*MEF2C*	F/18y	NA	Abnormal	Yes	Yes	Aided/3y	NA	No	Profound	Yes	No	NA
P37	*SYNGAP1*	F/7y 8m	No	Normal	Yes	Yes	Aided/22m	Yes	Few words	Moderate	No	No	Yes
P38	*SYNGAP1*	F/17y	Yes	NA	NA	Yes	Aided/NA	Yes	Few words	Moderate	Yes	NA	NA
P39	*SYNGAP1*	F/20y	Yes	Normal	Yes	Yes	Aided/4y 6m	Yes	NA	Moderate	Yes	No	Yes
P40	*SYNGAP1*	F/4y	No	Normal	Yes	Yes	Aided/3y 6m	Yes	NA	Moderate	Yes	No	Yes

Table 3 – Genes and phenotypic overlap with RTT. Abbreviations: EEIE = epileptic encephalopathy infantile-onset; PTHS = Pitt-Hopkins syndrome; MR = mental retardation; XLD = X-linked dominant; AD = autosomal dominant. Grey boxes are clinical characteristics that are not common with RTT.

	MECP2 MIM#312750	CDKL5 MIM#300672	FOXG1 MIM#613454	STXBP1 MIM#612164	TCF4 MIM#610954	SCN2A MIM#613720	KCNQ2 MIM#613720	MEF2C MIM#613443	SYNGAP1 MIM#612621
	RTT	EEIE2	RTT, congenital	EEIE4	PTHS	EEIE11	EEIE7	MR20	MR5
Developmental regression	+	+	+	+	–	+	–	–	+
Speech deficit/lost	+	+	+	+	+	+	+	+	+
Gait abnormalities	+	+	+	+	+	+	+	+	+
Hand use lost/absent	+	+	+	+	+	+	+	+	+
Stereotypic movements	+	+	+	+	+	+	+	+	+
Breathing dysfunction	+	+	+	–	+	–	–	–	–
Learning impairment	+	+	+	+	+	+	+	+	+
Seizures	+	+	+	+	+	+	+	+	+
Microcephaly	+	+	+	+	+	+	–	–	+
Dysmorphic facial features	–	+	–	–	+	–	–	+	–
Inheritance	XLD	XLD	AD	AD	AD	AD	AD	AD	AD

infantile-onset epileptic encephalopathy-11 (EEIE11; OMIM*613721) and benign familial infantile seizures-3 (BFIS3, OMIM*607745) and variants with diminished channel activity that leads to ASD/ID.[34]

We found six RTT-like patients carrying variants in the *SCN2A* gene: four missense, one nonsense and one frameshift variants. Two of them (p.R853Q and p.E1211K) have been associated with West syndrome and neonatal-infantile seizures.[35] Regarding the novel variants, the missense variant p.A1773T is predicted to be pathogenic by *in silico* analyses; in fact, another reported variant that affects the same codon (p.A1773V) has been related to ASD/ID.[36] Pathogenicity of the missense variant p.P569A is difficult to assess; despite the fact that it was considered damaging by 1 of 4 predictors, it had been inherited from the father. Moreover, this variant is located between the two first transmembrane domains in a region with unknown functional implications. Therefore, further studies are needed to define the effects of this variant. Although the frameshift variant is at the C-terminal end, outside the transmembrane regions, there are other frameshift deletions described in this region that are related with EEIE and ASD/ID.[37]

We had clinical information for five patients. We found that 3 of the 5 patients had a very early onset of seizures (neonatal or early infancy), as already reported by Kong et al.[38] The patient with the recurrent p.R853Q variant presented epilepsy onset at 3 months of life, similar to other patients described in the literature.[36] We did not find any of the abnormalities described by Kong and colleagues in brain MRI scans of four of our patients. Other patient series have found that variants located outside of the transmembrane domains were more likely to cause a severe phenotype.[35] However, Kong et al. did not find this correlation; in our limited cohort, we did not find this correlation either.

3.4. *KCNQ2* gene

We described five RTT-like patients with five different missense variants in the *KCNQ2* gene (potassium voltage-gated channel subfamily Q, member 2; OMIM#602235). In neuronal cells, *KCNQ2* and *KCNQ3* heterotetramerize to give rise to the M current (IM), a key player for the regulation of neuronal excitability.[39] Variants in this gene are responsible for a wide phenotypic spectrum of epileptic diseases, ranging from infantile-onset epileptic encephalopathy-7 (EIEE7, OMIM*613720) to benign familial neonatal seizures-1 (BFNS1, OMIM*121200).[40]

Two of the identified variants (p.A609T and p.R871G) are located outside of the known protein domains. The p.A609T variant is the only homozygous change identified in the present study that had been inherited from carrier parents (consanguineous family), and it is located close to the calmodulin binding distal domain. The 4 *in silico* predictors consider p.A609T as a benign variant since, in two of the alternative transcripts but not in the canonical one, it is a synonymous change. Though p.A609T seems to be benign, in our opinion, the absence of this variant in the control population and its homozygosity suggests that it could be producing a protein malfunction. Functional studies are needed to validate our hypothesis. Furthermore, p.R871G is located near

the C-terminal of the protein, and, as a missense variant, it is predicted to be pathogenic by *in silico* analyses; in fact, another reported variant affecting the same codon (p.R871S) has been related to EEIE7.[41] The other three variants are located in the transmembrane segments of the protein (TM6), the third segment (p.R144G) and the fourth segment (p.R198Q and p.R213W). The p.R144G and p.R198Q variants have been reported to exhibit a gain-of-function in heterologous expression studies, as p.R144G associated with BFNS1 and R198Q with EEIE7.[40,42] The *KCNQ2* p.R213W variant has been previously described and related to BFNS1[43] as well as in severely affected individuals.[41] In fact, it is known that *KCNQ2* mutation carriers, who have children affected with a severe epileptic phenotype, are mosaic for these variants and often present with BFNS1 associations. Therefore, it is important to look at the clinical history of the carrier mother and study the possibility of mosaicism in this case.[41]

We had the complete clinical information of patients 29 (p.R198Q), 30 (p.R213W), 31 (p.A609T) and 32 (p.R871G). All of them had the most characteristic features of RTT: a normal development during the first months of life, followed by a profound mental retardation, with developmental regression and autistic features. Specifically, patient 29 presented with similar clinical features to the patients previously described with the same variant.[42] Patients 29, 30 and 32 presented with generalized myoclonic or tonic-clonic seizures before the first 18 months of life. In previously described patients, the seizures resolved with treatment (ketogenic diet, carbamazepine or levetiracetam), suggesting that our patients, who are younger than those previously described, may become seizure-free with treatment.[44]

3.5. MEF2C *gene*

MEF2C haploinsufficiency syndrome has been recognized as a neurodevelopmental disorder. To date, fourteen patients with *MEF2C* variants have been identified, including three nonsense, three missense and three frameshift variants.[45–47] We have detected one missense, one no-stop and two frameshift variants. The missense variant is located in the MADS domain; to date, three other missense variants have been described. Although we did not perform functional studies, Zweier et al. (2010) demonstrated that mutations in this domain affect DNA binding specificity; thus, we hypothesize that the same mechanism may contribute to this case.[45] Both frameshift variants are predicted to generate a premature stop codon at amino acid positions 188 and 327, which would result in the loss of the functional TADII domain and an aberrant protein structure. Although the no-stop variant is *de novo* and results in a prolonged protein with 58 extra amino acids, it is predicted to be benign, and, in the gnomAD database, there is one no-stop variant reported (1/244656), p.*484Argext*57. When reviewing this single case, it appears to be a mosaicism, as the wild type is represented 170 times and the alternate variant 54 times. Transcriptional reporter assays have also indicated that *MEF2C* mutations diminish the synergistic transactivation of E-box promoters, including those from *MECP2* and *CDKL5*.[45]

By comparing the clinical descriptions of our patients, most of them have similar facial phenotypes (patients 33, 34 and 35, Supplementary Fig. S1) to those previously described.[46,47] Our patients also presented with epilepsy with no refractoriness that was controlled by anti-epileptic drugs (AEDs), except patient 36 (p.*473Lnext*58), who is seizure-free. Patients 34 and 36 had achieved independent walking, though patients 33 and 35 were able to walk with support despite their unstable wide-based gait. Patient 33 had been followed for a long period and had not improved and had not lost her ability to walk in the last 18 years. Hand stereotypies were present in all our patients and had not diminished during follow–up, as is seen in classic RTT.

Of the four patients who presented with severe intellectual disability with autistic features, three of them displayed content behaviours, which have been described in other patients.[47] Patients 33 and 34 were able to say only a few words, and patients 35 and 36 did not speak at all; these observations are not related to the mutation types observed in other studies, where microdeletions are not always associated with epilepsy.[47,48] Two of our patients had sleep problems (patients 33 and 35) and required pharmacological treatment.

The follow-up of our patients until adulthood will add new details to their phenotype. Our results provide more evidence to support the involvement of *MEF2C* in an RTT-like neurodevelopmental disorder that is characterized by severe intellectual disability, absent or delayed speech, motor and behavioural alterations, early onset of seizures, variable MRI abnormalities and facial dimorphisms.

3.6. SYNGAP1 *gene*

We found four RTT-like patients with four different variants in the *SYNGAP1* gene (synaptic RAS-GTPase-activating protein 1, OMIM#603384), one missense, one nonsense and two frameshift variants. The wild-type protein is localized to dendritic spines in neocortical pyramidal neurons, where it is able to positively or negatively regulate the density of NMDA and AMPA receptors at glutamatergic synapses and mediate signalling downstream of glutamate receptor activation.[49] Severe *de novo* variants in *SYNGAP1* resulting in haploinsufficiency lead to a defined phenotype characterized by ID with epilepsy [termed Mental Retardation-Type 5 (MRD5), OMIM#306684].[50]

The two frameshift variants (p.L595Cfs*55 and p.T674Pfs*36) are clearly deleterious, as they abolish functional domains and are located upstream of other truncating mutations previously reported in ID patients.[51] p.R967* is located in the C-terminus of the protein, which could go through NMD (Nonsense Mediated Decay), which is a missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.[51] Although p.R905C is predicted by *in silico* studies to be deleterious, its pathogenicity is difficult to assess. On the one hand, this variant is not located in any major domain of the protein; however, on the other hand, it has not been reported in any control databases. It is essential to determinate the inheritance of this variant to establish its causality.

We had complete clinical information from two of the four patients (p.L595Cfs*55 and p.R905C). In line with previous series, hypotonia and gait abnormalities were the main recurrent features. Corroborating previous results,[52] MRI scan in our patients did not show any specific features; thus, brain imaging is not helpful in the diagnoses of *SYNGAP1*-related disorders. Interestingly, our study is the first to report a group of patients with a moderate mental retardation; all of them are able to walk and communicate with few words.

4. Conclusion

In our findings, the limited number of patients grouped by altered genes do not allow to generate a clear genotype-phenotype correlation among them. We only found that RTT-like patients with *SYNGAP1* variants presented the most moderate phenotype, which consists of the possibility of learning, in contrast with the other RTT-like patients. A possible explanation for the heterogeneity in disease onset and/or severity of disease progression is the altered expression of a modifier gene or genes that can exacerbate or diminish the clinical syndrome.[34] Moreover, microenvironmental factors might account for skewing the genotype-phenotype relationship.

NGS methodologies have overcome some difficulties, and the process of diagnosis has moved from one of clinical assessment to one of genetic confirmation. Certainly, there are benefits of defining patients by clinical descriptions, such as grouping patients with similar features for the purpose of clinical management. For RTT molecular diagnosis, it is important to enlarge the study from the three classic genes (*MECP2*, *CDKL5* and *FOXG1*) to include those genes that have a clinical presentation that overlaps with RTT features, such as *STXBP1*, *TCF4*, *SCN2A*, *KCNQ2*, *MEF2C* and *SYNGAP1*. The detection of variants in RTT-like genes may modify the initial clinical diagnosis to other neurodevelopmental syndromes or determine new candidate genes related to RTT-like features, providing the clinician with more information and clues that could help in the prevention of future symptoms or in pharmacologic therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank all patients and their families who contributed to this study. The work was supported by grants from the Spanish Ministry of Health (Instituto de Salud Carlos III/FEDER, PI15/01159), the crowd-funding program PRECIPITA, the Spanish Ministry of Health (Fundación Española para la Ciencia y la Tecnología, Spain), the Catalan Association for Rett Syndrome, Fondobiorett and Mi Princesa Rett.

Appendix A

Working group

Hospital Sant Joan de Déu (Barcelona)
Maria del Mar O'Callaghan mocallaghan@sjdhospitalbarcelona.org
Àngels Garcia-Cazorla agarcia@sjdhospitalbarcelona.org
Hospital Sant Joan de Déu (Martorell)
Maria del Carmen Serrano Munuera cserrano@hmartorell.es
Silvia Cuso García scuso@hmartorell.es
Hospital San Borja Arriaran, Universidad de Chile Santiago (Chile)
Monica Troncoso monicatroncososch@gmail.com
Guillermo Fariña guillermofarina@gmail.com
Hospital Infantil Universitario Niño Jesús (Madrid)
Juan José García Peñas jgarciadelarape.1961@gmail.com
Hospital Universitario de Getafe (Madrid)
Belen Gil Fournier bgil.hugf@salud.madrid.org
Soraya Ramiro León soraya.ramiroleon@salud.madrid.org
Hospital Univesitari Parc Taulí (Sabadell)
Miriam Guitart MGuitart@tauli.cat
Neus Baena NBaena@tauli.cat
Hospital Universitario Araba-Txagorritxu (Vitoria)
Guiomar Perez de Nanclares gnanclares@osakidetza.eus
Intzane Ocio Ocio intzane.ocioocio@osakidetza.eus
Neurología, servicio de Medicina Interna. Hospital Universitario de Fuenlabrada (Madrid)
Eva Gutiérrez-Delicado eva.gutierrez@salud.madrid.org
Belén Abarrategui belen.abarrategui@salud.madrid.org
Instituto de Genética Médica y Molecular (INGEMM), IdiPAZ (Madrid)
Eva Barroso eva.barroso@salud.madrid.org
Fernando Santos-Simarro fsantossimarro@gmail.com
Pablo Lapunzina pablo.lapunzina@salud.madrid.org
Análisis Clínicos, Hospital Universitario Fundación Alcorcón (Madrid)
Francisco J. García fjgarciai@fhalcorcon.es
Juan M. Acedo jmacedo@halcorcon.es
Unidad Neuropediátrica, Hospital Universitario Fundación Alcorcón (Madrid)
Asunción García agarcia@fhalcorcon.es
Miguel A. Martinez MAMartinezg@fhalcorcon.es
Servicio de Neurología, Hospital Infantil, Hospital Universitario La Paz (Madrid)
Antonio Martínez-Bermejo ambermejo@salud.madrid.org.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpn.2019.04.006.

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