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"Characterization of a new epileptic syndrome due to PCDH19 gene mutation"

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Foreword

Protocadherin 19 (*PCDH19*) is an X-linked gene belonging to the protocadherin superfamily, whose members are predominantly expressed in the central nervous system and have been implicated in cell-cell adhesion, axon guidance and dendrite self-avoidance. Heterozygous loss-of-function mutations in humans result in the childhood epilepsy disorder with intellectual disability and autistic spectrum disorder.

PCDH19 gene mutations were firstly associated with epilepsy in 2008, when seven Australian families with Female-restricted Epilepsy and Mental Retardation (EFMR) were reported (Dibbens et al., 2008). Afterword, first sporadic cases were identified within a cohort of patients with Dravet Syndrome (DS) that had been resulted negative for *SCN1A* gene mutation (Depienne et al., 2009).

Since 2008, the number of patient with PCDH19-realted epilepsy has progressively grown-up and a more detailed phenotype came out (Depienne et al., 2009, 2011; Dibbens et al., 2008; Higurashi et al., 2012; Jamal et al., 2010; Marini et al., 2010, 2012; Specchio et al., 2011).

The first part of this doctoral work is based on an electro-clinical study, realized with the aim to better define the epileptic phenotype, identify genotype-phenotype correlation and predicting factors for outcome. Moreover, PCDH19-

related epilepsy was compared with Dravet Syndrome (DS) and find out a clear-cut distinction between the syndromes, in order to address the diagnosis toward one or the other at first clinical disease manifestations, other than for classification purposes.

The second part is focused on the pathogenetic mechanisms of epilepsy as well as intellectual disability and autism. The gene shows an unusual X-linked inheritance sparing the transmitting male and affecting only female subjects. A cellular interference mechanism has been hypothesized in which the coexistence of mixed neuronal populations in heterozygous mutated female results to be pathogenic, whereas homogeneous neuronal populations in hemizygous mutated males are not.

The unusual, gender reversed X-chromosome inheritance of PCDH19-FE led also to speculate that genes with different expression between the two sexes, may play a role in the pathogenesis. To support this hypothesis, it has been supposed that AKR1C1-3 genes, involved in the synthesis of a broad range of neuroactive steroids, could be dysregulated in these patients (Tan et al. 2015). Among neuroactive steroids, allopregnanolone is one of the most potent GABA receptor modulators, and may contribute to epilepsy, intellectual disability and autism. If this pathogenetic hypothesis would be verified, new opportunities for targeted therapeutic interventions may be available. The study that I conducted during the doctoral course was based on neuroactive steroids measurements in *PCDH19* mutated patients. The study confirmed *in vivo* the hypothesis by Tan

et al. (2015), however further studies needed to better understand the pathogenesis of this PCDH19-related epilepsy and to provide a better management and treatment of this syndrome.

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Abstract

Objective: PCDH19-related epilepsy is an epileptic syndrome, arising within the first three years of life and characterized by clustered and fever-induced seizures. In most of cases it is associated with Intellectual Disability (ID) and autistic features. Aim of this study is to analyze a large Italian population with PCDH19-related epilepsy in order to better define the epileptic phenotype, identify genotype-phenotype correlation, predicting factors for outcome, and markers for differential diagnosis with Dravet Syndrome (DS). The unusual, gender reversed X-chromosome inheritance of PCDH19-FE led also to speculate that genes with different expression between the two sexes may play a role in the pathogenesis and that AKR1C1-3 genes, could be dysregulated, thus resulting in allopegnanolone reduced blood levels.

Methods: We retrospectively collected genetic, clinical and EEG data of 61 patients affected by PCDH19-related Epilepsy, coming from 15 Italian hospitals. We stratified patients into two groups according the outcome. We analysed the following variables: mutation type, age at onset, age at study, seizure type, occurrence of status epilepticus, EEG abnormalities, cognitive and behavioural disorders. ROC curve analysis was performed in order to discriminate the age at which seizures could decrease in frequency. A group of

15 patients with PCDH19-related epilepsy were compared with 19 patients with DS. Comparisons were performed with Fisher's exact test or Student's t-test.

In order to ascertain allopregnanolone deficiency, we performed a prospective case-control study. We enrolled 12 patients affected by PCDH19-related epilepsy and 15 controls, age-and sex-matched. Controls were recruited among subjects evaluated for praecox puberty or hyperandrogenism. In both groups blood samples were taken at basal (T_0) and 60 min after (ACTH) administration (T_1). Quantitative analysis of neuroactive steroids in serum was performed by liquid chromatography-electrospray tandem mass spectrometry.

Results: At last follow-up (median 12 years; range 1.9-42.1), 13 patients (21.3%) had monthly-weekly seizures, 78.7% annual seizures/clusters or less frequent. Twelve patients (19.7%) were seizure-free since > 2 years. ROC analysis showed a significant decreasing of seizure frequency after the age of 10.5 years (sensitivity 81.0%; specificity 70.0%). Thirty-six patients (59.0%) had ID and behavioral disturbances. ID was moderate-severe in almost half of them. Autistic spectrum disorder was present in 31 patients. An earlier age at epilepsy onset resulted the only predictor factor for ID ($p=0.05$) and autistic spectrum disorder ($p<0.014$). Conversely, age at onset was not a predictor factor for seizure outcome ($p<0.214$). Epilepsy onset was earlier in DS (5.0+2.1 vs 11.2+7.0 months; $p<0.05$). The second seizure/cluster occurred after a longer latency in PCDH19-related epilepsy rather than in DS (10.1+13.6 vs 2.2+2.1 months; $p<0.05$). All neuroactive steroids resulted down produced in patients

with PCDH19-related epilepsy rather than controls and this data was confirmed after ACTH stimulus.

Conclusions: We found that an earlier age at epilepsy onset is linked with a significant risk for ID and autistic spectrum disorder. The decreasing of seizure frequency after the age of 10.5 years supports the hypothesis of a down-regulation of neurosteroid-metabolizing enzymes and allopregnanolone deficiency in PCDH19-related epilepsy. We also documented a down regulation of all steroidogenesis in PCDH19-related epilepsy. Particularly we found allopregnanolone and pregnenolone sulfate deficiency. Allopregnanolone is a GABA-A receptor modulator influencing the neuronal excitability, thus representing a realistic therapeutic target for PCDH19-related epilepsy. We failed to identify any genotype-phenotype correlation considering the site and type of PCDH19 mutations. We were able to find out some distinctive features, which could address the diagnosis towards DS or PCDH19-related epilepsy, since first manifestation. These considerations suggest to definitively considering PCDH19 gene as cause of a proper epileptic phenotype.

PART I: ELECTRO-CLINICAL STUDY

Objective: PCDH19-related epilepsy is an epileptic syndrome, arising within the first three years of life and characterized by clustered and fever-induced seizures. In most of cases it is associated with Intellectual Disability (ID) and autistic features. Aim of this study is to analyse a large Italian population with PCDH19-related epilepsy in order to better define the epileptic phenotype, identify genotype-phenotype correlation and predicting factors for outcome. Moreover we aim to find out differences with Dravet Syndrome (DS) in order between these two infantile epilepsies with fever sensitivity and allow a more precocious diagnosis.

Methods: We retrospectively collected genetic, clinical and EEG data of 61 patients affected by PCDH19-related Epilepsy, coming from 15 Italian hospitals. We stratified patients into two groups according the outcome. We analysed the following variables: mutation type, age at onset, age at study, seizure type, occurrence of status epilepticus, EEG abnormalities, cognitive and behavioural disorders. ROC curve analysis was performed in order to discriminate the age at which seizures could decrease in frequency. A group of 15 patients with PCDH19-related epilepsy were compared with 19 patients with DS. Comparisons were performed with Fisher's exact test or Student's t-test.

Results: At last follow-up (median 12 years; range 1.9-42.1), 13 patients (21,3%) had monthly-weekly seizures, 78,7% annual seizures/clusters or less frequent. Twelve patients (19.7%) were seizure-free since $\geq$ 2 years. ROC analysis showed a significant decreasing of seizure frequency after the age of 10.5 years (sensitivity 81.0%; specificity 70.0%). Thirty-six patients (59.0%) had ID and behavioral disturbances. ID was moderate-severe in almost half of them. Autistic spectrum disorder was present in 31 patients. An earlier age at epilepsy onset resulted the only predictor factor for ID ($p=0.05$) and autistic spectrum disorder ($p<0.014$). Conversely, age at onset was not a predictor factor for seizure outcome ($p<0.214$). Epilepsy onset was earlier in DS (5.0+2.1 *vs* 11.2+7.0 months; $p<0.05$). The second seizure/cluster occurred after a longer latency in PCDH19-related epilepsy rather than in DS (10.1$\pm$13.6 *vs* 2.2$\pm$2.1 months; $p<0.05$). Seizures were mainly single and prolonged seizures in DS, and brief and clustered in PCDH19-related epilepsy.

Conclusions: We found that an earlier age at epilepsy onset is linked with a significant risk for ID and autistic spectrum disorder. The decreasing of seizure frequency after the age of 10.5 years supports the hypothesis of a down-regulation of neurosteroid-metabolizing enzymes and allopregnanolone deficiency in PCDH19-related epilepsy. We failed to identify any genotype-phenotype correlation considering the site and type of *PCDH19* mutations. We were able to find out some distinctive features, which could address the diagnosis towards DS or PCDH19-related epilepsy, since first manifestation.

These considerations suggest to definitively considering *PCDH19* gene as cause of a proper epileptic phenotype.

CLINICAL AND ELECTROENCEFALOGRAPHIC CHARACTERIZATION OF PCDH19-RELATED EPILEPSY

Introduction

PCDH19-related epilepsy is an epileptic syndrome, arising within the first three years of life and characterized by mostly clustered and fever-induced seizures. The first association between *PCDH19* gene mutations and epilepsy dates back to 2008 (Sheffer et al., 2008; Dibbens et al., 2008). Since then, the number of patients with PCDH19-related epilepsy is grown and a more detailed clinical picture has been delineated (Depienne et al., 2009, 2011; Higurashi et al., 2012; Jamal et al., 2010; Marini et al., 2010, 2012; Specchio et al., 2011,Terracciano et al., 2016, Thiffault et al., 2016). Although *PCDH19* gene is located on chromosome Xq22, this condition has an unusual X-linked mode of inheritance sparing transmitting males and affecting only carrier females. Males are affected only in the case of a somatic mosaicism (Figure 1) (Depienne et al., 2009).

Epilepsy is characterized by focal and generalized seizures mainly induced by fever and recurring in clusters lasting from hours to days. In most of cases it is associated with intellectual disability (ID) and autistic features, but also patients

with normal psychomotor development are reported (Depienne et al., 2009, Marini et al., 2012). The presence of focal seizures mainly with affective symptoms emerged as quite a hallmark of this epileptic syndrome in a large study on 35 Italian patients (Marini et al., 2012). In the same study, generalized seizures (tonic seizures, myoclonic and absences) were also described.

EEG features are not still well defined. Interictal EEG is highly variable; it is reported normal in about half of patients or characterized by a slowing background activity and epileptiform and/or slow abnormalities the remaining cases (Marini et al., 2012).

On the basis of current knowledge, clinical phenotype appears to be highly variable, ranging from well-controlled epilepsy with normal cognitive development to drug-resistant epilepsy with severe cognitive delay (Depienne et al., 2009, 2011; Dibbens et al., 2008; Higurashi et al., 2012; Marini et al., 2010, 2012; Specchio et al., 2011) and genotype-phenotype correlation is not jet clearly defined.

Aim of this study is to analyse clinical and EEG features of a large Italian population with PCDH19-related epilepsy in order to better define the epileptic phenotype, identify genotype-phenotype correlation and prognostic factors for outcome in such a variable clinical spectrum. This would be of huge relevance both for clinical practice and classification purpose.

Materials and methods

This is a retrospective multicentre study. We collected 61 patients affected by PCDH19-related Epilepsy, coming from 15 Italian Epilepsy Centres. For each patient, the referring physicians filled out a detailed clinical questionnaire including neurologic and neuropsychological assessments, seizure semiology and frequency, brain neuroimaging, genetic mutation. The genetic report documenting the *PCDH19* gene mutation was available for all patients enrolled. For each patient, all consecutively performed EEG were analysed by an expert neurophysiologic team. An overall number of 551 EEG have been reviewed. A report form was applied to each EEG in order to collect data on parameters of recording, vigilance state, background activity; presence, type and localization of epileptiform and slow abnormalities, presence of Photo-Paroxysmal Response (PPR), electro-clinical characteristics of recorded seizures. In the cases in which the EEG trace was not available (52.2%), the analysis was based on EEG reports.

Informed consent for clinical data and video-EEG studies was obtained for all patients in each participating institution from parents or legal guardians. In our cohort are included also previously reported patients (Marini et al., 2010; Specchio et al., 2011; Leonardi et al., 2014, Terracciano et al., 2016; Trivisano et al., 2016).

Epileptic seizures were classified according to the International League Against Epilepsy criteria (Berg et al., 2010; Blume et al., 2001).

Other than the definition of the electro-clinical phenotype, aim of this study was the identification of prognostic factors. We stratified patients into two groups according the outcome: 1) patients with normal cognitive development and seizure-free since at least 2 years and 2) patients with cognitive delay and seizure persistence. The analysed variables were: gender, age at onset, age at study, Type of mutation, fever sensitivity, seizure type, fever sensitivity and cluster occurrence, SE occurrence, presence of EEG abnormalities, cognitive and behavioural disorders.

Statistical analysis

Statistical analysis was performed using R, version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

Prior to modelling, the data were summarized with descriptive statistics for each variable including means, medians, and range for continuous variables and frequencies for categorical variables.

An independent t-test and the Mann-Whitney U test with continuity correction were used for continuous variables; the analysis of variance (ANOVA) was used for the comparison of the mean of multiple groups with a Tukey's HSD post hoc analysis when necessary. For multiple testing, a correction was applied according to the Bonferroni rule. For dichotomous results, a chi-square test or Fisher's exact test was performed, as appropriate.

A p value < 0.05 was considered was considered to indicate statistical significance. All statistical tests were two-tailed.

ROC curve analysis was performed to discriminate the age at which seizures start to decrease in frequency.

Results

Clinical features

Median age at study was 12.0 years (7.4-16.0 years, 95%CI; range 1.9 -42.1). Two out 61 (3.3%) were males with a *PCDH19* gene mutation in mosaic status. Male patients have been already reported (Terracciano et al. 2016). We collected fifteen familiar cases, eleven inherited and two pair of twins with *de novo* mutation. Clinical and genetic details of all patients are included in the supplemental table.

Median age at epilepsy onset was 10.0 months (7.0-16.0 months, 95%CI; range 1.0-68.0). The majority of patients had clustered seizures (n=52; 85.4%) and/or fever sensitivity (n=57; 93.4%). Seizures were described with different semiology. Focal seizures were described in all patients. Focal seizures were classified into two types: motor (85.2%) and hypomotor (59.0%) according the semiology. Motor seizures were mainly tonic-vibratory seizures. Hypomotor seizures were characterized by psychomotor arrest, loss of tone, hypopnea, cyanosis and desaturation. In 82.0% of cases, both in motor and hypomotor seizures, a fearful expression was reported. Generalized seizures were described

in 7 patients (11.4%) and they were reported as absences (9.8%) and myoclonic seizures (4.9%); atonic and generalized tonic-clonic seizures were never reported nor recorded. Seizure frequency was highly variable, with the presence of multiple seizures in few hours (seizure clusters) and long seizure-free periods also lasting several years (8 years in two patients). In 19 patients (31.1%), seizure clusters resulted in a SE: in 7 cases (36.8%) it was a continuous NCSE while in 12 (63.2%) it consisted of repetitive seizures with motor or hypomotor seizures without regaining of consciousness between the episodes.

Long-term outcome

At last follow-up (median age 12 years; 7.40-16.0 years, 95%CI; range 1.9-42.1), 13 patients (21.3%) had monthly-weekly seizures, 48 patients (78.7%) annual seizures/clusters or less frequent, 12 patients (19.7%) were seizure-free since $\geq$ 2 years. ROC analysis showed a significant decreasing of seizure frequency after the age of 10.5 years (sensitivity 81.0%; specificity 70.0%). At last follow-up visit, 36 patients (59.0%) had a cognitive delay and behavioral disturbances, even not always associated. ID was moderate-severe in 21 patients. ASD was present in 31 patients. The earlier age at epilepsy onset resulted the only predictor factor for ID ($p=0.05$) and ASD ($p<0.014$). Conversely, age at onset was not a predictor factor for seizure outcome ($p<0.214$). Also considering the global outcome (combining seizures outcome

and cognitive-behavioral development) earlier age at onset was the unique predictor factor (*p=0.009*) (Table 1).

EEG features

A mean number of 9.0 EEG (range: 1-37) per patient have been analysed. Mean age at EEG recording was 6.2 years (range: 6 months- 38.3 years). In 135 EEG (28.7%) had also a video recording, and 177 EEG (37.6%) a poly-graphic recording. In 14 cases a long-term monitoring was performed (mean: 14 hours). Mean EEG follow-up period was 5.6 years (range: 0-37.4). The majority of EEG (79.8%) was recorded during awake.

Interictal EEG

Awake EEGs, showed a good organization and differentiation of background activity in 73.1%. The remaining part (26.9%) showed a diffuse slowing-down background activity. Among sleep EEGs, only about half (49.5%) had a good organization of sleep with the presence of physiological figures of NREM sleep. Epileptiform abnormalities were detected in two third of patients (66.6%; n=34) both focal (79.4%) and diffuse (23.5%). None of our patients had only properly generalized abnormalities. Prevalence of abnormalities was not different between awake (24.2%) and sleep (19.7%). A huge variability in abnormalities localization during follow-up was found, without a unique EEG pattern evolution for all the patients.

Slow abnormalities were found in 76.5% of patients, slighter more frequent during sleep (63.0% sleep *vs* 54.4% awake). They were focal in 85% of EEGs, mainly localized over temporal (56.1%) and central (26.9%) regions.
Photic stimulation induced a PPR in 6 patients (9.8%). It was transient EEG feature, in 5 patients from the age of 7 to 12.5 years; in one patient from 3.5 to 5 years. However, in about half of EEGs (51.2%), intermittent photic stimulation was not performed because the poor collaboration of patients. In one patient a transient fixation-off sensitivity from 11.4 to 13.4 years was found.

Ictal EEG

Overall 125 ictal EEGs belonging to 34 patients have been analyzed. Seventy-four ictal recordings were visually analyzed, while the remaining 51 seizures thought their EEG reports. Sixty-one seizures were also video recorded.
Focal seizures arose mainly from temporal regions (82.8%) followed by frontal (6.2%), parietal-occipital (6.2%), and central (4.7%) regions. Forty-nine seizures (39.2%) had a diffuse onset.
Seizures with a diffuse onset were characterized by a bilateral ictal discharge consisting of a diffuse slow spike followed by a low voltage theta activity followed by bilateral rhythmic fast activity of higher voltage increasing in voltage and decreasing in frequency, often asymmetric. From a clinical point of view these bilateral seizures could have a motor (Figure 2) or hypomotor

(Figure 3) semiology. Although these two types of seizures (motor and hypomotor) were stereotyped, they were not associated with a specific EEG pattern for each of them. In 78.9% of seizures a fearful expression or affective symptoms was detected. Properly generalized seizures were never recorded. The so-called "absence seizures" were not characterized by a generalized 3Hz spike and wave complexes, but had a frontal onset with a rapid synchronization (Fig. 4).

Moreover in about one fifth (19.4%) of recorded seizures, an asynchrony over the two hemispheres was found, as they were two independent seizures (Fig. 5). In the same cluster, seizures could start both from the right and the left hemisphere (Fig. 6).

Mean seizure duration was 65.3 seconds (range: 5-200 seconds). We analyzed 14 seizure clusters and 65 single seizures (not clustered). A higher incidence of seizures during sleep was observed (71.2% of recorded seizures).

In 7 patients a SE was recorded: in 3 patients it was a NCSE (Fig. 7) characterized by an impairment of consciousness associated with severe desaturation and cyanosis, oral automatisms and rare distal myoclonic jerks. Conversely, the other four patients had a motor SE characterized by repetitive focal motor seizures, without regaining of consciousness between seizures.

Discussion

In recent years, much effort has gone into molecular genetic studies on PCDH19-related epilepsy, with the purpose of discovering the pathogenic basis

of this syndrome. Multiple hypotheses have been considered for pathogenesis, particularly the mechanism of cellular interference (Depienne and LeGuern, 2012) and allopregnanolone deficiency due to neurosteroid-metabolizing enzymes down-regulation (Tan et al., 2015). Likewise, the phenotypic spectrum of patients with *PCDH19* mutations has been expanded (Higurashi et al., 2012; Marini et al., 2012, Terracciano et al., 2016).

Through a National multicenter study, we were able to collect the largest population with PCDH19-related epilepsy ever reported in order to try better define the clinical phenotype, identify genotype-phenotype correlation and predicting factors for outcome.

Electro-clinical phenotype

At the first description, PCDH19-related epilepsy was named *Epilepsy and mental retardation limited to females* (EFMR) (Sheffer et al., 2008; Dibbens et al., 2008) in order to underline the two main findings of this syndrome that were the ID and the exclusive expression in female subjects. In the latest years, and in our cohort, the clinical phenotype was expanded to male subjects, who present with clinical features absolutely corresponding with those of females (Table 2) (Terracciano et al., 2016). Nevertheless, mosaic males represent a very rare condition and up to now only two other cases have been described (Depienne et al., 2009; Thiffault et al., 2016).

Also ID is no more a constant finding and about one third of subjects of our cohort have a normal cognitive development at last follow-up. Differently, the occurrence of seizure in cluster and fever sensitivity have been confirmed the two main hallmarks of this epileptic syndrome (Dibbens et al., 2008; Depienne et al., 2009).

Seizures semiology resulted to be highly variable during the disease history with the prevalence of hypomotor seizures during the first years of disease. Through the analysis of the recorded seizures, we highlighted that all seizures have a focal onset. Despite the persistence of focal onset of seizures on EEGs, the rapid generalization that appears was misleading. The presence of seizures independently arising from both hemispheres, even during the same seizure cluster, and seizures characterized by asynchronous and independent discharges on both hemispheres, is typical of genetic epilepsies and for some aspects resembling the migrating seizure of MMPSI (Malignant Migrating Partial Seizures of Infancy (Fig. 4). The previous described generalized seizures (Marini et al., 2010, Specchio et al., 2011; Higurashi et al., 2012; Marini et al., 2012) were actually focal seizures with a rapid bilateral generalization. They represent the 40% of all seizures and, also form a clinical point of view, they were not properly generalized tonic-clonic seizures, but motor (tonic-vibratory) or hypomotor seizures, with focal signs (i.e. eye deviation, oral automatisms, asymmetric limb hypertonus).

Likewise, we did not identify a characteristic interictal EEG picture. Interictal abnormalities during a cluster period could be normal or showed a diffuse slowing of background activity and multifocal spikes, without disclose common specific features. The prevalence of interictal epileptiform abnormalities over frontal and temporal regions, has been hypothesized to be due to the possible prominent expression of protochaderin19 in the areas connected to the hippocampal formation, such as entorhinal cortex, lateral septum, and basolateral amygdaloid complex as has been demonstrated in rats (Kim et al., 2010). This data is also confirmed by the onset of seizures mainly form temporal regions and prevalence of affective symptoms during seizures, reported by Marini et al.in 2012 and confirmed also in our larger population. One third of patients do not show any interictal EEG abnormalities during the whole follow-up period, a lower rate respect the previous data from 48% to 57% (Marini et al., 2012; Higurashi et al., 2012).

We found a transient photosensitivity in about 10% of patients. PPR was not reported in the majority of reports on PCDH19-related epilepsy (Marini et al., 2010; Higurashi et al., 2012, Gagliardi et al. 2014) and up to now only few cases have been described (Specchio et al. 2011; Depienne 2009 et al., Depienne et al., 2010, Marini et al., 2012). However photosensitivity rate is much lower than in DS, for which it is reported around 64% (Bureau and Dalla Bernardina, 2011). This can be considered a distinctive feature from DS.

Brain MRI was normal in all patients, although recent studies identify a possible role of PCDH19 in neuronal cortical migration and few brain MRI abnormalities in mutated mouse (Thomas et al., 2016). Future studies with advanced brain MRI technologies, could better improve our knowledge in this field.

Predicting factors and Outcome

The long-term follow-up of our cohort provided original data. During the first decade, epilepsy tended to be active and resistant to multiple antiepileptic drugs (AEDs). Later on, we observed a tendency of seizure frequency to decrease regardless treatment. ROC analysis showed that after 10.5 years seizures start to decrease in frequency. This data could support the hypothesis of allopregnanolone deficiency (Tan et al, 2015). In fact, at the age of 10.5 years, hormonal changes for puberty start and this data might support the hypothesis of neurosteroid-metabolizing enzymes in PCDH19-related epilepsy. Recently Tan et al. summarized available seizure onset and offset ages of all published PCDH19-mutated females. The low frequency of seizures seems to be correlate to a low steroid hormone levels between the so-called 'mini-puberty' (0–6 months) and after puberty (Tan et al., 2015).

At last follow-up, patients had a median of 2 AEDs. A recent study showed that most effective drugs in patients with PCDH19 mutations were bromide and clobazam (Lotte et al., 2016). Actually clobazam was widely used in our cohort

both as chronic and for acute treatment of clusters while, bromide was not. However, assessing the effectiveness of AEDs in this epileptic syndrome is rather difficult because the highly variable seizure frequency and a possible age-dependent spontaneous seizure remission. Moreover, the retrospective nature of this study does not allow to find out any conclusions. Four patients (#5,#6,#7,#61) were treated with stiripentol associated with valproic acid and clobazam and three of them (#6,#7,#61) went into a long remission period (from 1 to 3 years) (Figure 8) (Trivisano et al 2014).

In PCDH19-realted epilepsy, a significant risk of ASD and ID might be present by the second year, without risks for motor deterioration (Camacho et al., 2014; Cappelletti et al., 2015). In fact, motor development is normal in contrast to patients with Dravet syndrome. At follow-up, about two third of patients developed ID and ASD and the younger age at epilepsy onset resulted to be a predictor factor. This data could be explained with a more severe expression of disease with early age at onset, drug resistant-epilepsy ID and ASD, probably due the genetic condition. Nevertheless, environmental factors such as precocious AED treatments and multiple hospitalizations could have a role in the delayed acquisition of psychomotor milestones. Interestingly, the severity of epilepsy did not correlate with a worse cognitive and behavioral outcome, meaning that these symptoms are due mainly to the disease itself and not to epilepsy severity. Also ID and ASD seemed to be independent thus confirming that ASD could be a comorbidity rather than a consequence of ID (Breuillard et

al., 2016). After puberty, when seizures become to decrease in frequency, ID and ASD that were in the background during first infancy become the most relevant symptoms. Therefore, a bigger effort should be done in the future for the identification of ASD and ID early in infancy in order to start a personalized rehabilitation program at first symptoms.

The predictor factor of age at onset was already reported for other epileptic syndromes, as recently for Dravet Syndrome (Cetica et al., 2017).

Genotype-phenotype correlation

We fail to identify any genotype-phenotype correlation considering the site and type of PCDH19 mutations. Thus, mutation does not appear to be a prognostic factor as in other epileptic syndromes in which deletions are associated with a worse outcome. This result is reasonable considering that a cellular interference mechanism has been hypothesized for PCDH19-related epilepsy. Based on this hypothesis, seizures and ID result from a malfunctioning of interaction between mixed (mutated and not-mutated) neuronal populations, independently from the type of mutation. Therefore the site (exon in which the mutation occurs) and the type (missense, splicing, nonsense, FS/rearrangements) of the *PCDH19* gene mutation resulted not relevant. Doubtless, the skewed X-inactivation may play the greater role in females with a mild phenotype or asymptomatic carrier together different neurosteroids expression (Tan et al. 2015). Other genes or

environmental factors may also be involved in the phenotypes associated with *PCDH19* gene mutations.

Even in familiar cases, we detected a huge phenotypic variability, including two pair of twins. As example, among three sisters (supplemental table, pt #17, #18, #19), we found different degree of cognitive outcome and epilepsy severity. Also in such cases, only age at epilepsy onset was confirmed as prognostic factor.

In this study we included only affected patients, not considering transmitted asymptomatic mother and asymptomatic mutated sisters, because we focused on epileptic phenotype. These subjects, asymptomatic for seizures, should probably be deeply tested as in some of them slight psychiatric symptoms could be find out (anxiety, panic attack, aggressive behaviors, etc..).

Overall this study allows us to expand and refine the clinical and electroencephalographic phenotype of patients with PCDH19-related epilepsy and identify an early age at onset as the unique prognostic factor identified ID and autistic spectrum disorder. After the age of eight years, seizures start to decrease in frequency and cognitive and behavioural disturbances become predominant. Genotype-phenotype correlation remained blurred.

We hope that a clearer definition of EEG and clinical features may significantly contribute to reach earlier diagnosis, improve management of seizures and cognitive and behavioral disturbances, and define the natural history and prognostic factor for outcome in such variable epileptic phenotype.

Table 1. Summary of demographic, clinical, genetic and EEG features of all 61 patients and correlation with outcome measures

			OUTCOME MEASURES			
	N	**%**	**ID**	**ASD**	**Seizure persistence**	**Seizure persistence & ID**
Demographic and clinical data						
Sex (female)	59	96.7%	*p=0.16*	*p=1*	*p=0.33*	*p= 0.247*
Age at follow-up (median ± SD)	12 ± 7.6 yrs		*p=0.44*	*p= 0.441*	*p= 0.148*	*p= 0.280*
Age at epilepsy onset (median ± SD)	10.0 ± 10.1mm		***p < 0.05***	***p=0.014***	*p=0.214*	***p= 0.009***
PCDH19 gene mutation						
Type						
- Missense	31	50.9%	*p= 0.431*	*p= 0.104*	*p= 0.83*	*p=0.618*
- Splicing	2	3.3%				
- Nonsense	8	13.1%				
- FS/rearrangements	19	31.1%				
Exon						
Exon 1 (extracellular domain)	54	88.5%	*p=0.51*	*p=0.64*	*p=0.34*	*p=0.84*
Exon 2 (transmembrane domain)	0	0.0 %				
Exon 3-4-5 (intracellular domain)	6	9.8%				
Seizures						
Fever sensitivity	57	93.4%	*p=0.016*	*p=0.28*	*p= 0.330*	*p= 0.581*
Clustered seizures	52	85.4%	*p= 0.267*	*p= 0.544*	*p=1*	*p=1*
Seizure type						
- Focal motor seizures	52	85.2%	*p= 0.725*	*p= 0.1023*	*p= 0.659*	*p= 0.335*
- Focal hypomotor seizures	36	59.0%	*p=1*	*p= 0.702*	*p= 0.316*	*p=1*
- Seizures with affective symptoms	50	82.0%	*p= 0.106*	*p= 0.644*	*p=1*	*p=1*
- Myoclonic	3	4.9%	*p=0.263*	*p=0.06*	*p=0.1*	*p=1*
- GTCS	0	0.0%	*n.a.*	*n.a.*	*n.a.*	*n.a.*

- Atypical absences	6	9.8%	*p= 0.386*	*p=1*	*p= 0.58*	*p=1*
Status epilepticus	19	31.1%	*p= 0.688*	*p= 0.436*	*p=1*	*p=1*
EEG features						
Interictal epileptiform abnormalities	34	66.6%	*p= 0.176*	*p=1*	*p=1*	*p= 0.686*
Photosensitivity	6	*9.8%*	*p= 0.386*	*p= 0.548*	*p= 0.580*	*p=1*
Cognitive and behavioural features at F-U						
ID	36	59.0%				
- Mild	15	24.6%	*n.a.*	*n.a.*	*p=0.262*	*n.a.*
- Moderate	11	18.3%	*n.a.*	*n.a.*	*p=0.438*	*n.a.*
- Severe	10	16.3%	*n.a.*	*n.a.*	*p=1*	*n.a.*
ASD	36	59.0%				
- Autistic traits	22	36.1%	*n.a.*	*n.a.*	*p=1*	*n.a.*
- Autism	9	14.7%	*n.a.*	*n.a.*	*p=1*	*n.a.*

N= number; n.a= not available; ASD= autistic spectrum disorder; ID=

Intellectual Disability; F-U = follow-up; mm= months; yrs= years

Table 2. : Clinical features of male and female PCDH19 related epilepsy patients

	Male patient (Depienne et al 2009)	Male patient #1 (Terracciano et al., 2016)	Male patient #2 (Terracciano et al., 2016)	Female Patients (Marini et al.2012)
Age at onset (months)	12	9	10	3-36
Present age	7 years	4 years	3,5 years	-
Previous genetic test	SCN1A negative	SCN1A negative	SCN1A negative	First choice in several cases
PMD before seizure onset	Normal	Normal	Normal	Normal
Fever sensitivity	yes	yes	yes	Most of cases
Cluster occurrence	yes	yes	yes	yes
Seizure Type	Focal with SG	Focal with SG	Focal with SG Focal with affective symptoms	Focal with SG;Focal with affective symptoms
Interictal EEG	-	normal	Rare fronto-central spikes	Bilateral SW, rare focal epileptiform abnormalities
Brain MRI	Normal	Normal	Normal	Normal
Cognitive assessment at follow-up	Moderate/Severe Mental Retardation	QDG 72	QDG 103	Normal to Severe Mental Retardation
Behavioral disturbances	yes	yes	no	Several cases
Drug Resistance at Follow-up	yes	No	Yes	Frequent but prolonged seizure free periods are reported in some patients

Figure Legends

Figure 1. Schematic illustration of the cellular interference mechanism associated with PCDH19 mutations. A) In normal individuals, characterized by a homogeneous population of PCDH19-positive cells, neurons are able to form normal neuronal networks; B) In mutated male patients, hemizygosity leads to a homogeneous population of PCDH19-negative cells; in this condition, neurons preserve the ability to form normal neuronal networks; C) In heterozygous mutated females, random X inactivation leads to the co-existence of two PCDH19-positive and PCDH19- negative cell populations. These two cell populations cause divergent cell sorting and migration (due to attractive or repulsive interactions) and lead to abnormal neuronal networks. Somatic mosaicism in mutated males gives rise to the same pathological situation (Depienne et al., 2009)

Figure 2. Focal seizure with motor symptoms (Patient#28 - Supplemental table). Seizure recorded at the age of 2 years and 6 months. The epileptic discharge consists of a diffuse recruiting theta activity, prevalent over bilateral central and parietal regions lasting 45 seconds. Seizure semiology is characterized by loss of contact, diffuse hypertonia, fix gaze, distal and asynchronous myoclonic jerks of limbs. EMG channels show the contraction of the deltoid muscles and the presence of high frequency asynchronous myoclonic jerks.

Figure 3. Focal seizure with hypomotor symptoms **(**Patient#11 - Supplemental table). Seizure recorded at the age of 21 months. The epileptic discharge consists of a diffuse recruiting theta activity, prevalent over bilateral frontal and central regions lasting 60 seconds. Seizure semiology is characterized by staring, slight and brief upper limb hypertonia (right > left), impairment of contact, hypopnea and desaturation (SO2 42%), lip cyanosis.

Figure 4. Atypical absence (Patient#26 - Supplemental table). Seizure recorded at the age of 14 years. The epileptic discharge consists of spike-and-wave complexes at 4.5 Hz, prevalent over the frontal regions.

Figure 5. Focal seizures with asynchronous discharge over hemispheres (Patient#5 - Supplemental table). The ictal discharge starts from the right temporal region, with a rhythmic theta activity. Later on it spreads to same region of the contralateral hemisphere. The ictal discharge persists mainly over bilateral temporal regions and stops independently firstly at right and later (after 25 seconds) on the left hemisphere. Seizure is characterized by fixe gaze, loss of contact, hypopnea and cyanosis without clinical evident motor signs. Seizure duration is about 160 seconds. This seizure is part of a cluster occurred at the age of eleven months (seizure onset).

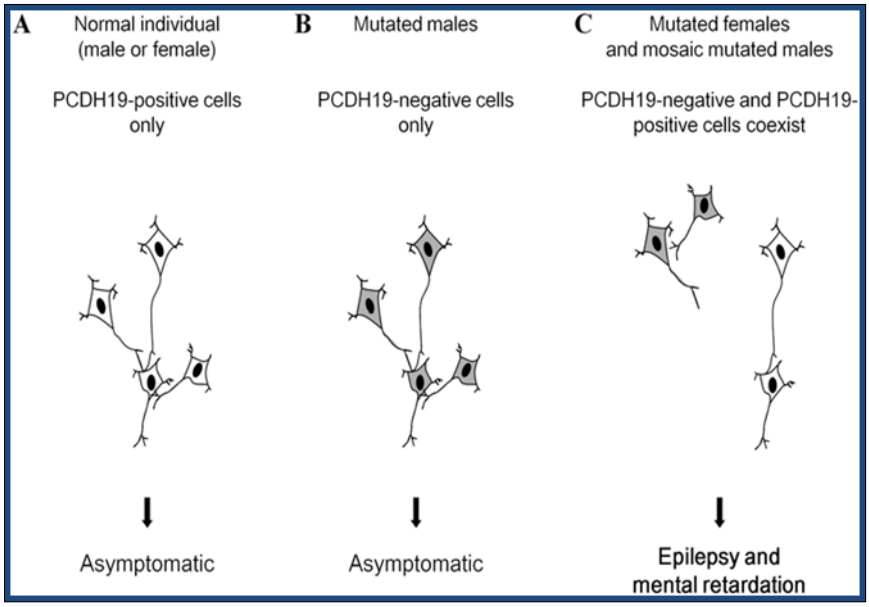
Figure 6. Cluster of focal seizures (Patient#2 - Supplemental table). Seizure cluster recorded during sleep in a 14 years old patient. It evident the presence of seizures with onset both from the right (first and second seizures) and left

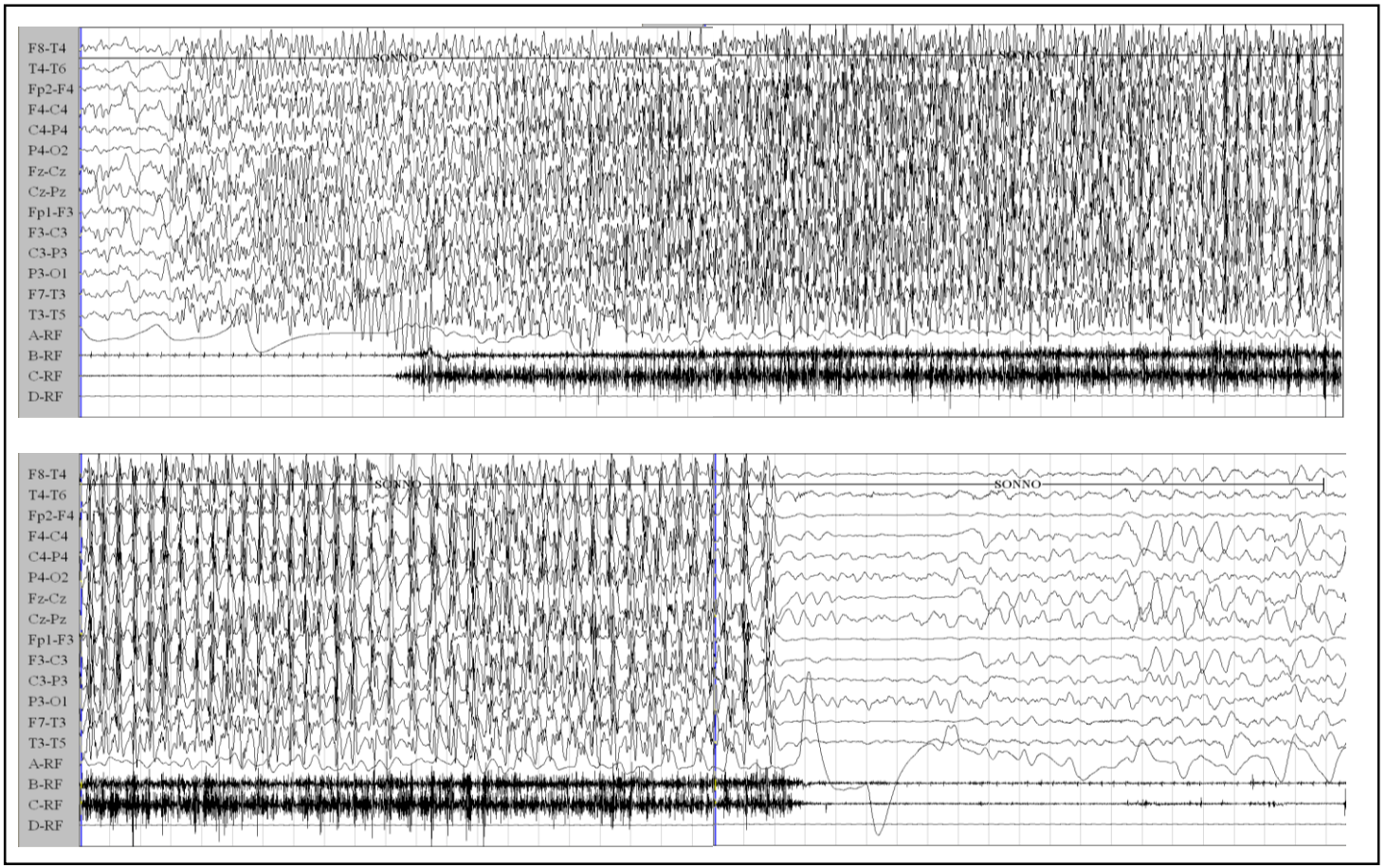
temporal (third two seizure) regions. No clinical signs were evident (only electrical discharge).

Figure 7. Non-convulsive Status Epilepticus (Patient#43, Supplemental table). The ictal EEG show the presence of a continuous delta activity, diffuse over both hemispheres and prevalent over the frontal and temporal bilateral regions, with a higher amplitude over the left hemisphere and intermingled with spikes of low voltage. Patients (at the age of 4 years and 5 months) has scarce interaction with fixe gaze and no clear motor signs.

Figure 8. Clinical and pharmacological course of a patient treated with stiripentol. The graphic shows seizure/cluster frequency and the antiepileptic drugs during the time, since epilepsy onset. After the beginning of stiripentol in add-on to valproic acid and clobazam, patient had long seizure-free period as never in her history. MDZ= midazolam; DZP= diazepam; PB= phenobarbital; CBZ= carbamazepine; VPA= valproic acid; CLB= clobazam; STP= stiripentol.

Figure 1


A
Normal individual
(male or female)
PCDH19-positive cells
only
Asymptomatic
B
Mutated males
PCDH19-negative cells
only
Asymptomatic
C
Mutated females
and mosaic mutated males
PCDH19-negative and PCDH19-
positive cells coexist
Epilepsy and
mental retardation


F8-T4
T4-T6
Fp2-F4
F4-C4
C4-P4
P4-O2
Fz-Cz
Cz-Pz
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F3-C3
C3-P3
P3-O1
F7-T3
T3-T5
A-RF
B-RF
C-RF
D-RF
SONNO
SONNO
F8-T4
T4-T6
Fp2-F4
F4-C4
C4-P4
P4-O2
Fz-Cz
Cz-Pz
Fp1-F3
F3-C3
C3-P3
P3-O1
F7-T3
T3-T5
A-RF
B-RF
C-RF
D-RF
SONNO
SONNO

Figure 2.

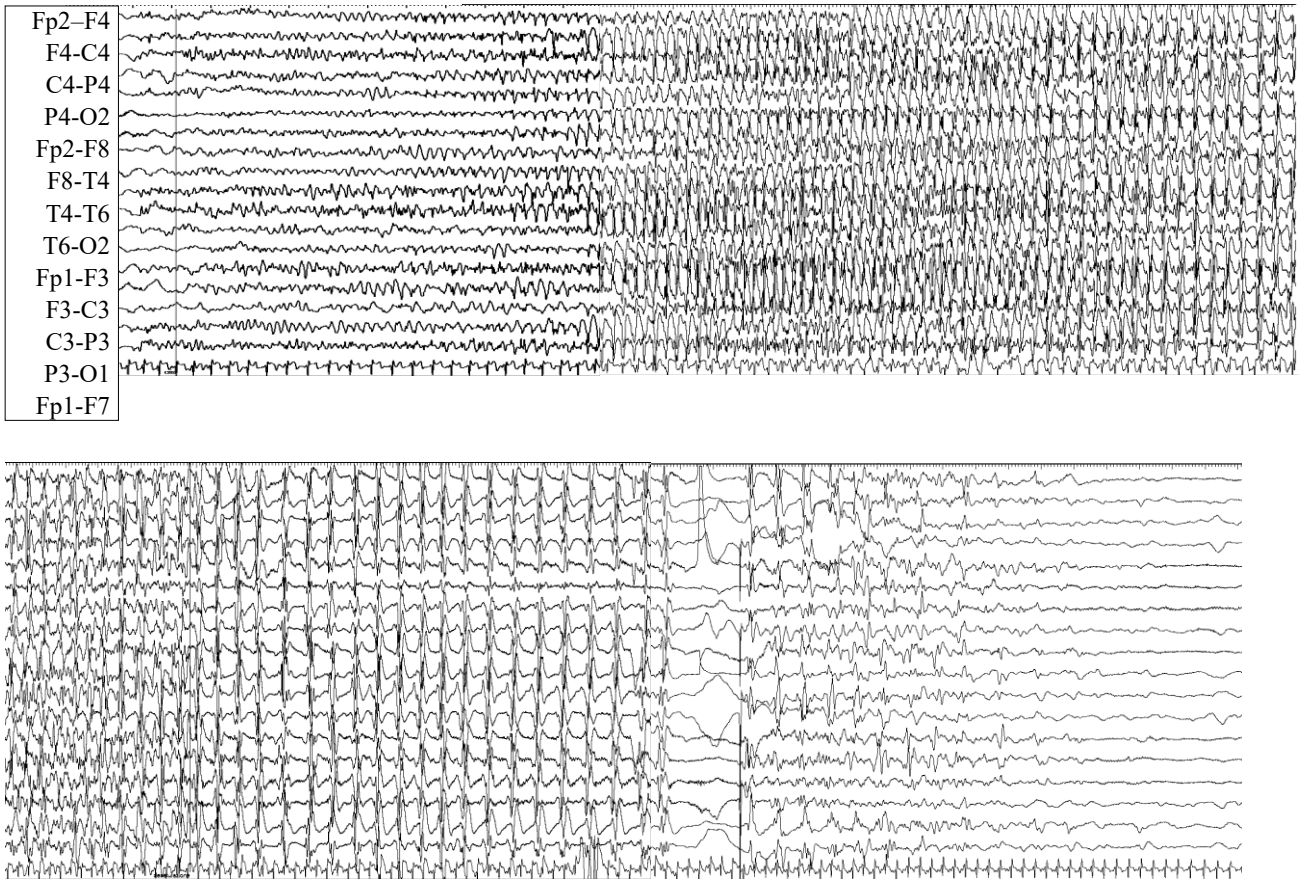

Fp2–F4
F4-C4
C4-P4
P4-O2
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F3
F3-C3
C3-P3
P3-O1
Fp1-F7

Figure 3

Figure 4

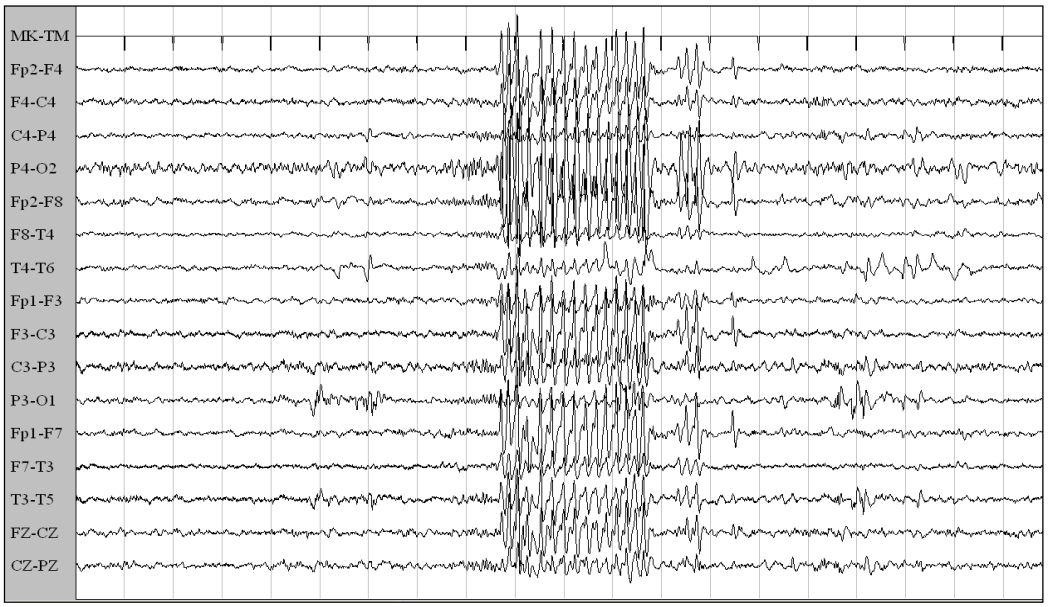

MK-TM
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp2-F8
F8-T4
T4-T6
Fp1-F3
F3-C3
C3-P3
P3-O1
Fp1-F7
F7-T3
T3-T5
FZ-CZ
CZ-PZ

Figure 5

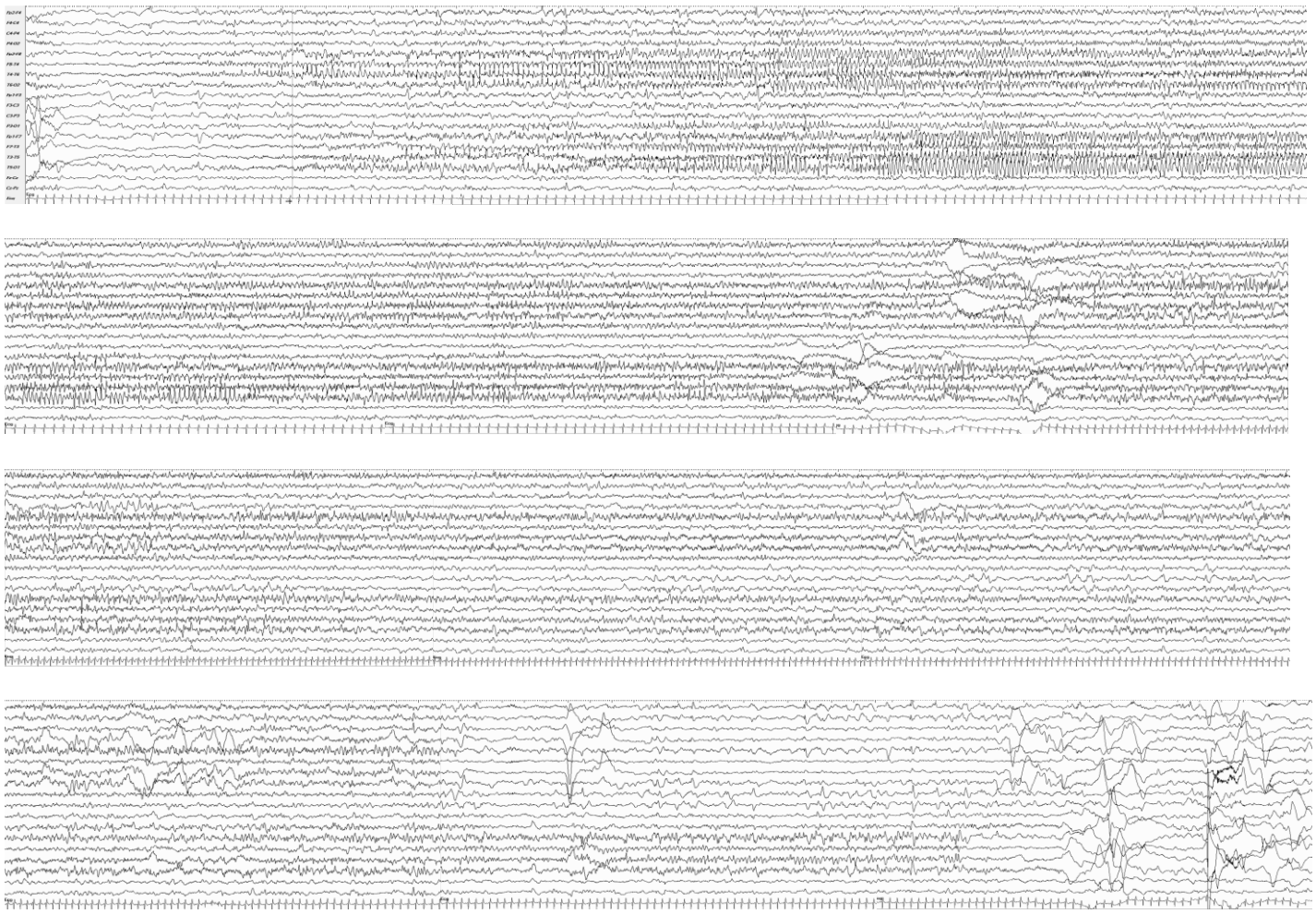


Figure 6

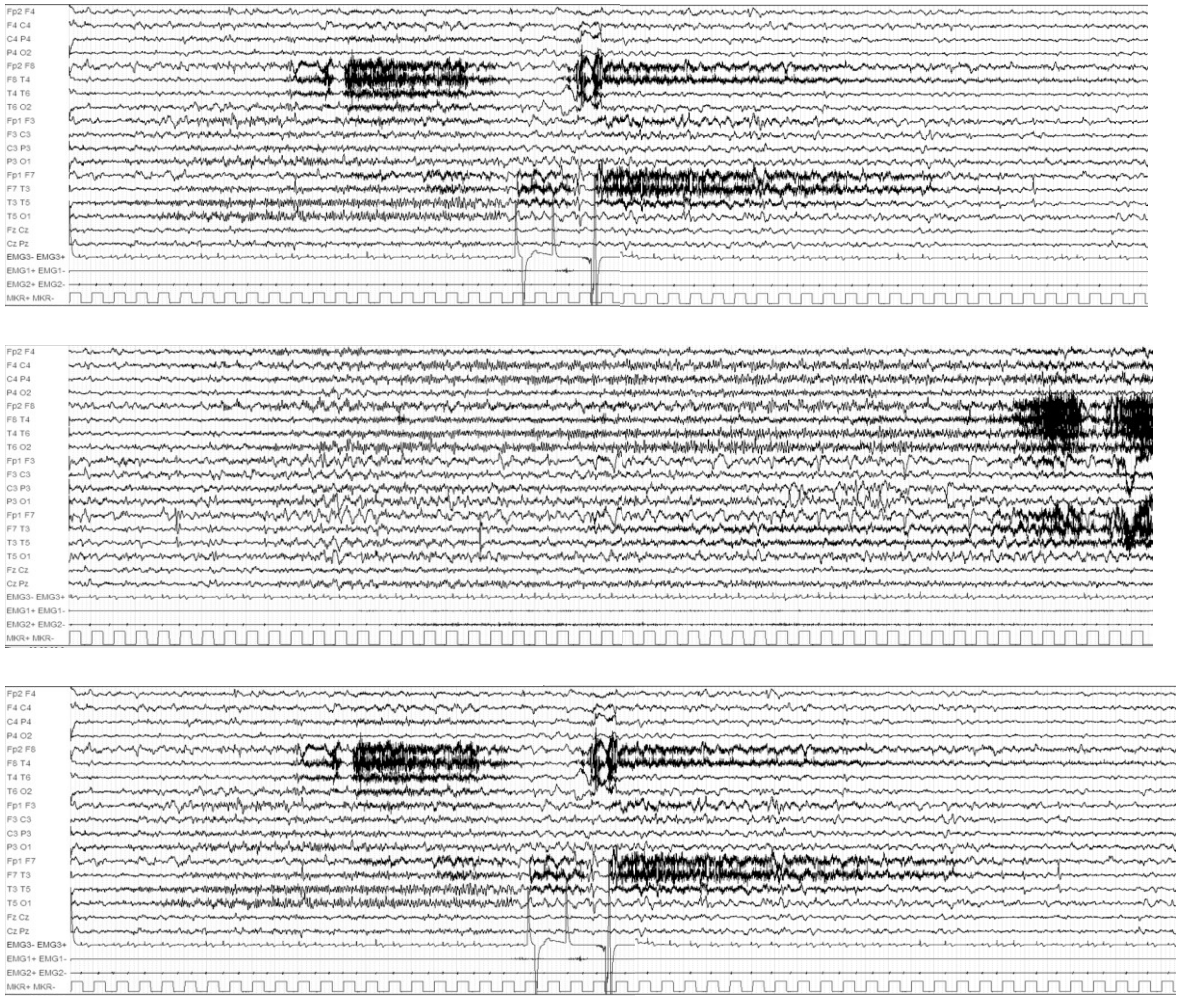

Fp2 F4
F4 C4
C4 P4
P4 O2
Fp2 F8
F8 T4
T4 T6
T6 O2
Fp1 F3
F3 C3
C3 P3
P3 O1
Fp1 F7
F7 T3
T3 T5
T5 O1
Fz Cz
Cz Pz
EMG3- EMG3+
EMG1+ EMG1-
EMG2+ EMG2-
MKR+ MKR-

Figure 7

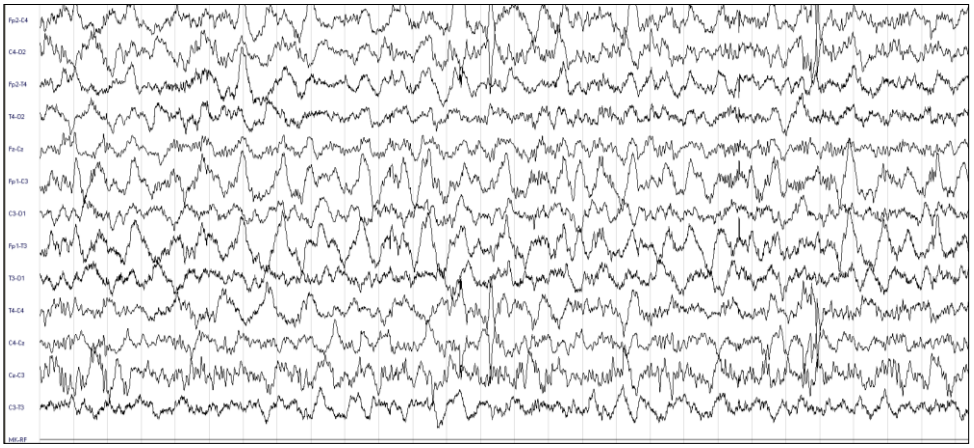
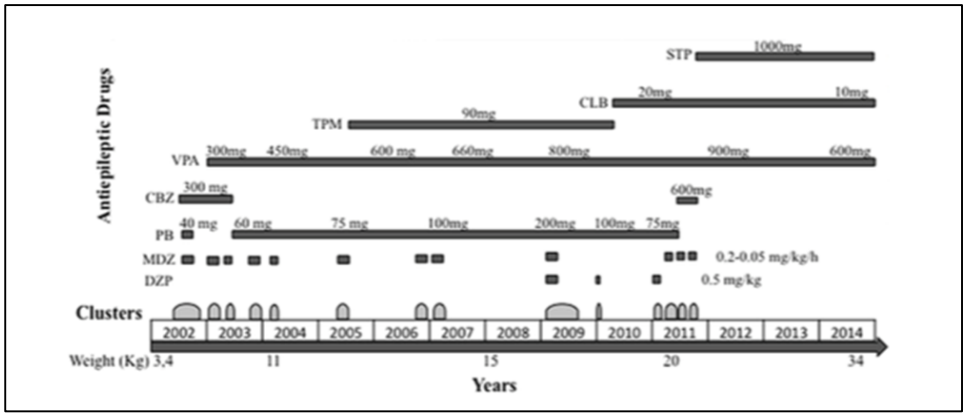


Figure 8



PCDH19-RELATED EPILEPSY AND DRAVET SYNDROME: DIFFERENTIAL DIAGNOSIS

Aim of this study is to compare PCDH19-related epilepsy and Dravet Syndrome (DS) in order to find out differences between these two infantile epilepsies with fever sensitivity.

We retrospectively reviewed the medical records of 15 patients with PCDH19-related epilepsy and 19 with DS. Comparisons were performed with Fisher's exact test or Student's t-test.

Females prevailed in PCDH19-related epilepsy. Epilepsy onset was earlier in DS (5.0+2.1 *vs* 11.2+7.0 months; $p<0.05$). The second seizure/cluster occurred after a longer latency in PCDH19-related epilepsy rather than in DS (10.1$\pm$13.6 *vs* 2.2$\pm$2.1 months; $p<0.05$). Seizures were mainly single and prolonged seizures in DS, and brief and clustered in PCDH19-related epilepsy. Myoclonic and clonic seizures have been found only in DS. Other types of seizures were found in both epilepsies with a prevalence of GTCS and atypical absences in DS, and focal motor and hypomotor seizures in PCDH19-related epilepsy. Seizures with affective symptoms have been confirmed to be typical of PCDH19-related epilepsy. Status Epilepticus equally occurred in both groups. Photosensitivity was detected only in DS. No differences were found about the presence of intellectual disabilities and behavioral disturbances.

We were able to find out some distinctive features, which could address the diagnosis towards DS or PCDH19-related epilepsy, since first manifestation. These considerations suggest to definitively considering *PCDH19* gene as cause of a proper epileptic phenotype.

Introduction

Protocadherin19 (PCDH19)-related epilepsy is a recently described epileptic syndrome, with onset in the first three years of life, characterized by clustered and fever induced seizures, often associated with intellectual disability (ID) and autistic traits (Marini et al., 2012). *PCDH19* gene is located on chromosome Xq22, and the condition has an unusual X-linked mode of inheritance affecting only carrier females, therefore a mechanism of "cellular interference" has been hypothesized (Depienne et al., 2012). Genotype-phenotype correlation is not jet clearly defined. Currently, clinical phenotype ranges from well-controlled epilepsy with normal cognitive development to drug-resistant epilepsy with severe cognitive delay (Depienne et al.; 2009; Depienne et al., 2011; Dibbens et al., 2008; Higurashi et al., 2012; Marini et al., 2010; Marini et al., 2012; Specchio et al., 2011).

PCDH19 gene mutations were firstly associated with epilepsy in 2008, when seven Australian families with *Female-restricted Epilepsy and Mental Retardation* (EFMR) were reported (Dibbens et al., 2008). Afterword, first

sporadic cases were identified within a cohort of patients with Dravet Syndrome (DS) that had been resulted negative for *SCN1A* gene mutation (Depienne et al.; 2009). Also DS is a genetic epileptic syndrome, with onset mainly in the first year of life and characterized by the occurrence of mostly fever-induced seizures. *SCN1A* is the major gene for DS, however in about 30% a causative gene has not yet been found (Depienne et al.; 2009).

Since 2008, the number of patient with PCDH19-realted epilepsy has progressively grown-up and a more detailed phenotype came out (Depienne et al., 2009; Depienne et al., 2011; Dibbens et al., 2008; Higurashi et al., 2012; Jamal et al., 2010; Marini et al., 2010; Marini et al., 2012; Specchio et al., 2011). Nevertheless, PCDH19-related epilepsy has been consistently linked with DS, both because first sporadic cases were identified among patients with a Dravet-like phenotype, and because of the occurrence of fever-induced seizures. Moreover, in some papers *PCDH19* gene has been considered as the second gene of DS (Akiyamaet al., 2012; Gaily et al., 2013; Kwong et al., 2012; Marini et al., 2010).

Aim of this study is to compare PCDH19-related epilepsy and DS in order to find out differences between these two epileptic syndromes that can address the diagnosis toward one or the other at disease presentation. Nevertheless, a clear-cut distinction between PCDH19-related epilepsy and DS, could be relevant also for classification purposes.

Material and methods

We retrospectively reviewed the medical records of all consecutive patients with a diagnosis of PCDH19-related Epilepsy or *SCN1A*-positive DS, who have been treated from 1 January 2012 to 30 June 2015 at the Neurology Unit of Bambino Gesù Children's Hospital in Rome, Italy. We identified 15 patients with PCDH19-related epilepsy and 19 patients with *SCN1A*-positive DS. We excluded patients with *SCN1A* gene mutation without a clinical diagnosis of DS (i.e. GEFS+ and others) and *SCN1A*-negative DS patients. For both groups of patients, genetic tests ascertaining the *PCDH19* or *SCN1A* gene mutation should have been available.

Informed consent for this study was obtained from patients' parents. We analysed familiar and personal medical antecedents, age at epilepsy onset, fever sensitivity, seizure semiology and frequency, EEG, treatment and neuropsychological outcome. All available ictal EEG have been analysed. Data on cognitive outcome have been collected retrospectively on cognitive assessment routinely performed during period of observation. Epileptic seizures were classified according the ILAE classification and terminology (Berg et al., 2009; Blume et al. 2001). We considered both classifications because they were complementary. Seizure cluster was defined as the incidence of seizures within a given period (usually one or a few days) that exceeds the average incidence over a longer period for the patient (Blume et al. 2001). Prolonged seizures were defined as seizure lasting more than 5 minutes (Shinnar et al., 2001).

Status Epilepticus (SE) was defined as a seizure lasting more than 30 minutes (Recommendations of the Epilepsy Foundation of America's Working Group on Status Epilepticus, 1993; Commission on Epidemiology and Prognosis, International League Against Epilepsy, 1993).

Statistical analysis

Comparisons were performed with Fisher's exact test or Student's t-test, as required. The software SPSS was used to store and analyzed the data. A p value <0.05 was considered statistically significant.

Results

Table 3 shows demographic, clinical and electrophysiological features for the two groups of patients, affected by DS or PCDH19-related epilepsy. Age at follow-up was similar in both groups. PCDH19-related epilepsy patients were all female. Epilepsy onset was earlier in patients with DS (5.0±2.1 *vs* 11.2±7.0 months; *p=0.001*) (Fig. 9A). The first seizure/cluster was fever-induced in both groups with almost the same rate (66.6% in PCDH19-related epilepsy *vs* 78.9% in DS; *p*= 0.68). Differently, the second seizure/cluster occurred after a longer latency in PCDH19-related epilepsy rather than in DS (10.1±13.6 *vs* 2.2±2.1 months; *p*= 0.04). Clustered seizures were much more frequent in PCDH19-related epilepsy than in DS (*p<0.001*). Myoclonic and clonic seizures occurred only in patients with DS. Generalized tonic-clonic seizures (GTCS) and

atypical absences were more frequent in DS *(p<0.05),* while focal motor and hypomotor seizures and seizures with affective symptoms mostly occurred in patients with PCDH19-related epilepsy *(p<0.05).* Patients with DS manifested more frequently prolonged seizures (*p=0.001*), while Status Epilepticus (SE) occurred in both groups with almost the same rate (36.8% *vs* 40.0%; *p=1.0*). Photosensitivity was detected only in DS *(p=0.005)*. Patients with PCDH19-related epilepsy had a smaller number of AEDs at last follow-up visit (1.9±0.6 vs 2.9±0.6; *p< 0.001*). No differences were found about the presence of ID and behavioural disturbances at the last follow-up (Fig. 9B).

Discussion

Comparing the phenotypes of patients with PCDH19-related epilepsy and DS, we tried to find out some key differences, that should be taken into account in the differential diagnosis of these two genetic epilepsies of infancy. At first clinical manifestation, both conditions share some overlapping features that could be misleading. The common clinical characteristics are the age of onset that is around the first year of life, a normal psychomotor development before the epilepsy onset and the fever sensitivity.

The first difference we found out is the only occurrence of PCDH19-related epilepsy in female patients. On the other hand, in DS both sex are almost equally affected. This is an expected result due to the unusual X-linked inheritance, affecting only females, in PCDH19-related Epilepsy (Depienne et

al. 2009). Nevertheless, male patients with a somatic mosaicism for *PCDH19* mutation have been recently reported (Depienne et al. 2009; Terracciano et al., 2016). This is a rare condition, and female prevalence in PCDH19-related epilepsy remains an important clinical feature to consider for the differential diagnosis.

Age at epilepsy onset resulted to be later in *PCDH19* mutated patients (Fig. 1A). An overlap window till the age of 10 months should be considered, while an older age at onset is significantly suggestive for PCDH19-related epilepsy. In previous cases series on DS, most authors indicate an age at onset mainly between 5 and 8 months (Dravet, 2011) and onset after 1 year only in rare cases (Kearney et al., 2006). Regarding PCDH19-related epilepsy, it is usually reported a wider range of age of onset, generally up to 36 months (Marini et al., 2012). Moreover the occurrence of a second seizure/cluster is delayed in PCDH19-related epilepsy rather than DS. In our series, PCDH19-related epilepsy a second seizure cluster usually occurs after a mean time of 10 months, with a peak after 45 months. This is concordant with the paper by Marini et al. (2012), which documented the occurrence of a second seizure after a mean time of 16 months (range 4-48 months) in PCDH19-related epilepsy patients. This could be considered one of the relevant differences with DS patients: this is useful, above all, during the early stages of the disease. We don't know the reason of this difference but we can speculate that probably seizure frequency

in DS in higher that in PCDH19-related epilepsy, almost during first years of disease.

With regards of seizure occurrence and duration, we detected a prevalence of single and prolonged seizures in DS, and brief and clustered seizures in PCDH19-related epilepsy. Clustered seizures might occur in several epileptic conditions and at any ages; however, since the first descriptions, cluster occurrence has been considered quite a hallmark of PCDH19-related epilepsy (Depienne et al., 2009; Dibbens et al, 2008; Marini et al., 2010). In our series, clusters occurrence was reported in all PCDH19-mutated patients, and only in about half of DS patients. Number of seizures for each cluster could be highly variable, from 3 to 22 seizures in 24h.

As regards seizure duration, patients with DS more frequently present with prolonged seizures rather than patients with PCDH19 mutations. Prolonged seizures are generally considered a risk factor for SE (Shinnar et al., 2001), nevertheless in both series of patients, we did not find any differences in the rate of SE occurrence.

Seizure semiology is one of the most relevant features that should be considered in the differential diagnosis: clonic and hemiclonic seizures have been exclusively reported in DS. Other types of seizures were found in both epilepsies with a prevalence of GTCS and atypical absences in DS and focal motor and hypomotor seizures in PCDH19-related epilepsy. Seizures with affective symptoms have been confirmed to be typical of PCDH19-related

epilepsy, even if they can occur, in about 10% of DS patients. Therefore, seizure semiology, both video-EEG recorded or reported by parents, represents a relevant feature to be deeply analysed in order to distinguish between DS and PCDH19-related epilepsy.

In the present report, no differences were found in epileptiform interictal EEG features that were poor in both conditions. Background activity remains normal in almost 50% of the cases of both groups. In the remaining cases, it was slow and poorly organized. A precise evaluation of the paroxysmal EEG abnormalities was not reliable, because the retrospective nature of this study as paroxysms can fluctuate according to the different conditions: spontaneous fluctuation, seizure frequency, and pharmacologic treatment. However, in PCDH19-related epilepsy it seems that interictal EEG could be poorer in epileptiform abnormalities. Abnormalities could be diffused or, more often, focal spikes or polyspikes involving asynchronously the fronto-central and occipital regions in DS and centro-temporal in PCDH19 related epilepsy. On the other hand, photosensitivity has frequently found in DS, while was never detected in any of the present PCDH19-mutated patients. Therefore, photoparoxysmal epileptiform response, except for few previously reported patients (Depienne et al., 2009; Marini et al., 2012; Specchio et al., 2011), could be considered an electrophysiological feature useful for the differential diagnosis.

Comparing treatments, both the epileptic syndromes resulted to be drug-

resistant. Patients with PCDH19-related epilepsy were treated with a higher number of AEDs in their life, but they resulted to be on a smaller number of AEDs at last follow-up. This difference, even if not relevant, might be explained with the fact that for PCDH19-related epilepsy, there are no evidences of a better treatment, and for this reason a greater number of drugs were tried, often including alternative treatments such as corticosteroids and intra venous IgG (Higurashi et al., 2015; Specchio et al., 2011). In DS patients there are evidences for efficacy of certain drug combinations, and most patients of our series, were on stiripentol, valproate and clobazam (Chiron et al., 2000; Wirrel et al., 2013). Previous papers showed that, in PCDH19-related epilepsy, seizures were, on average, less intractable rather than in DS patients (Depienne et al., 2009). We might hypothesize that this is not an issue of drug-resistance/sensitivity, but a natural evolution of the diseases. Time interval between seizures might be highly variable and sometimes longer in PCDH19-related epilepsy.

Intellectual disability and cognitive disturbances were found to be almost a constant feature in both conditions, as already reported (Depienne et al., 2011). However, about one third of PCDH19 mutated patients had a normal IQ and for those with cognitive delay IQ could ranges from mild to severe ID. Differently, in the group of patients with DS, we found a lower rate of patients with normal IQ, and patients with cognitive delay have a more severe ID. In DS is reported a prominent and faster impairment of motor abilities, since 2 years of age

(Ragona et al., 2011); on the contrary, a slower cognitive deterioration has been reported in PCDH19–related epilepsy, corresponding with a stagnation of development in the worst cases (Cappelletti et al., 2015).

The present study has some limitations: patients were recruited from a single institution, in a unit highly specialized in epilepsy in a setting of a reference, pediatric hospital. Furthermore, the sample size is limited, and can be considered underpowered. Nevertheless, we found multiple, striking differences between DS and PCDH19-related epilepsy, mostly with highly significant *p* values.

Even if DS and PCDH19-related epilepsy share some characteristics, observing the above-mentioned distinctive features, it could be possible to address the diagnosis towards the one or the other form of epilepsy since the occurrence of first clinical manifestation. The main marks are the earlier age at onset in DS, a major latency for the second seizure/cluster occurrence in PCDH19-relate epilepsy, different seizure semiology, the occurrence of single and prolonged seizures in DS, and brief and clustered in PCDH19-related epilepsy (Table 4).

Table 3. Demographic, clinical and electrophysiological features distinguished for the two groups of patients: Dravet syndrome and PCDH19-related epilepsy.

	Dravet Syndrome n=19	**PCDH19-related epilepsy n=15**	***p* value**
Demographic and clinical data	**n (%)**	**n (%)**	
Sex (female)	8 (42.1)	15(100.0)	<0.001
Age at follow-up (median ± SD)	8.1 ± 6.6 yrs	12.1 ± 9.3 yrs	0.256
Familiar for epilepsy and/or febrile seizures	6 (31.6)	1 (6.7)	0.104
Normal psychomotor development before epilepsy onset	18 (94.7)	15 (100)	1.000
Age at epilepsy onset (median ± SD)	5.0 ± 2.1 mths	11.2 ± 7.0 mths	0.001
Seizures	**n (%)**	**n (%)**	
Fever sensitivity	19 (100)	14 (93.3)	0.441
First-second seizure time interval	2.2±2.1 mths	10.1±13.6 mths	0.045
First seizure fever sensitivity	15 (78.9%)	10 (66.6)	0.685
Clustered seizures	9 (47.4)	15 (100)	<0.001
Seizure type			
- Myoclonic	15 (78.9%)	0 (0.0)	<0.001
- Hemiclonic	13 (68.4)	0 (0.0)	<0.001
- Clonic	7 (36.8)	0 (0.0)	0.011
- GTCS	14 (73.7)	3 (20.0)	0.005
- Atypical absences	12 (63.2)	4 (26.7)	0.045
- Focal motor seizures	6 (31.6)	11 (73.3)	0.037
- Focal hypomotor seizures	4 (21.1)	11 (73.3)	0.005
- Seizures with affective symptoms	2 (10.5)	8 (53.3)	0.010
Post-ictal paresis	8 (42.1)	0 (0.0)	0.005
Prolonged seizure (>5 minutes)	15 (78.9)	3 (20.0)	0.001
Status epilepticus	7 (36.8)	6 (40.0)	1.000
EEG features	**n (%)**	**n (%)**	
Interictal epileptiform abnormalities	12 (63.2)	7 (46.7)	0.489
Photosensitivity	8 (42.1)	0 (0.0)	0.005
Treatment	**n**	**n**	
Number of previously tried AEDs (mean ± SD)	4.9 ± 1.2	6.6 ± 3.4	0.071

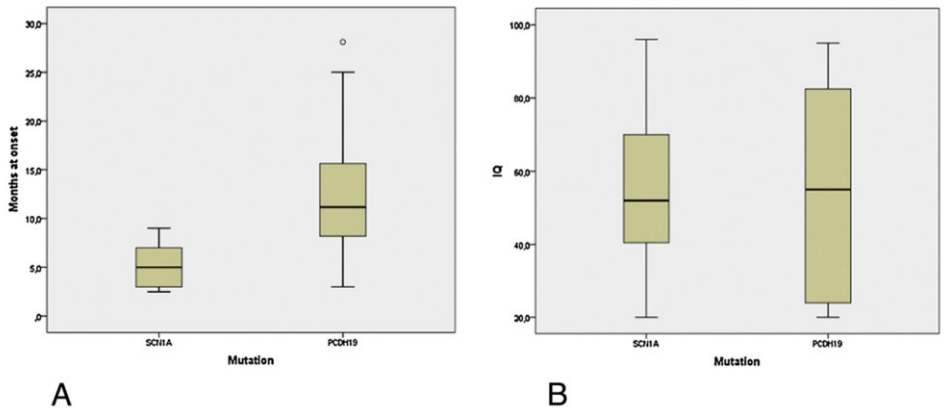
Number of AEDs at F-U (mean ± SD)	2.9 ± 0.6	1.9 ± 0.8	<0.001
Cognitive and behavioural features at F-U	**n (%)**	**n (%)**	
Intellectual disability	16 (84.2)	10 (66.6)	0.417
Behavioural disturbances and autistic traits	19 (100)	13 (86.7)	0.543

Table legend: mths=months; yrs= years; F-U= follow-up; SD= standard deviation; GTCS=generalized tonic-clonic seizure; AEDs= Anti-Epileptic Drugs; n= number

Table 4. Summary of the main differences between Dravet Syndrome and PCDH19-related epilepsy.

	Dravet Syndrome	**PCDH19-related epilepsy**
Sex (female)	+	+++
Familial history for FS or epilepsy	+	+
Normal development before seizure onset	+++	+++
Age at onset	5.2±2.1 m	13.1±7.0 m
Seizure semiology	Clonic/hemiclonic Post-ictal paresis	Hypomotor seizures/ seizures with affective symptoms
Cluster occurrence	+	+++
Prolonged seizures	+++	+
Status epilepticus	++	++
Developmental Disabilities	+++	++
Cognitive and behavioural disorders	+++	++
Interictal EEG	++	++
Photosensitivity	+++	+

Figure 9 (A) Age at onset by mutation; (B) Intelligence Quotient (IQ) by mutation.



PART II: EXPERIMENTAL STUDY

DETERMINATION OF ALLOPREGNANOLONE LEVELS PCDH19-RELATED EPILEPSY

Patients affected by protocadherin 19 (PCDH19)-female limited epilepsy (PCDH19-FE) present a remarkable reduction in allopregnanolone blood levels. However, no information is available on other neuroactive steroids and the steroidogenic response to hormonal stimulation. For this reason, we evaluated allopregnanolone, pregnanolone and pregnenolone sulfate by liquid chromatographic procedures coupled with electrospray tandem mass spectrometry in 12 unrelated patients and 15 age matched controls. We also tested cortisol, progesterone and 17OH-progesterone by standard immunoassays. These hormones were evaluated in basal condition and after stimulation with adrenocorticotropic hormone (ACTH). A generalized decrease in blood levels of almost all measured neuroactive steroids was found. While administration of ACTH increased neuroactive steroid levels in the PCDH19-FE girls, these fell short of the level of the age matched controls. Our findings point to multiple defects in steroidogenesis associated with and potentially relevant to PCDH19-FE

Introduction

Allopregnanolone (AP) belongs to the family of neuroactive steroids, peripherally-born molecules which are converted by the nervous tissue into other active steroidal compounds or that, alternatively, are directly synthesized in the brain. In this latter case they are usually referred to as "neurosteroids" (Mellon and Griffin, 2002; Melcangi and Panzica, 2014; Biagini et al., 2010). Notably, there is considerable published evidence pointing to AP as the most potent positive modulator of γ-aminobutyric acid receptor A ($GABA_A$) enhancing both tonic and phasic $GABA_A$-dependent inhibitory currents (Biagini et al., 2010; Belelli and Lambert, 2005). This biological activity of AP has been addressed in various seizure and epilepsy models leading to the suggestion that AP is a neuroprotective, antiepileptic agent with a mechanism of action potentially similar to that of benzodiazepines. In contrast, sulfated neuroactive steroids such as pregnenolone sulfate (PS) negatively modulate $GABA_A$-mediated inhibitory currents, thus promoting seizures (Kokate et al., 1999; Harteneck, 2013).

Recently, it has been found that girls with mutations in the protocadherin 19 (*PCDH19*) gene, known to cause female-limited epilepsy (PCDH19-FE), have significantly reduced levels of AP in their blood (Tan et al. 2015). In agreement with this hypothesis, *AKR1C1-3* genes, which are dysregulated in the PCDH19-FE girls (Tan et al. 2015) and are likely responsible for the AP deficiency, have multiple other activities in the synthesis of a broad range of neuroactive

steroids. As a consequence, it is plausible, that the PCDH19-FE patients are deficient for a range of neuroactive steroids. This observation led us to postulate that the remarkable deficit of production of AP and other neurosteroids could be responsible for an imbalance in the ratio of anticonvulsant and proconvulsant neuroactive steroids, so to favor the occurrence of recurrent, difficult to treat seizures in PCDH19-FE patients.
To address these important issues, we aimed to investigate the production of a variety of neuroactive steroids in the PCDH19-FE patients. In particular, we replicate AP deficiency, and further the study into other neuroactive steroids such as PS, pregnanolone, progesterone, 17OH-progesterone and cortisol, both at the basal level as well as after stimulation with adrenocorticotropic hormone (ACTH), in order to exclude peripheral gland disorders.

Methods

Experimental design

PCDH19-FE group

We prospectively enrolled 12 unrelated patients affected by PCDH19-FE from April 2014 to May 2015. Genetic report assessing *PCDH19* gene mutations was available for all patients. The age of PCDH19-FE patients was 8.3±5.7 years (range 2.5-18.9). Other than epilepsy, 6 patients had intellectual disability. Relevant clinical details of all patients studied are reported in Table 5.

Clinical control group

The controls (n=15) were a group of individuals evaluated for precocious puberty or hyperandrogenism within the same period. All controls were age-matched females (9.2±4.0 years; range 6.1-17.9) with a normal cognitive profile. Nine of these individuals received a diagnosis of praecox puberty and 6 of hyperandrogenism, but their hormonal profile was completely within the normal range (Table 6).

Treatment

Blood samples of the PCDH19-FE patients and controls were obtained twice. Firstly at 9 a.m., which represented the basal levels (T^0) and secondly at 60 min (T^1) after a bolus of 0.25 mg of ACTH delivered intravenously. For post-pubertal patients, test was performed from the 5^{th} to 9^{th} days of the menstrual cycle, in order to minimize hormonal variations. Blood samples were maintained at room temperature for 30 min and then centrifuged at 3500 rpm for 10 min to collect sera, which were then stored at -80 °C. Standard immunoassays were used to test for progesterone, 17OH-progesterone, and cortisol levels. After that the samples were shipped on dry ice from the Bambino Gesù Children's Hospital in Rome to the Laboratory of Experimental Epileptology in Modena for analytic determination of the AP, PS, and pregnanolone levels.

The research protocol applied was approved by the Bambino Gesù Hospital Institutional Review Board according to the local regulations. Informed written consent was obtained from all studied subjects or their relatives.

Quantitative analysis of AP, PS and pregnanolone in serum by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS)

Chemicals and reagents

Allopregnanolone and PS standards were purchased from Sigma (St. Louis, MO). Internal standards with isotopes labelling were: 5α-pregnan-3α-ol-20-one-17α,21,21,21-d4 (AP-D4) and sodium pregnenolone-17α,21,21,21-d4 sulfate (PS-D4), purchased from CDN Isotopes (Quebec, Canada). All solvents for HPLC-ESI-MS/MS were LC-MS purity grade, while other solvents used for sample preparation were analytical grade (Sigma-Fluka, St. Louis, MO).

LC-MS/MS analysis

The chromatographic separation was performed on an Agilent 1200 Series Binary Pump (Agilent, Waldbronn, Germany). Mass spectrometric detection was performed using an Agilent QQQ-MS/MS (6410B) triple quadrupole mass analyzer equipped with an ESI ion source (Agilent, Waldbronn, Germany), operating in the positive mode, as previously described.[8]

Statistics

Data were compared using a repeated measure two-way analysis of variance (ANOVA), considering time intervals as the within factor and groups as the between factor, and subgroups were compared by *post hoc* Holm-Šídák test. The only exception to this design was for progesterone that was analyzed at T^0 with the Student's t-test. All statistical analyses were carried out using Sigmaplot 11 (Systat Software, San Jose, CA). Data are presented as mean±sem, and they were regarded significantly different at $p<0.05$.

Results

Chromatographic assessment of AP levels in the serum of PCDH19-FE patients and controls allowed detection of this analyte in all samples (Fig. 10A). Quantification of AP revealed significantly lower levels in the PCDH19-FE patients (approximately -50%; $p<0.05$, Holm-Šídák test) at the baseline. Induction of adrenal steroidogenesis with ACTH resulted in significantly ($p<0.05$) increased AP in both groups (Table 3). However, the increase in AP levels was not large enough to compensate for the baseline difference among the PCDH19-FE patients and controls.

Peaks corresponding to PS were also detected in all samples, as shown in Fig. 1B. Quantification of PS demonstrated significantly lower levels of this neuroactive steroid in the sera of PCDH19-FE, compared to controls (approximately -90%; $p<0.001$). Similarly to AP, the administration of ACTH

resulted in the increase of PS levels in both groups (p<0.001) (Table 3). However, the serum levels of PS of the PCDH19-FE patients did not reach the same values as in controls (p<0.001).

Apart from these two neuroactive steroids, significant differences (p<0.05) were found for cortisol, for which the PCDH19-FE girls had approximately 20% reduced levels to that of controls. Even in this case, the administration of ACTH did not result in an increase comparable to that observed for controls (p<0.05) (Table 7). At the baseline, less pronounced differences were found for 17OH-progesterone. However, and again, under ACTH stimulation the PCDH19-FE patients' levels of 17OH-progesterone were lower when compared to controls (p<0.001). No significant differences were found for progesterone and pregnanolone (data no shown).

We also considered the possibility that our results could be affected by differences in development of the two groups. Thus, we divided the PCDH19-FE girls and controls in two subgroups composed by the respective pre-pubertal and post-pubertal individuals. Statistical comparisons of the four subgroups consistently confirmed the presence of differences independent of pubertal development in basal and stimulated AP and PS levels, with the only exception of post-pubertal AP for which there was a trend for lower levels in PCDH19-FE girls (Table 8). However, because of the larger variability, differences found for the other hormones did not reach a statistically significant level in most of cases.

Discussion

We aimed to investigate neuroactive steroids in PCDH19-FE patients, at the baseline as well as after stimulation of adrenal steroidogenesis. We found that AP levels were significantly reduced, both at the baseline as well as after stimulation with ACTH, thus confirming the previous findings (Tan et al. 2015) and suggesting that PCDH19-FE patients present a significantly reduced ability to synthesize AP. We determined that this defect was not limited to AP, but involved other neuroactive steroids, such as PS, 17OH-progesterone and cortisol. This result was confirmed for AP and PS also when considering pre and post-pubertal patients separately. Overall, our investigations provide evidence for a significantly compromised ability to synthesize various adrenal steroids in PCDH19-FE.

The finding of reduced PS levels is however against our initial hypothesis of a possible imbalance in the AP/PS ratio leading to seizure facilitation in PCDH19-FE. Surprisingly, PS production was reduced even more than that of AP. This left open an important question of whether and how these neuroactive steroids play a role in the seizures observed in patients suffering from PCDH19-FE. Although the role of PS in the human brain has still to be clearly defined (Schumacher M et al, 2008), few cases of a successful use of allopregnanolone for the treatment of status epilepticus in children suggests that AP potently modulates seizures during development (Broomall et al., 2010). In view of these findings, it can be hypothesized that a therapeutic approach aimed at

restoring AP levels in patients affected by PCDH19-FE could result in beneficial effects. Interestingly, our results indicate that such a goal could be attained by administering ACTH. In this study, the ACTH dose was not sufficient to give a neurological effect. However, corticosteroid administration have been reported to be efficacious in some pilot studies of PCDH19-FE (Higurashi et al., 2015; Bertani et al., 2015). Thus, we tentatively suggest that administration of ACTH may be a strategy to get a therapeutic effect on the whole steroidogenesis by restoring neuroactive steroid levels.

Restoring adrenal steroidogenesis in patients affected by PCDH19-FE could be beneficial also for the expected increase in cortisol production. It is a common observation that exposure to stress may facilitate the occurrence of seizures, especially in patients affected by epilepsy (van Campen et al., 2014). It has recently been shown that patients who present an association between acute stress and seizures are characterized by reduced adrenal response when compared to those less sensitive to stress (van Campen et al., 2015). Thus, a reduced capability to produce cortisol apparently results in enhanced seizure susceptibility to environmental challenges. Although studies on the adrenal response to acute stress in patients with PCDH19-FE are lacking, the observed response to ACTH suggests that also this type of epilepsy may be aggravated by exposure to stressors, thus indicating that an optimal cortisol production could be a therapeutic goal in this condition.

A limitation of this study was the small sample size. However, we found multiple, striking differences between the two groups, mostly with highly significant *p* values and also in agreement with previously published work on a separate PCDH19-FE cohort (Tan et al., 2015). A second limitation of the study is the controls: we enrolled only individuals who underwent ACTH stimulation, thus reducing the size of controls. On the other hand, this choice was also made in order to verify normal peripheral production of steroids. Elevated AP levels have been reported in patients affected by disorders of sexual development, such as in the case of precocious puberty.[12] For this reason, we could have overestimated the deficit in AP production occurring in our PCDH19-FE patients. On the other hand, we also found that adrenal AP production did not completely recover after ACTH stimulation, suggesting a reduced capacity of hormonal synthesis in the PCDH19-FE in the first instance. Additionally, cortisol levels were also different in the two considered groups, definitely indicating that steroidogenesis was dysfunctional in at least the adrenal gland of patients suffering from PCDH19-FE. Finally, the presence of marked differences in PS levels suggests that an overall altered processing of neuroactive steroids takes place in PCDH19-FE and might be considered as a therapeutic target.

Figure 10. Allopregnanolone, pregnanolone and pregnenolone sulfate chromatograms.

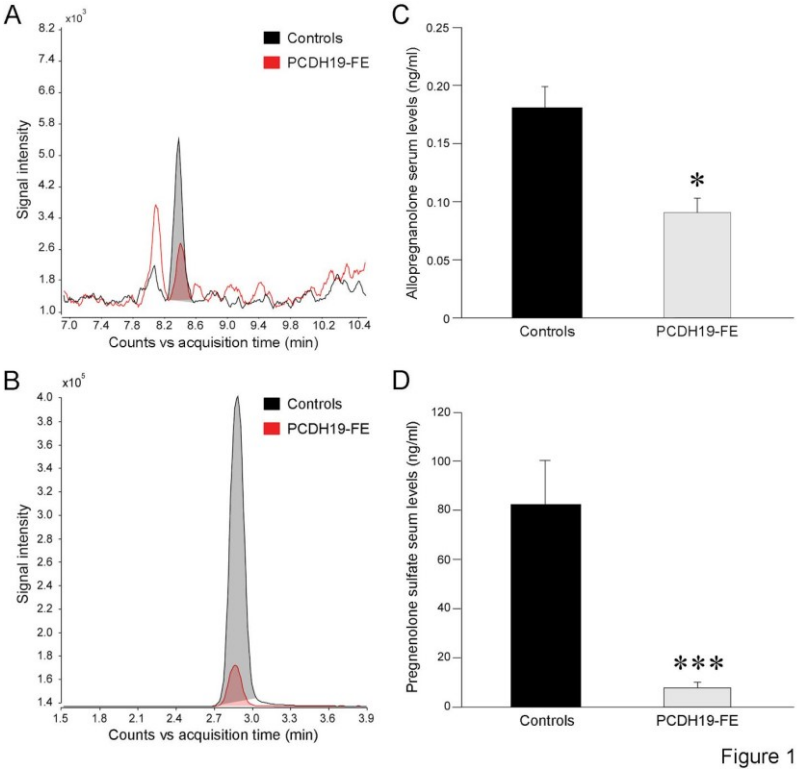


Table 5. Clinical, electrophysiological and genetic features of patients with PCDH19-female limited epilepsy.

Patient	Age at study (y)	Age at onset (mm)	Seizure type	Seizure frequency (follow-up)	Interictal EEG	Treatment (follow-up)	Last cognitive evaluation (age)	Behavioral disturbances	PCDH19 gene mutation
P1	3.1	11	Hypomotor, tonic posturing	Monthly clusters	Normal	VPA	IQ 54 (3 y)	Yes	c.1098 C>G p.Y366X
P2	2.5	25	Hypomotor, tonic posturing	1-2 clusters/y	Normal	CBZ	IQ 88 (2.4 y)	Yes	c.1710_1716delCGGCACT; p.Asn570fs*12
P3	4.4	14	Hypomotor, tonic posturing	Rare cluster	Normal	VPA	IQ 91 (5 y)	Yes	c.2341dupA p.Ile781Asnfs*3
P4	5.1	16	Tonic posturing, focal seizures with fear	Weekly seizures	Bil F theta W	VPA, LEV	IQ 79 (4.3 y)	Yes	c.1091dupC p.Tyr366Leufs*10
P5	4.4	20	Hypomotor, tonic posturing	1-2 clusters/y	Normal	VPA	IQ 95 (4.4 y)	Yes	c.1178 C>T p.Pro393Leu
P6	6.8	7	Hypomotor, tonic posturing	Weekly seizures	Bil F delta W	OXC, CLB	IQ 20 (6.7 y)	Autistic trait	c.2617-1G>A
P7	9.8	7	Atypical absences, tonic posturing	Monthly clusters	Normal	VPA	IQ 20 (9.8 y)	Autistic trait	c.799T>G; p.Leu260Arg.
P8	12.3	7	Atypical absences, tonic posturing	1-2 clusters/y	Bil F theta W	VPA, CLB, STP	IQ 20 (12 y)	Autistic trait	c.2676-6A>G
P9	11.3	28	Focal seizures with fear, tonic posturing	No seizures since 3y	Normal	VPA, CBZ	IQ 82 (11 y)	No	c.958dupG p.Asp320Glyfs*22
P10	14.4	15	Tonic posturing	Annual clusters	Bil Right F ShW	PB, VPA, LCM	IQ 83 (14 y)	No	Deletion
P11	17.6	8	Tonic posturing	Annual clusters	Bil T theta W	ZNS, CLB	IQ 50 (16 y)	Yes	p.ASp375Tyr
P12	18.9	5	Atypical absences, tonic posturing	3-4 clusters/y	Bil F-T ShW	LCM, TPM, CNZ, deflazacort	Moderate delay (unknown IQ)	Yes	c.1973T>G

Abbreviations: Bil, bilateral; CBZ, carbamazepine; CLB, clobazam; CNZ, clonazepam; F, frontal; LCM, lacosamide; LEV, levetiracetam; mm, months; OXC, oxcarbazepine; PB, phenobarbital; ShW, Sharp waves; STP, stiripentol; T, temporal; TPM, topiramate; VPA, valproic acid; W, waves; y, years; ZNS, zonisamid

Table 6. Age and hypothesized pathological disorder are illustrated for each subject enrolled in the control group. *Post-pubertal individuals. Abbreviations: AGS, adrenogenital syndrome; Cort T^0, cortisol basal values; E2 T^0, estradiol basal values; n.a., not assessed; P T^0, progesterone basal values; 17P T^0, 17OH-progesterone basal values

Patient	Age (years)	Diagnostic hypotheses	Cort T^0	E2 T^0	P T^0	17P T^0	Confirmed diagnosis
C1	6.1	AGS (precocious puberty)	67	<0.012	<0,2	0.5	No
C2	6.9	AGS (precocious puberty)	98	<0.012	1.18	0.4	No
C3	6.7	AGS (precocious puberty and obesity)	132	0.018	<0.15	1.0	No
C4	7.4	AGS (precocious puberty)	70	0.012	n.a.	0.7	No
C5	7.9	AGS (precocious puberty)	68	0.029	<0.2	0.3	No
C6	9.2	AGS (precocious puberty and obesity)	n.a.	0.016	0.39	0.5	No
C7	10.6	AGS (acne)	n.a.	0.041	0.27	0.8	No
C8	7.1	Adrenal insufficiency)	78	0.023	n.a.	0.8	No
C9	8.1	AGS (hirsutism)	87	n.a.	n.a.	0.3	No
		Pre-pubertal normal values (ng/ml)	**40-220**	**0.019 -0.214**	**<1.4**	**0.02-1.78**	
C10*	12.5	AGS (hirsutism)	81	0.021	<0,2	0.5	No
C11*	13.1	AGS (hirsutism and acne)	79	0.036	0.42	1.1	No
C12*	13.6	AGS (hirsutism and acne)	77	n.a.	n.a.	1.0	No
C13*	17.8	AGS (hirsutism)	237	0.025	0.79	1.0	No
C14*	17.9	AGS (precocious puberty)	172	0.059	0.97	4.9	No
C15*	14.1	AGS (precocious puberty)	81	0.232	0.78	1.3	No
		Post-pubertal normal values (ng/ml)	**40-220**	**0.019 -0.214**	**<1.4**	**0.11-5.0**	

Table 7. Determination of hormonal serum levels (ng/ml) for allopregnanolone (AP), pregnenolone sulfate (PS), 17OH-progesterone (17P), and cortisol (Cort), in patients affected by protocadherin 19 mutation associated with female-limited epilepsy (PCDH19-FE; n=12) and their respective controls, investigated for presumptive adrenogenital syndrome characterized by precocious puberty (n=9) or hyperandrogenism (n=6), then found to be healthy. Hormonal levels were determined in basal condition (T^0) or 60 min (T^1) after adrenocorticotropic hormone administration. Statistical analysis was performed with a repeated measure two-way analysis of variance followed by Holm-Šídák test. Few samples were missing for 17OH-progesterone. NS, not significant.

	CONTROLS			CONTROLS vs PCDH19		PCDH19-FE		
	T^0	T^1	n	T^0vsT^0	T^1vsT^1	T^0	T^1	n
AP	0.19±0.02	0.23±0.02	15	p<0.05	p<0.05	0.09±0.02	0.13±0.02	12
	p<0.05					p<0.05		
PS	95.37±13.03	198.75±13.03	15	p<0.001	p<0.001	8.09±14.07	65.04±14.07	12
	p<0.001					p< 0.001		
17P	0.73±0.12	2.40±0.12	14	NS	p<0.001	0.52±0.13	1.44±0.13	10
	p<0.001					p<0.001		
Cort	139.00±13.72	367.40±13.72	15	p<0.05	p<0.05	29.48±14.28	277.37±14.28	12
	p<0.001					p<0.001		

Table 8. Serum levels (ng/ml) of allopregnanolone (AP), pregnenolone sulfate (PS), 17OH-progesterone (17P), and cortisol (Cort), in patients affected by protocadherin 19 mutation associated with female-limited epilepsy (PCDH19-FE) and their respective controls, re-analyzed in relation to pubertal development. Hormonal levels were

determined in basal condition (T^0) or 60 min (T^1) after adrenocorticotropic hormone (ACTH) administration. Statistical analysis was performed with a repeated measure three-way analysis of variance (factors: PCDH19-FE, ACTH administration, puberty) followed by Holm-Šídák test. NS, not significant.

		PRE-PUBERTAL LEVELS			POST-PUBERTAL LEVELS		
		Patients (n=8)	Controls (n=9)		Patients (n=4)	Controls (n=6)	
T^0	**Cort**	30.34±8.81	85.83±8.86	NS	27.77±13.76	121.28±27.61	p<0.05
	17P	0.30±0.14	0.59±0.08	NS	0.91±0.25	1.63±0.66	NS
	AP	0.08±0.01	0.15±0.03	NS	0.12±0.03	0.22±0.04	NS
	PS	5.42±1.13	54.61±11.80	NS	12.53±2.93	123.82±19.66	p<0.01
T^1	**Cort**	279.47±12.03	358.70±32.29	p<0.05	279.63±16.41	297.82±16.61	NS
	17P	1.51±0.16	2.38±0.18	p<0.05	1.30±0.23	2.44±0.20	NS
	AP	0.12±0.02	0.19±0.01	NS	0.14±0.02	0.26±0.04	NS
	PS	61.67±7.76	166.50±23.04	p<0.001	72.22±24.80	230.62±33.75	p<0.001

CONCLUSIONS

This doctoral work allowed me to better define the epileptic phenotype of PCDH19-related epilepsy. The most relevant result is the identification of age at onset as the main and only predicting factor for outcome. The research of a genotype-phenotype correlation failed but I could design the natural history of epilepsy, finding out a gradual reduction of seizure frequency with puberty. The experimental part on neurosteroid measurements confirmed, firstly *in vivo,* the hypothesis of allopregnanolone deficiency, due to the interaction of PCDH19 gene with AKRC1-3 genes. This is relevant result as open to new-targeted treatments for epilepsy such as allopregnanolone and ganaxolone.

Other than the scientific results, this doctoral study allowed me to be in contact with the best scientists in the filed of PCDH19-related epilepsy. First of all, the geneticist who firstly described the gene, Josef Gecz, Professor of Human Genetics at the University of Adelaide, who actively contributed in the design of the experimental part and interpretation of the results. other international basic scientists involved in IPS and models and obviously clinician both neurologists and endocrinologists.

I think that during these years a well-consistent study group was born and the hope is that all of them could continue to work together in order to reach a better targeted-treatment of this syndrome.

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Supplemental Table. Demographic, clinical and genetic features detailed for patient

Pt#/ Sex	Age at onset (mm)	Age at study (yrs)	Seizure semiology	Cluster occurrence	Fever sensitivity	Seizure/cluster frequency (F-U)	Treatment (F-U)	Last cognitive evaluation (age)	Behavioural disturbances	PCDH19 gene mutation	Inheritance
1/F	13,8	6,0	Hypomotor, tonic posturing	Y	Y	Annual clusters	VPA, CBZ	DQ= 97	Autistic traits	c.2341dupA p.Ile781Asnfs*3	*de novo*
2/F	3	18,0	Hypomotor	Y	N	Monthly clusters	VPA, LEV, Diamox	IQ= 46	Isolation traits	c.1700C>T p.Pro567Leu	*de novo*
3/F	8,3	24,0	Hypomotor	Y	Y	Seizure-free (8 yrs)	CBZ, LEV	IQ= 64	Autistic traits	c.1298T>C p.Leu433Pro	*de novo*
4/F	10,5	42,0	Hypomotor	Y	Y	Several per year	CBZ	IQ= 48	Autistic traits	c.706C>T p.Pro236Ser	*de novo*
5/F	16,2	8,0	Tonic posturing	Y	Y	Weekly-monthly seizures	VPA, CLB	Mild ID	Autistic traits	c.1091dupC p.Tyr366Leufs*10	*de novo*
6/F	8	16,0	Tonic posturing	Y	Y	Seizure-free (2 yrs)	VPA, STP, CLB	Severe ID	Autism	c.1129G>C p.Asp377His	*de novo*
7/F	7	15,0	Hypomotor, tonic posturing	Y	Y	Several per year	VPA, CLB, STP	Severe ID	Autism	c.2676-6A>G?	*de novo*
8/F	11,4	15,1	Hypomotor, tonic posturing	Y	Y	Seizure-free (3 yrs)	CBZ, CLB, LEV	IQ= 83	No	c.136 G>C p.Ala46Pro	Father
9/F	16,7	19,8	Tonic posturing	Y	Y	Seizure-free (2 yrs)	CBZ, CLB	IQ= 51	Hyperactivity, attention deficit	c.136 G>C p.Ala46Pro	Father
10/F	11,1	6,0	Hypomotor, tonic posturing	Y	N	Annual clusters	VPA, LEV, CLB	Mild ID	Autistic traits	c.1098 C>G p.Y366X	*de novo*
11/F	20,4	7,0	Hypomotor, tonic posturing	Y	N	Annual clusters	VPA, MDZ, CLB,	DQ= 95	Hyperactivity, attention deficit	c.1178 C>T p.Pro393Leu	*de novo*
12/F	11,6	9,0	Hypomotor, tonic posturing	Y	N	Seizure-free (2 yrs)	OXC	Severe ID	Autism	c.2617-1G>A?	*de novo*
13/F	28,1	14,0	Tonic posturing	Y	Y	Seizure-free (5 yrs)	VPA, CBZ,	IQ= 98	No	c.958dupG p.Asp320Glyfs*22	*de novo*
14/F	8	4,5	Tonic posturing	Y	Y	Several per year	VPA, LEV	Moderate ID	No	c.2885G>A p.R962Q	*de novo*
15/F	8	13,6	Tonic posturing, myoclonic seizures	Y	N	Several per year	VPA, LEV, NZP	IQ= 32	Autism	c.1183C>T p.Arg395*	*de novo*
16/F	5,5	14,7	Tonic posturing	Y	Y	Annual clusters	TPM, LEV	IQ= 40	Autism	c.1019A>G p.Asn340Ser	*de novo*
17/F	18	9,4	Hypomotor, tonic posturing	Y	Y	Annual clusters	TPM	Severe ID	Autistic traits	c. 3431-3434del	Father

18/F	8	11,2	Tonic posturing	Y	Y	Seizure-free (2 yrs)	VPA, TPM, NZP	Moderate ID	Autistic traits	c. 3431-3434del	Father
19/F	8	12,7	Tonic posturing	Y	Y	Seizure-free (8 yrs)	No drugs	IQ= 98	No	c. 3431-3434del	Father
20/F	14	14,5	Tonic posturing, absence, myoclonic seizures	Y	Y	Several per year	VPA, TPM, CLB	IQ= 35	Autistic traits	c.1300_1301delCA p.Gln434Glufs*11	*de novo*
21/F	24	14,5	Hypomotor	Y	Y	Seizure-free (4 yrs)	no AED	Normal	No	c.1300_1301delCA p.Gln434Glufs*11	*de novo*
22/F	4,5	12,8	Tonic posturing	Y	Y	Monthly clusters	LTG, CLB; PHT	Mild ID	Autistic traits	c.1521dupC p.Ile508Hisfs*15	*de novo*
23/F	10	12,8	Tonic posturing	Y	Y	Several per year	CZP, VPA, LEV	Moderate ID	No	c.1019A>G p.Asn340Ser	*de novo*
24/F	17	29,5	Tonic posturing	Y	Y	Several per year	TPM, PRG, CZP	Normal	dist di personalità dissociativo	c.1786G>C p.Asp596His	Father
25/F	8	33,5	Tonic posturing	N	Y	Annual clusters	VPA, PB	Mild ID	No	c.154_176del p.Ala52Serfs*29	*de novo*
26/F	12	20,9	Tonic posturing, absences	Y	Y	Weekly seizures	CZP, PB, ESM	Moderate ID	No	c.1804C>T p.Arg602*	*de novo*
27/F	32	5,1	Tonic posturing	Y	Y	Several per year	VPA, LEV, TPM	Normal	No	c.1676_1682delinsGGTGCG p.Ans559fs*10	*de novo*
28/F	10,2	9,0	Hypomotor, tonic posturing	Y	Y	Annual clusters	VPA, PB, LEV	Severe ID	Autism	c.1019A>G p.Asn340Ser	*de novo*
29/F	16	10,8	Tonic posturing	Y	Y	Several per year	GVG, OXC, LTG	IQ= 69	No	c.83C>A p.Ser28*	*de novo*
30/F	5	9,3	Tonic posturing	Y	N	Weekly-monthly seizures	LEV, LTG	Mild ID	Autistic traits	c.1464_1466del p.Ser489del	*de novo*
31/F	17	9,4	Hypomotor	Y	Y	Monthly clusters	VPA, TPM, CBL	Mild ID	No	c.1091dupC p.Tyr366Leu fs*10	*de novo*
32/F	8	21,7	Hypomotor	Y	Y	monthly seizures	VPA, LCM	Borderline	Autistic traits	c.83C>A p.Ser28*	*de novo*
33/F	19	11,4	Hypomotor, tonic posturing	Y	Y	Weekly-monthly seizures	LEV	Borderline	No	c.2903dupA p.Asp968Glufs*18	*de novo*
34/F	6	9,0	Hypomotor, tonic posturing, absences, myoclonic seizures	Y	Y	Several per year	VPA, LEV	Severe ID	Autism	c.695A>G p.Asn232Ser	*de novo*
35/F	7	11,9	Tonic posturing	Y	Y	Several per year	VPA	Normal	No	c.1211C>T p.Thr404Ile	*de novo*
36/F	6	18,1	Hypomotor	Y	Y	Several per year	CBZ, LEV, LCM	Moderate ID	No	c.1019A>G p.Asn340Ser	Mother
37/F	7	4,0	Hypomotor	Y	Y	Weekly-monthly seizures	CBZ, CLB	Mild ID	No	c.2697dupA p.Glu900Argfs*8	*de novo*

38/F	6	13,4	Hypomotor, tonic posturing, absences	Y	Y	Weekly-monthly seizures	LCM, CLB	Severe ID	Autistic traits	c.242T>G p.Leu81Arg	*de novo*
39/F	6	13,4	Hypomotor, tonic posturing, absences	Y	Y	Weekly-monthly seizures	LCM, CLB	Severe ID	Autistic traits	c.242T>G p.Leu81Arg	*de novo*
40/F	3	6,7	Hypomotor, tonic posturing	Y	Y	Several per year	VPA, CLB,	Mild ID	Autistic traits	c.456T>A	*de novo*
41/F	17	22,1	Hypomotor, tonic posturing	Y	Y	Several per year	PB, CBZ	Normal	No	c.445C>T p.Pro149Ser	*de novo*
42/F	68	8,0	Tonic posturing	Y	Y	Annual clusters	VPA, CBZ, LEV	Normal	No	c.937G>A p.Glu307Lys	Father
43/F	8	6,0	Hypomotor, tonic posturing	Y	Y	Annual clusters	CBZ, LEV, PB, LTG, VPA	IQ= 67	No	c.1765_1766delTG p.Val589CysfsX8	*de novo*
44/F	32	4,8	Hypomotor, tonic posturing	Y	Y	Several per year	CBZ, LEV	Normal	No	c.919G>A p.Glu313Lys	*de novo*
45/F	12	16,0	Hypomotor, tonic posturing	Y	Y	Annual clusters	CBZ	Mild ID	No	c.608A>C/c.617T> p.His203Pro	*de novo*
46/F	2,5	8,9	Tonic posturing	N	N	Monthly seizures	CBZ,TPM, PB, NZP	DQ= 70	No	c.1091dupC p.Tyr366Leufs*10	*de novo*
47/F	9	19,0	Tonic posturing	Y	Y	Seizure-free (2 yrs)	TPM, CLB	Normal	No	c.790G>C p.Asp264His	*de novo*
48/F	5	9,8	Hypomotor	Y	Y	Annual clusters	PB, LEV, DZP	DQ= 72	Autistic traits	c.1019A>G p.Asn340Ser	*de novo*
49/F	1	1,9	Tonic posturing	N	N	Annual seizure	VPA, CLB	DQ= 70	No	c.152dupTp. Ala52Argfs*37	*de novo*
50/F	12	8,1	Tonic posturing	Y	Y	Seizure-free (2 yrs)	TPM, CLB	Normal	No	c.1019A>G p.Asn340Ser	Mother
51/F	10	2,0	Tonic posturing	Y	Y	Annual clusters	PB, LEV, CLB	Normal	No	c.1537G>C p.Gly513Arg	*de novo*
52/F	25	4,1	Hypomotor, tonic posturing	Y	Y	Annual seizure	CBZ	DQ= 93 (4 y)	No	c.1710_1716delCGGCACT; p.Asn570fs*12	*de novo*
53/F	7	12,0	Hypomotor, tonic posturing	Y	Y	monthly clusters	VPA	IQ=20 (9,8 y)	Autism	c.799T>G; p.Leu260Arg	*de novo*
54/F	15	14,4	Tonic posturing	Y	Y	Annual clusters	PB, VPA, LCM	IQ= 83 (14 y)	No	Xq21.33q22.1 deletion encompassing PCDH19 gene	*de novo*
55/F	8	17,6	Hypomotor, tonic posturing	Y	Y	Annual clusters	ZNS, CLB	IQ= 50 (16 y)	Yes	c.1123G>T; p.ASp375Tyr	*de novo*
56/F	8	26,0	Hypomotor, tonic posturing	Y	Y	Weekly-monthly seizures	ZNG, LTG	Moderate ID	Autistic traits	c.1159delC; p.R387Vfs135X	*de novo*
57/F	15	6,0	Hypomotor, tonic posturing	Y	Y	Seizure-free (2 yrs)	LEV	DQ= 90 (4,10)	Isolation traits	c.671 T>A; p.Leu224His	*de novo*

58/F	26	14,0	Hypomotor, tonic posturing	Y	Y	Annual clusters	VPA, CLB	IQ= 83	Attention deficit, anxiety and depression traits	c 1456 G>C; p.G486R	Father
59/F	5	21,0	Hypomotor, tonic posturing, absences	Y	Y	Several per year	LCM, TPM, CNZ, deflazacort	Moderate ID	Autistic traits, depression	c.1973T>G; pVal658Gly	Mother
60/M	9	6,4	Tonic posturing	Y	Y	Several per year	CBZ	DQ= 72 (4,3 y)	Compulsive and stereotyped behaviours.	c.918C>G; p.(Tyr306*)	*de novo* mosaicism
61/M	10	5,2	Tonic posturing	Y	Y	Annual clusters	VPA, STP, CLB	DQ= 91(4,3 y)	Isolation traits	c.1352 C>T; p. (Pro451Leu)	*de novo* mosaicism

Legend: F=female; M=male; y= yrs; mm=months; Y=yes; N=no; VPA= valproic acid; CBZ= carbamazepine; LEV= levetiracetam; OXC=oxcarbazepine; CLB=clobazam; STP=stiripentol; CNZ=clonazepam; PB= phenobarbital; LCM= lacosamide; TPM= topiramate; CNZ= clonazepam; ZNS= zonisamide; F-U= Follow-Up; IQ= Intelligent Quotient; DQ= Developmental Quotient; F-U = follow-up;

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List of abbreviation:

AED: Antiepileptic Drug

ASD: Autistic Spectrum Disorder

EFMR: Epilepsy and mental retardation limited to females

ID: Intellectual Disability

MMPSI: Malignant Migrating Partial Seizures of Infancy

NCSE: Non Convulsive Status Epilepticus

PCDH19-FE: PCDH19- Female Epilepsy

PPR: Photo-Paroxysmal Response

SE: Status Epilepticus

LC-MS/MS: liquid chromatography-electrospray tandem mass spectrometry