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HCN ion channels and accessory proteins in epilepsy: genetic analysis of a large cohort of patients and review of the literature

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ABSTRACT

The Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels are highly expressed in the Central Nervous Systems, where they are responsible for the I_h current. Together with specific accessory proteins, these channels finely regulate neuronal excitability and discharge activity. In the last few years, a substantial body of evidence has been gathered showing that modifications of I_h can play an important role in the pathogenesis of epilepsy. However, the extent to which HCN dysfunction is spread among the epileptic population is still unknown. The aim of this work is to evaluate the impact of genetic mutations potentially affecting the HCN channels' activity, using a NGS approach. We screened a large cohort of patients with epilepsy of unknown etiology for mutations in *HCN1, HCN2* and *HCN4* and in genes coding for accessory proteins (MiRP1, Filamin A, Caveolin-3, TRIP8b, Tamalin, S-SCAM and Mint2). We confirmed the presence of specific mutations of *HCN* genes affecting channel function and predisposing to the development of the disease. We also found several previously unreported additional genetic variants, whose contribution to the phenotype remains to be clarified. According to these results and data from literature, alteration of HCN1 channel function seems to play a major role in epilepsy, but also dysfunctional HCN2 and HCN4 channels can predispose to the development of the disease. Our findings suggest that inclusion of the genetic screening of HCN channels in diagnostic procedures of epileptic patients should be recommended. This would help pave the way for a better understanding of the role played by I_h dysfunction in the pathogenesis of epilepsy.

1. Introduction

The diffusion of Next Generation Sequencing (NGS) in the clinical practice has improved the search and understanding of the pathogenesis of several genetic diseases. In the last few years, NGS has allowed to identify novel genes associated with a specific disease and enabled the screening of a large number of patients, with considerable savings in time and cost, compared to direct gene analysis. Investigation of large cohorts of epilepsy patients by NGS has changed significantly our knowledge of the genetic causes of the disease (Thomas and Berkovic,

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2014; Mei et al., 2017).

Along with the discovery of an increasing number of novel genes whose dysfunction contributes to epilepsy, a growing amount of data shows that a tight genotype-phenotype association linking causative gene dysfunctions to the disease does not normally occur, with the exception of a few specific cases, such as for example the Dravet syndrome (DS), known to be caused mainly by *SCN1 A* mutations (Dravet, 2011). The dysfunction of a specific gene can in fact be associated with different epileptic phenotypes. This is the case for example of the *KCNQ2* gene, whose alteration is known to cause neonatal epilepsies with a wide phenotypic heterogeneity, ranging from benign familial neonatal seizures to epileptic encephalopathy with severe pharmaco-resistant seizures and cognitive delay (Miceli et al., 2013). Conversely, patients with a similar epileptic phenotype can have mutations in different genes with totally different function and expression patterns, such as those causing the spectrum of the "DS-like syndromes" (Steel et al., 2017).

Despite the growing knowledge of the pathogenic mechanisms of epilepsy, however, the etiology of several epileptic syndromes remains unknown in the majority of patients. Several studies have shown that genetic alterations responsible for the disease are detectable only in a small proportion of cases, even when more subjects of the same family are involved, indicating an autosomal dominant type of inheritance (Helbig et al., 2008). In patients with generalized epilepsy, the most frequent mutations linked to the disease have been identified in the *SCL2 A1* gene (Arsov et al., 2012; Striano et al., 2012). In familial temporal lobe epilepsy (TLE), the most frequent form of focal epilepsy, about half of the cases are linked to *LGI1* mutations (Nobile et al., 2009).

In addition to the set of proteins already known to have a role in the pathogenesis of the disease, one group recently emerging in the field of epilepsy is the family of the Hyperpolarization-activated Cyclic-Nucleotide-gated (HCN) ion channels. Four HCN isoforms (HCN1–4), each with different kinetic and voltage characteristics, represent the molecular correlates of native hyperpolarization-activated f channels carrying the "funny" current in cardiomyocytes and the "h" current in neurons (I_f/I_h). Isoforms 1, 2 and 4 are widely expressed in neurons, each with a diverse distribution in brain areas (Seo et al., 2015).

The role of HCN channels in neurons has been only partially clarified. It has been established that HCN channels contribute to the control of neuronal discharge and that their dysfunction can lead to hyperexcitability and uncontrolled action potential firing, thus predisposing to seizures (Robinson and Siegelbaum, 2003; Biel et al., 2009; Baruscotti et al., 2010; Benarroch, 2013; DiFrancesco and DiFrancesco, 2015). Further evidence for a relevant role of HCN channels in the pathogenesis of epilepsy has been recently obtained (DiFrancesco and DiFrancesco, 2015; Oyrer et al., 2018). *HCN1*-knockout animal models show a significant increase of hyperexcitability, with a predisposition to the development of seizures (Huang et al., 2009; Santoro et al., 2010) and epilepsy (Nishitani et al., 2018). *HCN2* loss-of-function causes spontaneous generalized epilepsy, both in genetically modified models (Ludwig et al., 2003) and in animals carrying a spontaneous mutation (Chung et al., 2009).

The evidence for HCN mutations in human epilepsy has rapidly grown in the last few years. A study investigating patients with generalized epilepsy appeared in 2008 and reported a single point mutation in *HCN2*, characterized by a partial reduction of activity of the mutant channel (Tang et al., 2008). Subsequently, Dibbens and colleagues identified a gain-of-function mutation of *HCN2*, due to a triple proline deletion (delPPP), more frequent in children with either febrile seizures (FS) or genetic epilepsy with febrile seizures plus (GEFS +) than in controls (Dibbens et al., 2010). In 2011, our group identified in a patient with generalized epilepsy the first recessive mutation in *HCN2* (p.Glu515Lys), causing a nearly complete loss of function of the channel, together with a significant increase in the activity of neuronal discharge and excitability (DiFrancesco et al., 2011). In a later study, two unrelated children with FS resulted carriers of the *HCN2* p.Ser126Leu mutation, characterized by an increased I_h availability only at high temperatures (Nakamura et al., 2013). Recently, two variants of *HCN2* (p.Ser632Trp and p.Val246Met), both characterized by a gain-of-function effect on the channel activity, have been identified in genetic epilepsy with variable phenotypes (Li et al., 2018).

HCN4, the isoform typically described as the "pacemaker" channel in cardiac cells (Baruscotti and Difrancesco, 2004), is also involved and a loss-of-function mutation of *HCN4* associated with familiar epilepsy has been recently described (Campostrini et al., 2018). This work identified the p.Arg550Cys heterozygous mutation of *HCN4* in two brothers affected by benign myoclonic epilepsy of infancy, and showed that the mutation determines a loss of function of the channel, leading to increased neuronal excitability and predisposition to epilepsy (Campostrini et al., 2018). The p.Pro1117Leu and p.Gly153Glu variants of *HCN4* have also been identified in patients with different subtypes of generalized epilepsy. The functional characterization of these variants showed a loss-of-function effect on channel function (Becker et al., 2017).

So far, the HCN isoform most clearly involved in the pathogenesis of epilepsy appears to be HCN1. In 2014, *de novo HCN1* mutations were reported in early infantile epileptic encephalopathy (EIEE), characterized by pharmaco-resistant seizures and very poor prognosis (Nava et al., 2014). Functional studies demonstrated that some of these mutations have a dominant gain-of-function effect, even if with different degrees of severity. More recently, our group reported the first evidence linking the dysfunction of HCN1 to generalized epilepsy, without developmental and encephalopathic characteristics (Bonzanni et al., 2018). In one patient with generalized epilepsy we identified the p.Leu157Val novel mutation in *HCN1*. The characterization of its properties in CHO cells and neuronal models showed that this mutation exerts a dominant negative loss-of-function effect, leading to the increase of neuronal firing rate and excitability, predisposing the proband to the development of epilepsy. In line with this result, a recent cooperative study of a large cohort of patients analyzed with NGS, identified several other mutations of *HCN1* in patients with different phenotypes of generalized epilepsy. Apparently in contrast with the previous evidence on EIEE (Nava et al., 2014), one of the most interesting results of this study was the observation that the majority of patients had a mild epileptic phenotype, mainly generalized epilepsy and GEFS + . Moreover, along with *de novo* mutations, this work identified for the first time families with an autosomal dominant inheritance of the genetic defect (Marini et al., 2018). The electrophysiological characterization, however, could not fully clarify the existence of a tight correlation between the genetic defects of this channel and the different phenotypes, which range from severe encephalopathy to mild epilepsy. In fact, these mutations are scattered along the protein and can have both a gain- or loss-of-function effects on channel function.

For their correct function, the HCN channels interact with accessory proteins that finely regulate their activity. Even if this has never been demonstrated in patients, it is also possible that the alteration of the accessory proteins can contribute to modifying the properties of the I_h current and thus potentially predispose to the development of seizures. Different proteins are known to interact with these ion channels. *KCNE2,* encoding for the MinK-related peptide 1 (MiRP1) protein, was first reported as a β-subunit for the HCN family able to enhance the expression and to alter the kinetics of the various HCN channels (Yu et al., 2001; Qu et al., 2004; Brandt et al., 2009). MiRP1 is highly expressed in the cardiac tissue, where the dysfunction of MiRP1 is known to suppress the pacemaker current and is associated with sinus bradycardia (Nawathe et al., 2013), but is also present in the brain (Tinel et al., 2000). Filamin A (*FLNA*) is an actin-cross-linking protein, required for neuronal migration to the cortex, whose mutations are linked to human periventricular heterotopia (Fox et al., 1998). It is known that this protein interacts with HCN1, but not with the HCN2 and HCN4

isoforms (Gravante et al., 2004). Caveolin-3 (*CAV3*) affects the function of I_f/HCN4 (Barbuti et al., 2007; Ye et al., 2008; Barbuti et al., 2012) and is expressed in the brain (Ikezu et al., 1998). One of the most studied proteins interacting with HCN ion channels is TRIP8b (TPR-containing Rab8b interacting protein), expressed by *PEX5L*. TRIP8b regulates the surface expression of HCN1, markedly decreasing I_h current density (Santoro et al., 2004). More recently, it was demonstrated that the scaffold proteins Tamalin (*GRASP*), S-SCAM (*MAGI2*) and Mint2 (*APBA2*) are assembled with HCN2 in the rat brain (Kimura et al., 2004). However, their functional interaction with the channel has not been investigated.

In this work we screened the genes coding for the HCN ion channels and the related accessory proteins in a large population of patients with epilepsy of unknown etiology, in order to identify new variants and to evaluate their potential contribution to the development of the disease.

2. Materials and methods

2.1. Patient recruitment and data collection

From January 2010 to December 2016, we recruited patients with different types of epilepsy (generalized, focal, combined generalized-focal), according to definition (Fisher et al., 2014; Scheffer et al., 2017). Patients were recruited from different Epilepsy Centers distributed throughout Italy (North, Center and South). We included subjects with onset of seizures within the infantile-pediatric age, normal psychomotor development or normal cognitive functioning (according to age) and normal neurological examination. We excluded patients with developmental and epileptic encephalopathies and/or symptomatic epilepsy. For all the cases included, we collected information about gender, type of epilepsy (generalized, focal and combined generalized-focal) and inheritance of the disease. We considered the disease sporadic when the patient was the only affected of the family, or familial when at least one member of the proband's family was affected by epilepsy with similar features. In order to exclude a possible symptomatic etiology of the seizures, clinical and instrumental data of recruited patients were carefully analyzed. Structural etiology of the disease (cerebrovascular, tumor, infectious, metabolic, immune, trauma and malformation of cortical development) were investigated with 1.5 or 3 T brain MRI with proper sequences (T1, T1 with Gadolinium, T2/FLAIR, Inversion Recovery). Biochemical and hematological tests were performed to exclude metabolic causes. Common seizure-provoking factors (antipsychotic or antidepressant therapy, alcohol or drug dependency, infection of the central nervous system) were excluded. EEG was used to characterize the features of the disease. A written informed consent was obtained from all patients and/or from their parents, as approved by the local Institutional Review Board of the Besta Institute and S. Gerardo Hospital and by the Italian Ministry of Health. Upon acceptance of the informed consent, patients underwent a small blood withdrawal in EDTA anticoagulant for DNA extraction.

2.2. DNA extraction and genetic screening

Genomic DNA was prepared from peripheral-blood lymphocytes using standard procedures, as previously reported (DiFrancesco et al., 2014, 2015). We used a TruSeq Custom Amplicon (Illumina), with a Studio Design software (Illumina Inc., San Diego, CA, USA) to customize a gene panel for the analysis of the genes coding for HCN ion channels [*HCN1* (NM_021072; NP_066550), *HCN2* (NM_001194; NP_001185), *HCN4* (NM_005477; NP_005468)] and HCN-accessory proteins [*APBA2* (NM_005503; NP_005494), *CAV3* (NM_033337; NP_203123), *MAGI2* (NM_012301; NP_036433), *FLNA* (NM_001110556; NP_001104026), *KCNE2* (NM_172201; NP_751951), *PEX5L* (NM_016559; NP_057643), *GRASP* (NM_181711; NP_859062)]. All the variants identified were confirmed by Sanger analysis. When possible, the segregation of the identified variants was verified within the probands' families.

2.3. Variants' classification and evaluation of pathogenicity

Among all variants identified by the genetic screening, those with MAF > 1% reported in the dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genome (browser.1000genomes.org), EVS database (evs.gs.washington.edu), ExAC database (http://exac.broadinstitute.org/) and gnomAD browser (http://gnomad.broadinstitute.org/) were considered of benign significance and excluded from the report. Following this filtering, the remaining variants were evaluated according to different parameters: frequency in the main references databases and in the cohort of patients analyzed, *in silico* and splicing predictions, segregation within the proband's family (when available). These parameters were used to evaluate the pathogenicity of the variants. According to the guidelines of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015), the identified variants were classified in different classes: clearly not pathogenic (class 1), unlikely to be pathogenic (2), unknown significance (3), likely to be pathogenic (4), clearly pathogenic (5).

3. Results

3.1. Patients

We recruited 597 patients from different Epilepsy Centers distributed throughout the Country: Fondazione IRCCS Istituto Neurologico Carlo Besta (Milan), San Gerardo Hospital (Monza), Ospedale S. Maria della Misericordia (Perugia), IRCCS Neuromed (Pozzilli), University "Magna Graecia" (Catanzaro).

Patients were classified as affected by generalized epilepsy in 321 cases (54%), with focal origin in 211 (35%) and combined generalized-focal in the remaining 65 (11%). Within all cases, 310 were females (52%). The disease was associated with familial inheritance in 210 cases (35%) and was sporadic in 167 (28%). For the remaining 220 cases (37%), we could not retrieve this information. These data are reported in Table 1.

3.2. Genetic screening of HCN ion channels

The screening of HCN ion channels identified several missense variants, all in heterozygosis and each present in a single subject (Table 2).

3.2.1. HCN1

Four variants (p.Pro42Ser, p.Gly47Val, p.Arg88Gly, p.Leu157Val) of *HCN1* were identified in patients with generalized epilepsy, and one (p.Ile206Val) in a patient with focal epilepsy. The segregation of p.Pro42Ser and p.Ile206Val could not be completed due to the unavailability of biological samples from probands' families. Analysis of

Table 1
Characteristics of epileptic patients.

GENDER	Number of patients	%
Female	310	52
Male	287	48
TYPE OF EPILEPSY		
Generalized	321	54
Focal	211	35
Combined generalized-focal	65	11
INHERITANCE		
Familial	210	35
Sporadic	167	28
Undetermined	220	37
TOTAL	597	100

Table 2
Genetic and clinical features of patients with HCN ion channels variants. F/M Female/Male, G/P/C Generalized/Partial/Combined generalized-focal epilepsy, F/S/U Familial/Sporadic/Unknown.

GENETIC DATA						*IN SILICO* PREDICTION			CLINICAL DATA			
GENE	EXON	NUCLEOTIDE VARIANT	PROTEIN CHANGE	dbSNP	ALLELE FREQUENCY	SIFT	POLYPHEN	CLASS OF PATHOGENICITY	NUMBER OF PATIENTS	GENDER	TYPE OF EPILEPSY	INHERITANCE
HCN1	1	Heterozygous c.124C > T	p.Pro42Ser	rs56164833	0.0003734	0.24 tolerated	0.034 benign	2	1	M	G	U
	1	Heterozygous c.140 G > T	p.Gly47Val	rs544994462	0.002875	0.14 tolerated	0.084 benign	2	1	F	G	S
	1	Heterozygous c.262C > G	p.Arg88Gly	rs774375241	0.00003665	0.38 tolerated	0.007 benign	2	1	M	G	F
	2	Heterozygous c.469C > G	p.Leu157Val	not reported		0.68 tolerated	0.005 benign	4	1	M	G	F
	2	Heterozygous c.616 A > G	p.Ile206Val	rs1444972054	0.00000407	0.17 tolerated	0.12 benign	2	1	M	P	U
HCN2	5	Heterozygous c.1543 G > A	p.Glu515Lys	rs746420784	0.000008263	0 deleterious	0.917 possibly damaging	4	1	M	G	S
HCN4	2	Heterozygous c.881C > G	p.Pro294Arg	rs1216261955	0,000004062	0.08 tolerated	0.905 possibly damaging	3	1	M	P	F
	2	Heterozygous c.989C > T	p.Pro330Leu	rs370442588	0,00003249	0 deleterious	0.999 possibly damaging	3	1	F	G	F
	5	Heterozygous c.1648C > T	p.Arg550Cys	rs150691273	0,000008122	0 deleterious	0.993 possibly damaging	4	2	2M	2G	2F
	8	Heterozygous c.1703 G > C	p.Ser568Thr	rs138714806	0.0009502	0.02 deleterious	0.174 benign	2	1	M	G	F
	8	Heterozygous c.1840 G > A	p.Glu614Lys	rs201319883	0.000012	0 deleterious	0.996 probably damaging	3	1	F	COMB	S
	8	Heterozygous c.2211 G > T	p.Gln737His	rs755502946	0,000008436	0.12 tolerated	0.011 benign	2	1	M	P	U
	8	Heterozygous c.2275 G > A	p.Val759Ile	rs62641689	0.003204	0.41 tolerated	0.003 benign	2	1	M	C	F
	8	Heterozygous c.2648C > G	p.Pro883Arg	rs148398509	0,007727	0.11 tolerated	0.011 benign	2	2	1M/1F	1G/1P	1F/1U
	8	Heterozygous c.2831C > T	p.Ala944Val	rs144450232	0,001656	0.51 tolerated	0 benign	2	1	M	G	S
	8	Heterozygous c.3350C > T	p.Pro1117Leu	rs140402087	0,00199	0 deleterious	0.005 benign	2	1	M	G	S
	8	Heterozygous c.3490C > G	p.Pro1164Ala	rs750255859	0,00002944	0.01 deleterious	0.003 benign	2	1	F	P	U

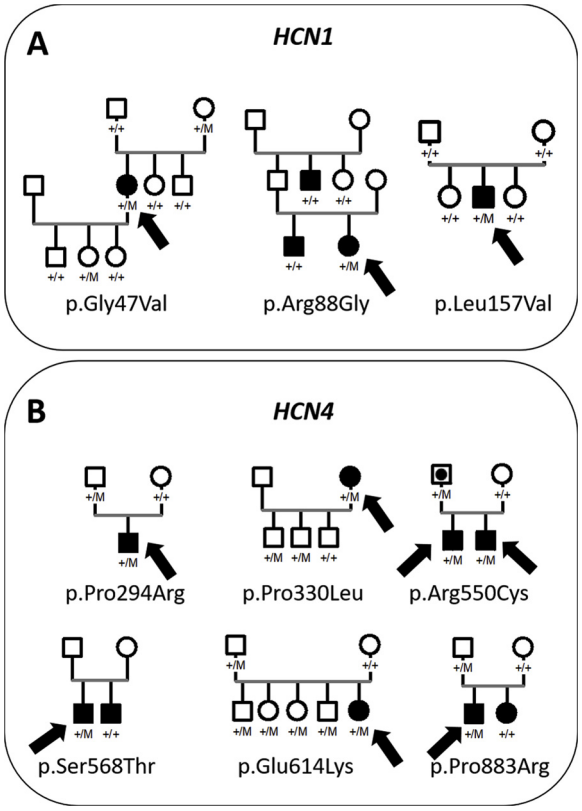

Fig. 1. Family trees of patients with *HCN1* (**A**) and *HCN4* (**B**) variants. "+"wild-type, "M" mutation. Full symbol: affected, empty: unaffected. Arrows indicate the probands. As already reported (Campostrini et al., 2018), we lacked anamnestic young-age data for the probands' father in the p.Arg550Cys family (dotted symbol), and we could therefore neither confirm nor exclude infantile seizures in this subject.

the variants p.Gly47Val and p.Arg88Gly were instead extended to the relatives of the probands. We observed that these two variants do not segregate with the disease, since in one case the mutation was carried by two unaffected subjects (p.Gly47Val; Fig. 1A), and in the other case the mutation was absent in one epileptic relative (p.Arg88Gly; Fig. 1A). The p.Leu157Val novel variant resulted *de novo* in the proband (Fig. 1A). According to the ACMG guidelines (Richards et al., 2015), the variants p.Pro42Ser, p.Gly47Val, p.Arg88Gly and p.Ile206Val all result unlikely to be pathogenic (class 2), while the variant p.Leu157Val is classified as likely to be pathogenic (class 4).

Indeed, as we have reported previously, functional characterization of the p.Leu157Val mutation shows that this has a loss-of-function dominant negative effect on the channel function, causing a reduced contribution of HCN1 activity to the net inward current; this is associated with increased neuronal firing rate and excitability, conditions that potentially predispose to the development of epilepsy (Bonzanni et al., 2018).

3.2.2. HCN2

The screening of *HCN2* confirmed the presence of the p.Glu515Lys recessive mutation in one patient with generalized epilepsy, as already reported (DiFrancesco et al., 2011). However, we could not identify any additional variants of potential interest in other patients.

3.2.3. HCN4

We identified the variants p.Pro330Leu, p.Arg550Cys, p.Ser568Thr, p.Pro883Arg, p.Ala944Val and p.Pro1117Leu in patients with generalized epilepsy, p.Pro294Arg, p.Gln737His, p.Pro883Arg and p.Pro1164Ala in patients with focal epilepsy, p.Glu614Lys and p.Val759Ile in patients with combined generalized-focal epilepsy. Four of these variants did not show to segregate with the disease, being present also in unaffected members of the probands' families (p.Pro294Arg, p.Pro330Leu, p.Glu614Lys) or absent in affected relatives (p.Ser568Thr) (Fig. 1B). The p.Pro883Arg variant was identified in two unrelated patients. In one case, this variant did not segregate with the disease (Fig. 1B), while in the other we could not extend the analysis to the proband's family. For the same reason, we could not investigate the variants p.Gln737His, p.Val759Ile, p.Ala944Val, p.Pro1117Leu and p.Pro1164Ala. The variants p.Ser568Thr, p.Gln737His, p.Val759Ile, p.Pro883Arg, p.Ala944Val, p.Pro1117Leu, p.Pro1164Ala were classified as unlikely to be pathogenic (class 2); p.Pro294Arg, p.Pro330Leu, p.Glu614Lys as VUS (Variants of Uncertain Significance, class 3); p.Arg550Cys resulted to be likely to be pathogenic (class 4). None of the subjects carrying an HCN4 variant reported conduction cardiac disease in medical history.

As previously reported (Campostrini et al., 2018), the p.Arg550Cys mutation was identified in two brothers with generalized epilepsy (benign myoclonic epilepsy of infancy) with very similar clinical and instrumental phenotypes. The functional characterization in heterologous expression system (CHO cells) and neurons showed that this mutation determines a loss-of-function effect on HCN4 contribution to I_h activity and a significant increase of neuronal discharge, compatible with the onset of seizures during infancy.

3.3. Genetic screening of HCN-accessory proteins

The results of the genetic screening of HCN-accessory proteins are summarized in Table 3.

3.3.1. APBA2

The p.Ser55Asn variant was identified in two unrelated patients, affected by combined generalized-focal epilepsy and focal epilepsy, respectively. The variants p.Pro168Leu and p.Asp216Asn were found in two patients with generalized epilepsy, and the variants p.Thr445Met and p.Leu571Val in patients with focal epilepsy. In none of these cases was full segregation achieved within the families investigated. The variants p.Pro168Leu and p.Leu571Val were classified as unlikely to be pathogenic (class 2), p.Ser55Asn, p.Asp216Asn and p.Thr445Met were classified as VUS (class 3).

3.3.2. CAV3

The screening of *CAV3* led to the identification of the p.Thr78Met variant in seven unrelated patients, all affected by generalized epilepsy, except one with focal epilepsy. As already reported (Spadafora et al., 2012), this variant is very frequent in Italy. Compatibly with the high expression of caveolin-3 in the heart, we have previously reported this mutation to be found at a higher frequency in patients with cardiac arrhythmias (sick sinus syndrome, atrial fibrillation and stillbirths) and to affect HCN4 current (Campostrini et al., 2017). We also identified the p.Cys116Gly novel variant in one male patient with generalized epilepsy; however, it was not possible to fully investigate the segregation profile of the variant within the proband's family. Both variants were classified as VUS (class 3).

3.3.3. FLNA

The screening of this gene revealed several variants in patients affected by generalized epilepsy: p.Arg443Cys, p.Val579Leu, p.Val752Ile, p.Val985Ala, p.Gln1488Arg, p.Arg2049Cys, p.Asn2117Ser, p.Met2490Leu, p.Pro2545Leu. Segregation analysis showed that, in most cases, these variants do not segregate with the disease, either because they are present in unaffected relatives (p.Val579Leu, p.Val752Ile, p.Val985Ala, p.Gln1488Arg, p.Asn2117Ser) or because they are lacking in affected members of the same family (p.Arg2049Cys). We could not complete segregation analysis of the variants p.Arg443Cys and p.Met2490Leu because samples of the

Table 3
Genetic and clinical features of patients with HCN accessory proteins variants. Legend: F/M Female/Male, G/P/C Generalized/Partial/Combined generalized-focal epilepsy, F/S/U Familial/Sporadic/Unknown.

GENETIC DATA						*IN SILICO* PREDICTION			CLINICAL DATA			
GENE	EXON	NUCLEOTIDE VARIANT	PROTEIN CHANGE	dbSNP	ALLELE FREQUENCY	SIFT	POLYPHEN	CLASS OF PATHOGENICITY	NUMBER OF PATIENTS	GENDER	TYPE OF EPILEPSY	**INHERITANCE**
APBA2	1 A	Heterozygous c.164 G > A	p.Ser55Asn	rs142678624	0.005339	0.06 tolerated	0.977 probably damaging	3	2	1F/1M	1C/1P	1F/1S
	1B	Heterozygous c.503C > T	p.Pro168Leu	rs148760039	0.001924	0.2 tolerated	0.004 benign	2	1	M	G	S
	1B	Heterozygous c.646 G > A	p.Asp216Asn	NR	0	0 deleterious	0.822 possibly damaging	3	1	F	G	S
	10	Heterozygous c.1334C > T	p.Thr445Met	rs765758239	0.00004137	0.01 deleterious	0.984 probably damaging	3	1	M	P	S
	13	Heterozygous c.1711C > G	p.Leu571Val	rs370580206	0.00002908	0.52 tolerated	0.042 benign	2	1	M	P	U
CAV3	2	Heterozygous c.233C > T	p.Thr78Met	rs72546668	0,002674	0.12 tolerated	0.861 possibly damaging	3	7	4F/3M	6G/1P	4F/1S/2U
	2	Heterozygous c.346 T > G	p.Cys116Gly	NR	0	0.07 tolerated	0.969 possibly damaging	3	1	M	G	S
FLNA	6	Hemizygous c.958 G > A	p.Val320Met	rs781889196	0,0001002	0 deleterious	0.989 probably damaging	3	1	M	P	U
	6	Heterozygous c.958 G > C	p.Val320Leu	NR	0	0 deleterious	0.989 probably damaging	3	1	F	P	U
	9	Heterozygous c.1327C > T	p.Arg443Cys	rs782673341	0,0001157	0 deleterious	0.954 probably damaging	3	1	F	P	U
	9	Heterozygous c.1399C > T	p.Arg467Cys	rs367948333	0.00001207	0 deleterious	0.764 possibly damaging	3	1	F	G	S
	12	Heterozygous c.1735 G > C	p.Val579Leu	rs1320726395	0,00004606	0.01 deleterious	0.963 probably damaging	3	1	F	P	U
	15	Heterozygous c.2254 G > A	p.Val752Ile	rs1297013254	0	0.07 tolerated	0.915 possibly damaging	3	1	F	G	F
	19	Heterozygous c.2662 G > T	p.Glu888X	NOT REPORTED	0	premature stop codon		3	1	F	G	S
	19	Heterozygous Hemizygous c.2845 G > A	p.Val949Ile	rs201656372	0,0003102	0.11 tolerated	0.907 possibly damaging	3	2	1F/1M	1G/1P	2S
	21	Heterozygous c.2954 T > C	p.Val985Ala	rs782804253	0,0001849	0.04 deleterious	0.978 probably damaging	3	1	F	G	F
	21	Hemizygous c.3094C > T	p.Arg1032Cys	rs782546714	0,00002502	0.01 deleterious	0.828 possibly damaging	3	1	M	P	U
	26	Hemizygous c.4463 A > G	p.Gln1488Arg	NOT REPORTED	0	0.64 tolerated	0.048 benign	2	1	M	G	S
	32	Heterozygous c.5251C > T	p.Pro1751Ser	rs56102764	0,0007188	0.75 tolerated	0.003 benign	2	6	6F	3G/3P	3F/1S/2U
	33	Heterozygous c.5342 A > G	p.Asn1781Ser	rs373089783	0,00007006	0.38 tolerated	0 benign	2	1	F	P	U
	38	Hemizygous c.6145C > T	p.Arg2049Cys	rs782740450	0,0000056	0 deleterious	0.972 probably damaging	3	1	M	G	F
	38	Hemizygous c.6350 A > G	p.Asn2117Ser	rs375205247	0.0001175	0.41 tolerated	0.251 benign	2	1	M	G	S
	46	Heterozygous c.7450C > T	p.Arg2484Cys	rs782557713	0,0001651	0.01 deleterious	0.828 possibly damaging	3	2	2F	2P	2U
	46	Hemizygous c.7468 A > C	p.Met2490Leu	NOT REPORTED	0	0.41 tolerated	0.509 possibly damaging	3	1	M	G	F
	47	Heterozygous c.7634C > T	p.Pro2545Leu	rs1377674951	0.0000079	0.35 tolerated	0.0 benign	2	1	F	G	U

(*continued on next page*)

Table 3 (*continued*)

GENETIC DATA						*IN SILICO* PREDICTION			CLINICAL DATA			
GENE	EXON	NUCLEOTIDE VARIANT	PROTEIN CHANGE	dbSNP	ALLELE FREQUENCY	SIFT	POLYPHEN	CLASS OF PATHOGENICITY	NUMBER OF PATIENTS	GENDER	TYPE OF EPILEPSY	**INHERITANCE**
GRASP	2	Heterozygous c.271c > T	p.Leu91Phe	rs73104710	0.009375	0.08 tolerated	0.01 benign	2	5	1F/4M	5G	3F/2S
	5	Heterozygous c.499c > T	p.Arg167Trp	rs201792271	0,00003247	0 deleterious	0.997 probably damaging	3	1	M	G	F
	6	Heterozygous c.565 G > A	p.Gly189Arg	NOT REPORTED	0	0 deleterious	0.999 probably damaging	3	1	F	P	U
KCNE2	2	Heterozygous c.22 A > G	p.Thr8Ala	rs2234916 CM003449	0,003752	0 deleterious	0.991 probably damaging	3	9	4F/5M	8G/1P	2F/4S/3U
	2	Heterozygous c.29C > T	p.Thr10Met	rs199473648 CM055291	0.0002273	0.02 deleterious	0.557 possibly damaging	3	1	F	P	U
	2	Heterozygous c.161 T > C	p.Met54Thr	rs74315447 CM993508	0.0002381	0 deleterious	0.557 possibly damaging	3	1	F	G	F
	2	Heterozygous c.170 T > C	p.Ile57Thr	rs74315448 CM993509	0.0009594	0.04 deleterious	0.918 probably damaging	3	7	5F/2M	3G/4P	1F/3S/3U
	2	Heterozygous c.193 G > C	p.Val65Met	rs199473364 CM21618	0.00002030	0.01 deleterious	0.999 probably damaging	3	2	2M	2G	1F/1S
	2	Heterozygous c.229C > T	p.Arg77Trp	rs141423405 CM064063	0.00007716	0.02 deleterious	0.727 possibly damaging	3	1	F	P	U
MAGI2	5	Heterozygous c.874C > T	p.Pro292Ser	rs757912009	0,0000528	0.44 tolerated	0 benign	2	1	F	P	S
	10	Heterozygous c.1658 G > A	p.Arg553Gln	rs759667457	0,0000163	0.51 tolerated	0.766 possibly damaging	3	1	M	P	U
	10	Heterozygous c.2023 A > G	p.Thr675Ala	rs760890102	0.00000407	0.27 tolerated	0.037 benign	2	1	M	P	S
	12	Heterozygous c.2213 G > A	p.Arg738Gln	rs145722885	0,001923	0.1 tolerated	0.232 benign	2	2	1F/1M	1G/1C	1F/1S
	16	Heterozygous c.2723C > G	p.Pro908Arg	rs150080418	0,0003083	0.07 tolerated	0.708 possibly damaging	3	1	F	G	S
	22	Heterozygous c.3911 G > A	p.Gly1304Asp	rs145648453	0,001552	0.13 tolerated	0.276 benign	2	1	M	G	S
PEX5L	6	Heterozygous c.535 G > A	p.Asp179Asn	rs746551798	0.00002526	0.09 tolerated	0.023 benign	2	1	M	G	F
	8	Heterozygous c.758 G > A	p.Ser253Asn	NOT REPORTED	0	0.081 tolerated	0.521 benign	2	1	M	C	U

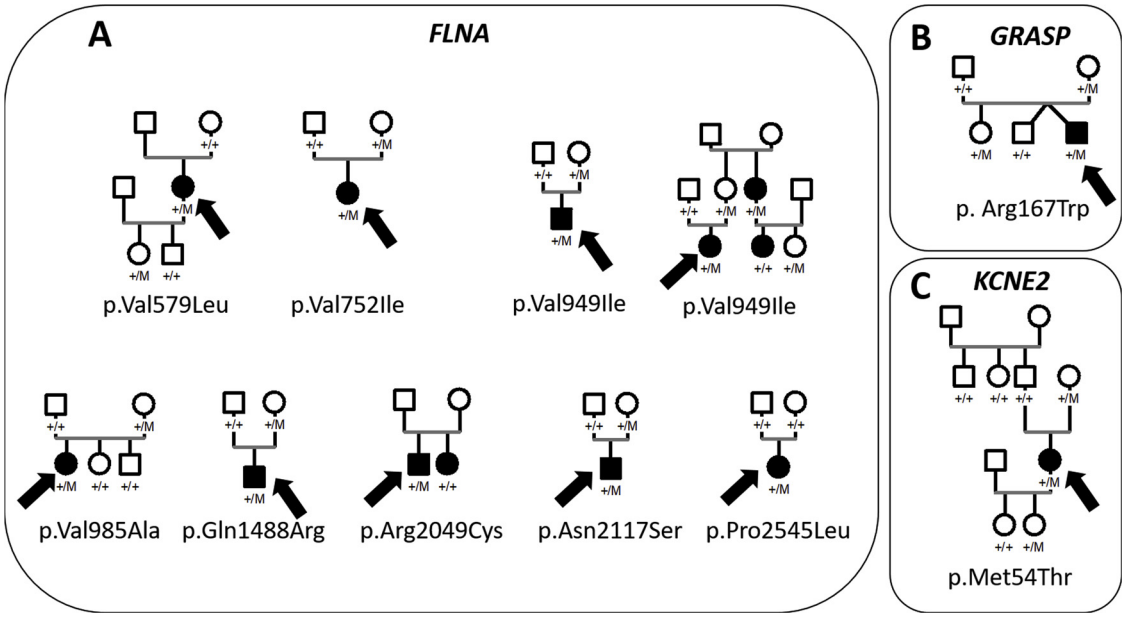

Fig. 2. Family trees of patients with *FLNA* (**A**), *GRASP* (**B**) and *KCNE2* (**C**) variants. "+"wild-type, "M" mutation. Full symbol: affected, empty: unaffected. Arrows indicate the probands.

probands' relatives were not available. The p.Pro2545Leu resulted a *de novo* variant since it was not present in the proband's parents (Fig. 2A). In patients with focal epilepsy, we identified the variants p.Val320Met, p.Val320Leu, p.Arg467Cys, p.Glu888X, p.Arg1032Cys, p.Asn1781Ser and p.Arg2484Cys. We could not complete the segregation analysis of these variants due to the unavailability of probands' family samples. Two variants were found both in patients with generalized and focal epilepsy. The p.Val949Ile was found in two unrelated patients, one with generalized epilepsy and one with focal epilepsy. Segregation analysis in the families of the two probands showed that this variant does not segregate with the disease in either of these two cases (Fig. 2A). The p.Pro1751Ser was identified in six unrelated patients, equally distributed between generalized and focal epilepsy (three cases for each phenotype). Due to the unavailability of samples from probands' relatives, we could not complete the segregation study.

In order to exclude the presence of nodular periventricular heterotopia, the brain MRI of all patients carrying *FLNA* variants were re-evaluated and a subset of them (subjects carrying the variants p.Val579Leu, p.Val752Ile, p.Glu888X and p.Val949Ile) underwent 3 T brain MRI. In all the cases analyzed, this specific investigation resulted negative for nodular periventricular heterotopia.

The variants p.Gln1488Arg, p.Pro1751Ser, p.Asn1781Ser and p.Asn2117Ser were classified as unlikely to be pathogenic (class 2), p.Val320Met, p.Val320Leu, p.Arg443Cys, p.Arg467Cys, p.Val579Leu, p.Val752Ile, p.Glu888X, p.Val949Ile, p.Val985Ala, p.Arg1032Cys, p.Arg2049Cys, p.Arg2484Cys and p.Met2490Leu as VUS (class 3).

3.3.4. GRASP

We identified the p.Leu91Phe variant in five unrelated patients and the p.Arg167Trp variant in one patient, all with generalized epilepsy. The p.Gly189Arg variant was found in one patient with focal epilepsy. The segregation analysis of the p.Arg167Trp variant within the family revealed that this variant does not segregate with the disease, being present both in the unaffected proband's mother and sister (Fig. 2B). For the remaining variants, the segregation analysis could not be completed. The variant p.Leu91Phe was classified as unlikely to be pathogenic (class 2), p.Arg167Trp and p.Gly189Arg were classified as VUS (class 3).

3.3.5. KCNE2

The analysis of *KCNE2* brought to the identification of the p.Thr8Ala variant in nine unrelated patients, all affected by generalized epilepsy, except one with focal epilepsy. Due to the relatively high frequency of this variant (Table 3), segregation analysis within the probands' families was not completed. We also identified the p.Ile57Thr variant in seven unrelated patients with either generalized or focal epilepsy, the p.Thr10Met and p.Arg77Trp variants in two unrelated patients with focal epilepsy and the p.Val65Met variant in two unrelated male patients affected by generalized epilepsy. For all these variants, we could not complete the segregation analysis within probands' family members. We further identified the p.Met54Thr variant in one patient affected by generalized epilepsy. The segregation analysis showed that this variant does not segregate with the disease (Fig. 2C). All the variants identified in *KCNE2* were classified as VUS (class 3).

3.3.6. MAGI2

Screening of the *MAGI2* gene led to the identification of the variants p.Pro292Ser, p.Arg553Gln and p.Thr675Ala in patients with focal epilepsy, and of the variants p.Pro908Arg and p.Gly1304Asp in patients with generalized epilepsy. The p.Arg738Gln variant was identified in two unrelated patients, one affected by generalized epilepsy and the other by combined generalized-focal epilepsy. For these variants, segregation analysis could not be completed. The variants p.Pro292Ser, p.Thr675Ala, p.Arg738Gln, p.Gly1304Asp were classified as unlikely to be pathogenic (class 2), the variants p.Arg553Gln and p.Pro908Arg as VUS (class 3).

3.3.7. PEX5L

The p.Asp179Asn variant was identified in one patient with generalized epilepsy, and the p.Ser253Asn variant in one subject with combined generalized-focal epilepsy. For these variants, the segregation could not be completed within probands' family members. Both variants were classified as unlikely to be pathogenic (class 2).

4. Discussion

In this work, we have searched for variants in the genes coding for HCN ion channels and accessory proteins in a large population of patients affected by epilepsy with unknown etiology. The principal aim of

the study was to evaluate the presence and the impact of mutations potentially affecting HCN channels' activity in the pathogenesis of epilepsy. To this aim, we performed NGS analysis on a large cohort of patients with heterogeneous epileptic phenotypes, excluding those with symptomatic epilepsy and clinical-instrumental features of developmental and epileptic encephalopathies. To widen our range of investigation, together with the genes coding for the HCN channels, we also screened the genes coding for neuronal accessory proteins that are known to cooperate for proper functional activity with the HCN channel proteins (Yu et al., 2001; Gravante et al., 2004; Kimura et al., 2004; Santoro et al., 2004; Ye et al., 2008; Brandt et al., 2009).

Overall, our results indicate that different mutations of the HCN channels can be found in patients with epilepsy with unknown etiology. Previous characterization of some of the mutations reported here (DiFrancesco et al., 2011; Bonzanni et al., 2018; Campostrini et al., 2018) and of other HCN mutations (Dibbens et al., 2010; Nakamura et al., 2013; Nava et al., 2014; Becker et al., 2017; Li et al., 2018; Marini et al., 2018), has shown that, when a mutation alters significantly the channel function, increased neuronal excitability potentially associated with the development of epilepsy can arise. However, causative epilepsy-linked mutations appear to be rare in patients. As our data show, in the 597 patients analyzed, only a small fraction revealed a genetic alteration of one HCN channel bearing an association, either potential or shown on the basis of increased neuronal excitability, with epilepsy. These data agree with previous reports showing the presence of *HCN* mutations in a reduced number of epileptic patients (Nava et al., 2014; Li et al., 2018).

According to our results and data from the literature, it is difficult to draw a clear genotype-phenotype correlation between the dysfunction of the HCN channels and epilepsy. So far, missense mutations of the *HCN1* gene have been found to contribute to the pathogenesis of epilepsy (Nava et al., 2014; Bonzanni et al., 2018; Marini et al., 2018). However, there appears to be a relatively large spectrum of epileptic disorders associated with HCN1 dysfunction. For example, recent evidence demonstrated that *HCN1* missense mutations can be identified both in EIEE and in patients with mild generalized epilepsy (Nava et al., 2014; Bonzanni et al., 2018; Marini et al., 2018). This scenario is even more complicated, since the various mutations reported until now are spread throughout the channel structure and there appears to be no obvious correspondence between the location in the HCN1 protein and the severity of symptoms. Interestingly, some mutational hot spots seem to exist, such as the residue G391 where three distinct *de novo* mutations (p.Gly391Ser, p.Gly391Cys and p.Gly391 Asp) have been identified. Unrelated patients carrying the same mutation on this residue exhibit a strikingly concordant phenotype, suggesting that the clinical feature is largely determined by the mutation (Marini et al., 2018). Furthermore, the electrophysiological properties of the mutated HCN1 channels did not allow to clarify the correlation between the genetically determined channel dysfunction and the variable epileptic phenotype. In fact, missense mutations can exert both gain-of-function and loss-of-function effects on the channel activity (Nava et al., 2014; Bonzanni et al., 2018; Marini et al., 2018).

The evidence presently available suggests that the contribution of the isoforms HCN2 and HCN4 to the pathogenesis of epilepsy is limited (Dibbens et al., 2010; DiFrancesco et al., 2011; Nakamura et al., 2013; Becker et al., 2017; Campostrini et al., 2018; Li et al., 2018). An established role of HCN4 channels is that of generating and controlling heart rhythm, and several mutation of this gene have been found to be associated with cardiac arrhythmias (DiFrancesco, 2010, 2015). It is interesting to note that patients with HCN4-related arrhythmias have not been reported to be affected by epileptic disorders (DiFrancesco, 2013). Moreover, none of the *HCN4* variants here identified in the epileptic patients have been associated with heart disease (DiFrancesco, 2015). It appears therefore that, while several dysfunctional mutations of HCN4 have been found, in agreement with its role in cardiac rhythm, to be associated with arrhythmias, a few other mutations may be more specifically linked to neurological phenotypes. In view of the expansion of the NGS analysis in the clinical practice, our data and results from other laboratories (Becker et al., 2017; Li et al., 2018) suggest that, along with *HCN1*, also *HCN2* and *HCN4* should be targeted in the screening of patients with genetic epilepsy.

Together with the mutations predisposing to epilepsy, we also identified several genetic variants in class of pathogenicity 3 (Variants of Uncertain Significance, VUS), both in the genes coding for HCN channels and in those for accessory proteins. Notably, no more than one variant of HCN and accessory proteins was identified in a single patient. Although their significance remains to be clarified, these VUS could partially predispose to the development of the epileptic phenotype. For instance, the *FLNA* variants identified in patients without radiological evidence of periventricular nodular heterotopias could exert a still unknown effect predisposing to the development of epilepsy. The significance of these variants is complicated, since in the majority of cases they do not segregate within the proband's family, with the exception of the variant p.Pro2545Leu, which was lacking in the proband's parents. Unfortunately, we could not further extend the segregation analysis of this variant within other relatives, although it should be noted that the *in silico* prediction does not consider this variant of possible pathogenic significance (class of pathogenicity 2). Since *FLNA* is an X-linked gene, the p.Pro2545Leu mutation could have arisen *de novo* in the proband or been inherited from the germline maternal line as a mosaicism. None of these aspects however could be evaluated. In this complex scenario, future studies are needed to understand the impact of these variants.

We are aware that the lack of segregation analysis of some of the variants identified does not allow a complete evaluation of their pathogenicity. At the same time, we think that inclusion of a wide cohort of patients as the one comprised in this study and the extensive genetic screening by NGS strengthen the quality of our work. Moreover, the recruitment of patients from Centers widely distributed within the national territory guarantees access to subjects with a heterogeneous genetic background, thus avoiding a potential bias due to the selection of patients native of specific geographical areas.

5. Conclusions

The screening of a large population of patients has shown that rare mutations in the genes coding for HCN ion channels predispose to the development of epilepsy in humans. Furthermore, besides these mutations, several genetic VUS involving both the HCN and their accessory proteins have been identified, although their potential contribution to the disease remains to be clarified. Overall, these results suggest to include the analysis of the genes coding for the HCN ion channels in the diagnostic process, in order to allow in the future a better understanding of the role played by their dysfunction in the pathogenesis of epilepsy.

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