Pathogenic variants in KCNQ2 and KCNQ3, paralogous genes encoding Kv7.2 and Kv7.3 voltage-gated K⁺ channel subunits, are responsible for early—onset developmental/epileptic disorders characterized by heterogeneous clinical phenotypes ranging from benign familial neonatal epilepsy (BFNE) to early—onset developmental and epileptic encephalopathy (DEE). KCNQ2 variants account for the majority of pedigrees with BFNE and KCNQ3 variants are responsible for a much smaller subgroup, but the reasons for this imbalance remain unclear. Analysis of additional pedigrees is needed to further clarify the nature of this genetic heterogeneity and to improve prediction of pathogenicity for novel variants. We identified a BFNE family with two siblings and a parent affected. Exome sequencing on samples from both parents and siblings revealed a novel KCNQ3 variant (c.719T>G; p.M240R), segregating in the three affected individuals. The M240 residue is conserved among human Kv7.2-5 and lies between the two arginines (R5 and R6) closest to the intracellular side of the voltage-sensing S4 transmembrane segment. Whole cell patch-clamp recordings in Chinese hamster ovary (CHO) cells revealed that homomeric Kv7.3 M240R channels were not functional, whereas heteromeric channels incorporating Kv7.3 M240R mutant subunits with Kv7.2 displayed a depolarizing shift of about 10 mV in activation gating. Molecular modeling results suggested that the M240R substitution preferentially stabilized the resting state and possibly destabilized the activated state of the Kv7.3 subunits, a result consistent with functional data. Exposure to β-hydroxybutyrate (BHB), a ketone body generated during the ketogenic diet (KD), reversed channel dysfunction induced...
by the M240R variant. In conclusion, we describe the first missense loss-of-function (LoF) pathogenic variant within the S4 segment of Kv7.3 identified in patients with BFNE. Studied under conditions mimicking heterozygosity, the M240R variant mainly affects the voltage sensitivity, in contrast to previously analyzed BFNE Kv7.3 variants that reduce current density. Our pharmacological results provide a rationale for the use of KD in patients carrying LoF variants in Kv7.2 or Kv7.3 subunits.

Keywords: KCNQ, BFNE, encephalopathy, channelopathies, ketogenic diet

INTRODUCTION

Voltage-gated potassium (K⁺) channels (Kv channels) regulate the resting membrane potential and set the threshold and duration of the action potential in excitable cells. Among these, Kv7.2 and Kv7.3 voltage-gated K⁺ subunits, encoded by the KCNQ2 and KCNQ3 genes, are expressed in the central and peripheral nervous system (Brown and Adams, 1980; Wang et al., 1998). These subunits form homo- and heterotetrameric channels underlying the M-current (I_KM), a non-inactivating K⁺ current with slow activation and deactivation kinetics that activates at the threshold potential of about −60/−50 mV, thus regulating the resting membrane potential and suppressing repetitive neuronal firing (Brown and Adams, 1980).

Pathogenic variants in KCNQ2 cause early-onset epilepsies with wide phenotypic heterogeneity (Allen et al., 2014; Miceli et al., 2018). Indeed, some variants have been identified in patients with benign familial neonatal epilepsy (BFNE), an autosomal-dominant epilepsy with seizures affecting otherwise healthy infants in the first days of life and spontaneously disappearing over the next several months, with mostly normal neurocognitive development (Jentsch, 2000; Singh et al., 2003; Soldovieri et al., 2006). At the severe end of the KCNQ2 spectrum is an early-onset developmental and epileptic encephalopathy (DEE) characterized by recurrent seizures starting in the neonatal period and neurodevelopmental disability (Weckhuysen et al., 2012). While more than 300 pathogenic variants have been described in KCNQ2, few variants in KCNQ3 have been detected, mostly in families with BFNE. In addition, de novo variants in KCNQ3 have been rarely described in children with DEE (Allen et al., 2013; Grozeva et al., 2015; Miceli et al., 2015; Ambrosino et al., 2018; Lauritano et al., 2019), intellectual disability (ID) apparently without epilepsy (Rauch et al., 2012; Deciphering Developmental Disorders, 2017), cortical visual impairment (Bosch et al., 2016), and in patients with ID and autism (Sand et al., 2019).

In most children affected with KCNQ2- or KCNQ3-related BFNE, seizures are controlled with conventional antiepileptic drugs, including sodium channel blockers (Sand et al., 2016). Instead, few options are available for patients with the most severe forms of KCNQ2- or KCNQ3-related disorders; in addition to sodium-channel blockers such as carbamazepine and phenytoin which appear to be highly effective (Pisano et al., 2015), ezogabine, a selective Kv7 channel activator, has been shown to improve seizure control and development in patients with Kv7.2 loss-of-function (LoF) variants (Millichap et al., 2016). Unfortunately, because of its unfavorable risk/benefit ratio, ezogabine has been withdrawn from the market. Among non-pharmacological therapies, ketogenic diet (KD) has been recently shown to be particularly effective in children with DEE caused by KCNQ2 variants (Ko et al., 2018), but the mechanisms of action are not completely understood and there are no data on the effects on KCNQ3 related disorders. KD is a low carbohydrate, high-fat, adequate-protein diet regimen that shifts the primary fuel source for neuronal activity from glucose to endogenous ketones: acetone, acetoacetate, and β-hydroxybutyrate (BHB). KD likely improves seizure control through a variety of mechanisms such as inhibition of the glycolysis, disruption of glutamatergic synaptic transmission, and activation of ATP-sensitive potassium channels (Lutas and Yellen, 2013). Recently, BHB has been shown to directly and specifically activate Kv7 channels containing Kv7.3 subunits by increasing current sensitivity to voltage (Manville et al., 2018, 2020).

In the present manuscript, we report the clinical, molecular and functional properties of a BFNE family carrying a novel KCNQ3 variant (c.719T>G; p.M240R) segregating with epilepsy in the three affected individuals. Whole cell patch-clamp recordings in Chinese hamster ovary (CHO) cells revealed that homomeric Kv7.3 M240R channels were not functional, whereas heteromeric channels incorporating Kv7.3 M240R mutant subunits with Kv7.2 and Kv7.3 displayed a depolarizing shift of about 10 mV in activation gating, consistent with a LoF in vitro effect. Consistent with these functional data, molecular modeling suggested that the M240R substitution preferentially stabilized the resting state and possibly destabilize the activated state of the Kv7.3 subunits. Finally, we demonstrate that BHB was able to reverse the functional alterations observed in heteromeric channels carrying Kv7.3 M240R subunits.

MATERIALS AND METHODS

Patients

The BFNE family was referred for an epilepsy genetics research study by their treating clinician and written informed consent was obtained. Exome sequencing was performed in both parents and each sibling, and the presence of c.719T>G (p.M240R) in affected members was confirmed by Sanger sequencing in each sibling, and the presence of c.719T>G (p.M240R) in affected members was confirmed by Sanger sequencing in all members of the family. The study was approved by the human research ethics committee of Columbia University Irving Medical Center.
Mutagenesis and Heterologous Expression of KCNQ2 and KCNQ3 cDNAs

Mutations were engineered in KCNQ3 human cDNA cloned into pcDNA3.1 by QuikChange site-directed mutagenesis (Agilent Technologies Italia SpA, Milan, Italy), as previously described (Miceli et al., 2013). Channel subunits were expressed in CHO cells by transient transfection. CHO cells were grown in 100 mm plastic Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS), L-glutamine (0.1 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere at 37°C with 5% CO2. For electrophysiological experiments, cells were seeded on glass coverslips (Carolina Biological Supply Company, Burlington, NC, United States) and transfected on the next day with the appropriate cDNAs using Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer’s protocol. A plasmid encoding for enhanced green fluorescent protein (Clontech laboratories, Inc., Palo Alto, CA, United States) was used as transfection marker; total cDNA in the transfection mixture was kept constant at 4 μg.

Whole-Cell Electrophysiology

Currents from CHO cells were recorded at room temperature (20–22°C) 1–2 days after transfection, using a commercially available amplifier (Axopatch 200A, Molecular Devices, Union City, CA, United States) and the whole-cell configuration of the patch-clamp technique, with glass micropipettes of 3–5 MΩ resistance. The extracellular solution contained (in millimolar): 138 NaCl, 2 CaCl2, 5.4 KCl, 1 MgCl2, 10 glucose, and 10 HEPES, pH 7.4 with NaOH. The pipette (intracellular) solution contained (in millimolar): 140 KCl, 2 MgCl2, 10 EGTA, 10 HEPES, 5 Mg-ATP, pH 7.3–7.4 with KOH. The pCLAMP software (version 10.0.2) was used for data acquisition and analysis. Current densities (expressed in picoamperes per picofarad) were calculated as peak K+ currents divided by C. Data were acquired at 0.5–2 kHz and filtered at 1–5 kHz with the four-pole lowpass Bessel filter of the amplifier. No corrections were made for liquid junction potentials. To generate conductance-voltage curves, the cells were held at −80 mV, then depolarized for 1.5 s from −80 mV to +20/+80 mV in 10 mV increments, followed by an isopotential pulse at 0 mV of 300 ms duration; the current values recorded at the beginning of the 0 mV pulse were measured, normalized, and expressed as a function of the preceding voltages. Data were fit to a Boltzmann distribution of the following form \[ y = \max \left[ 1 + \exp \left( V_{1/2} - V \right) / k \right] \], where \( V \) is the test potential, \( V_{1/2} \) the half-activation potential, and \( k \) the slope factor.

For current-activation kinetics analysis, the current traces recorded in response to incremental voltage steps were fitted to a double-exponential function and then a single time constant, representing the weighted average of the slow and fast components, was obtained by using the following equation:

\[
\tau = (\tau_f A_f + \tau_s A_s) / (A_f + A_s),
\]

where \( \tau_f \) and \( \tau_s \) indicate the amplitude of the fast and slow exponential components, \( \tau_f \) and \( \tau_s \) the time constants of each component (Miceli et al., 2013).

Structural Modeling

Three-dimensional models of K7.3 subunits in different gating states were generated by using as templates the coordinates of resting and activated states of the K1.2/2.1 chimera (Long et al., 2007; PDB accession number 2R9R; 26% of sequence identity with K7.3) subjected to long (>200 μs) molecular dynamic simulations (Jensen et al., 2012). Modeling of the S1–S4 VSD in each state was performed with SWISS-MODEL, as described (Sands et al., 2019). The models were optimized through all-atom energy minimization by using the GROMOS96 implementation of Swiss-PDBViewer, and analyzed using both the DeepView module of Swiss-PDBViewer (version 4.0.1)1 and PyMOL.2

Statistics

Data are expressed as the mean ± SEM. Statistically significant differences between the data were evaluated with the Student’s t-test (\( p < 0.05 \)).

RESULTS

Clinical and Genetic Features of the BFNE Pedigree With a Novel KCNQ3 Variant

Case II-1

A term male neonate was hospitalized for seizures starting in the first week of life. The seizures lasted less than 1 min, recurring every 2 h. Electroencephalography (EEG) recorded variable lateralization of ictal onset. Clinically, there was bilateral arm stiffening, rightward head version, and right leg stiffening, lasting 45 s. Workup for acute etiologies, including an MRI of the brain, was unrevealing. The child was treated with phenobarbital, but continued to have seizures after hospital discharge. He was cross-titrated onto levetiracetam from 3 weeks of age and seizures stopped around that time. Levetiracetam was weaned at 12 months. At 18 months he was diagnosed with isolated language delay (one standard deviation below expected) and started speech therapy. There was no concern for autism.

Case II-2

Nineteen months later, a female sibling was born. She began having episodes on the 5th day of life, characterized by limb stiffening and eye rolling. EEG confirmed seizures. Workup included a normal MRI of the brain. She was treated with phenobarbital and levetiracetam, which were weaned off at 4 and 6 months, respectively. She had recurrence with two back-to-back seizures at 22 months, and she was started on oxcarbazepine with seizure freedom. Follow-up EEGs have been normal. Developmental milestones have all been met on time as of 26 months.

Family history was notable for an isolated seizure in the father (case I–1) during infancy. The father’s interictal EEG was normal, the seizure did not recur, and he was not treated with anti-seizure

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1http://spdbv.vital-it.ch/
2http://www.pymol.org/
medication. No seizure was ever described in the mother (case I-2; Figure 1A).

Exome sequencing, performed in all four family members, demonstrated a novel missense variant in KCNQ3, c.719T > G (p.M240R), in the two siblings and the affected father (Figure 1A), thus segregating with the phenotype. The variant is absent from gnomAD and predicted to be deleterious (polyphen-2, 0.989; SIFT, 0.9122; CADD score 27.3). The M240 residue lies between the R5 (R239) and R6 (R242) positions of the voltage-sensing S4 transmembrane segment within the voltage-sensing domain (VSD; Figure 1B). This non-polar residue is conserved among human Kv7.2-5 subunits, but not in Kv7.1 and other Kv channels such as Kv1.1, Kv1.2, and Kv2.1, although residues with similar physicochemical properties are present at this position (Figure 1C).

**Functional and Pharmacological Properties of Homomeric and Heteromeric Channels Carrying Kv7.3 M240R Subunits**

Heterologous expression of wild-type Kv7.3 subunits led to the appearance of voltage-dependent K+ –selective currents characterized by a rather slow time-course of activation and deactivation and a threshold for current activation of around −50 mV; the macroscopic K+ currents density at +20 mV was 12.6 ± 1.1 pA/pF, similarly to previously reported values (Wang et al., 1998; Sands et al., 2019). By contrast, no measurable currents were recorded in cells expressing Kv7.3 M240R subunits, consistent with the variant causing a complete LoF effect (Figure 1D and Table 1).

Kv7.3 subunits assemble with Kv7.2 subunits to form $I_{KM}$ (Wang et al., 1998). Co-expression of Kv7.2 and Kv7.3 subunits generated currents which were considerably larger than those recorded upon expression as Kv7.2 or Kv7.3 subunits alone; in addition, currents from Kv7.2 + Kv7.3 heteromeric channels displayed an increased sensitivity to blockade by tetroethylammonium (TEA) when compared to homomeric Kv7.3 channels (Table 1). When compared to currents from wild-type Kv7.2 + Kv7.3 subunits, co-expression of Kv7.3 M240R with Kv7.2 subunits caused a marked rightward shift (by about +40 mV) of the activation gating, a significant decrease of slope of the G/V curve (see section Materials and Methods for details), a reduced current density by about 50% at depolarized potential, with no change in sensitivity to TEA blockade (Figures 2A–C and Table 1). The marked rightward shift of the G/V curves of the heteromeric Kv7.2 + Kv7.3 M240R channels markedly reduced current density at the physiologically
relevant potentials of −50/−40 mV (Figure 2C). The activation kinetics of Kv7.2 + Kv7.3-M240R currents were slower than those of Kv7.2 + Kv7.3, particularly at more depolarized potentials. In fact, $t_{\text{activation}}$ at +20 mV were 142 ± 19 ms and 274 ± 71 ms, for Kv7.2 + Kv7.3 and Kv7.2 + Kv7.3-M240R, respectively. In order to assess the influence of the depolarizing pulse length on the current steady-state properties from Kv7.2 + Kv7.3-M240R-expressing channels, additional experiments in which the pulse length was increased from 1.5 to 3 s were performed. The results obtained revealed no significant difference in the $V_{1/2}$ and $k$ values on Kv7.2 + Kv7.3-M240R current using the two protocols; indeed, the $V_{1/2}$ and the $k$ values were, respectively, +12.2 ± 7.6 and +10.2 ± 5.4 mV (n = 4; p > 0.05), and 23.0 ± 3.6 and 23.5 ± 1.4 mV/e-fold (n = 4; p > 0.05) for Kv7.2 + Kv7.3- and Kv7.2 + Kv7.3-M240R-expressing cells.

Altogether these data suggest that Kv7.3 M240R subunits are able to form heteromeric channels with Kv7.2 subunits, although their currents display gating properties very different from Kv7.2 + Kv7.3 heteromeric channels. To replicate in vitro the genetic combination occurring in the affected family members who are heterozygous for the pathogenic allele (Individuals I-1, I-2, and I-1), functional studies were also carried out upon transfection of cDNAs for Kv7.2 + Kv7.3 + Kv7.3 M240R at a cDNA ratio of 1:0.5:0.5. The current density measured in CHO cells expressing heteromeric channels formed by the described subunit combinations was very similar to that recorded in cells expressing Kv7.2 + Kv7.3 + Kv7.3 currents at a transfection ratio 1:1 (mimicking a healthy individual) (Figures 2A,B and Table 1) and no difference in the activation kinetics was observed between the Kv7.2 + Kv7.3 and Kv7.2 + Kv7.3 + Kv7.3 M240R currents; indeed the $t_{\text{activation}}$ at +20 mV were 142 ± 19 ms and 128 ± 28 ms, respectively. By contrast, the midpoint of activation of the currents recorded upon Kv7.2 + Kv7.3 + Kv7.3 M240R subunit co-expression was right-shifted by about 10 mV when compared to Kv7.2 + Kv7.3 (Figures 2A–C and Table 1), thus confirming LoF effects of the mutant subunits also when incorporated into channel tetramers with Kv7.2 + Kv7.3 subunits.

### Structural Basis for the LoF Effect by the Kv7.3 M240R Substitution

The herein identified M240R variant introduces an extra positively charged residue into the S4 segment of Kv7.3 subunits, between R5 and R6. To achieve a better understanding of the possible role of the M240 residue in the gating process and of the structural consequences of its replacement with an R, we built homology models of a Kv7.3 subunit in both resting and activated states, as previously described (Jensen et al., 2012; Sands et al., 2019). Our structural models suggested that, no electrostatic interaction between the M240 residue side chain and surrounding protein residues occurs in both the resting and the activated VSD configurations (Figure 3A); in particular, in the resting VSD state and in two of the four subunits, the M240 side chain points toward the N-terminal region of the same subunits, whereas in the other two subunits it points in the opposite direction, namely toward the S3 segment (Figure 3B). Substitution of the non-polar M residue at position 240 with an R introduces a novel electrostatic interaction between R240 side chain and a highly conserved negatively charged N-terminal glutamate (E116) in one Kv7.3 subunit. This interaction only occurred in the resting state of the VSD (Long et al., 2007) (Figure 3C). Instead, VSD movement during the activation process translated the R240 side chain in a tight pocket surrounded by non-polar residues, possibly destabilizing the tight network of hydrophobic interactions within this pocket. As a result, our model suggests that the M240R substitution may preferentially stabilize the closed state and possibly destabilize the activated state of the Kv7.3 subunits.

### Pharmacological Effects of BHB Exposure on Heteromeric Channels Incorporating the Kv7.3 Epilepsy-Causing Variant

The results described suggest that heteromeric channels incorporating Kv7.3 M240R subunits display a reduced sensitivity to voltage, strongly suggesting a LoF pathogenic mechanism. Given that BHB has been recently shown to potentiate Kv7.3 and Kv7.2 + Kv7.3 currents by a mechanism opposite to that introduced by the M240R variant (Manville et al., 2018, 2020), we further evaluated its ability to counteract in vitro the described functional alteration. We tested the effects of the BHB using a voltage protocol in which Kv7.2 + Kv7.3 and Kv7.2 + Kv7.3 + Kv7.3 M240R currents were activated by 3 s voltage ramps from −80 to +20 mV. Perfusion with 100 μM BHB enhanced ramp-evoked Kv7.2 + Kv7.3 and Kv7.2 + Kv7.3 + Kv7.3 M240R currents; this effect was reversible since the currents returned to basal values after about 10 s upon BHB removal from the bath (Figure 4A). In addition,
steady-state experiments revealed that at $-40$ mV, a membrane potential value close to the activation threshold, 100 µM BHB increased $Kv7.2 + Kv7.3$ and $Kv7.2 + Kv7.3 + Kv7.3$ M240R currents (Figure 4B) and also caused a 10 mV negative shift in the $G/V$ curve (Figure 4C). Interestingly, the $V_{1/2}$ value of $Kv7.2 + Kv7.3 + M240R$ currents upon BHB exposure was similar to that of $Kv7.2 + V_{1/2}$-expressing cells (Figure 4C and Table 1), suggesting the ability of the BHB to restore $Kv7.2 + Kv7.3 + M240R$ currents to that of wild-type.

**DISCUSSION**

Inherited variants in KCNQ2 or KCNQ3 cause BFNE, a neonatal-onset familial epilepsy, characterized by recurrent focal tonic seizures in otherwise well infants (Sands et al., 2016). While seizure onset is most often in the first days of life, seizures can present later in infancy (Zara et al., 2013), as demonstrated by the father in our pedigree, or not at all, as penetrance is incomplete. Seizures tend to remit over the first year, but $\sim 30\%$ of individuals have seizures later in life (Grinton et al., 2015), as illustrated by case II-2.

Most BFNE families carry pathogenic variants in KCNQ2, whereas only a small percentage carry KCNQ3 variants (Grinton et al., 2015; Sands et al., 2016; Miceli et al., 2017, 2018). KCNQ2 variants responsible for BFNE are missense, stop-gain, frameshift, splice variants, and deletions randomly distributed along the entire primary sequence of the subunit. In contrast, BFNE-causing KCNQ3 variants are all missense, affecting specific residues located in the pore region of the channel ($S_5$–$S_6$ and intervening loop; Table 2). Prior studies have reported 13 such variants, each affecting a different $Kv7.3$ residue located in and around the pore (Table 2); in addition, four $Kv7.3$ variants affecting residues in the long C-terminus have also been described, although the pathogenic role of these variants appears questionable due to their ample representation in the gnomAD population database without the benefit of supportive functional data (N821S, Bassi et al., 2005; R780C, Zara et al., 2013), or with
functional data that fail to support a disease association (N468S, Singh et al., 2003; P574S, Miceli et al., 2009). Out of the 13 KCNQ3 variants causing BFNE, those nine with supportive functional data lie within a span of 51 residues (V279 to R330) in this region. Functional analyses of these variants to date, e.g., V279F, I317T, R330C, and R330L, have mostly demonstrated a reduction in maximal current with no effect on the voltage-dependence of activation (Soldovieri et al., 2014; Miceli et al., 2015; Maljevic et al., 2016), consistent with these variants being in the pore. Only W309R induced both a reduction in currents (by about 60%) and a rather small (3–4 mV) rightward shift in the $V_{1/2}$ value, when expressed together with Kv7.2 and Kv7.3 (Uehara et al., 2008).

In the present work, we report a BFNE family carrying the first variant in KCNQ3 located in the S4 helix of the VSD (M240R). Functional studies revealed that the M240R variant abolishes channel function in homomeric configuration, but that this is partially rescued in heteromeric channels with wild-type Kv7.2 and/or Kv7.2/3 subunits. Differently from all other Kv7.3 BFNE variants characterized to date, M240R subunits, when expressed with Kv7.2 and Kv7.3 subunits, show a significant decrease of channel sensitivity to voltage of about 10 mV, without major changes in pore properties and heteromerization. These functional results suggest that the introduction of an additional positively charged residue at the bottom of S4 in Kv7.3 subunits destabilizes the activated conformation of the voltage sensor, thereby impeding pore opening. Structural modeling provides a potential explanation for such a conclusion; indeed, introduction of an R at position 240 stabilizes the VSD resting state by forming a novel strong ionic interaction with an highly conserved residue in the N-terminus; in addition, the larger R side chain may destabilize the tight network of hydrophobic interaction occurring in the activated state. Further studies using residue swapping or charge reversion are needed to confirm the potential interaction between the R240 and E116 residues. The available data do not allow us to determine whether the G/V shift introduced by the mutation originates from the participation of the 240 residue in voltage-sensing or in the subsequent steps along the activation pathway leading to pore opening. However, the fact that recent structural data from human Kv7.1 (Sun and MacKinnon, 2020), reveal that the V241 residue, corresponding to the M240 residue in Kv7.3, is located within a key region for VSD-pore electro-mechanical coupling (Hou et al., 2020), raises the possibility that this residue may play a similar functional role also in Kv7.3. Therefore, a LoF mechanism appears mainly responsible for BFNE pathogenesis in our family. Similar conclusions have been reached for BFNE-causing variants in Kv7.2, where haploinsufficiency, corresponding to an $I_{KM}$ reduction of only $\sim$25%, appears responsible for disease pathogenesis (Jentsch, 2000).
Benign familial neonatal epilepsy with normal neurocognitive development is not the only phenotype associated with variants in Kv7.3. In fact, individuals with mild/moderate ID have been described in BFNE pedigrees (I317T, R330L; Soldovieri et al., 2014; Miceli et al., 2015); moreover, de novo variants in KCNQ3 have been described in children with DEE (Allen et al., 2013; Grozeva et al., 2015; Miceli et al., 2015; Ambrosino et al., 2018; Lauritano et al., 2019), ID apparently without epilepsy (Rauch et al., 2012; Deciphering Developmental Disorders, 2017), cortical visual impairment (Bosch et al., 2016), and in patients with ID and autism (Sands et al., 2019). Such phenotypic heterogeneity is at least in part correlated with variant-specific functional effects, by yet unknown mechanisms; in fact, opposite to BFNE variants causing LoF, gain-of-function (GoF) variants cause non-verbal ID, autism, and prominent sleep-activated multifocal epileptiform EEG discharges without neonatal seizures (Singh et al., 2003; Sands et al., 2019).

The observation that epilepsy-associated variants in KCNQ2 are more than 10 times more frequent than those in KCNQ3, suggests that KCNQ3 tolerates variation better than KCNQ2. Several pieces of evidence support this view; as an example, while heterozygous frameshift variants in KCNQ2 are frequent causes of BFNE (Miceli et al., 2018), no heterozygous pathogenic frameshift KCNQ3 variant has ever been associated with a human phenotype. In addition, no individual carrying KCNQ2 frameshift variants in homozygosity has ever been described, as minimal KCNQ2 residual activity is probably essential for survival (Lauritano et al., 2019); by contrast, two recent studies reported the occurrence of homozygous frameshift variants in KCNQ3 (each inherited from an asymptomatic parent) in patients...
TABLE 2 | Missense variants reported for KCNQ3.

| Residue | Variant | Region | Phenotype | Effect | gnomAD | References |
|---------|---------|--------|-----------|--------|--------|------------|
| R227    | R227Q   | S4 (R1) | ID/ASD    | GoF    | 0      | Sands et al., 2019 |
| R230    | R230C/H/S | S4 (R2) | ID/ASD    | GoF    | 1 (3.98E−06)* | Sands et al., 2019 |
| M240    | M240R   | S5     | BFNE      | LoF    | 0      | Current work |
| Y266    | Y266C   | S5     | BFNE      | LoF    | 0      | Symonds et al., 2019 |
| V279    | V279F   | Pore loop | BFNE    | LoF    | 0      | Maljevic et al., 2016 |
| E299    | E299K   | Pore loop | BFNE    | LoF    | 0      | Neubauer et al., 2008 |
| D305    | D305G   | Pore loop | BFNE    | LoF    | 0      | Singh et al., 2003 |
| W308    | W308S   | Pore loop | BFNE    | LoF    | 0      | Sands et al., 2016 |
| W309    | W309R   | Pore loop | BFNE    | LoF    | 1 (3.98E−06) | Hirose et al., 2000; Uehara et al., 2008 |
| G310    | G310V   | Pore loop | BFNE    | LoF    | 0      | Charlier et al., 1998; Schroeder et al., 1998 |
| I317    | I317T   | Pore loop | BFNE*   | LoF    | 0      | Soldovieri et al., 2014 |
| R330    | R330L   | Pore loop | BFNE*   | LoF    | 0      | Miceli et al., 2015 |
| R330C   | R330C   | Pore loop | BFNE    | LoF    | 0      | Li et al., 2008; Fister et al., 2013; Miceli et al., 2015 |
| G340    | G340V   | S4     | BFNE      | LoF    | 0      | Allen et al., 2014 |
| R364    | R364H   | Prox C-term | BFIE   | 0      | Qintron et al., 2015 |
| N468    | N468S   | C-terminus | BFIE   | No effect | 1 (3.18E−05) | Fusco et al., 2015 |
| P574    | P574S   | C-terminus | BFIE    | No effect | 39 (1.38E−04) | Singh et al., 2003 |
| R780    | R780C   | C-terminus | BFIE    | No effect | 586 (2.07E−03) | Miceli et al., 2009 |
| N821    | N821S   | C-terminus | BFNE    | 0      | 26 (9.20E−05) | Zara et al., 2013 |
|        |         |         |           |        | 60 (2.12E−04) | Bassi et al., 2005 |

Table gives number of observations of each variant (frequency) in gnomAD, associated phenotype, region within Kv7.3, and functional consequences in vitro if known; blue, intellectual disability (ID) with or without autism spectrum disorder (ASD); purple, benign familial neonatal epilepsy (BFNE) or benign infantile epilepsy (BFIE) phenotypes; gray, variants of questionable pathogenicity; LoF, loss-of-function; GoF, gain-of-function; * individuals with mild/moderate ID reported; ˆ mosaic individual.

with developmental delay and neonatal seizures (Kothur et al., 2018; Lauritano et al., 2019), demonstrating that, homozygous variants in KCNQ3 are compatible with life. Moreover, a distinct functional role of these two genes emerging from developmental and genetic studies in humans, appears also to be recapitulated in mice. In fact, while KCNQ2 homozygous KO mice died at birth, homozygous KCNQ3 KO mice showed no seizures and survived until adulthood (Tzingounis and Nicoll, 2008; Kim et al., 2016); similarly, conditional deletion of KCNQ2 in cortical pyramidal neurons increased neuronal excitability and decreased lifespan, whereas deletion of KCNQ3 neither increased pyramidal neurons excitability nor affected mice survival (Soh et al., 2014).

Notably, in heteromeric channels, the functional effects of the M240R variant in Kv7.3 herein described in a BFNE family are quantitatively and qualitatively very similar to those triggered by the R213Q variant in Kv7.2, the latter identified in sporadic patients with DEE (Weckhuysen et al., 2012; Millichap et al., 2016). The fact that similar, dramatic in vitro functional consequences are associated to a severe phenotype in Kv7.2 and to self-limiting epilepsy in Kv7.3, further supports the hypothesis that Kv7.3 is more tolerant than Kv7.2 to genetic changes causing LoF effects. Among many others, such as inclusion/exclusion of both genes in panels for DEE, diagnostic NGS coverage, and epistatic compensation, an attractive explanation for such distinct functional role lies in the different developmental pattern of expression, with KCNQ2 being expressed at earlier stages of development when compared to KCNQ3, in both mice and humans (Tinel et al., 1998; Kanaumi et al., 2008).

In patients with KCNQ2- and KCNQ3-related disorders, seizures are controlled with sodium channel blockers in most patients (Pisano et al., 2015; Sands et al., 2016). However, a percentage continues to have seizures, which may contribute to cognitive impairment. In addition, successful management of seizures does not address the neurodevelopmental disability that occurs in KCNQ2 and KCNQ3 DEEs. Efficacy of the KD, with greater than 90% seizure reduction, has been reported in patients with monogenic DEEs, including KCNQ2-DEE (Ko et al., 2018) and Dravet syndrome (Knupp and Wirrell, 2018). Our finding that the endogenous ketone BHB can activate heteromeric Kv7.2 + Kv7.3 channels containing Kv7.3 M240R subunits to the same extent as wild-type Kv7.2 + Kv7.3 channels (Manville et al., 2018, 2020), thereby counteracting the underlying pathophysiology, suggests that the KD could represent precision medicine for more severe phenotypes caused by KCNQ2 and KCNQ3 LoF variants. Further work is required to identify additional patients carrying KCNQ3 or KCNQ2 pathogenic LoF variants retaining responsiveness to BHB in vitro and who may benefit from KD treatment in vivo.

CONCLUSION

In conclusion, we describe the first missense LoF pathogenic variant located in the S4 segment of KCNQ3 as a cause of BFNE. In contrast to BFNE-KCNQ3 variants previously described,
functional and modeling data suggest that the M240R variant primarily affects the voltage sensitivity of heteromeric channels. Our study provides a pharmacological rationale for investigating the use of KD in patients with DEE caused by KCNQ3 and KCNQ2 LoF variants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Columbia University Irving Medical Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FM, MT, and TS conceived the study, analyzed the data, and wrote the manuscript. EC, MC, and DG analyzed the data and wrote the manuscript. FM, LC, VB, MS, EH, AM, NL, and LB performed the research and analyzed the data. All authors contributed to the article and approved the submitted version.

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Funding

The present work was supported by the Telethon Foundation (GGP15113) and by the Italian Ministry for University and Research (PRIN 2017ALCR7C) to MT, the Italian Ministry for University and Research (Project Scientific Independence of Researchers 2014 BBSI1444EM and PRIN 2017YH3SXK) to FM, the Italian Ministry of Health Research Finalizzata Giovani Ricercatori 2016 (project GR-2016-0236337), the Italian Ministry for University and Research (PRIN 2017ALCR7C), and Research Funds from University of Molise to MS. This publication was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Number UL1TR001873. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Acknowledgments

We thank Thomas J. Jentsch, Department of Physiology and Pathology of Ion Transport, Leibniz-Institut für Molekulare Pharmakologie, Berlin for sharing Kv7.2 and Kv7.3 cDNAs and David E. Shaw, D. E. Shaw Research, New York, for the coordinates of the Kv1.2/2.1 chimera.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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