Original Research

Transcriptomic profile of epileptic children treated with ketogenic therapies

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DOI:10.31083/j.jin2101031

Submitted: 5 August 2021 Revised: 6 September 2021 Accepted: 22 September 2021 Published: 28 January 2022

Background: Ketogenic dietary therapies (KDT) are used as a treatment in childhood epilepsy. However, their mechanism has not yet been established. The main objective of this study was to determine the changes in the transcriptomic profile induced by KDT in children with epilepsy in order to shed light on its possible mechanisms. *Methods*: Eight children with refractory epilepsy were enrolled in the study. Peripheral blood mononuclear cells were obtained before and after the children were treated with KDT for a minimum of 6 months. RNA was extracted and mRNA and miRNA profiling were performed and analyzed. *Results*: Our intervention with KDT significantly reduced the seizure number in seven of the eight paediatric patients treated and caused important changes in their gene expression profile. Our study reveals modifications in the transcription of 4630 genes and 230 miRNAs. We found that the genes involved in the protection against epileptic crises were among those mainly changed. These genes collectively encode for ion channels, neurotransmitter receptors, and synapse structural proteins. *Conclusions*: Together our results explain the possible mechanisms of KDT and reinforce its clinical importance in the treatment of epilepsy.

Keywords

Epilepsy; Ketogenic diet; Ketogenic dietary therapies; Transcriptome; Anticonvulsant; Synapsis; miRNome

1. Introduction

The global incidence of epilepsy is about 60 per 100,000 inhabitants per year, and up to a third of patients may suffer from refractory epilepsy [1, 2]. An alternative treatment to antiseizure medications (ASM) are the ketogenic dietary therapies (KDT), which have been used as a treatment for refractory epilepsy in children since the 1920s [3]. The most commonly-used KDT are Classic KD (CKD), KD (ketogenic diet) with medium chain triglycerides (MCT, MCT-KD) and the modified Atkins diet (MAD) [4]. CKD is a high-fat diet (about 90% of the energy intake) with mostly long chain triglycerides (LCT), very low carbohydrate content and adequate amount of proteins [5]. The most commonly recommended ratios are 3:1 or 4:1, which means that for every 3 or 4 g of fat, 1 g of protein and carbohydrates is administered. The MCT-KD was proposed in 1971 by Huttenlocher and co-workers [6]. In this diet, part of the fat is supplied in the form of MCT oil. MCT are quickly metabolized, ketosis is achieved faster, and ratios 1.2:1 can be used. The MAD is less restrictive than the previous ones because, although carbohydrates are limited to 10 g/day, a free intake of proteins and lipids is allowed [7]. In clinical practice it is also common to supplement the MAD with preparations containing CKD components to increase ketosis [8]. Regardless of the type of KDT, they all seem to be neuroprotective and there is no clear evidence for different efficiency between CKD and MCT-KD [9] or MAD [10]. However, the molecular pathways involved in these antiseizure mechanisms have not yet been established.

Recent research highlights the role of ketone bodies and polyunsaturated fatty acids in the modulation of the KD-induced neuroprotection [4, 11–13]. The neuroprotective mechanisms include an increase in the cellular mitochondrial function and biogenesis [14], a decrease in oxidative stress [15], changes in potassium channels [15, 16], an increase in the brain-derived neurotrophic factor (BDNF) [17] and in the purinergic and GABAergic neurotransmission [18], attenuation of neuroinflammation, or even changes in gut microbiota [13, 19].

Epigenetic processes are related to changes in neuronal activity and epileptogenesis. Molecular mediators of epigenetics include non-coding RNAs, methylation of DNA and histone modifications [20]. Therapies for epilepsy, including KDT, may bear an influence on epigenetic processes [21].

Transcriptomic analysis allows the study of changes in gene expression in different human diseases as well as the effect of potential interventions with clinical relevance [22]. A

Journal of Integrative Neuroscience

massive analysis of gene expression allows the detection of broad coordinated trends which cannot be discerned by more targeted assays.

Our major aim was to determine the changes in gene expression induced by a 6-month treatment with KDT in children with epilepsy to shed light on the potential mechanisms of this type of diets. This is, as far as we are aware, the first transcriptomic study in epileptic children who have followed KDT.

2. Materials and methods

All methods were performed in accordance with the relevant guidelines [23] and regulations. All experimental procedures were approved by the Committee on Ethics in Clinical Research and an informed consent was signed before obtaining the samples.

2.1 Patients and intervention

Eight children with refractory epilepsy and treated with KDT were recruited for our study at the University Children's Hospital Niño Jesús of Madrid (Spain). The effect of KDT on epilepsy was quantified according to the reduction in the number of seizures comparing to the baseline state [24]. The parents quantified the number of seizures suffered by the children in the month prior to starting the diet and this was considered the number of baseline seizures. In each follow-up evaluation the parents reported the number of seizures per month that the children had suffered. The percentage of seizure reduction in each medical check-up was calculated taking into account the baseline value, as follows: 100% (seizure-freedom), 90%–100% improvement, 50%–90%, <50%, 0% (no improvement), or increase of seizures.

2.2 Sample collection

Blood (approximately 5 mL) was draw into a VACUTAINER CPT (BD, Franklin Lakes, NJ) containing sodium heparin as anticoagulant and was gently inverted five times. Blood was immediately processed according to the manufacturer's instructions by centrifugation at 3000 × g at room temperature for 15 minutes. The supernatant (plasma) was removed and the buffy coat containing mononuclear cells collected. Mononuclear cells were washed twice in PBS and frozen at –80 °C for subsequent RNA isolation.

2.3 RNA extraction

Total RNA was isolated from mononuclear cells using the Trizol reagent (TRIzol, Invitrogen, Thermo-Fisher Scientific, Carlsbad, CA, USA). RNA integrity number (RIN) was tested with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA concentration and purity were determined using a spectrophotometer (GeneQuant, GE Healthcare Biosciences, German).

2.4 Gene expression profiling

mRNA profiling was performed using Clariom D-Human Array (Life technologies, SIF: A28139434). This array comprises >542,000 transcripts constituting over 134,000 gene-level probe sets. Microarray experiments were conducted according to the manufacturer's instructions (Life technologies, SIF: A28139434). 300 ng of total RNA were labeled using Genechip WT Plus Reagent Kit. The labeling reaction was hybridized on the Human Gene Array in Hybridization Oven 645 at 45 °C for 16 hours. The sixteen arrays for each group were stained with Fluidics Station 450 using fluidics script FS450_0001, then scanned on GeneChip Scanner 3000 7G. GeneChip Command Console software supplied by Life technologies was used to perform the gene expression analysis.

2.5 Small non-coding RNAs expression profiling

Small non-coding RNA expression profiling was performed by using GeneChip miRNA 4.0 Array (Life technologies, SIF: A28139434). Microarray experiments were conducted according to the manufacturer's instructions. 300 ng of total RNA were labelled with FlashTag Biotin HSR RNA Labeling Kit (Life technologies, SIF: A28139434). The labelling reaction was hybridized on the miRNA Array in Affymetrix Hybridization Oven 645 at 48 °C for 18 h. The arrays were stained with Fluidics Station 450 using fluidics script FS450_0002, and then scanned on GeneChip Scanner 3000 7G. GeneChip Command Console software supplied by Life technologies (Thermo Fisher Scientific, OR, USA) was used to perform expression analysis. miRNA probe outliers were defined as per the manufacturer's instructions (Life technologies) and further analyzed for data summarization, normalization, and quality control by using the web-based miRNA QC Tool software (Affymetrix Inc., Germany).

2.6 Microarray data analysis

Data (.CEL files) were analyzed and statistically filtered using software Partek Genomic Suite 6.4 (Partek Inc., St. Louis, MO). Input files were normalized with the RMA algorithm for gene array on core metaprobesets. A one-way ANOVA was performed with the Partek Genomics Suite across all samples. A p value ≤ 0.05 was considered statistically significant. The imported data were analyzed by Principal Components Analysis (PCA) to determine the significant sources of variability in the data. The selected genes were analyzed by using the Pathway Studio v8 software (Ariadne software provided by Elsevier, Netherlands) to perform a Biological Enrichment Analysis, which is a computational method that determines if a defined set of genes shows statistically significance between two different biological states, in our study, before and after the treatment with KDT. This method, as described by Mootha *et al.* [25], works by ranking the complete gene set using the fold change values (omitting the corrected p-values), providing significant processes, in which most genes related share the same expression profile. The gene set enrichment analysis allows detecting those genes describing a concrete phenotype in small sample size experiments.

Table 1. Patients and KD intervention characteristics.

Patient	A	B	C	D	E	F	G	H
Gender	Male	Female	Male	Male	Female	Male	Female	Female
Age of onset of epilepsy	9 m	15 d	4 m	6 m	3 m	19 m	9 y, 11 m	3 m
Aetiology of epilepsy	Down synd.	CDKL5 mut.	RBFOX1 mut.	Severe asphyxia	SCN1A mut.	Anti-NMDA receptor encephalitis	SCN9A mut.	UK
Duration from seizure onset to initiation of the KDT	12 d	2 m	11 d	6 y, 3 m	2 y, 1 m	12 m	3 y, 4 m	2 m
Age at the beginning of the intervention	9 m, 12 d	2 m, 15 d	4 m, 11 d	6 y, 9 m	2 y, 4 m	2 y, 7 m	13 y, 3 m	5 m
Type of KDT	CDK 3:1	CDK 3:1	CDK 3:1	CDK 4:1	MAD	CDK 3:1	MAD	CDK 3:1
Efficacy in seizure reduction:								
3 months	100%	-	-	90–100%	-	<50%	-	100%
6 months	100%	50–90%	<50%	90–100%	0%	90–100%	90–100%	100%
12 months	100%	90–100%	100%	90–100%	0%	50–90%	100%	100%
24 months	100%	100%	-	-	0%	50–90%	-	100%
BHB blood levels (µmol/L)								
3 months	3.8	6	-	1.4	2.1	5.4	0.4	3.9
6 months	2.6	-	5.4	0.4	2	4.4	1.1	4.2
12 months	5	3.8	-	0.1	2.4	3.3	1.5	3.7
24 months	5.9	-	-	1.6	1.8	4.3	-	2.6
Length of the KDT	29 m	20 m	9 m	26 m	22 m	24 m	23 m	29 m

m, months; y, years; d, days; UK, unknown; synd, syndrome; mut, mutation; CDKL5, cyclin dependent kinase-like 5; RBFOX1, RNA binding fox-1; SCN1A, sodium voltage-gated channel alpha subunit 1; NMDA, N-methyl-D-aspartate; SCN9A, sodium voltage-gated channel alpha subunit 9; KDT, ketogenic dietary therapies; CKD, classic ketogenic diet; MAD, Modified Atkins Diet; BHB, β-Hydroxybutyrate.

Table 2. Biological processes regulated by the mRNA differently expressed in the patients treated with KDT.

	Biological process	*p*-value	Number of genes
1	Multicellular organismal development	5.61×10^{-9}	36
2	Synaptic transmission	3.40×10^{-7}	19
3	Protein homooligomerization	5.97×10^{-6}	11
4	Cell differentiation	5.00×10^{-5}	22
5	Response to denervation involved in regulation of muscle adaptation	5.54×10^{-5}	3
6	Potassium ion transmembrane transport	6.99×10^{-5}	8
7	Transmembrane transport	7.11×10^{-5}	20
8	Protein ubiquitination	8.62×10^{-5}	13
9	Nervous system development	1.09×10^{-4}	16
10	Small molecule metabolic process	1.12×10^{-4}	30

Top ten biological processes whose gene expression change after intervention with KDT (>6 months) in epileptic children according to PS analysis.

3. Results

Eight children with refractory epilepsy were treated with a KDT (Table 1 and **Supplementary Table 1**). The age at seizure onset was very variable and seizure type was as follows: 3 focal onset, 2 generalized tonic-clonic, 2 tonic, 1 multiple. Six children had seizures daily (1–50 seizures/day) and 2 of them had seizures monthly (1–2 seizures/month). The median number of ASM used before the onset of KDT was 4 (range 2–8) and no patient was treated with epilepsy surgery prior to KDT. The average age of the patients at the beginning of the diet was 19 months, while an average of 7 months elapsed from the onset of the seizures to the onset of the treatment.

Six patients followed a CKD and two of them a MAD. ASM were prescribed concomitantly with KDT in all cases. The nutritional routes were: nasogastric tube (one patient), gastrostomy tube (one patient) and oral feeding (six patients). The onset of KDT was carried out in hospital admission in 6 patients who started CKD, without fasting or caloric restriction, and the fat content of the diet was progressively increased in all cases. Effectiveness of KDT in seizure reduction is in Table 1. Only one patient did not reduce the number of seizures with KDT (Patient E). However, she achieved a faster recovery from the seizures and her cognitive status subjectively and significantly improved during the treatment. Four patients had early secondary effects. Throughout the

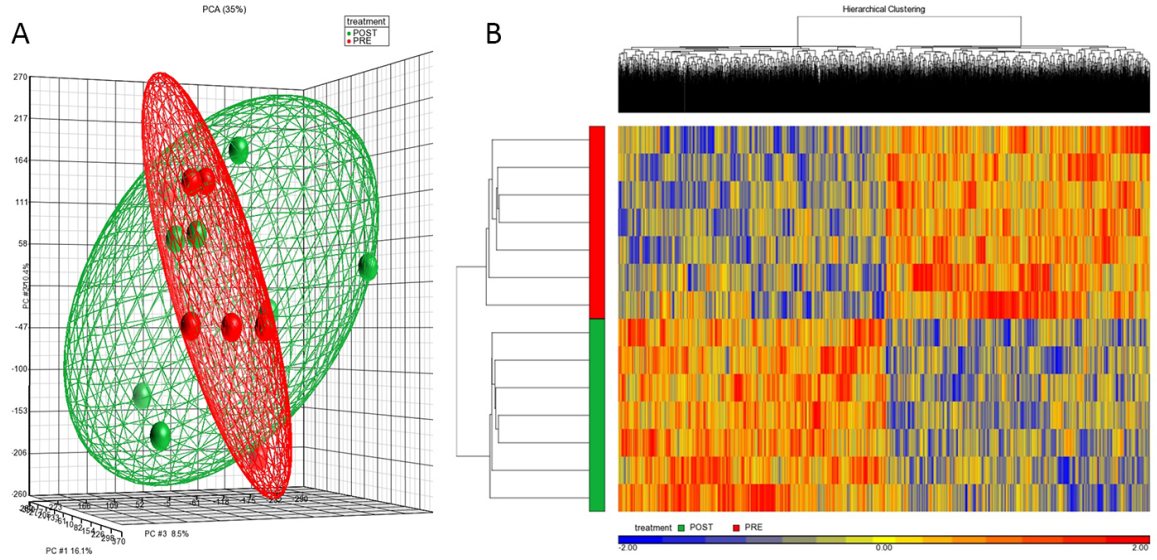

Fig. 1. Transcriptomic analysis of PBMCs before and after a KD intervention in epileptic paediatric patients. (A) Principal components analysis (PCA) displays clear spatial separation in the expression between the two groups of samples identified by unsupervised hierarchical clustering. In the 3-dimensional plot, the three principal components PC#1, #2 and #3 of all samples with over 20,000 well-annotated genes and their respective variations are expressed on the x-, y- and z-axis. The total percentage of PCA mapping variability is 35%. Each data point represents one sample. The ellipsoids highlight the portioning of the different samples. Assignment of samples by colour: pre-intervention (red) and post-intervention (green). (B) Heatmap of pre-intervention (red bar) and post-intervention (green bar) samples and 4630 genes derived from ANOVA. Each line represents a patient and each column a gene. Up-regulated genes are represented in red and down-regulated genes in blue. This shows a complete separation of transcriptomic profile pre- (red) and post- (green) intervention. Images obtained by using The Pathway Studio Software (Ariadne software, https://www.pathwaystudio.com).

follow-up, these side effects occurred in some patients, most of them were considered mild: hypercholesterolemia, hypertriglyceridemia, constipation, hypercalciuria, hypocitraturia, proteinuria, acidosis, and elevation of plasma gamma-glutamyl transferase (GGT) activity. The duration of KDT ranged from 9 to 24 months. Three patients discontinued the diet throughout the follow-up (one due to side effects and two because the diet became ineffective in the long-term).

3.1 Treatment with the KDT changes the mRNA expression profile in epileptic paediatric patients

The peripheral blood mononuclear cells (PBMCs) gene expression profile from the paediatric patients treated with the KDT for at least 6 months, significantly differed from the pre-intervention one. 4630 mRNAs differentially expressed ($p < 0.05$) were found. Three-dimensional unsupervised PCA showed two clustered groups that included the pre- and post-intervention samples (Fig. 1A). The unsupervised hierarchical clustering (heatmap) also showed significant differences in the gene expression from the two groups of samples (Fig. 1B).

According to Pathway Studio, of the 10 biological pathways that show the highest statistically significant treatment-induced change in their gene expression, we can highlight the following ones: synaptic transmission (19 genes involved, p-value 3.40×10^{-7}), potassium ion transmembrane transport (8 genes involved, p-value 6.99×10^{-5}), and nervous system development (16 genes involved, p-value 1.09×10^{-4}). Table 2 shows the top ten modulated biological processes according to Pathway Studio.

3.2 Synaptic transmission genes modified by the KDT are mainly down-regulated

Most of the genes involved in synaptic transmission are down-regulated after KDT (16 vs 3 genes). Potassium channels KCNJ12, KCNMA1, KCNK6 and KCNA10 are down-regulated, while only a Ca^{2+} channel (CACNA1C) is up-regulated in the treated patients (see Table 3). The receptors for some of the main neurotransmitters are down-regulated after KDT: orexin receptor (HCRT1 and HCRT2), melatonin receptor (MTNR1B), serotonin receptor (HTR6), glutamate receptor (GRM3), and cholinergic receptors (CHRNB3).

3.3 Different miRNA expression profile in epileptic paediatric patients before and after the treatment with KDT

A global analysis of miRNA expression (miRNome) indicates that there are 230 miRNAs which are expressed differently before and after KDT. Fig. 2 shows the heatmap of the expression profile of these mRNA regulators. If additionally the miRNAs are restricted with a fold change $> |1.3|$, 11 relevant miRNAs whose expression differs after KDT in PMBCs are found: miR-3978, miR-6726-3p, miR-130a-3p, miR-4758, miR-6745, miR-532, and miR-185-5p (down-regulated after the KDT); and miR-4538, miR-602, miR-330-5p, and miR-4673 (up-regulated after the KDT). As with mRNA analysis, the miRNA expression profile before and after KDT intervention allowed complete separation of each case (no green bars intertwined with the red ones or vice versa).

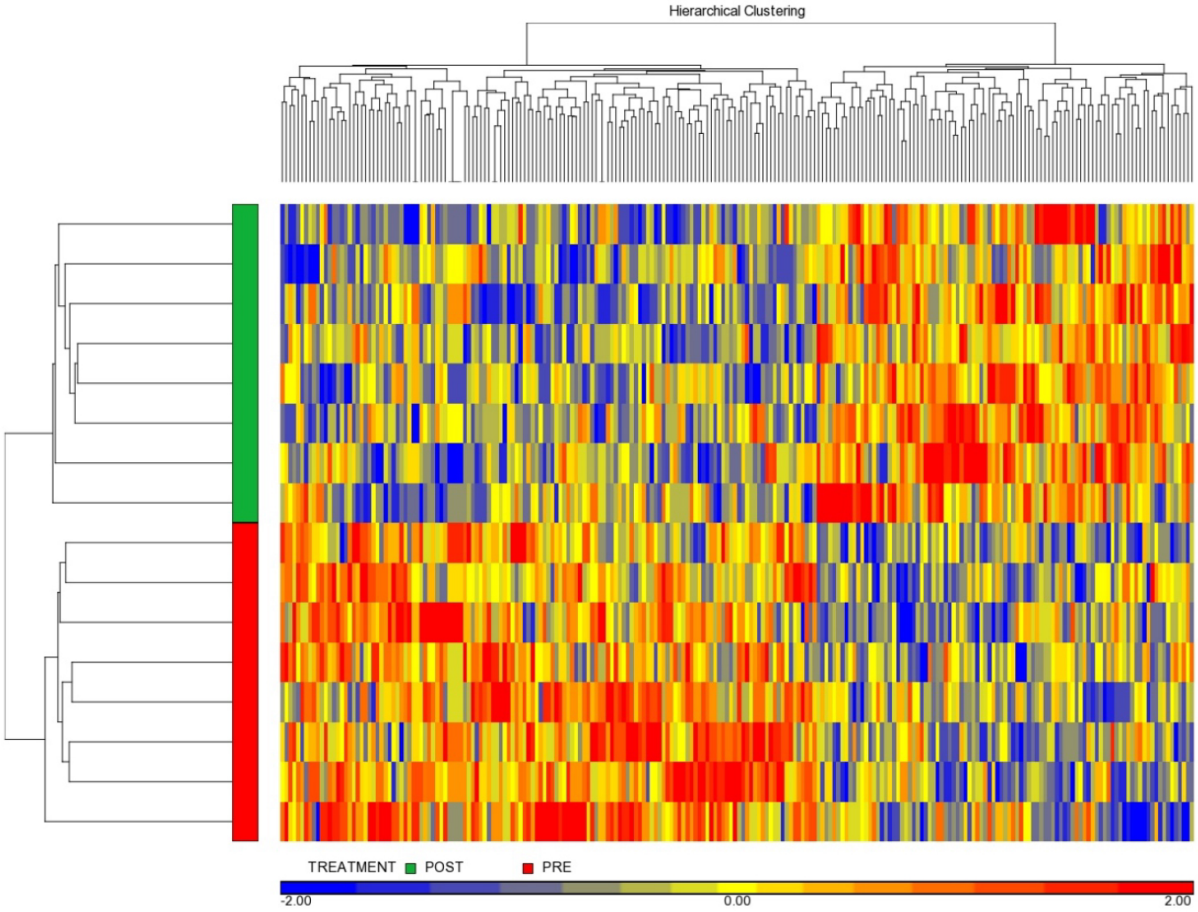

Fig. 2. Heatmap of pre-intervention (red bar) and post-intervention (green bar) samples of 230 miRNA derived from ANOVA. Each line represents a patient and each column a miRNA. Up-regulated miRNAs are represented in red and down-regulated miRNAs in blue. Image obtained by using The Pathway Studio Software (Ariadne software, Elsevier, https://www.pathwaystudio.com).

Table 3. Fold-change in the expression of genes involved in synaptic transmission after KDT.

Gene	Protein encoded	Fold change
KCNJ12	Kir2.2. ATP-sensitive inward rectifier potassium channel 12	–1.164
KCNMA1	KCa1.1. Ca^{2+}-activated potassium channel subunit alpha-1	–1.279
KCNK6	$K_{2P}6.1$. potassium channel subfamily K member 6	–1.17
SLC22A2	Solute carrier family 22 member 2	–1.175
CACNA1C	Ca^{2+} voltage-gated channel subunit alpha1 C	1.183
KCNA10	KCNA10. potassium voltage-gated channel subfamily A member 10	–1.277
MAOA	Monoamine oxidase A	–1.196
FGF12	Fibroblast Growth Factor 12	1.193
PRKCG	Protein kinase C gamma	–1.175
GNG10	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-10	–1.175
HCRTR1	Hypocretin receptor type 1	–1.168
HCRTR2	Hypocretin receptor type 2	–1.183
HTR6	5HTR6. 5-Hydroxytryptamine Receptor 6	–1.331
MTNR1B	Melatonin Receptor 1B	–1.193
GRM3	Glutamate Metabotropic Receptor 3	–1.175
DOC2A	Double C2-like domain-containing protein alpha	1.21
CHRNB3	Neuronal acetylcholine receptor subunit beta-3	–1.231
DLGAP1	Disks large-associated protein 1 (DAP-1)	–1.166
BSN	Bassoon presynaptic cytomatrix protein	–1.084

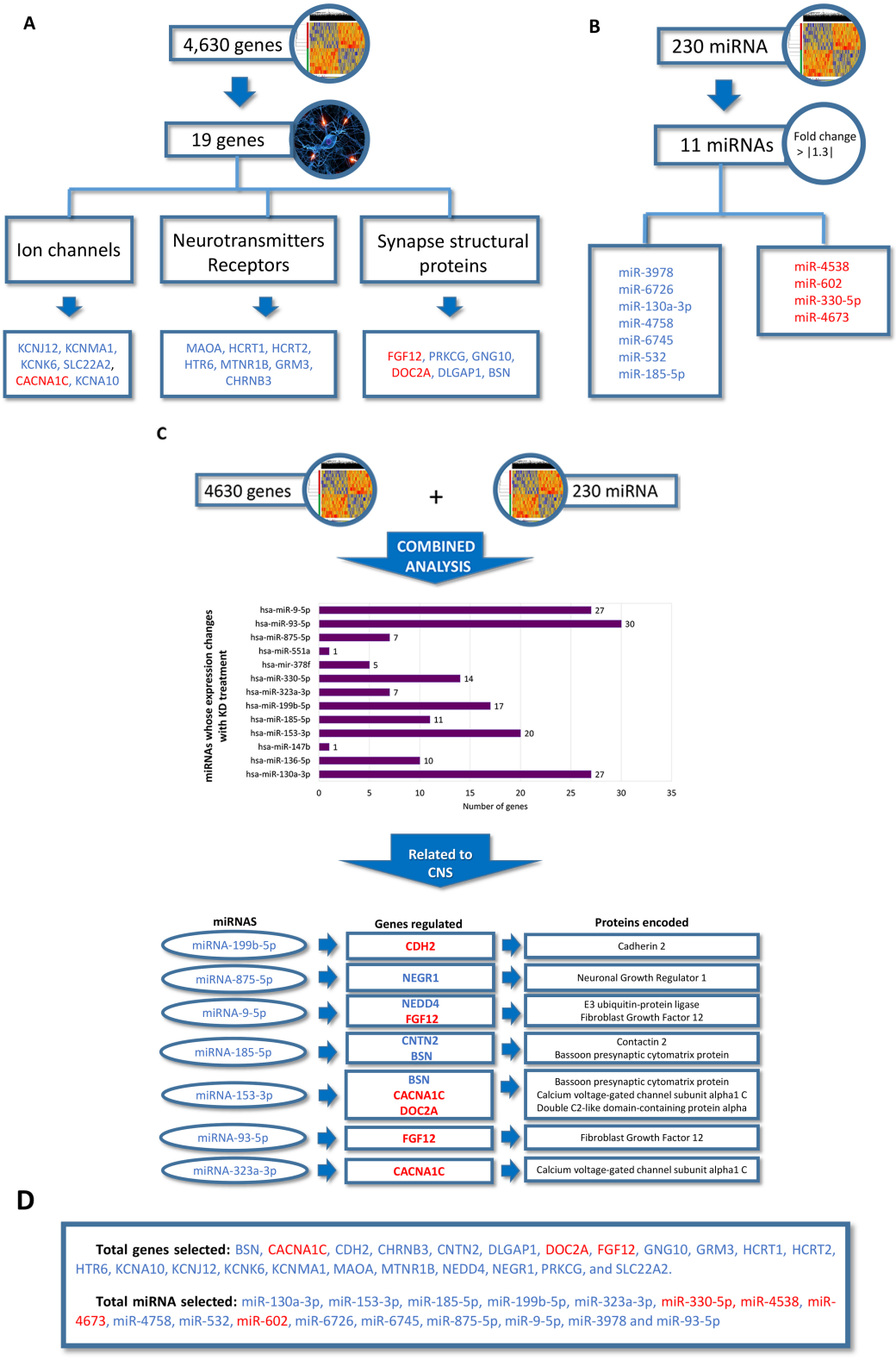

Fig. 3. Genes and miRNAs with biological relevance in our study. In all panels, blue indicates genes or miRNAs that are down-regulated and red those that are up-regulated. Each panel shows our selection strategy. (A) From 4630 genes differently expressed after KDT, the 19 involved in the synaptic transmission pathway have been selected to be discussed. Protein encoded by these genes can be found in Table 2. (B) From 230 miRNAs differently expressed after KDT, the 11 which have a fold-change $> |1.3|$ have been selected to be discussed. (C) From the combine analysis of miRNAs and genes whose expression changes with KDT, those related to the central nervous system (CNS) were selected. (D) List of genes and miRNAs selected through strategy A, B and C, and which finally are discussed.

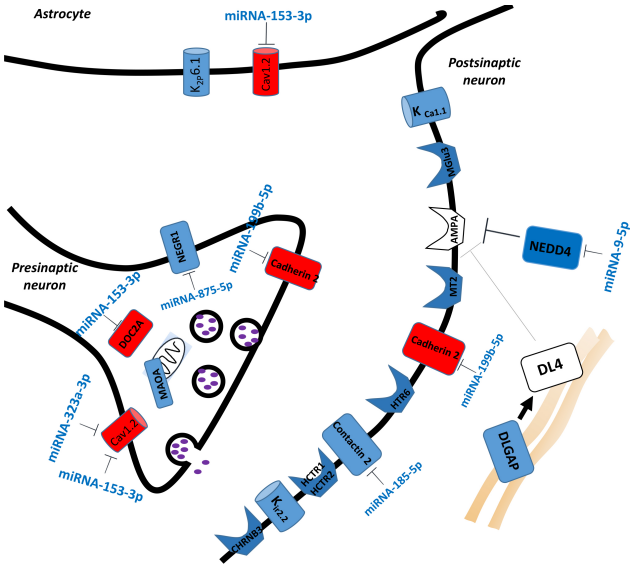

Fig. 4. Treatment with KDT (>6 months) in refractory epileptic children change the transcriptomic profile of genes involved in epileptic processes that encode ion channels, neurotransmitter receptors, and synapse structural proteins. The down-regulated genes and miRNAs are shown in blue, whereas those up-regulated are shown in red.

3.4 Gene and miRNAs expression combined analysis

A combined analysis of the miRNAs and mRNAs that change significantly with KDT intervention was also carried out (see the list of genes and miRNAs in the Supplementary Information). Several of the selected miRNAs regulate genes whose expression change with the intervention (see Fig. 3). All the relevant changes in mRNA and miRNAs (and their physiopathological significance) are summarized in Fig. 4. The results show that 8 genes related to the nervous system modulate their expression after KDT and are known to be regulated by the miRNAs modified during the intervention. These genes are: CDH2 (regulated by miRNA-199b-5p), NEGR1 (regulated by miRNA-875-5p), NEDD4 (regulated by miRNA-9-5p), FGF12 (regulated by miRNA-9-5p and miRNA-93-5p), CNTN2 (regulated by miRNA-185-5p), BSN (regulated by miRNA-185-5p and miRNA-153-3p), CACNA1C (miRNA-323a-3p and miRNA-153-3p), and DOC2A (regulated by miRNA-153-3p).

4. Discussion

The results show that synaptic transmission is the most relevant biological pathway related to epilepsy, targeted by our intervention with KDT in epileptic children. In general, genes which encode proteins involved in this pathway are down-regulated after the intervention. These genes are basically: ion channels, neurotransmitter receptors, and structural synapse proteins.

Regarding the changes in the expression of ion channels, of the four potassium channels whose expression varies with KDT, three of them have been previously linked to epilepsy: Kir 2.2, whose up-regulation has been described in epilepsy [26]; KCNMA1, a Ca^{2+}-dependent potassium channel of the family known as BK proposed to be responsible of higher neuronal excitability [27]; and KCNK6, whose up-regulation contributes to epileptic seizures [28]. CACNA1C, which encodes an L-type Ca^{2+} voltage-gated Ca^{2+} channel (Cav 1.2), is the only ion channel up-regulated after KDT. It has been described mutations in this gene lead to epileptic phenotypes [29], and its dysfunction has been also associated to other neurological disorders such as autism and bipolar disease [30].

The results show a KD-induced down-regulation of several genes encoding neurotransmitters' receptors in epileptic children: orexin, serotonin, melatonin, glutamatergic and cholinergic receptor. Antagonists of the orexin receptors, HCTR1 and HCTR2, can reduce seizures in animal models [31]. Serotonin receptor 5-HT6 seems to be involved in the regulation of cognition, feeding, and, possibly, affective state and seizures [32]. Blockade of the 5-HT6/mTOR pathway can be used as a target for epileptic treatment [33]. It has been found that the expression of melatonin receptors, MT1 and MT2, is increased in the hippocampus of pilocarpine-induced epileptic rats during the epileptic state, while it decreases in a chronic state [34]. Glutamatergic systems are involved in the control of excitation and inhibition. Higher expression of mGlu2/3 receptors have been found in the hippocampus of patients with temporal lobe epilepsy [35]. Finally, the results also show a decrease in the expression of CHRNB3, a cholinergic receptor, already been described as a possible gene associated with epilepsy [36].

On the other hand, MAOA is the enzyme of the external mitochondrial membrane that degrades neurotransmitters. Its inhibition has anticonvulsant effects [37]. The KDT down-regulated the MAOA expression.

Some structural synapse proteins have shown also different gene expression after the KDT. DLGAP1 indirectly interacts with a voltage-dependent Ca^{2+} channel subunit that can modulate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptors and slow their deactivation, thereby increasing neuronal excitability [38]. Our results show that the KDT down-regulates this signalling pathway and therefore decreases excitability. Several studies in animal models indicate that the deletion of the chromosome where the *DOC2A* gene is encoded [39, 40] causes seizures. The treatment, by inducing an overexpression of this gene may explain the reductions in the number of seizures in our patients. PRKCG encodes the gamma subunit of PRKC, a protein kinase enzyme involved in many metabolic pathways, which has been associated to epilepsy [41]. It has been described that its inhibition has anticonvulsant effects [42]. KDT down-regulate this gene in our study.

In general, the changes we have seen in the gene expression related to synaptic transmission fit with a decrease in neuronal excitability, explaining at least part of the therapeutic effect of KDT in epilepsy.

Changes in miRNAs expression are especially important since they regulate the translation of mRNA and therefore control gene expression. Some of the miRNAs whose expression changed more significantly after KDT are involved in antioxidant pathways. It has been previously shown that glucose-induced oscillations of miR-185-5p impairs the cellular antioxidant response in humans through the deregulation of GPx-1 [43]. KDT reduce the oscillation in glucose levels and consequently, in our study, we have found a down-regulation in the expression of miR-185-5p.

On the other hand, miR-330 has been related to the stability of hippocampal neurons [44]. A protective role of this miRNA has been assigned in neurological disorders as Alzheimer's disease [45]. miR-330-3p is up-regulated by PUFA enriched diets [46] and our results also show an up-regulation of this miRNA after fat enriched KDT.

The combined analysis of both (miRNAs and mRNA) transcriptomic results shows that miR-330-5p regulates the expression of 14 genes and miR-185-5p regulates the expression of 11 genes, two of them related to the central nervous system (CNS): CNTN2 and BSN. Other miRNAs which regulate CNS related genes are miRNA-199b-5p, miRNA-875-5p, miRNA-9-5p, miRNA-93-5p, miRNA-153-3p, and miRNA-323a-3p. Almost all the CNS-genes regulated by these miRNAS have been previously discussed. There are four additional genes regulated by these miRNAs that deserve to be briefly mentioned: CDH2, NEGR1, NEDD4 and CNTN2. Cadherin 2 and contactin 2, encoded by the CDH2 and the CNTN2 genes, respectively, are membrane proteins whose gene deletion has been linked to epilepsy [47, 48]. The KD-induced down-regulation of miRNA-199-5p (CDH2 expression regulator) and miRNA-185-5p (CNTN2 regulator) should be accompanied with an increased expression of these membrane proteins. However, this was the case only for CDH2. CNTN2 was slightly down-regulated in our patients after the treatment. A paradoxical result regarding the NEGR1 gene that is regulated by miRNA-875-5 was also found. It encodes a neuronal growth regulator related to axon regeneration whose deficiency increases seizures susceptibility [49]. The results show a down-regulation of miRNA-875-5 after KDT that is not accompanied with an up-regulation of NEGR1 expression. Finally, we studied a well-known epilepsy associated gene, i.e., NEDD4, known to be regulated by miRNA-9-5p [50]. Using loss of function studies, it has been found that mice deficient in Nedd4-2 increase their seizure susceptibility [50]. Our results show a down-regulation of miRNA-9-5p by KDT in the epileptic children. However, this was not accompanied with a concomitant increase in NEDD4 expression in the patients. Collectively our data show the complexity of the gene regulation and strengthen the idea that it involves not only miRNAs.

5. Limitations of the study

We have performed the transcriptional study in the epileptic paediatric patients in PBMCs to reduce the invasiveness of the study. We are assuming that KDT may have a similar effect on the nervous system cells than that found in blood mononuclear cells. We are aware that changes in gene expression varies from one tissue to another, but we would like to emphasize that the vast majority of transcriptomic human studies, especially in patients, are usually performed in blood cells [51, 52]. In addition, despite the coherence between the different individuals in the study and the fact that our results are consistent with the clinical effects of KDT, the number of patients analyzed is small, and this makes it difficult to generalize our results.

6. Conclusions

Treatment with KDT for more than 6 months reduces the number of seizures in epileptic paediatric patients and induces significant changes in their gene expression profile. Our study reveals changes at the transcriptomic level in genes encoding ion channels, neurotransmitter receptors, and synapse structural proteins. These findings may explain the effect of the diet on the epileptic crises. Our results contribute to understand the mechanisms of action of KDT and reinforce their clinical importance in the epilepsy treatment.

Abbreviations

ASM, antiseizure medications; BDNF, brain-derived neurotrophic factor; BHB, β-Hydroxybutyrate; BMI, body mass index; BSN, bassoon presynaptic cytomatrix protein; CACNA1C, Ca^{2+} voltage-gated channel subunit alpha1 C; CDKL5, cyclin dependent kinase-like 5; CHRNB3, Neuronal acetylcholine receptor subunit beta-3; DLGAP1, Disks large-associated protein 1; DOC2A, Double C2-like domain-containing protein alpha; CKD, classic ketogenic diet; FGF12, fibrfoblast growth factor 12; GGT gamma-glutamyl transferase; GNG10, Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-10; GRM3, Glutamate Metabotropic Receptor 3; HCRTR1, hypocretin receptor type 1; HCRTR2, hypocretin receptor type 2; KCNA10, potassium voltage-gated channel subfamily A member 10; KCNJ12, Kir2.2. ATP-sensitive inward rectifier potassium channel 12; KCNK6, K2P6.1. potassium channel subfamily K member 6; KCNMA1, KCa1.1. Ca^{2+}-activated potassium channel subunit alpha-1; KD, ketogenic diet; KDT, ketogenic dietary therapies; LCT, long chain triglycerides; MAD, modified Atkins diet; MAOA, monoamine oxidase A; MCT-KD, ketogenic diet with medium chain triglycerides; MTNR1B, Melatonin Receptor 1B; NMDA, N-methyl-D-aspartate; PBMCs, peripheral blood mononuclear cells; PCA, principal components analysis; PRKCG, Protein kinase C gamma; RBFOX1, RNA binding fox-1; SCN1A, sodium voltage-gated channel alpha subunit 1; SCN9A, sodium voltage-gated channel alpha subunit 9; SLC22A2, solute carrier family 22 member 2.

Author contributions

JRH, GOG, ECV, CPG, and JV planned the experiments. ES, AGC and GOG performed the transcriptomic experiments and JG, MCGC and JV supervised the experiment. CPG supervised each clinical step. GOG and JV wrote the first draft and all authors commented criticized, and reviewed the manuscript. All authors accepted the final version of the manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Committee on Ethics in Clinical Research of the University Children's Hospital Niño Jesús of Madrid (R-0002/15). All patients or their relatives were fully informed of the aims and scope of the research and signed an informed consent.

Acknowledgment

We thank Marilyn Noyes for her kind help in reviewing the manuscript.

Funding

This work was supported by Sira Carrasco Grant 2015 from SEGHNP, Instituto de Salud Carlos III and FEDER [grant number PIE15/00013], SAF2016-75508 -R from the Spanish Ministry of Education and Science (MEC), CB16/10/00435 (CIBERFES), PROMETEOII2014/056 from Conselleria de Sanitat de la Generalitat Valenciana and EU Funded CM1001 and FRAILOMIC-HEALTH.2012.2.1.1-2, ADVANTAGE-724099 Join Action (HP-JA) 3rd EU Health Program and DIALBFRAIL-LATAM (825546 H2020-SC1-BHC).

Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/JIN/21/1/10.31083/j.jin2101031.

References

[1] Fiest KM, Sauro KM, Wiebe S, Patten SB, Kwon C, Dykeman J, *et al.* Prevalence and incidence of epilepsy. Neurology. 2017; 88: 296–303.

[2] Beghi E. The Epidemiology of Epilepsy. Neuroepidemiology. 2020; 54: 185–191.

[3] Wilder R. The effects of ketonemia on the course of epilepsy. Mayo Clinic Proceedings. 1921; 2: 307–308.

[4] Gasior M, Rogawski MA, Hartman AL. Neuroprotective and disease-modifying effects of the ketogenic diet. Behavioural Pharmacology. 2006; 17: 431–439.

[5] Kossoff EH. International consensus statement on clinical implementation of the ketogenic diet: agreement, flexibility, and controversy. Epilepsia. 2009; 49: 11–13.

[6] Huttenlocher PR, Wilbourn AJ, Signore JM. Medium-chain triglycerides as a therapy for intractable childhood epilepsy. Neurology. 1971; 21: 1097–1097.

[7] Kossoff EH, Dorward JL. The modified Atkins diet. Epilepsia. 2008; 49: 37–41.

[8] Kossoff EH, Dorward JL, Turner Z, Pyzik PL. Prospective study of the modified Atkins diet in combination with a ketogenic liquid supplement during the initial month. Journal of Child Neurology. 2011; 26: 147–151.

[9] Neal EG, Chaffe H, Schwartz RH, Lawson MS, Edwards N, Fitzsimmons G, *et al.* The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. The Lancet. Neurology. 2008; 7: 500–506.

[10] Kim JA, Yoon J, Lee EJ, Lee JS, Kim JT, Kim HD, *et al.* Efficacy of the classic ketogenic and the modified Atkins diets in refractory childhood epilepsy. Epilepsia. 2016; 57: 51–58.

[11] Bough KJ, Wetherington J, Hassel B, Pare JF, Gawryluk JW, Greene JG, *et al.* Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. Annals of Neurology. 2006; 60: 223–235.

[12] Maalouf M, Rho JM, Mattson MP. The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. Brain Research Reviews. 2009; 59: 293–315.

[13] Boison D. New insights into the mechanisms of the ketogenic diet. Current Opinion in Neurology. 2017; 30: 187–192.

[14] Bough KJ, Rho JM. Anticonvulsant Mechanisms of the Ketogenic Diet. Epilepsia. 2007; 48: 43–58.

[15] Kim DY, Abdelwahab MG, Lee SH, O'Neill D, Thompson RJ, Duff HJ, *et al.* Ketones prevent oxidative impairment of hippocampal synaptic integrity through KATP channels. PLoS ONE. 2015; 10: e0119316.

[16] Ma W, Berg J, Yellen G. Ketogenic diet metabolites reduce firing in central neurons by opening K(ATP) channels. The Journal of Neuroscience. 2007; 27: 3618–3625.

[17] Genzer Y, Dadon M, Burg C, Chapnik N, Froy O. Effect of dietary fat and the circadian clock on the expression of brain-derived neurotrophic factor (BDNF). Molecular and Cellular Endocrinology. 2016; 430: 49–55.

[18] Freeman J, Veggiotti P, Lanzi G, Tagliabue A, Perucca E. The ketogenic diet: from molecular mechanisms to clinical effects. Epilepsy Research. 2006; 68: 145–180.

[19] Newell C, Bomhof MR, Reimer RA, Hittel DS, Rho JM, Shearer J. Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder. Molecular Autism. 2016; 7: 37.

[20] Kobow K, Reid CA, van Vliet EA, Becker AJ, Carvill GL, Goldman AM, *et al.* Epigenetics explained: a topic "primer" for the epilepsy community by the ILAE GeneticsEpigenetics Task Force. Epileptic Disorders. 2020; 22: 127–141.

[21] Schoeler NE, Leu C, Balestrini S, Mudge JM, Steward CA, Frankish A, *et al.* Genome-wide association study: Exploring the genetic basis for responsiveness to ketogenic dietary therapies for drug-resistant epilepsy. Epilepsia. 2018; 59: 1557–1566.

[22] Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. PLoS Computational Biology. 2017; 13: e1005457.

[23] Kossoff EH, Zupec-Kania BA, Auvin S, Ballaban-Gil KR, Christina Bergqvist AG, Blackford R, *et al.* Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group. Epilepsia Open. 2018; 3: 175–192.

[24] Huttenlocher PR. Ketonemia and seizures: metabolic and anticonvulsant effects of two ketogenic diets in childhood epilepsy. Pediatric Research. 1976; 10: 536–540.

[25] Mootha VK, Lindgren CM, Eriksson K, Subramanian A, Sihag S, Lehar J, *et al.* PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature Genetics. 2003; 34: 267–273.

[26] Young CC, Stegen M, Bernard R, Müller M, Bischofberger J, Veh RW, *et al.* Upregulation of inward rectifier K+(Kir2) channels in dentate gyrus granule cells in temporal lobe epilepsy. The Journal of Physiology. 2009; 587: 4213–4233.

[27] N'Gouemo P. Targeting BK (big potassium) channels in epilepsy. Expert Opinion on Therapeutic Targets. 2011; 15: 1283–1295.
[28] Kim J, Kwak S, Kang T. Upregulated TWIK-related acid-sensitive K+channel-2 in neurons and perivascular astrocytes in the hippocampus of experimental temporal lobe epilepsy. Epilepsia. 2009; 50: 654–663.
[29] Bozarth X, Dines JN, Cong Q, Mirzaa GM, Foss K, Merrit JL, *et al.* Expanding clinical phenotype in CACNA1C related disorders: From neonatal onset severe epileptic encephalopathy to late-onset epilepsy. American Journal of Medical Genetics. 2018; 176: 2733–2739.
[30] Gargus JJ. Genetic calcium signaling abnormalities in the central nervous system: seizures, migraine, and autism. Annals of the New York Academy of Sciences. 2009; 1151: 133–156.
[31] Roundtree HM, Simeone TA, Johnson C, Matthews SA, Samson KK, Simeone KA. Orexin Receptor Antagonism Improves Sleep and Reduces Seizures in Kcna1-null Mice. Sleep. 2016; 39: 357–368.
[32] Quiedeville A, Boulouard M, Da Silva Costa-Aze V, Dauphin F, Bouet V, Freret T. 5-HT6 receptor antagonists as treatment for age-related cognitive decline. Reviews in the Neurosciences. 2014; 25: 417–427.
[33] Wang L, Lv Y, Deng W, Peng X, Xiao Z, Xi Z, *et al.* 5-HT6 Receptor Recruitment of mTOR Modulates Seizure Activity in Epilepsy. Molecular Neurobiology. 2017; 51: 1292–1299.
[34] Rocha AKADA, de Lima E, Amaral F, Peres R, Cipolla-Neto J, Amado D. Altered MT1 and MT2 melatonin receptors expression in the hippocampus of pilocarpine-induced epileptic rats. Epilepsy & Behavior. 2017; 71: 23–34.
[35] Tang FR, Chia SC, Chen PM, Gao H, Lee WL, Yeo TS, *et al.* Metabotropic glutamate receptor 2/3 in the hippocampus of patients with mesial temporal lobe epilepsy, and of rats and mice after pilocarpine-induced status epilepticus. Epilepsy Research. 2004; 59: 167–180.
[36] Wang J, Lin Z, Liu L, Xu H, Shi Y, Yi Y, *et al.* Epilepsy-associated genes. Seizure. 2017; 44: 11–20.
[37] Löscher W, Lehmann H, Teschendorf HJ, Traut M, Gross G. Inhibition of monoamine oxidase type a, but not type B, is an effective means of inducing anticonvulsant activity in the kindling model of epilepsy. The Journal of Pharmacology and Experimental Therapeutics. 1999; 288: 984–992.
[38] Rasmussen AH, Rasmussen HB, Silahtaroglu A. The DLGAP family: neuronal expression, function and role in brain disorders. Molecular Brain. 2017; 10: 43.
[39] Walsh KM, Bracken MB. Copy number variation in the dosage-sensitive 16p11.2 interval accounts for only a small proportion of autism incidence: a systematic review and meta-analysis. Genetics in Medicine. 2011; 13: 377–384.
[40] McCammon JM, Blaker-Lee A, Chen X, Sive H. The 16p11.2 homologs fam57ba and doc2a generate certain brain and body phenotypes. Human Molecular Genetics. 2017; 26: 3699–3712.
[41] Liu J, Schenker M, Ghiasvand S, Berdichevsky Y. Kinase Inhibitors with Antiepileptic Properties Identified with a Novel in Vitro Screening Platform. International Journal of Molecular Sciences. 2019; 20: 2502.
[42] Gajda Z, Török R, Horváth Z, Szántai-Kis C, Orfi L, Kéri G, *et al.* Protein kinase inhibitor as a potential candidate for epilepsy treatment. Epilepsia. 2011; 52: 579–588.
[43] La Sala L, Cattaneo M, De Nigris V, Pujadas G, Testa R, Bonfigli AR, *et al.* Oscillating glucose induces microRNA-185 and impairs an efficient antioxidant response in human endothelial cells. Cardiovascular Diabetology. 2016; 15: 71.
[44] Cohen JE, Lee PR, Fields RD. Systematic identification of 3'-UTR regulatory elements in activity-dependent mRNA stability in hippocampal neurons. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 2014; 369: 20130509
[45] Zhou Y, Wang Z, Li W, Hong H, Chen J, Tian Y, *et al.* Protective effects of microRNA-330 on amyloid β-protein production, oxidative stress, and mitochondrial dysfunction in Alzheimer's disease by targeting VAV1 via the MAPK signaling pathway. Journal of Cellular Biochemistry. 2018; 119: 5437–5448.
[46] Ortega FJ, Cardona-Alvarado MI, Mercader JM, Moreno-Navarrete JM, Moreno M, Sabater M, *et al.* Circulating profiling reveals the effect of a polyunsaturated fatty acid-enriched diet on common microRNAs. The Journal of Nutritional Biochemistry. 2016; 26: 1095–1101.
[47] Verhoeven WM, Egger JI, Knegt AC, Zuydam J, Kleefstra T. Absence epilepsy and the CHD2 gene: an adolescent male with moderate intellectual disability, short-lasting psychoses, and an interstitial deletion in 15q26.1-q26.2. Neuropsychiatric Disease and Treatment. 2016; 12: 1135–1139.
[48] Stogmann E, Reinthaler E, Eltawil S, El Etribi MA, Hemeda M, El Nahhas N, *et al.* Autosomal recessive cortical myoclonic tremor and epilepsy: association with a mutation in the potassium channel associated gene CNTN2. Brain. 2013; 136: 1155–1160.
[49] Singh K, Loreth D, Pöttker B, Hefti K, Innos J, Schwald K, *et al.* Neuronal Growth and Behavioral Alterations in Mice Deficient for the Psychiatric Disease-Associated Negr1 Gene. Frontiers in Molecular Neuroscience. 2018; 11: 30.
[50] Zhu J, Lee KY, Jewett KA, Man H, Chung HJ, Tsai N. Epilepsy-associated gene Nedd4-2 mediates neuronal activity and seizure susceptibility through AMPA receptors. PLoS Genetics. 2017; 13: e1006634.
[51] Borras C, Abdelaziz KM, Gambini J, Serna E, Inglés M, de la Fuente M, *et al.* Human exceptional longevity: transcriptome from centenarians is distinct from septuagenarians and reveals a role of Bcl-xL in successful aging. Aging. 2016; 8: 3185–3208.
[52] Serna E, Gambini J, Borras C, Abdelaziz KM, Belenguer A, Sanchis P, *et al.* Centenarians, but not octogenarians, up-regulate the expression of microRNAs. Scientific Reports. 2012; 2: 961.