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Original article

AFG2A-related encephalopathy: Effectiveness of ketogenic diet in epilepsy and mitochondrial dynamics modulation

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A B S T R A C T

AFG2A-related encephalopathy (AFG2A-RE) is a neurodevelopmental disorder that may present with drug-resistant epilepsy (DRE). Our aims were: to evaluate the clinical response to a ketogenic diet (KD) in a series of patients with AFG2A-RE and DRE, and to describe the mitochondrial effects in patient's fibroblasts cultured in a KD mimicking medium (KD-MM). This was a collaborative, descriptive, and experimental study involving a total of five patients. The primary outcomes assessed following ketogenic diet (KD) treatment were the percentage of seizure reduction and the parents' global impression of change. Additionally, patient-derived fibroblasts ($n = 3$) were cultured in a KD-MM to evaluate effects on mitochondrial dynamics and metabolism. The mean age of the patients was 7.9 years, and four were males. All patients presented with developmental and epileptic encephalopathy with DRE, motor impairment, severe intellectual disability, deafness, and microcephaly. In all but one case, the initial epilepsy presentation was infantile epileptic spasms syndrome (IESS), with a mean age at onset of 13.6 months. Four patients received KD treatment for DRE, with seizure reduction rates of 0 %, 30 %, 70 % and 100 %, respectively. Improvement in social interaction improvement was observed in one patient, while improvements in attentional and motor function were noted in two. *In vitro* studies demonstrated that AFG2A-deficient fibroblasts exhibited altered mitochondrial morphology and dynamics, as well as reduced ATP production and ROS levels. These abnormalities were significantly reversed when the fibroblasts were cultured in KD-MM. In conclusion, this small series of patients with AFG2A-RE showed beneficial effects from KD treatment. Greater seizure control was achieved when the ketogenic diet was initiated during early childhood. These findings are preliminary and validation in multicenter prospective study is required.

1. Introduction

AFG2A-related encephalopathy (AFG2A-RE) is a rare autosomal recessive neurodevelopmental disorder characterized by the triad of epilepsy, microcephaly and deafness, typically associated with intellectual disability and spasticity (OMIM #616577).

Forty cases have been previously reported [1–9]. Although the epilepsy phenotype is poorly described, up to 70 % of the individuals with AFG2A-RE present with epilepsy. The most frequently reported seizure types are epileptic spasms [1,2,4–6,9] and tonic seizures [1,3]. Other commonly observed seizure types include tonic-clonic and myoclonic seizures [3,5,6,9]. Infantile epileptic spasms syndrome (IESS) is reported in 32 % of patients. Moreover, epilepsy is usually drug-resistant [1,4,9].

The disorder is caused by homozygous or compound heterozygous variants in the *AFG2A* gene (AFG2 AAA ATPase HOMOLOG A) (OMIM *613940), located in the chromosomal region 4q28.1. *AFG2A* gene encodes the AAA ATPase HOMOLOG A, previously known as *SPATA5* (spermatogenesis-associated protein 5), and *SPAF* (spermatogenesis-associated factor). *AFG2A* is expressed in testes, spleen, skin, intestines, brain, and skeletal muscle [7]. Although the role of *AFG2A* is not yet fully understood, there is some evidence that it is involved in the mitochondrial morphogenesis during spermatogenesis. Other studies reported the implication of *AFG2A* in the mitochondrial function in rat cortical neurons, mononuclear cells, and platelets [3,9].

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AFG2A-deficient rat cortical neurons presented altered mitochondrial morphology and dynamics, decreased cellular ATP, and a delay in neuronal development [3]. Monocytes deficient in *AFG2A* exhibited less mitochondrial mass (including mitochondrial number, volume and surface area) without differences in the mitochondrial morphology [9]. Platelets deficient in *AFG2A* showed less mitochondrial function with less respiratory complex activity and ATP synthesis, and more ATP consumption [9]. Moreover, a role of AFG2A in global protein synthesis and ribosome assembly has recently been reported [10,11]. Among the non-pharmacological treatments available for pediatric drug-resistant epilepsy (DRE), the ketogenic diet (KD) has been shown in randomized clinical trials to be an effective treatment option [12,13]. KD mimics the biochemical effects of fasting, with various mechanisms implicated in its anti-seizure effect. During ketosis, the body reduces glycolysis and enhances energy production through oxidative phosphorylation of fatty acids via β-oxidation, leading to the production of ketone bodies. Ketosis may protect against epileptic seizures by increasing GABAergic output and modulating ion channel activity [14]. Furthermore, KD exerts direct effects on mitochondrial function, influencing mitochondrial bioenergetics, reducing oxidative stress, modulating mitochondrial dynamics, and increasing mitochondrial mass [15,16]. Given that AFG2A-deficiency is associated to mitochondrial dysfunction in various cells types [3,9], we hypothesized that KD could be a potential treatment option for patients with AFG2A-RE and DRE.

The aims of this study were: (1) to evaluate the clinical response to KD in a series of AFG2A-RE patients with DRE, and (2) to describe the mitochondrial effects *in vitro* through functional studies in patient's fibroblasts cultured in a KD mimicking medium (KD-MM).

2. Subjects and methods

This is a collaborative, descriptive, and experimental study involving five patients with AFG2A-RE and DRE, of whom Patients 1, 2, 3, and 4 received KD treatment, while Patients 1, 3, and 5 were included in the fibroblasts analysis. The patients were recruited from our center and through collaborative calls within European Reference Network for all rare and complex epilepsies (EpiCARE) and the Spanish Society of Pediatric Neurology (SENEP).

2.1. Clinical effect of the ketogenic diet

Indication for KD treatment followed standard clinical practice [17]. Although KD treatment was clinically indicated, the family of Patient 5 declined its initiation.

The initiation of the KD followed the clinical practice recommendations, which are outlined below [17]. Prior to initiating the KD, a consultation with a nutrition specialist was conducted to inform the family about the treatment and to rule out contraindications, such as fatty acid β-oxidation defects. Accordingly, initial blood and urine tests were conducted to rule out potential contraindications and to facilitate ongoing monitoring for adverse effects. The blood panel includes complete blood count, coagulation profile, free carnitine, acylcarnitines, prealbumin, total protein, electrolytes, albumin, iron parameters, lipid profile, calcium, ferritin, phosphate, and creatinine. Urinalysis includes sediment examination, electrolyte assessment, organic acids, calcium, citrate, oxalates, proteins, and creatinine. In the absence of KD contraindications and upon parental consent, patients initiated treatment with the classic KD. The classic KD was initiated gradually up to a 4:1 ratio during a 5 day hospital admission. During hospitalization, blood glucose and serum ketone levels were monitored, with target **β-hydroxybutyrate** levels of 2.4–5 μmol/L. Following discharge, the family continued punctually home monitoring through urinary ketones determination, with a target level between 2+ and 4+. Ongoing follow-up was conducted by nutrition specialists and dietitians to monitor potential adverse effects of KD.

The evaluation of the clinical effect of the KD was conducted based on the epilepsy improvement and the parent global impression of improvement. Baseline seizure type and frequency over the four weeks preceding KD initiation were documented by the parents for treatment monitoring purposes. Evaluation of epileptic outcomes (seizure frequency and percentage of seizure reduction), and non-epileptic outcomes (attention, interaction and motor skills) according to parent global impression of improvement was performed at least three months after the initiation of KD treatment. In patients where seizures occur with high frequency that precise quantification is not feasible, the seizure frequency was classified as uncountable. For uncountable seizures, improvement was assessed based on the parents' impression of the percentage of reduction. A video electroencephalogram (vEEG) was performed before initiating the KD and repeated 3–4 months after its initiation. Subsequent follow-up visits were conducted in accordance with standard clinical practice and tailored to each patient's clinical status, ensuring intervals of no more than six months between visits.

2.2. Fibroblasts response to a ketogenic diet mimicking medium

The study was conducted using primary cultures of skin fibroblasts obtained from three patients and age-matched controls. Dermal fibroblasts were grown following standard culture conditions in minimal essential medium (MEM) supplemented with 1 % glutamine, 10 % foetal bovine serum (FBS) and 1 % penicillin-streptomycin. Unless stated otherwise, experiments were accomplished when fibroblasts were at 80 % confluence and between the 7th and 14th passages. KD-MM was achieved by using complete MEM low glucose (1 g/L) with a final concentration of 5 mM of acetoacetate (MW: 108.02 g/mol) and β-hydroxybutyrate (MW: 126.09 g/mol). Prior to experiments, cells were treated for five consecutive days with this medium, changing for fresh medium every 48 h.

In vivo time-lapse confocal microscopy: Cells were incubated with 300 nM MitoTracker Green (Invitrogen) for 30 min at 37 °C and 5 % CO_2. Immunofluorescence was visualized using Leica TCS SP8 microscope (Leica, Wetzlar, Germany) with a 63x, 1.4 N A. oil-immersion objective, 2.75 electronic zoom, and at a frame size of 512x512 pixels. We used a 495-nm white laser, and recorded for 3 min with 0.7 s between each frame, maintaining cell culture conditions. Mitochondrial dynamics and shape analyses were performed with the MATLAB add-in Mitometer [18].

Western blotting: Proteins were extracted from cultured fibroblasts through a 30′ cold incubation with RIPA and protease inhibitors. Following extraction, proteins were quantified by Bradford method and prepared at a homogenous concentration in Laemmli Buffer in reductive denaturing conditions and subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked with TBS-Tween (0.05 %): milk 5 % for 1h at room temperature. Primary antibodies were incubated O/N at 4 °C in blocking buffer, at the following concentrations: MnSOD (1:80,000, ADI-SOD-111-F, Enzo Life Sciences) and GPx1 (1:1,000, ab108427, Abcam). Vinculin (1:20,000, sc-59803, Santa Cruz Biotechnologies) was used as a loading control. For the OXPHOS complexes' detection, proteins were denatured in a non-reductive buffer, and incubated after SDS-PAGE and Western blot with OXPHOS Human WB Antibody Cocktail (45–8199, Thermo Fisher). The secondary antibodies used were HRP-conjugated goat anti-Rabbit and goat anti-Mouse IgG antibodies (Thermo Fisher) and were detected using the PierceTM ECL Western Blotting substrate (Thermo Fisher). Quantification of protein expression was performed using Fiji software.

ATP content measurement: Cells were harvested and re-suspended in boiling ATP extraction buffer (100 mM Tris–HCl, 4 mM EDTA pH 7.7). ATP concentration was assayed by bioluminescence using a luciferin-luciferase system (ATP Bioluminescence Assay Kit CLS II, Roche) according to manufacturer's instructions. ATP concentration was corrected per mg of protein.

Analysis of intracellular $O_2^{\cdot-}$ by flow cytometry: Superoxide anion concentration was measured using MitoSOX™ Red probe (Invitrogen).

Alive fibroblasts were incubated with the dye at a concentration of 5 μM for 10 min at 37 °C according to manufacturer's instructions. Cells were subsequently resuspended in complete Hanks Balanced-Buffered Saline (HBSS) and analyzed by flow cytometry in a NovoCyte Flow Cytometer (ACEA Biosciences).

GraphPad Prism 9.0 software was used for statistical analysis in *in vitro* studies. First, outliers were identified by ROUT test and discarded, and then data normality was determined. ANOVA for parametric data or Kruskal-Wallis for non-parametric data were performed, with posterior multiple comparisons analysis correction when appropriate. Equal standard deviations were not assumed. Two-way ANOVA was used for comparing groups with two or more variables. Differences were considered statistically significant when *p-value <0.05, **p-value <0.01, ***p-value <0.001, and ****p-value <0.0001.

2.3. Ethics approval

All parents or legal guardians of the patients provided written informed consent to participate in the study. This study was approved by the local institutional ethics committee (Sant Juan de Déu Hospital ID number: PIC-131-18). All research work has been carried away following the principles under the Helsinki declaration.

3. Results

A total of 5 patients were included in the study, with a mean age of 7.9 years (range: 4.2–13.4), four of whom were males. All patients presented with developmental and epileptic encephalopathy (DEE) with DRE, severe neurodevelopmental delay, and severe intellectual disability (non verbal). Additionally, they presented with motor impairment (hypotonia and spasticity) classified according to the Gross Motor Function Classification System as ranging from III to V, as well as deafness and microcephaly. Other clinical features observed in the patients included visual impairment (4 patients), movement disorder (dystonia in 2 patients, chorea in 1 patient, and dyskinesia in 1 patient), and gastrointestinal symptoms (dysphagia in all 5 patients and

Table 1
Clinical features, genetic findings, and response to ketogenic diet in patients with AFG2A-Related encephalopathy.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex (last follow up age)	Male (7 years)	Female (4 years)	Male (7 years)	Male (8 years)	Male (13 years, death)
Perinatal History	IGR III, Late preterm, fetal distress	Uneventful	Uneventful	Uneventful	Uneventful
Microcephaly	+	+	+	+	+
Neurodevelopmental Delay	Severe	Severe	Severe	Severe	Severe
Intellectual Disability	Severe (non-verbal)	Severe (non-verbal)	Severe (non-verbal)	Severe (non-verbal)	Severe (non-verbal)
Hypotonia	+	+	+	+	+
Spasticity	+ more in lower limbs	+	+ more in upper limbs	+	+
Movement Disorder	+ Generalized Choreo-dystonia	–	+ Dystonia in lower limbs	+ Dyskinesia	–
GMFCS	Level III	Level V	Level V	Level V	Level V
Deafness	+	+	+	+	+
Visual Impairment	+	+	+	–	+
Dysphagia	+	+	+	+	+
Epilepsy onset (Seizure type/age)	GES - IESS (27 m)	GES - IESS (7 m)	GES – IESS (14 m)	Generalized motor tonic seizures (6 m)	GES – IESS (onset 14 m).
Other types of seizures	–	Generalized motor tonic seizure with right cephalic version. Generalized hypotonia with upward gaze deviation	Generalized motor atonic	GESS – IESS .	–
KD Initiation Age	33 months	18 months	6 years	7 years	Not treated ([a])
vEEG pre-KD	Clusters of GES. Multifocal epileptiform discharges. Slow and poorly organized baseline cerebral activity	Clusters of GES. Multifocal epileptiform discharges. Slow and poorly organized baseline cerebral activity	Focal epileptiform discharges (Bilateral fronto-central/fronto-temporal). Slow and poorly organized baseline cerebral activity	Multifocal epileptiform discharges. Slow and poorly organized baseline cerebral activity	Multifocal epileptiform discharges. Slowed and poorly organized baseline cerebral activity
vEEG post-KD	No epileptiform abnormalities. Baseline cerebral activity appropriate for age	Significantly less frequent, brief, and subtle GES clusters. Multifocal epileptiform discharges. Slow and poorly organized baseline cerebral activity	No changes observed.	No changes observed.	Not treated ([a])
ASM received	ACTH, ESM, VPA	ACTH, VGB, VPA,ZNS, CZP, PER	VGB, ACTH, CLB	Not available	VPA, VGB, CLB, PB, LTG
ASM pre-KD	VGB + CLB	LEV + LCM + CBZ	PER + TPM + VPA + LTG	VPA + LTG + RFM	Not treated ([a])
ASM post-KD	KD (Atkins Diet)	KD + LEV + LCM + PER	KD + PER + VPA + LTG	KD + VPA + LTG + RFM	Not treated ([a])
Brain MRI (age)	Normal (2.9 y)	Diffuse CA and leukoencephalopathy (4y)	Normal (1.2 y)	Diffuse CA and leukoencephalopathy (2.5 y)	White matter CA and hypomyelination (7.75 y)
***AFG2A* variants**	c.251G > A; p.Arg84Gln c.251G > A; p.Arg84Gln	c.251G > A; p.Arg84Gln c.1586G > A; p.Arg529Gln	c.251G > A; p.Arg84Gln c.1064T > C; p.Ile355Thr	c.251G > A; p.Arg84Gln c.2174T > C; p.Phe725Ser	c.327_328del; p.Lys110fs c.1631T > G; p. Leu544Arg
Genetic variation (*in silico* pathogenicity)	Missense (P) Missense (P)	Missense(P) Missense(P)	Missense (P) Missense (P)	Missense (P) Missense (LP)	Frameshift (LP) Missense (LP)

Abbreviations: Yes (+), No (–), Months (m), Ketogenic Diet (KD), Intrauterine Growth Restriction (IGR), Generalized Epileptic Spasms - Infantile Epileptic Spams Syndrome (GES-IESS), Gross Motor Function Clinical Scale (GMFCS), Adrenocorticotropic hormone (ACTH), Ethosuximide (ESM), Valproate (VPA), Vigabatrin (VGB), Zonisamide (ZNS), Clonazepam (CZP), Perampanel (PER), Clobazam (CLB), Phenobarbital (PB), Lamotrigine (LTG), Years (y), Cerebral Atrophy (CA), Pathogenic (P), Likely Pathogenic (LP).

[a] KD was not initiated due to the family's preferences.

gastroesophageal reflux in 3 patients). Brain MRI scans revealed non-specific abnormalities, such as diffuse cerebral atrophy and leukoencephalopathy in 2 patients, and hypomyelination and white matter atrophy in 1 patient.

Regarding epilepsy, all patients were diagnosed with IESS, with a mean age of onset of 13.6 months (range: 7–27 months). Interictal multifocal epileptiform discharges were observed in all patients except for Patient 3, in whom focal epileptiform discharges were observed. Hypsarrhythmia was registered in one patient (Patient 4).

Patient 5 passed away at the age of 13.4 years due to respiratory failure associated with a lung infection, while under palliative care.

Table 1 summarizes the clinical, neuro-imaging, and genetic findings for the patients cohort.

3.1. *Clinical effect of the ketogenic diet*

Four patients with DRE were treated with classic KD based on standard clinical practice indication (Patients 1, 2, 3, 4). Three were males and one female, all of whom experienced countless daily seizures.

Patient 1 experienced seizure onset at 27 months of age, presenting with late-onset IESS, which proved to be drug-resistant. At 33 months of age, he began treatment with the KD in combination with vigabatrin and clobazam. Prior to this, he had been treated with adrenocorticotropic hormone (ACTH), valproate and ethosuximide. Seizure remission was achieved one month after starting the KD. He is currently 7 years old and remains seizure-free on KD monotherapy with modified Atkins diet.

Patient 2 presented with IESS at 7 months of age. Subsequently, she developed two new seizure types: (1) generalized motor tonic seizure with right cephalic version, and (2) generalized hypotonia with upward gaze deviation. The KD was initiated at 18 months of age, in combination with ongoing treatment with levetiracetam, lacosamide, and carbamazepine. Previous therapies with ACTH, vigabatrin, valproate and zonisamide had proven ineffective. The patient experienced a rapid 95 % reduction in seizure frequency. At the last follow up, at 4 years of age, she remained on KD in combination with levetiracetam, lacosamide, and perampanel, and reported experiencing up to two brief seizures per week, characterized by right-sided mouth deviation lasting a few seconds. The family also reported improvements in social interaction.

Patient 3 presented with IESS at 14 months of age. The KD was initiated at 6 years of age, due to persistent atonic seizures, despite treatment with a combination of perampanel, topiramate, valproate and lamotrigine. At the most recent follow-up, at 7 years of age, the patient demonstrated a 30 % reduction in seizure frequency since the initiation of the KD, with the seizure semiology remaining unchanged from prior episodes. According to family reports, improvements were observed in attention and motor skills, which were associated with a change in GMFCS level from V to IV. Topiramate was subsequently discontinued, with maintenance of the other three antiseizure medications.

Patient 4 presented with generalized motor tonic seizures at 6 months of age. Shortly after, he presented with IESS. At 7 years of age, the KD was initiated due to DRE, characterized by countless tonic spasms despite treatment combination of valproate, lamotrigine and rufinamide. At last follow-up, at 8 years of age, seizure frequency and semiology remained unchanged under treatment combination that included KD, valproate, brivaracetam and rufinamide. Nevertheless, the family wanted to continue with the KD, as they observed improvements in attention and motor function.

All patients exhibited VEEG patterns with epileptiform discharges and background activity consistent with developmental and epileptic encephalopathy. Generalized epileptic spasms were observed in Patients 1 and 2, who also showed VEEG improvement after initiating the KD, more notably in Patient 1 (see Table 1).

KD tolerance and adherence were satisfactory in all patients, with no reports of severe side effects related to the treatment. At 4 years of age, Patient 1 developed asymptomatic persistent hypercalciuria, which was managed with oral potassium citrate.

3.2. *Fibroblasts response to a ketogenic diet mimicking medium*

To understand the effect of KD on mitochondrial physiology, we cultured patient's fibroblasts by growing them for five days in a low-glucose medium supplemented with 5 mM acetoacetate and β-hydroxybutyrate (KD-MM), as described in materials and methods. We first analyzed the effect of KD-MM on mitochondrial dynamics using in vivo time-lapse image acquisition via confocal microscopy, recording during 3 min with 0.7 s between each frame (Fig. 1A, Video 1–3), and subsequent analysis of the fusion and fission events and mitochondrial network morphology with the MATLAB add-in Mitometer. AFG2A-deficient cells exhibited a decrease in the fusion:fission events ratio, which was recovered by KD-MM (Fig. 1B). Additionally, the mitochondria of the patients displayed slower "burst events" (i.e. rapid movements of mitochondria) and lowered directionality on their movements. Both of these aspects were restored upon culturing with KD-MM. Regarding the mitochondrial network morphology, significant differences between *AFG2A* patients and controls fibroblasts were observed in several parameters, outstanding the decreased area and mitochondria perimeter and mitochondrial aspect ratio (i.e. the major: minor axis ratio). All three parameters were restored to control levels upon growth with KD-MM (Fig. 1C).

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.ejpn.2025.07.007

After analyzing the effect of KD-MM on mitochondrial shape and dynamics, we further examined its ability to modulate the bioenergetic function in the context of AFG2A deficiency. Two of the three patients (Patient 1 and 5) exhibited decreased ATP concentrations, which were significantly restored by KD-MM in both cases. Opposite to that, Patient 3 showed a normal ATP levels, which was unaffected by KD-MM growing (Fig. 1D). Both Patient 1 and 5 had decreased expression of mitochondrial Complex IV, which was significantly increased with KD-MM growth, while the third patient had normal levels of the complex, which were not altered by KD-MM (Fig. 1E).

Finally, we analyzed the impact of KD-MM on oxidative stress metabolism by measuring both the concentration of the reactive oxygen species (ROS): superoxide anion ($O_{2.-}$), and the ROS-detoxifying enzymes superoxide dismutase and glutathione peroxidase. Analysis of ROS levels revealed that all three patients-derived fibroblasts were exposed to significantly higher levels of $O_2^{\bullet-}$, which can lead to oxidative damage. In all three cases, culturing with KD-MM resulted in a reduction of oxidative stress (Fig. 1F). Interstingly, only fibroblasts from Patient 3 showed increased levels of mitochondrial superoxide dismutase (MnSOD), which decreased upon KD-MM growth, whereas the other two patients exhibited no differences in MnSOD levels under either basal or treated conditions (Fig. 1G).

4. Discussion

This article describes, for the first time, the effects of the KD in a cohort of patients with AFG2A-RE, and the associated changes in mitochondrial dynamics and metabolism in patient-derived fibroblasts cultured in a KD-MM. The most significant effects on seizure control were noted when KD therapy was initiated at an early age, with no serious adverse events reported. No additional factors were identified as predictors of KD responsiveness. The epileptic phenotype associated with AFG2A deficiency remains poorly characterized in the literature, despite approximately 40 previously published cases [1–9]. All patients described in this study presented early onset seizures and DRE, and IESS. The epilepsy phenotype observed in our patients appears more severe compared to previous articles, which describe a lower incidence of IESS and DRE. However, many of those articles lack detailed information on seizure types, EEG abnormalities, and anti-seizure medications used [1–9]. This contrast with our findings, where all AFG2A deficiency patients, presented with IESS and showed poor response to antiseizure medication. Further research is necessary to determine the prevalence

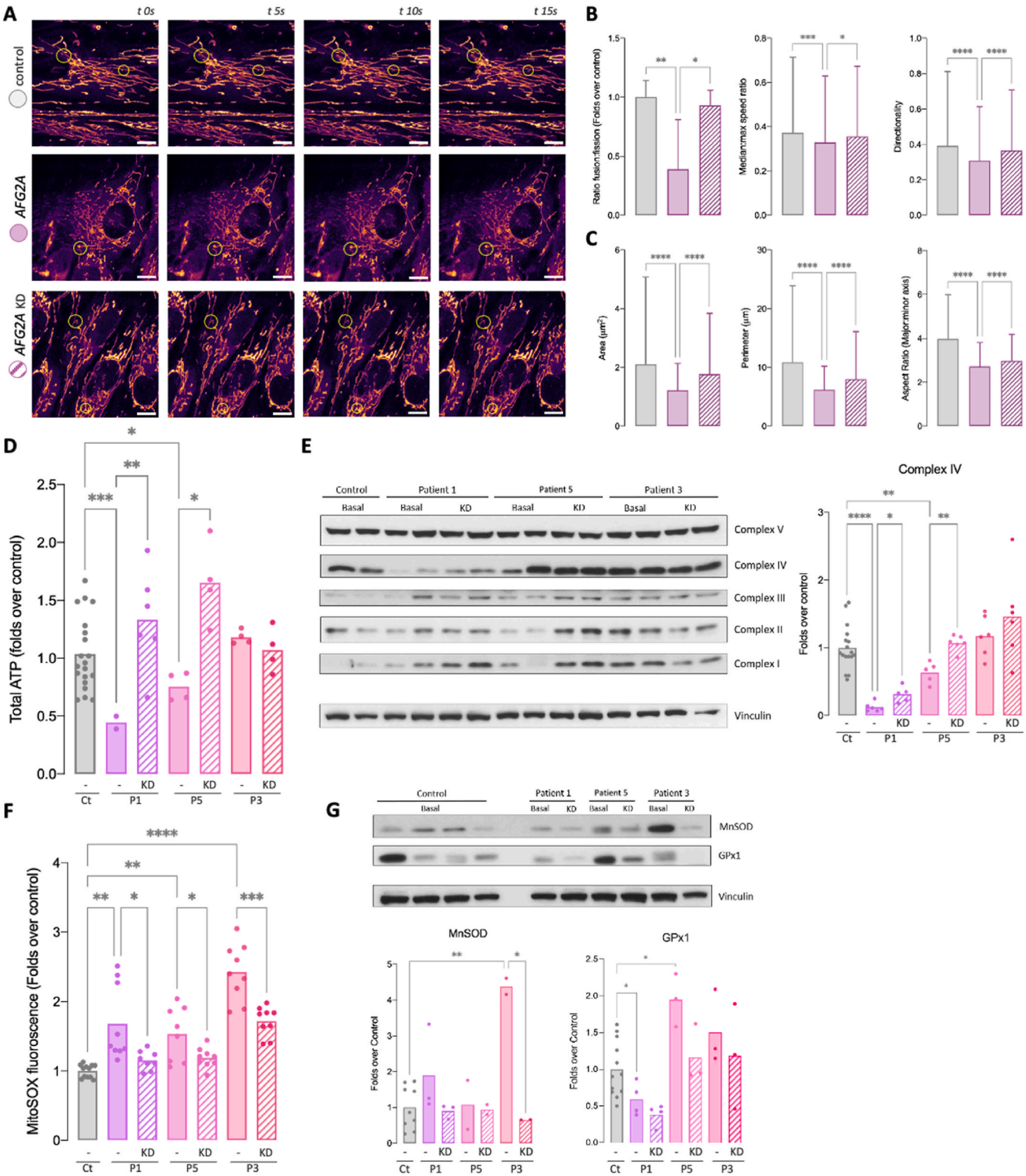

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Fig. 1. Mitochondrial dynamics and bioenergetic dysfunction in three *AFG2A* individuals' fibroblasts. (**A-C**) Mitochondrial dynamics and shape characterization of control and individuals' fibroblasts using MATLAB add-in Mitometer. (**A**) Frames obtained from time-lapse images at different time points (0s, 5s, 10s, 15s) showing in yellow the fusion-fission dynamics in the mitochondrial network, detected with MitoTracker Green (ImageJ LUT inferno, scale bar 10 μm). (**B**) Quantification of the dynamics parameters: fusion:fission ratio ($N = 4$–9 cells in each group), median:max speed ratio (capacity of burst movement), and directionality (defined as the total distance traveled divided by the maximum displacement of the track) ($N = 2$–6 cells in each group). (**C**) Quantification of mitochondrial shape parameters: area occupied by signal, perimeter and aspect ratio (calculated as the ratio between the major and minor axes of mitochondria) ($N = 2$–6 cells in each group). (**D**) Total ATP production in fibroblasts from the different *AFG2A* individuals, detected by bioluminescence assay ($N = 2$ replicates). (**E**) OXHOS complexes expression detected by Western blot and complex IV quantification ($N = 5$ replicates). (**F**) Superoxide anion generation detected by flow cytometry with MitoSOX probe ($N = 3$ replicates). (**G**) Antioxidant enzymes (MnSOD and GPx1) expression by Western blot and quantification in the three *AFG2A* individuals' fibroblasts ($N = 2$ replicates).
Statistical analysis was performed as described in Materials and Methods. * = p-value<0.05, ** = p-value<0.01, *** = p-value<0.001, **** = p-value<0.0001. Control (Ct), Patient 1 (P1), Patient 3 (P3), Patient 5 (P5), Ketogenic diet mimicking medium (KD). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and spectrum of epilepsy in larger cohorts of patients with AFG2A-RE.

The pathophysiology of AFG2A deficiency is characterized by dysfunction of mitochondrial dynamics (decrease in the fusion and fission events along with mitochondrial movement speed), lowered mitochondrial mass, altered production and metabolism of ROS and respiratory activity, with a subsequently decreased cellular level of ATP [3,9]. Given that the KD has a modulatory role in mitochondrial dynamics and neuronal energy metabolism [15,16], we hypothesized that it could improve mitochondrial homeostasis and thus be useful as a potential therapeutic option. To data, only one patient with AFG2A-RE treated with KD has been reported [6]; however, no clinical effects were described, and details regarding the age at KD initiation, the type of KD used, and the duration of treatment were not provided.

In our series of AFG2A-RE patients treated with the KD, we observed a significant seizure reduction or neurological non-epileptic symptoms improvement. The two patients who began KD at an early age (before 3 years of age), demonstrated marked seizure reduction of 95 % and 100 %, respectively (Patients 1 and 2). In contrast, patients with initiated KD later experienced more limited seizure reduction, 0 % and 30 %, respectively (Patients 3 and 4). Despite the lack of substantial seizure control in these latter cases, improvements were noted in social interaction, awareness, and motor function. As previously reviewed by van Berkel et al., improvements in alertness and attention have been observed in objective neuropsychological assessments of both adults and children with epilepsy treated with the KD [19]. In this systematic review, such cognitive benefits were not consistently associated with seizure reduction, suggesting a potential independent effect of the KD on cognitive function. In our cases (Patients 3 and 4), improvements in attention were noted; however, it is not possible to determine whether these changes were solely attributable to the KD, to the natural course of the disease, or, in the case of Patient 3, also influenced by the simplification of the antiseizure medication. The differences in epilepsy treatment response underscore the importance of timing in epilepsy management, highlighting the need for early diagnosis and prompt initiation of disease-modifying treatments. Although further validation is needed, early initiation of KD may help prevent secondary energy failure associated with recurrent seizures at cellular level and promote synaptic stabilization. Moreover, the observed neurobehavioral improvements, regardless of seizure reduction, suggest that KD's antioxidant effects, reduction of neuroinflammation, and modulation of mitochondrial function may confer additional neuroprotective benefits [20,21].

KD tolerance and adherence were satisfactory, with no severe adverse effects reported. Patients were regularly monitored by expert dieticians and nutritionists. The only side effect observed was asymptomatic persistent hypercalciuria, which was managed with oral potassium citrate, allowing the continuation of the KD treatment [17].

Traditionally, it is recommended that KD treatment should be maintained for approximately two years if tolerance is optimal. However, in cases of significant or complete response, it may be continued for longer periods, with close monitoring of potential side effects. Furthermore, for long-term treatment, the classical KD can be transitioned to other modalities, such us the modified Atkins diet, as was done in Patient 1 due to the complete response to KD.

In vitro analysis of AFG2A-deficient fibroblasts demonstrated that after 5-days exposure to a KD-MM led to a recovery of mitochondrial dynamics and an improvement in their bioenergetic profile. These results highlight the potential role of KD as a treatment option for patients with AFG2A-RE.

These preliminary findings underscore the need for larger, prospective, multicenter studies to further evaluate the efficacy and safety of KD in patients with DRE epilepsy due to *AFG2A* variants.

Author contributions

Conception of the study: CF. Design of the study: CF and AO. Acquisition and analysis of data: all authors. Statistical analysis: UM and AO. Drafting a significant portion of the manuscript or figures: LNF, UM, AO and CF. All authors revised the work. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejpn.2025.07.007.

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