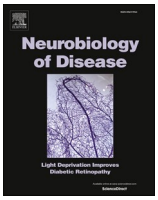
Neurobiology of Disease 163 (2022) 105594

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy

Rebecca Bornstein [a,1], Katerina James [b,1], Julia Stokes [b,1], Kyung Yeon Park [b,1], Ernst-Bernhard Kayser [b], John Snell [b], Angela Bard [b], Yihan Chen [b], Franck Kalume [b,c,*], Simon C. Johnson [a,b,d,e,*]

[a] Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, United States of America
[b] Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA, United States of America
[c] Department of Neurological Surgery, University of Washington, Seattle, WA, United States of America
[d] Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, United States of America
[e] Department of Neurology, University of Washington, Seattle, WA, United States of America

ARTICLE INFO

Keywords:
Mitochondrial disease
Epilepsy
mTOR
Ketosis
Ketogenic diet
Seizure

ABSTRACT

Genetic mitochondrial diseases are the most frequent cause of inherited metabolic disorders and one of the most prevalent causes of heritable neurological disease. Leigh syndrome is the most common clinical presentation of pediatric mitochondrial disease, typically appearing in the first few years of life, and involving severe multi-system pathologies. Clinical care for Leigh syndrome patients is difficult, complicated by the wide range of symptoms including characteristic progressive CNS lesion, metabolic sequelae, and epileptic seizures, which can be intractable to standard management. While no proven therapies yet exist for the underlying mitochondrial disease, a ketogenic diet has led to some reports of success in managing mitochondrial epilepsies, with ketosis reducing seizure risk and severity. The impact of ketosis on other aspects of disease progression in Leigh syndrome has not been studied, however, and a rigorous study of the impact of ketosis on seizures in mitochondrial disease is lacking. Conversely, preclinical efforts have identified the intracellular nutrient signaling regulator mTOR as a promising therapeutic target, with data suggesting the benefits are mediated by metabolic changes. mTOR inhibition alleviates epilepsies arising from defects in TSC, an mTOR regulator, but the therapeutic potential of mTOR inhibition in seizures related to primary mitochondrial dysfunction is unknown. Given that ketogenic diet is used clinically in the setting of mitochondrial disease, and mTOR inhibition is in clinical trials for intractable pediatric epilepsies of diverse causal origins, a direct experimental assessment of their effects is imperative. Here, we define the impact of dietary ketosis on survival and CNS disease in the *Ndufs4(KO)* mouse model of Leigh syndrome and the therapeutic potential of both dietary ketosis and mTOR inhibition on seizures in this model. These data provide timely insight into two important clinical interventions.

1. Introduction

Genetic mitochondrial diseases are estimated to impact over 1 in 4000 individuals, making them the most common form of inherited metabolic disorders and one of the most common causes of heritable neurological disease (Gorman et al., 2016). Mitochondrial diseases are extraordinarily variable in genetic cause; over 250 loci, including both nuclear and mitochondrial variants, have been causally linked to genetic mitochondrial disease (Alston et al., 2017; Chinnery, 1993a; Viscomi et al., 2015).

Mitochondrial disorders are highly variable in clinical manifestation, clustering into clinically defined syndromes. Some forms of mitochondrial dysfunction primarily impact one organ, such as Leber hereditary optic neuropathy (LHON), while others result in complex multi-system disease including Mitochondrial Myopathy with Encephalomyopathy, Lactic acidosis, And Stroke-like symptoms (MELAS) or subacute necrotizing encephalomyelopathy, also known as Leigh syndrome (LS) (El-Hattab et al., 2015; Hilo et al., 2013; Yu-Wai-Man and Chinnery, 1993;

* Corresponding authors at: 1900 9th Ave, JMB-925, Seattle, WA 98101, United States of America.
E-mail addresses: fkalume@uw.edu (F. Kalume), simoncj@uw.edu (S.C. Johnson).
[1] Authors contributed equally to this work.

https://doi.org/10.1016/j.nbd.2021.105594
Received 21 May 2021; Received in revised form 27 September 2021; Accepted 17 December 2021
Available online 20 December 2021

Thorburn et al., 1993; Rahman and Thorburn, 1993; Chinnery, 1993b). The clinical and genetic heterogeneity of mitochondrial diseases have hampered research, and effective clinical therapies have been elusive; clinical management is primarily palliative. Dietary supplements, such as the so-called 'mitochondrial-cocktail' of vitamins and co-factors, and nutritional interventions, including ketogenic diets, are often used in mitochondrial disease patients, but there is a notable lack of experimental evidence to suggest efficacy (Tarnopolsky, 2008; Rahman, 2012).

LS is the most common clinical presentation of pediatric onset mitochondrial disease, and over 75 causal genes have been identified in LS alone (Schubert Baldo and Vilarinho, 2020; Ruhoy and Saneto, 2014; Gerards et al., 2016; Rahman et al., 2017). LS typically presents by two years of age, with early symptoms including hypotonia, respiratory dysfunction, neurodevelopmental defects, ataxia, lactic acidosis, and epileptic seizures. LS progression involves characteristic progressive symmetric necrotizing lesions in the central nervous system (CNS), most prominent in the brainstem, identified by hyperintensity and lactate peaks in MRI imaging. Other regions, including the cerebellum and basal ganglia, may also show signs of bilateral degeneration. Respiratory insufficiency resulting from brainstem degeneration is the most common cause of death in LS (Sofou et al., 2014).

Epileptic seizures are an important clinical feature of LS, presenting in a significant fraction of patients - estimates vary, but range from 35 to 60% (Desguerre et al., 2014; Finsterer and Zarrouk Mahjoub, 2012; Lee et al., 2019). Seizures significantly impact quality of life, can be difficult to manage in LS due to drug resistance and contraindications, and can progress to medically intractable epilepsy. Seizures have not been directly studied in the premier model of pediatric genetic mitochondrial disease, the *Ndufs4(KO)* mouse model of LS; however, it has been shown that *Ndufs4* deletion restricted to GABAergic, but not cholinergic or glutamatergic, neurons is sufficient to cause severe seizures phenotypes (Bolea et al., 2019; Johnson et al., 2020a).

Ketogenic diets have been utilized clinically to manage intractable epilepsy resulting from mitochondrial dysfunction, with some reports suggesting the greatest benefits occur in patients with electron transport chain complex I (ETC CI) defects (Paleologou et al., 2017). However, clinical data is limited by the rarity and heterogeneity of LS. Pre-clinical studies have been completely absent, and the precise mechanism of action has not been established.

Recent pre-clinical studies have identified new targets for intervention in the progression of LS, including inhibition of the intracellular nutrient sensor mTOR (the mechanistic target of rapamycin) (Johnson et al., 2013; Johnson et al., 2015; Sage-Schwaede et al., 2019). mTOR inhibitors have been shown to attenuate symptoms in a number of animal models of mitochondrial disease, including the *Ndufs4(KO)* model of LS, and recent clinical trials suggest the benefits may extend to humans (Sage-Schwaede et al., 2019; Johnson et al., 2019). Specifically, mTOR inhibitors have shown success in managing epilepsy in pre-clinical models and human trials, reducing both seizure frequency and duration (Sadowski et al., 2015; Curatolo and Moavero, 2013; Goldstein and Hauptman, 2021). However, these studies have largely focused on seizures related to dysfunction of the mTOR regulating tuberous sclerosis complex (TSC). The impact of mTOR inhibitors on epilepsies related to LS has not been studied.

mTOR inhibition and ketone metabolism are mechanistically linked; ketosis has been shown to inhibit mTOR (McDaniel et al., 2011), while hepatic mTOR activation impairs fasting induced ketogenesis (Sengupta et al., 2010). However, it is not known if chronic high-dose rapamycin leads to a ketotic state, or if there is mechanistic convergence between mTOR inhibition and a ketogenic diet in the setting of mitochondrial disease.

Here, we test the impact of dietary ketosis on survival and CNS disease in the *Ndufs4(KO)* mouse model of Leigh syndrome. We then examine the therapeutic potential of both dietary ketosis and mTOR inhibition on seizures arising from primary genetic mitochondrial dysfunction. For the latter, we utilize the nanoparticle-albumin bound rapamycin (Nab-rapamycin, ABI-009) formulation currently undergoing clinical study in the Rapamycin for Surgically Refractory Epilepsy (RaSuRE) trial (ClinicalTrials.gov Identifier: NCT03646240), which is testing this compound in intractable pediatric epilepsies of mixed etiology. These experiments provide critical new insight into the therapeutic potential of both ketogenic diet and mTOR inhibition in LS.

2. Methods

2.1. Ethics statement and animal use

All experiments were approved by the Institute Animal Care and Use Committee of Seattle Children's Research Institute (Seattle, WA) under protocols ACUC00611 (PI – Johnson) and ACUC000108 (PI - Kalume). Experiments utilize the *Ndufs4(KO)* strain, originally obtained from laboratory of Dr. Richard Palmiter at the University of Washington (Kruse et al., 2008). All treatment group assignments were random. Animal numbers for each dataset are noted in the associated figure legends, and wherever possible all individual datapoints are shown. *Ndufs4(KO)* mice were bred by heterozygous mating and genotyped using the Jackson laboratory PCR method. *Ndufs4* deletion is a recessive defect, and heterozygosity results in no reported or observed phenotypes, including no detectable defects in ETC CI activity, so controls for this dataset include both heterozygous and wildtype mice.

2.2. Animal diets

Breeders were fed PicoLab Lab Diet 5053, control-fed adult mice were fed PicoLab Diet 5058. Mice were gradually acclimated to the ketogenic diet (Envigo, Teklad TD.96335) starting at weaning (P21) using the following protocol: 3 days (starting at weaning) on a 50/50 mix (by weight) of ketogenic diet and ground normal mouse diet (PicoLab Diet 5058), followed by 3 days of 75/25 ketogenic/normal, then finally to 85/15 ketogenic/normal.

2.3. Point of care blood data

Point-of-care blood data (glucose and β-HB) was collected using a minimally invasive tail-prick method. Each datapoint represents one animal, with values shown the median of multiple samples taken throughout the respective time period (in general three times per week). All values were taken from ad-lib fed mice at approximately the same time of day (early afternoon) with no synchronization. Except where indicated otherwise, blood glucose was measured using a Prodigy Autocode glucose meter (product #51850–3,466,188), blood β-HB was assessed using a Precision Xtra XEGW044 meter with β-HB assay strips, and blood lactate was measured using Nova Biomedical assay meter (Product #40828).

2.4. ABI-009

Rapamycin was provided in the form of ABI-009 (nab-rapamycin), an albumin encapsulated water-soluble formulation. ABI-009 was provided by Aadi, LLC in lyophilized form. ABI-009 was resuspended to 1.2 mg/mL rapamycin in 1× PBS. This solution was sterile filtered and stored in aliquots at −80 degrees Celsius. ABI-009 was administered at 66 uL per 10 g for a final dose of 8 mg/kg/day rapamycin, as in our prior studies (Johnson et al., 2013; Johnson et al., 2015).

2.5. Rotarod and rotarod seizures

Rotarod assays were performed using a Med Associates Inc. ENV-571 M single lane rotarod with a black mat installed in the floor of the lane to reduce visibility of the bottom. Assays were performed by placing animals onto an already rotating rod and timing latency to fall, with a

steady rotation speed set to 6 rpm (controlled by attached laptop and Med Associates software). The maximum time of each trial was 10 min, with the trial ending at that time if mice were still on the rotarod. For each assay, three trials were performed, with a minimum of 5 min between each assay. The best of the three trials was reported for the rotarod data in Fig. 2.

Mice were monitored throughout each assay for seizure activity, and rotarod performance trials were ended if a seizure was observed. Mice were considered to be showing seizure activity if any symptoms on the Racine behavioral scale or Pinel and Rovner scale were observed: abnormal oroalimentary movements (dropping of the jaw repeatedly, atypical gnawing or chewing movements), repeat head nodding, anterior limb clonus (twitching/jumping while making no contact with the face), dorsal extension/rearing, loss of balance and falling (observable only after mouse has exited the rotarod), violently running/jumping ('popcorning').

While blinding was impossible (ketogenic diet results in severely greasy hair, while ABI-009 treatment leads to small body size), the experiments were scored by technicians with no expectations regarding the outcomes. Technicians trained together and cross-compared observed symptoms to ensure consistent scoring, and analysis of each treatment group was roughly evenly split among technicians involved in these studies to prevent any unaccounted-for observer bias from impacting any individual treatment group.

2.6. Heat-induced seizure assay

We performed the temperature induced seizure assay as previously reported (Bolea et al., 2019; Oakley et al., 2009). Briefly, rectal thermometers probes (Physitemp RET-5, attached to Physitemp TCAT-2DF monitor/controller) were gently, using aqueous lubricant, inserted rectally into experimental animals and secured to the tail with medical tape. Animals were placed in a small plexiglass chamber and baseline temperatures were recorded for 10–15 min following placement into the containment area to allow for stabilization. For all mice, body temperature was increased by 0.5 °C every 2 min until a seizure occurred or 42 °C was reached. After two minutes at 42 °C or immediately after a seizure, the heat lamp was removed, and a small fan was directed toward the subject. The mouse was returned to their cage once their temperature reached their original baseline or 37 °C, which ever was higher. Seizures were defined as described above.

2.7. Immune staining and microscopy

Brains were fixed for 48 h in 10% formalin at 4 °C. Following fixation, brains were moved to a cryoprotectant solution (30% sucrose, 1% DMSO, 100 μM glycine, 1× PBS, 0.45 μm filtered, pH 7.5), and stored for over 48 h, until the fixed tissues sank to the bottom. Tissues were then placed in OCT media (Tissue-Tek OCT compound, Sakura 0004348–01), frozen in cryoblock holders on dry ice, and stored at −80 until sectioned for staining. Cryoblocks were cut at 50 μm thickness using a Leica CM30505 cryostat set at −40 degrees C. Slices were moved to 1× PBS and stored at 4 degrees C until used for staining. Prior to staining, slices were mounted on slides and briefly dried to adhere.

Antibody staining was performed as follows: slides were first incubated in an antigen retrieval and permeabilization buffer (0.05% Triton X-100, 50 μM digitonin, 10 mM Tris-HCl, 1 mM EDTA, pH 9.0) at 60 °C overnight in a white-light LED illuminated box (to promote photobleaching of tissue autofluorescence). To reduce formaldehyde induced background fluorescence, slides were treated with sodium borohydride in ice cold PBS, added at 1 mg/mL immediately before incubation, on ice for 30 min, then moved to 10 mM glycine 1×PBS, pH 7.4, for 5 min at room temperature. Lipid background fluorescence was then blocked by incubating slides in 0.2 um filtered Sudan Black B solution (5 mg/mL in 70% ethanol) overnight at room temperature. Slides were then rinsed twice, 5 min each, in 1× PBS. Excess fluid was wiped from the slide, and the tissue was circled using Liquid Blocker PAP pen (Fisher Scientific, NC9827128) to hold staining solutions. Slides were blocked for 15 min at room temperature in 1× PBS with 10% rabbit serum (Gibco, 16,120–099) then stained overnight at 4° C in a mixture of rabbit anti-IBA1-fluorochrome 635 conjugated (WAKO 013–26,471) at 1:300, mouse anti-GFAP, Alexa555 conjugated (Cell Signaling, 3656S) at 1:300, and DAPI (Sigma, D9542) at 1 μg/mL. The following day, slides were washed 3 × 5 min in 1× PBS then mounted in aqueous mounting media (Fluoromount-G), coverslipped, and stored at 4° C protected from light until imaging.

Slices were imaged on a Zeiss LSM 710 confocal microscope. Images were collected using a 10× dry objective at 0.6× optical zoom, resulting in images of 1417 × 1417 μm in physical area. Images were collected with 8–16 line averages and a line scan speed of 6–7. Channels were set to an optical thickness of 50 nm. DAPI was excited at 405 nm, with emission light collected using a sliding filter with the range setting at 424–503 nm. GFAP-Alexa555 was excited with a 543 nm laser, emissions collected at 548–587 nm. Iba-1-635 was excited at 633 nm and emitted light was collected at 641–661 nm.

2.8. Surgery and EEG recordings

Mice underwent survival surgery to implant EEG electrodes under isoflurane general anesthesia with 1 mg/kg subcutaneous bupivacaine for analgesia, as previously described (Bolea et al., 2019). A midline incision was made anterior to posterior to expose the cranium using aseptic technique. EEG electrodes consist of micro-screws attached to a 130 μm diameter (bare, 180 μm coated) silver wire. Screw electrodes were placed through small cranial burr holes at visually identified locations in the left and right frontal cortex, approximately 1 mm anterior to bregma and 3 mm lateral the sagittal suture. A reference electrode was placed at the midline cerebellum, and a ground electrode was placed over the back subcutaneously. All electrodes connected to a micro-connector system with impedances typically <10 kΩ. Following electrode placement the skin was closed using sutures and mice were allowed to recover for 2–3 days.

Recordings were performed as described (Kalume et al., 2013). Simultaneous video-EEG recordings were collected in conscious mice on a PowerLab 8/35 data acquisition unit using Labchart 7.3.3 software (AD Instruments, Colorado Spring, Co). All bio-electrical signals were acquired at 1 KHz sampling rate. EEG signals were processed off-line with a 1–80 Hz bandpass filter and the EMG signals with a 3 Hz high-pass filter. Video-EEG-EMG data collected were analyzed using Labchart software.

2.9. Western blotting

Tissues were collected from control animals raised on control conditions, treated with ABI-009, or raised on a ketogenic diet as detailed above. Tissues were collected at P30. On the day of euthanasia, animals were fasted for four hours then re-fed for one hour to synchronize nutrient signaling status. Animals were euthanized by cervical dislocation and tissues flash-frozen in liquid nitrogen, followed by storage at −80 degrees C until used. Tissues were cryohomogenized using a Covaris cryohomogenizer according to manufacturer directions. 1× RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease and phosphatase inhibitors (ThermoScientific, cat. #A32959) was added while frozen. Samples were sonicated on ice until no tissue pieces were visible, then centrifuged for 10 min at 4 degrees C. Supernatant was moved to a new tube, and this step was repeated until no insoluble pellet was produced. s. At this point, an aliquot was removed for protein quantification via bicinchoninic acid (BCA) assay (ThermoFisher, cat. #23225, as per manufacturer protocol).

Protein samples were run on 26 well 4–12% Tris-EDTA NuPage gels with MOPS running buffer (ThermoFisher, NP0001). Equal protein as

loaded in each well in 1× protein loading dye (Licor, 4× Protein Loading Dye) with 50 mM TCEP (BondBreaker, ThermoFisher cat. # 77720), in a total volume of 15 μL. Samples were denatured prior to loading by heating to 95 degrees for 2 min. Blots were transferred to nitrocellulose membranes (BioRad, 1,704,271) using a BioRad Turbo-Transfer system set to manufacturer preset 'turbo' settings.

Following transfer, blots were briefly stained with Ponceau S (Sigma, P7170) to assess quality and protein loading. Blots were blocked in 10% milk in TBS with 0.05% tween-20 for 1 h, then primary antibody for 24–72 h. Phospho- S6 and actin antibodies were directly conjugated to HRP, whereas total S6 required incubation in a secondary antibody solution for 1 h at room temperature. Following 4–5 washes after antibody incubation, blots were briefly dried, saturated with ECL reagent (ThermoScientific cat # 32209), then exposed to film (ThermoScientific cat # 34089). Developed films were scanned and integrated densities of detected bands were quantified using ImageJ (https://imagej.net/software/fiji/).

Antibodies used for western blotting are as follows: mouse mAb to beta-actin-HRP conjugate - Abcam, cat. #ab49900, 1:5000 dilution; Rabbit mAb to p-S6 ribosomal protein (Ser235/236) – Cell Signaling, cat. #4858S, 1:1000 dilution; Rabbit mAb to p-S6 ribosomal protein (Ser240/244) – Cell Signaling, cat. #5364S, 1:1000 dilution; Mouse mAb to S6 ribosomal protein (total)-HRP conjugate – Cell Signaling, 14662S, 1:1000 dilution. Goat anti-rabbit IgG – HRP conjugate (secondary antibody) – ThermoScientific cat. #31460, 1:5000.

2.10. Statistical analyses

All statistical analyses were performed using GraphPad Prism as detailed in figure legends. Unless otherwise stated, error bars represent standard error of them mean (SEM), and $p < 0.05$ is considered statistically significant.

3. Results

3.1. Achieving ketosis in the Ndufs4(KO) mouse model of Leigh syndrome

In preliminary studies, we found that *Ndufs4(KO)* mice do not tolerate being placed on a full ketogenic diet at weaning; this led to rapid weight loss and necessitated a halt in treatment. To establish a ketogenic diet paradigm in this model, we used a tiered dietary modification program where animals were fed weight/weight mixtures of control chow and ketogenic diet chow (see ***Methods***) at 50%/50% ratio from post-natal day 21 (P21) to P24, 75%/25% (ketogenic diet to control diet) from P24 to P27, and an 85%/15% (ketogenic diet to control diet) ratio thereafter (see Fig. 1A–B), with blood beta-hydroxybutyrate (BHB) and glucose monitored by point-of-care measurement (see ***Methods***). *Ndufs4(KO)* animals showed robust ketosis on the 85% diet by the end of the ramp-up period (see Fig. 1C), so this ratio was used for our ketosis studies, and ketogenic diet treated animals were maintained on this chow for the duration of treatment after P27.

3.2. The effects of treatment with a ketogenic diet or mTOR inhibition on blood BHB and glucose

In the *Ndufs4(KO)* model, animals are overtly healthy until after weaning. Signs of disease including cachexia, ataxia, and forelimb clasping all develop shortly after P37. Disease progression is rapid, with 100% mortality in untreated animals by approximately P80 (see Figs. 1B and 2).

Treatment of *Ndufs4(KO)* mice with the mTOR inhibitor rapamycin has been shown to significantly attenuate disease; shifts in metabolism have been proposed to mediate the benefits, at least in part, and chronic mTOR inhibition leads to a shift away from glucose metabolism (Johnson et al., 2020a; Johnson et al., 2013). Accordingly, we expected a compensatory rise in BHB in ABI-009 (a water-soluble formulation of rapamycin, see ***Methods***) treated animals. In *Ndufs4(KO)* mice treated with a ketogenic diet, ketosis was maintained at early (P38-45) and late (P46-60) periods of disease (Fig. 1) but, to our surprise, BHB was not elevated at any age in *Ndufs4(KO)* mice treated with ABI-009, demonstrating that chronic mTOR inhibition does not lead to ketosis, and that ketosis does not play any role in mediating the benefits of mTOR inhibition (Fig. 1C–D and K–M).

A ketogenic diet can lead to lowered baseline blood glucose levels in some situations, such as in patients with metabolic syndrome or type-2 diabetes (Yuan et al., 2020; Gershuni et al., 2018). mTOR inhibition has been proposed to rescue disease by shifting tissue metabolism away from glucose utilization to other carbon sources (Johnson et al., 2013). We considered the possibility that mTOR inhibitors and a ketogenic diet converge at blood glucose levels. Accordingly, we compared blood glucose in control and *Ndufs4(KO)* mice in control, dietary ketosis, and ABI-009 treatment groups. We found blood glucose levels significantly decrease as a function of disease progression in untreated *Ndufs4(KO)* mice (Fig. 1E–F). Ketogenic diet and ABI-009 both lower glucose, but the impact of the two therapeutic strategies is distinct when examined longitudinally – a ketogenic diet reduces glucose at all ages, exacerbating disease-associated changes to blood glucose, while ABI-009 treatment leads to a more modest lowering of blood glucose but a prevention in disease-associated hypoglycemia during overt disease (see Fig. 1E–F and N–P).

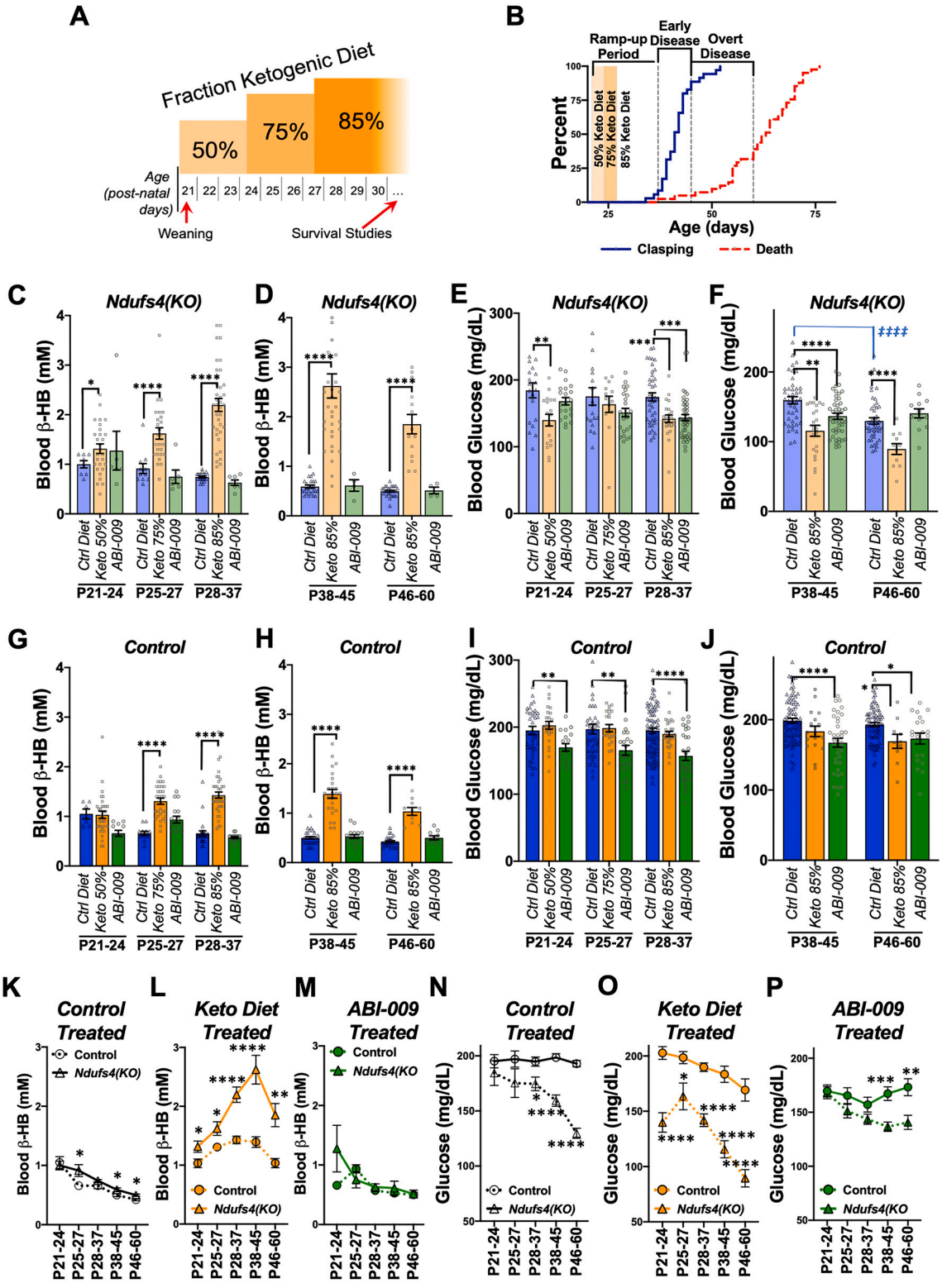
In order to uncouple the effects of these interventions from the progressive disease model we treated control mice with the ketogenic diet regimen or ABI-009. Intriguingly, the impact of the ketogenic diet on BHB and blood glucose was significantly more robust in the *Ndufs4 (KO)* mice compared to controls, indicating that they are metabolically primed for entering into a ketotic state (Fig. 1C–P). Control animals in dietary ketosis showed a very modest reduction in blood glucose only at the latest ages, while ABI-009 treatment significantly lowered blood glucose at all ages tested (Fig. 1I–J).

Comparing the trajectories of blood glucose and BHB in the *Ndufs4 (KO)* and control groups confirms that untreated *Ndufs4(KO)* mice become increasingly hypoglycemic as disease progresses, ketosis lowers glucose levels at all ages without altering the rate of decrease, and mTOR inhibition reduces glucose at all ages but appears to prevent disease-associated reductions (see Fig. 1K–P). Together, these data support the hypothesis that mTOR inhibition shifts metabolism from glucose, but also definitively show that mTOR inhibition does not lead to an induction of ketosis.

3.3. Dietary ketosis fails to attenuate disease in the Ndufs4(KO) model of Leigh syndrome

While our ketogenic diet approach was successful in leading to dietary ketosis, we found that the intervention had no impact on survival in the *Ndufs4(KO)* model (Fig. 2A – the previously published median survival for rapamycin treated animals shown for reference). The overall distribution of cause of death was also unchanged (Fig. 2B).

Additionally, ketosis failed to attenuate symptoms of disease in the *Ndufs4(KO)* model, including the onset of cachexia (Fig. 2C), the onset of neurologic symptoms including clasping (Fig. 2D), ataxia (Fig. 2E), and circling (Fig. 2F). Ketosis also failed to attenuate the progressive loss of ability to perform on the rotarod assay, which requires muscle function and motor coordination, providing an assessment of overall health status (Fig. 2G). Finally, neuroinflammatory lesions characterized by the accumulation of microglia and astrocytes were present in both untreated and ketogenic diet treated *Ndufs4(KO)* mice, consistent with the behavioral and lifespan findings (Fig. 2H). Together, these findings demonstrate that ketosis alone is insufficient to alter the course of disease in this mouse model of LS, while further demonstrating that ketosis cannot play a role the benefits of mTOR inhibition; mTOR inhibition through treatment with high-dose rapamycin significantly attenuates each of the outcomes measured here (see (Johnson et al., 2013; Johnson



(caption on next page)

Fig. 1. Implementation of a ketogenic diet in the Ndufs4(KO) mouse model of Leigh syndrome. (A) Ketogenic diet ramp-up strategy. Animals assigned to the ketogenic diet treatment were fed 50% ketogenic diet from weaning (P21) to P24, 75% ketogenic diet from P24-P27, and 85% ketogenic diet for the remaining duration of their lifespan (see Methods). (B) Diagram of the course of treatment, onset of neurological disease, and survival in untreated Ndufs4(KO) model, and the age bins used for analysis of blood glucose and beta-hydroxybutyrate (BHB) levels. Forelimb clasping (clasping) is one of the first visual signs of central nervous system degeneration in this mouse model. Mice are free from symptoms in the 'ramp-up' period. Animals begin to show signs of CNS disease but there is no significant mortality during the 'early-disease' period. By early in the 'overt-disease' period 100% of mice show overt signs of neurologic decline, and this period includes the first third of mortality in untreated animals. Animal to animal variability increases dramatically after the 'overt-disease' period. (C-D) Blood BHB in Ndufs4(KO) animals without intervention (Ctrl treated) or treated with a ketogenic diet or 8 mg/kg/day ABI-009 (rapamycin). (C) BHB levels in Ndufs4(KO) mice during the ketogenic diet 'ramp-up' ages. (D) Blood BHB in Ndufs4(KO) mice during early and late disease. (E-F) Blood glucose for Ndufs4(KO) mice without intervention (Ctrl treated) or treated with a ketogenic diet or 8 mg/kg/day ABI-009 (rapamycin). (E). Blood glucose levels in Ndufs4(KO) mice during the ketogenic diet 'ramp-up' ages. (D) Blood glucose in Ndufs4(KO) mice during early and late disease. (G-H) Blood BHB in control animals without intervention (Ctrl treated) or treated with a ketogenic diet or 8 mg/kg/day ABI-009 (rapamycin). (G) BHB levels in control mice during the ketogenic diet 'ramp-up' ages. (H) Blood BHB in control mice during ages associated with early and late disease in Ndufs4(KO) animals. (I-J) Blood glucose in control mice without intervention (Ctrl treated) or treated with a ketogenic diet or 8 mg/kg/day ABI-009 (rapamycin). (I). Blood glucose levels in control mice during the ketogenic diet 'ramp-up' ages. (J) Blood glucose in control mice during early and late disease. (K-P) Summary data from (C-J) plotted to show differences by genotype. (K) Blood BHB as a function of age in control treated control and Ndufs4(KO) mice. (L) Blood BHB as a function of age in ketogenic diet treated control and Ndufs4(KO) mice. (M) Blood BHB as a function of age in ABI-009 treated control and Ndufs4(KO) mice. (N) Blood glucose as a function of age in control treated control and Ndufs4(KO) mice. (O) Blood glucose as a function of age in ketogenic diet treated control and Ndufs4(KO) mice. (P) Blood glucose as a function of age in ABI-009 treated control and Ndufs4(KO) mice. (C-J) Individual datapoints represent biological replicates (individual animals, see Methods). (C-P) All error bars represent standard error of the mean (SEM). $*p \leq 0.05$, $***p \leq 0.0005$, $****p \leq 0.00005$ by Welch's unequal variances *t*-test. ‡‡‡‡ – $p < 0.00005$ P46-60 vs P38-45 Ndufs4(KO) Welch's unequal variances t-test. (K-P) Pairwise tests performed between control and Ndufs4(KO) mice at each age. All datapoints provided in supplemental data.

et al., 2015)). Notably, while others have suggested that ketosis reduces mTOR signaling (McDaniel et al., 2011), we found that dietary ketosis had no impact on mTOR signaling in mouse cortex, determined by western blotting for phosphorylation of ribosomal protein S6, a well-established downstream target of mTOR signaling (Fig. 2I). Conversely, ABI-009 treatment was associated with a complete depletion of S6 phosphorylation in cortex, as expected.

3.4. Electroencephalographic brain activity in the Ndufs4(KO)

To determine whether *Ndufs4(KO)* animals show seizure activity, as we have previously reported in mice with *Ndufs4* deleted only in GABAergic neurons, we measured electroencephalographic (EEG) brain activity in unstressed (in the absence of an overt epileptogenic stressor) conditions (Fig. 3). Electrographic spikes occurring without a seizure (interictal spikes) in EEG recordings are common in both normal and epileptic animals (Purtell et al., 2018), but increased frequency is a biomarker of a brain susceptibility to seizures. Electrographic spike events were seen in 60% of mutants compared to 40% of the controls in a small sample set (Fig. 3C). However, the frequency of spikes was strikingly and significantly elevated in mutants compared to controls (Fig. 3D).

3.5. Ketosis and mTOR inhibition significantly reduce exercise induced seizure frequency

As discussed, both ketosis and mTOR inhibition have been shown to provide benefits in at least some forms of seizures. To define the impact of dietary ketosis and mTOR inhibition on seizure risk in the *Ndufs4(KO)* model of LS, we first utilized data from the rotarod assay, which provides a model for exercise or stress induced seizures (see ***Methods***). Our protocol, optimized for detecting changes to health status in the *Ndufs4 (KO)* model, consists of three trials of up to 10 min each, at a constant steady low rotational speed of 6 rpm. In *Ndufs4(KO)* animals, we observe seizure activity in ~30% of untreated *Ndufs4(KO)* animals during this assay when performed at P30 (Fig. 4A), prior to the onset of symptoms related to degenerative CNS disease or the appearance of CNS lesions (see Figs. 1–2 and previous reports (Johnson et al., 2020a; Johnson et al., 2013; Johnson et al., 2015)).

We found that both a ketogenic diet and treatment with ABI-009 significantly reduced overall rotarod assay seizure incidence in the *Ndufs4(KO)* mice at P30 (Fig. 4A). Furthermore, in addition to overall seizure incidence, we assessed the probability of seizure activity as a function of trial time by summing the time spent on the rotarod over the course of the three trials (trials are ended if a seizure occurs, see ***Methods***). At P30, ketogenic diet and ABI-009 treatment both significantly reduced risk of seizures as a function of time on rotarod (Fig. 4B).

Repeating these experiments at P40, around the onset of overt symptoms (see Fig. 2C-G), we found that neither a ketogenic diet nor ABI-009 significantly reduced overall seizure frequency or probability of seizing on rotarod at this age (Fig. 4C–D), although a non-significant trend was observed with ABI-009 and a larger cohort of animals may reveal a modest effect (see ***Discussion***).

3.6. Temperature-induced seizures occur in the Ndufs4(KO) but are not attenuated by ketosis or ABI-009

Febrile seizures have been reported amongst mitochondrial disease patients (Saneto, 2017; Waruiru and Appleton, 2004). Recently, we demonstrated that disruption of mitochondria in GABAergic neurons results in sensitivity to temperature-induced seizures, while deletion in glutamatergic (VGlut2-Cre driven targeting) does not lead to seizure susceptibility in this setting (Bolea et al., 2019). Using the thermal seizure induction paradigm, we assessed thermal seizures in the whole-body *Ndufs4(KO)*, finding that these animals are susceptible to temperature induced seizures (Fig. 4E). EEG videography demonstrated that epileptic activity assessed by visual observation (clonic seizure) corresponds to epileptic activity in the electroencephalogram, with epileptic EEG activity beginning prior to, and continuing beyond, the visible seizure (Fig. 4F).

To assess whether ketosis or mTOR inhibition can attenuate temperature induced seizures in the *Ndufs4(KO)*, we tested treated animals in this paradigm. Neither intervention altered seizure frequency or temperature of onset in the face of this epileptogenic stress at the age tested (Fig. 4E).

4. Discussion

4.1. Therapeutic ketosis in mitochondrial disease

Here, we provide direct evidence that both a ketogenic diet and mTOR inhibition are viable therapeutic strategies for prevention of seizures arising from primary mitochondrial disease. We also demonstrate that ketosis alone fails to attenuate any other measured sequelae of disease in the mouse model for LS. While ketosis has been shown benefit mouse models of mitochondrial myopathy (Pitceathly and Viscomi, 2016; Ahola-Erkkila et al., 2010), and may have therapeutic value in other forms of mitochondrial disease, our findings demonstrate that ketogenic diets do not represent a panacea, as ketosis fails to alter the majority of disease sequelae in a model for the LS, which is the most

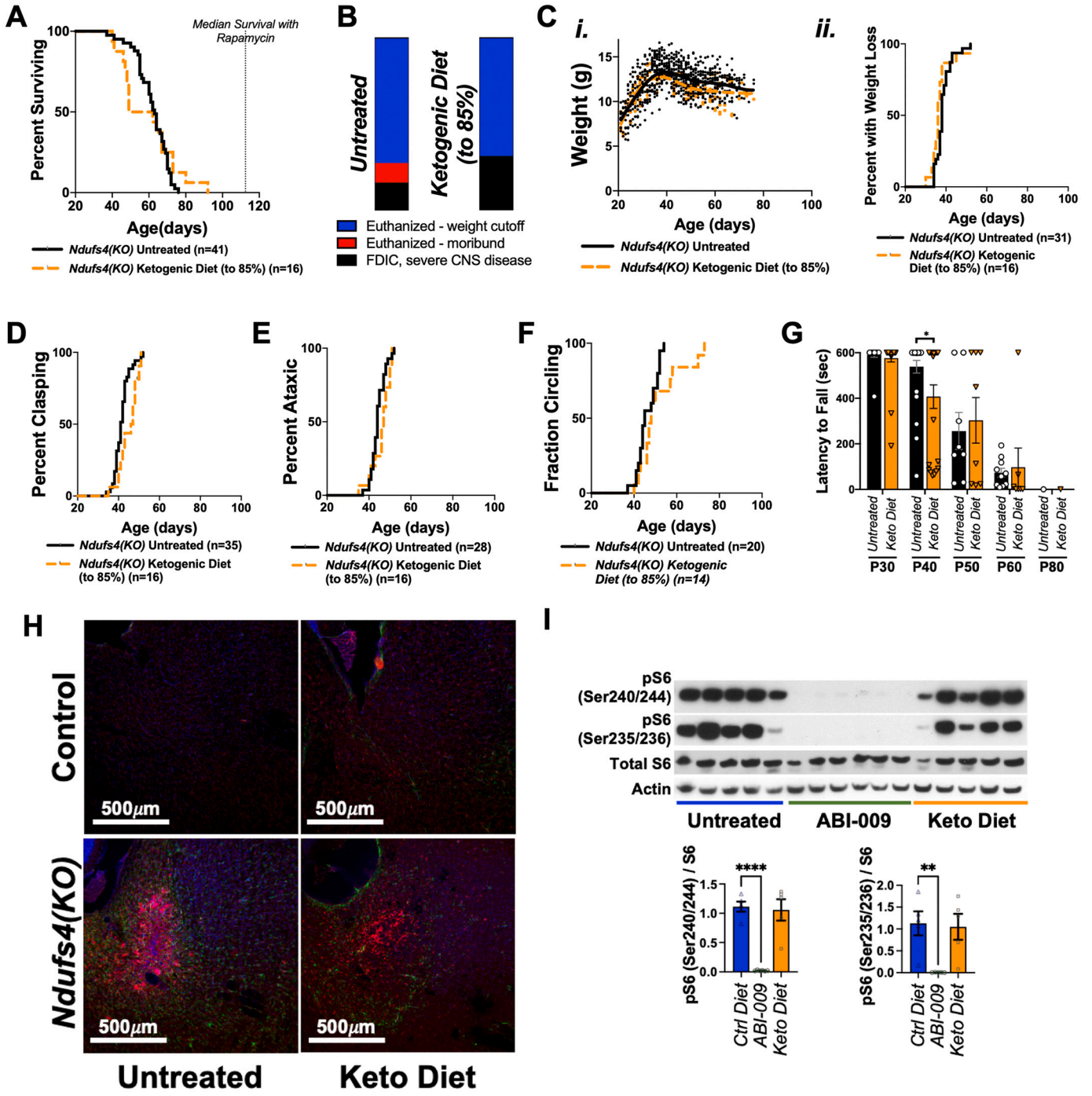


Fig. 2. Impact of ketogenic diet on survival and disease onset in *Ndufs4(KO)* mice. (A) Survival of *Ndufs4(KO)* mice fed either a standard mouse chow (untreated) or ketogenic diet. A ketogenic diet fails to extend survival in the *Ndufs4(KO)* model of Leigh syndrome. (B) Cause of death in *Ndufs4(KO)* mice fed either a standard mouse chow (untreated) or ketogenic diet. The majority of animals are euthanized upon reaching designated euthanasia criteria (see *Methods*). Animals fed a ketogenic diet were more likely to be found dead in cage (FDIC) than found euthanized due to moribund condition, but in both conditions the majority of mortality was the result of euthanasia from reaching the designated weight loss cutoff. (C) i. Weight plots of control fed and ketogenic diet fed *Ndufs4(KO)* mice. Local polynomial regression (LOWESS) shown for running average. ii. Onset of weight loss in control and ketogenic diet fed *Ndufs4(KO)* mice. A ketogenic diet did not impact the onset of weight loss. (D-F) Onset of clasping (D), ataxia (E), and circling (F) in control diet and ketogenic diet fed *Ndufs4(KO)* mice. A ketogenic diet did not impact any of these symptoms of CNS degeneration. (G) Rotarod performance of control diet and ketogenic diet fed *Ndufs4(KO)* mice as a function of post-natal age from post-natal day 30 (P30) to 80 (P80). Nearly 100% of *Ndufs4(KO)* animals perish by P80. Control diet fed *Ndufs4(KO)* mice show a progressive decline in performance on rotarod from P40-80. Ketogenic diet fed *Ndufs4(KO)* mice performed significantly worse than those on a control diet at P40, but performance was similar in both treatment groups at every other age. $*p < 0.05$ by Welch's unequal variances *t*-test. (H) Neuroinflammatory lesions were detected in both untreated and ketogenic diet treated *Ndufs4(KO)* mice by immunohistological staining for microglia and astrocytes. Images show representative staining at the site of brainstem lesions in the *Ndufs4(KO)* model. Red – Iba1, a microglia specific marker; green – GFAP, an astrocyte specific marker; blue – DAPI, a DNA co-staining dye. (I) Western blotting of cortex homogenates from control animals treated with ABI-009 or ketogenic diet probed for phosphorylation of ribosomal protein S6 at two established mTOR regulated sites. $**p < 0.005$, $****p < 0.00005$ by pairwise t-test. Comparisons between control and ketogenic diet treated animals were non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

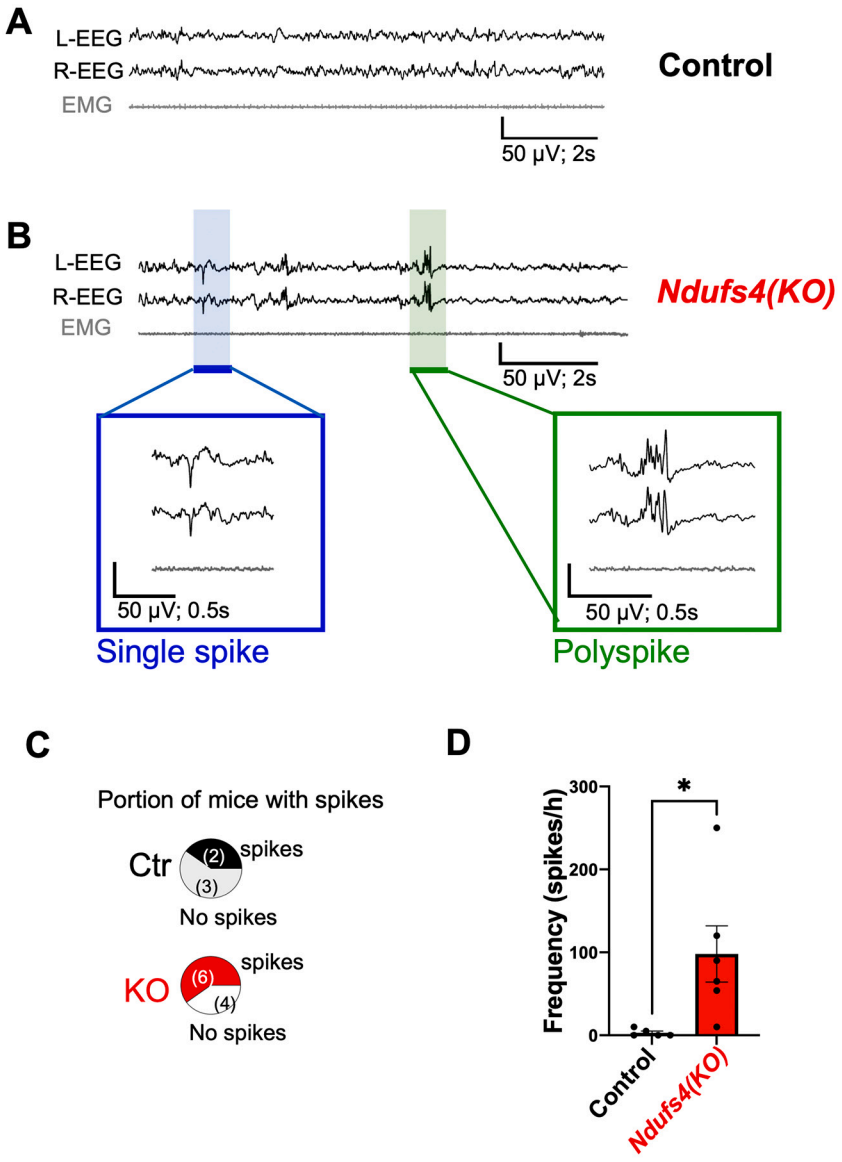


Fig. 3. Epileptic activity in the *Ndufs4(KO)* mouse. (A) Representative EEG recordings of control animals. (B) Representative EEG recordings of *Ndufs4(KO)* mice. Spontaneous interictal spikes presented in the *Ndufs4(KO)* in two forms, as either single spikes or as bursts of spikes (polyspikes). A representative EEG region with both forms is shown. (C) In the relatively small sample size assessed, there were no significant differences in the fraction of mice showing any spikes, however (D) interictal spike frequency was strikingly and significantly elevated in the *Ndufs4(KO)* animals compared to control mice. $*p < 0.05$ by pairwise t-test.

common form of pediatric mitochondrial disease. These findings are timely and important given the great deal of popular interest in the therapeutic potential of ketogenic diets, the current use among clinicians managing mitochondrial disease patients, and the ongoing excitement among scientific reviews (Pitceathly and Viscomi, 2016; Ahola-Erkkila et al., 2010; Konarikova et al., 2020; Verrotti et al., 2017; Branco et al., 2016; Wijburg et al., 1992; Kanabus et al., 2016; Laugel et al., 2007; Leung et al., 1998; Fraser et al., 2014).

Further defining the sequelae which are, and are not, attenuated by dietary ketosis will provide important information for clinicians, while clustering responsive and non-responsive symptoms may lead to a better understanding of the underlying pathogenesis of mitochondrial diseases. Clinical data is sparse, but little clinical evidence exists to support the use of ketogenic diets in treating mitochondrial disease. Our data here further indicates that benefits to overall mitochondrial disease progression may be limited. Greater evidence supports the use of ketogenic diets in intractable epilepsies, including those arising from primary mitochondrial dysfunction, but this setting is also complicated by the variable etiologies of individual patients (Paleologou et al., 2017; Neal et al., 2008). Perhaps unsurprisingly, ketogenic diets appear to most significantly benefit epilepsies arising from primary dysfunction of glucose metabolism, such as in patients with defects in glucose transporter 1, phosphofructokinase, or pyruvate dehydrogenase (PDH, which also causes LS) (Paleologou et al., 2017). It seems possible that disruption of glucose metabolism is the mechanistic point in which many diverse forms of intractable epilepsy, including mitochondrial disease, and ketogenic diets converge.

It is important to note that our study was designed to investigate the therapeutic potential of ketosis, not a specific ketogenic diet. While often regarded as a single strategy, ketogenic diets vary widely. We cannot rule out that some form of ketogenic diet may attenuate disease in the *Ndufs4(KO)* model; however, we can conclude that ketosis alone is insufficient.

It is intriguing to note that ketosis was more robust in the *Ndufs4 (KO)* mice than control mice fed the same diet, perhaps indicating that these animals are metabolically primed for ketosis. While the significance of this observation is unknown, it warrants further study.

4.2. *Evidence that mTOR inhibition and ketosis are mechanistically distinct*

The mechanisms underlying the benefits of mTOR inhibition in mitochondrial disease are unknown, but some evidence has supported a role for metabolic remodeling, including a shift away from glucose

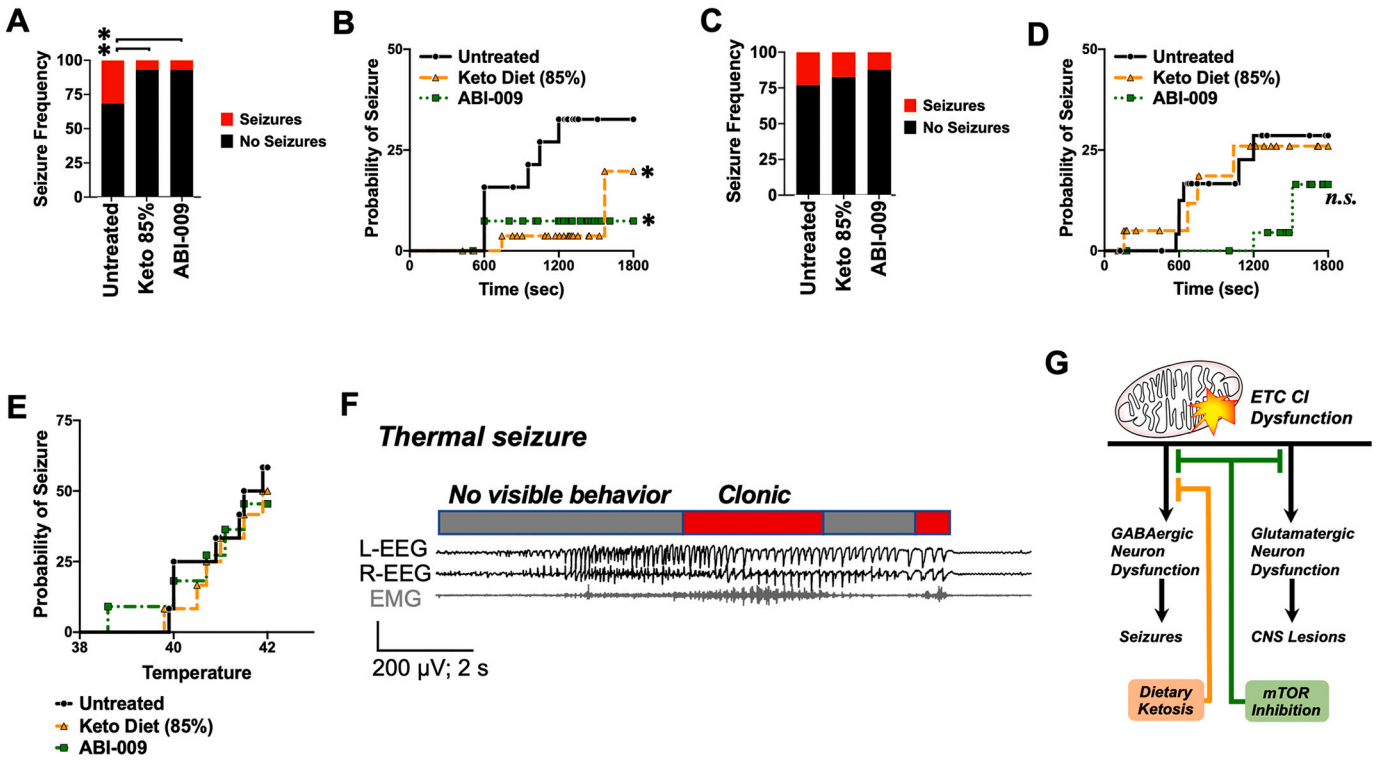


Fig. 4. Ketosis and mTOR inhibition significantly reduce seizure susceptibility of Ndufs4(KO) mice, but the benefits are restricted to young mice and an exercise-induced seizure model. (A) Exercise induced seizure incidence in P30 control treated Ndufs4(KO) mice or Ndufs4(KO) animals treated with 8 mg/kg/day rapamycin or ketogenic diet (Keto diet) (see Methods). $n = 19$, 29, and 28 animals in control ketogenic diet, and rapamycin treated conditions, respectively. Both rapamycin treatment and ketogenic diet significantly reduced seizure incidence at P30. *$p < 0.05$ by one-sided Fisher's exact test. (B) Rapamycin and ketogenic diet both significantly shifted the time to seizure in rotarod induced seizures. *$p < 0.05$ by Log-rank test. n = 19, 29, and 28 animals in control, ketogenic diet, and rapamycin treated conditions, respectively. (C) Seizure incidence was not significantly reduced by rapamycin or ketogenic diet at P40, early in disease onset. $n = 26$, 23, and 24 for control, ketogenic diet, and rapamycin, respectively. (D) Time to seizure was not significantly reduced by rapamycin or a ketogenic diet at P40, early in disease onset. n.s. – not significant. (E) Seizure onset and rate were not altered by rapamycin or a ketogenic diet in the temperature-sensitive seizure paradigm in P45 Ndufs4(KO) mice. $n = 12$, 12, and 11 for control, ketogenic diet, and rapamycin, respectively. (A-E) Control animals show no seizure activity in any of these assays. (F) Representative EEG recordings during a thermal-induced seizure with corresponding videographic analysis of behavior, annotated with red and grey bars above EEG lines. Behavioral seizure activity corresponds to electrographic seizure activity, while seizure activity is longer by EEG than visible clonic behavior. (G) A simplified model for the relationship between ketosis, mTOR inhibition, and disease pathogenesis in LS. Published data shows that ETC CI dysfunction leads to seizures through GABAergic neuron specific effects, whereas defective ETC CI in glutamatergic neurons drives lesions (see Discussion). In the simplest model, dietary ketosis benefits GABAergic, but not glutamatergic, neuronal dysfunction, while mTOR inhibition attenuates dysfunction in both sets of neurons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metabolism in peripheral tissues (Johnson et al., 2013). Here, we find that mTOR inhibition *does* lead to a significant decrease in average blood glucose in control and *Ndufs4(KO)* mice, but, notably, there is no concomitant induction of ketosis. In contrast, average blood glucose levels were reduced in ketogenic diet fed mice, but benefits of this intervention were limited to seizures. Together, these data indicate that neither an induction of ketosis or a reduction in blood glucose are sufficient to reproduce the benefits of mTOR inhibition on CNS lesions or lifespan in the *Ndufs4(KO)* model of Leigh syndrome.

In addition, while prior published studies have suggested that ketosis acts through modulation of mTOR, our data collected here indicate that dietary ketosis alone has no impact on cortical mTOR activity. Accordingly, while it is possible that these interventions converge on one or more downstream mechanistic targets, our results clearly reveal that ketosis and mTOR inhibition are largely mechanistically distinct.

4.3. Age and paradigm specific attenuation of seizures

mTOR inhibition and ketosis both significantly attenuated epileptic seizures in P30 animal in the rotarod-induced seizure assay, providing encouraging pre-clinical evidence supporting their use in the setting of primary mitochondrial disease. However, their failure to significantly modify seizures at older ages or in the temperature-induced seizure paradigm raise important questions. A simple explanation is that the relative epileptogenic stress of the rotarod assay increases between P30 and P40. Overall rotarod performance is reduced by P40 (Fig. 2G), consistent with this idea, but seizure frequency is not increased in control treated animals, and time to seizure onset is not shifted in control animals (Fig. 4B, D). The onset of CNS lesions is another possibility, i.e. overt CNS degeneration may worsen epileptic activity. The reduced efficacy of ABI-009 might suggest otherwise, as this treatment prevents lesions to a late age (see (Johnson et al., 2013; Johnson et al., 2015)).

Available data seems to suggest a more direct a developmental cause. CNS lesions do not develop in the GABAergic neuron specific *Ndufs4 (KO)* mice, yet lifespan limiting spontaneous seizures do not occur at high frequency until after ~P35. This is also the approximate age of CNS disease onset in the whole-body *Ndufs4(KO)* and VGlut2 specific *Ndufs4* knockout mice. We suspect that developmental events occurring around ~P35 precipitate lesions and significantly worsen seizure risk. It is worth noting that, in untreated knockout mice, hypoglycemia is only present after ~P35, and may indicate a metabolic state that exacerbates seizures. Metabolic derangement in older knockout animals may impede the efficacy of ketosis and ABI-009. Further exploration of these models will require substantial additional experimental effort, but may provide new insight into the origins of seizures in the setting of primary mitochondrial disease.

Similarly, it is possible that the failure of these interventions in the temperature-induced seizure paradigm simply indicate that they are only effective against relatively weak epileptogenic stressors, but they could also be the result of the age of animals tested (P45). Further studies are needed to test these possibilities. Testing additional seizure-inducing insults, and determining whether the benefits of mTOR inhibition and ketosis are additive, will provide additional insight into these questions.

4.4. Pathobiology of symptoms in Leigh syndrome and mechanisms of interventions

Experimental data from the *Ndufs4(KO)* model demonstrates that seizures are mechanistically distinct from CNS lesions: lesions, and associated symptoms, occur in mice with *Ndufs4* loss restricted to glutamatergic neurons, while seizures are restricted to mice with GABAergic loss of *Ndufs4* (Bolea et al., 2019; Johnson et al., 2020b). In light of this, the simplest interpretation of our findings is that mTOR inhibition and ketosis converge on attenuating GABAergic dysfunction, while ketosis provides no benefit to defects in glutamatergic neurons. Future studies aimed at assessing additivity may provide some insight, but our ability to test this model is ultimately limited by the lack of a clear mechanism for the benefits of either mTOR inhibition or ketosis. A dissection of the cell and molecular mechanisms underpinning the beneficial effects of these intervention strategies is badly needed. Our findings that ketosis attenuates seizures, but not other sequelae of LS, is further evidence that seizures in LS are mechanistically distinct from other sequelae. Further elucidation of the mechanisms underpinning the interventions we describe here may lead to a better understanding of the pathobiology of individual symptoms in LS.

Declaration of Competing Interest

None.

Acknowledgements

This work was funded by an NIH/GM R00 126147, an NIH/NS R01 102796, and seed funds provided by the Northwest Mitochondrial Research Guild.

We dedicate this study to Logan O'Connell and his family, and to all those living with the consequences of mitochondrial disease. They continue to inspire us.

References

Ahola-Erkkila, S., et al., 2010. Ketogenic diet slows down mitochondrial myopathy progression in mice. Hum. Mol. Genet. 19, 1974–1984.
Alston, C.L., Rocha, M.C., Lax, N.Z., Turnbull, D.M., Taylor, R.W., 2017. The genetics and pathology of mitochondrial disease. J. Pathol. 241, 236–250. PMC5215404.
Bolea, I., et al., 2019. Defined neuronal populations drive fatal phenotype in a mouse model of Leigh syndrome. Elife 8. PMC6731060.
Branco, A.F., et al., 2016. Ketogenic diets: from cancer to mitochondrial diseases and beyond. Eur. J. Clin. Investig. 46, 285–298.
Chinnery, P.F., 1993a. in *GeneReviews(R)*, R. A. Pagon *et al.*, Eds. (Seattle (WA)).
Chinnery, P.F., 1993b. in *GeneReviews((R))*, M. P. Adam *et al.*, Eds. (Seattle (WA)).
Curatolo, P., Moavero, R., 2013. mTOR inhibitors as a new therapeutic option for epilepsy. Expert. Rev. Neurother. 13, 627–638.
Desguerre, I., Hully, M., Rio, M., Nabbout, R., 2014. Mitochondrial disorders and epilepsy. Rev. Neurol. (Paris) 170, 375–380.
El-Hattab, A.W., Adesina, A.M., Jones, J., Scaglia, F., 2015. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. Mol. Genet. Metab. 116, 4–12.
Finsterer, J., Zarrouk Mahjoub, S., 2012. Epilepsy in mitochondrial disorders. Seizure 21, 316–321.
Fraser, J.L., et al., 2014. Thiamine pyrophosphokinase deficiency causes a Leigh Disease like phenotype in a sibling pair: identification through whole exome sequencing and management strategies. Mol. Genet. Metab. Rep. 1, 66–70. PMC5121315.
Gerards, M., Sallevelt, S.C., Smeets, H.J., 2016. Leigh syndrome: Resolving the clinical and genetic heterogeneity paves the way for treatment options. Mol. Genet. Metab. 117, 300–312.
Gershuni, V.M., Yan, S.L., Medici, V., 2018. Nutritional ketosis for weight management and reversal of metabolic syndrome. Curr. Nutr. Rep. 7, 97–106. PMC6472268.
Goldstein, H.E., Hauptman, J.S., 2021. The putative role of mTOR inhibitors in non-tuberous sclerosis complex-related epilepsy. Front. Neurol. 12, 639319. PMC7907183.
Gorman, G.S., et al., 2016. Mitochondrial diseases. Nat. Rev. Dis. Primers. 2, 16080.
Hilo, W., Jabaly-Habib, H., Modi, N., Briscoe, D., 2013. Leber's hereditary optic neuropathy. Harefuah **152**, 486–489, 498, 497.
Johnson, S.C., et al., 2013. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. Science 342, 1524–1528. PMC4055856.
Johnson, S.C., et al., 2015. Dose-dependent effects of mTOR inhibition on weight and mitochondrial disease in mice. Front. Genet. 6, 247. PMC4510413.
Johnson, S.C., et al., 2019. mTOR inhibitors may benefit kidney transplant recipients with mitochondrial diseases. Kidney Int. 95, 455–466.
Johnson, S.C., et al., 2020a. Regional metabolic signatures in the Ndufs4(KO) mouse brain implicate defective glutamate/alpha-ketoglutarate metabolism in mitochondrial disease. Mol. Genet. Metab. 130, 118–132. PMC7272141.
Johnson, S.C., et al., 2020b. Regional metabolic signatures in the Ndufs4(KO) mouse brain implicate defective glutamate/alpha-ketoglutarate metabolism in mitochondrial disease. Mol. Genet. Metab. 130 (2), 118–132.
Kalume, F., et al., 2013. Sudden unexpected death in a mouse model of Dravet syndrome. J. Clin. Invest. 123, 1798–1808. PMC3613924.
Kanabus, M., et al., 2016. The pleiotropic effects of decanoic acid treatment on mitochondrial function in fibroblasts from patients with complex I deficient Leigh syndrome. J. Inherit. Metab. Dis. 39, 415–426. PMC4851692.
Konarikova, E., Markovic, A., Korandova, Z., Houstek, J., Mracek, T., 2020. Current progress in the therapeutic options for mitochondrial disorders. Physiol. Res. 69, 967–994.
Kruse, S.E., et al., 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. Cell Metab. 7, 312–320. PMC2593686.
Laugel, V., et al., 2007. Early-onset ophthalmoplegia in Leigh-like syndrome due to NDUFV1 mutations. Pediatr. Neurol. 36, 54–57.
Lee, S., Na, J.H., Lee, Y.M., 2019. Epilepsy in leigh syndrome with mitochondrial DNA mutations. Front. Neurol. 10, 496. PMC6518976.
Leung, T.F., Hui, J., Yeung, W.L., Goh, K., 1998. A Chinese girl with Leigh syndrome: effect of botulinum toxin on dystonia. J. Paediatr. Child Health 34, 480–482.
McDaniel, S.S., Rensing, N.R., Thio, L.L., Yamada, K.A., Wong, M., 2011. The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. Epilepsia 52, e7–11. PMC3076631.
Neal, E.G., et al., 2008. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. Lancet Neurol. 7, 500–506.
Oakley, J.C., Kalume, F., Yu, F.H., Scheuer, T., Catterall, W.A., 2009. Temperature- and age-dependent seizures in a mouse model of severe myoclonic epilepsy in infancy. Proc. Natl. Acad. Sci. U. S. A. 106, 3994–3999. PMC2656193.
Paleologou, E., Ismayilova, N., Kinali, M., 2017. Use of the ketogenic diet to treat intractable epilepsy in mitochondrial disorders. J. Clin. Med. 6. **PMC5483866**.
Pitceathly, R.D., Viscomi, C., 2016. Effects of ketosis in mitochondrial myopathy: potential benefits of a mitotoxic diet. EMBO Mol. Med. 8, 1231–1233. PMC5090656.
Purtell, H., et al., 2018. Electrographic spikes are common in wildtype mice. Epilepsy Behav. 89, 94–98. PMC7325561.
Rahman, S., 2012. Mitochondrial disease and epilepsy. Dev. Med. Child Neurol. 54, 397–406.
Rahman, S., Thorburn, D., 1993. in *GeneReviews((R))*, M. P. Adam *et al.*, Eds. (Seattle (WA)).
Rahman, J., Noronha, A., Thiele, I., Rahman, S., 2017. Leigh map: A novel computational diagnostic resource for mitochondrial disease. Ann. Neurol. 81, 9–16. PMC5347854.
Ruhoy, I.S., Saneto, R.P., 2014. The genetics of Leigh syndrome and its implications for clinical practice and risk management. *Appl.* Clin. Genet. 7, 221–234. PMC4235479.
Sadowski, K., Kotulska-Jozwiak, K., Jozwiak, S., 2015. Role of mTOR inhibitors in epilepsy treatment. Pharmacol. Rep. 67, 636–646.
Sage-Schwaede, A., et al., 2019. Exploring mTOR inhibition as treatment for mitochondrial disease. Ann. Clin. Transl. Neurol. 6, 1877–1881. **PMC6764630**.
Saneto, R.P., 2017. Epilepsy and mitochondrial dysfunction:a single center's experience. J. Inborn Errors Metabol. Screen. 5, 2326409817733012.
Schubert Baldo, M., Vilarinho, L., 2020. Molecular basis of Leigh syndrome: a current look. Orphanet J. Rare Dis. 15, 31. **PMC6990539**.
Sengupta, S., Peterson, T.R., Laplante, M., Oh, S., Sabatini, D.M., 2010. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. Nature 468, 1100–1104.
Sofou, K., et al., 2014. A multicenter study on Leigh syndrome: disease course and predictors of survival. Orphanet J. Rare Dis. 9, 52. **PMC4021638**.
Tarnopolsky, M.A., 2008. The mitochondrial cocktail: rationale for combined nutraceutical therapy in mitochondrial cytopathies. Adv. Drug Deliv. Rev. 60, 1561–1567.
Thorburn, D.R., Rahman, J., Rahman, S., 1993. in *GeneReviews((R))*, M. P. Adam *et al.*, Eds. (Seattle (WA)).
Verrotti, A., Iapadre, G., Pisano, S., Coppola, G., 2017. Ketogenic diet and childhood neurological disorders other than epilepsy: an overview. Expert. Rev. Neurother. 17, 461–473.

Viscomi, C., Bottani, E., Zeviani, M., 2015. Emerging concepts in the therapy of mitochondrial disease. Biochim. Biophys. Acta 1847, 544–557.
Waruiru, C., Appleton, R., 2004. Febrile seizures: an update. Arch. Dis. Child. 89, 751–756. PMC1720014.
Wijburg, F.A., et al., 1992. Leigh syndrome associated with a deficiency of the pyruvate dehydrogenase complex: results of treatment with a ketogenic diet. Neuropediatrics 23, 147–152.
Yuan, X., et al., 2020. Effect of the ketogenic diet on glycemic control, insulin resistance, and lipid metabolism in patients with T2DM: a systematic review and meta-analysis. Nutr. Diabet. 10, 38. **PMC7705738**.
Yu-Wai-Man, P., Chinnery, P.F., 1993. in *GeneReviews((R))*, M. P. Adam *et al.*, Eds. (Seattle (WA)).