Protective effects of MCT diet in a mouse model of Dravet syndrome

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ABSTRACT

Objective: Dravet syndrome (DS) is a severe developmental and epileptic encephalopathy with early childhood onset. Patients with DS do not respond well to antiepileptic drugs and have only a few treatment options available. Here, we evaluated the effect of medium chain triglyceride (MCT) diet therapy in a mouse model of DS.

Methods: Scn1a<sup>R1407X/+</sup> DS mice were given diets supplemented with MCTs with varying ratios of decanoic (C10) and octanoic (C8) acid or a control diet for four weeks. Video monitoring was performed to evaluate spontaneous convulsive seizure frequency. Susceptibility to hyperthermia induced seizures was also examined. Medium-chain fatty acids, mitochondrial and antioxidant markers were assessed in brain homogenate.

Results: Dietary intervention with MCTs significantly prolonged survival and reduced convulsive seizure frequency during the critical period of highest seizure occurrence in the Scn1a<sup>R1407X/+</sup> DS mice. Moreover, MCT diet therapy showed protective effects against hyperthermia-induced seizures. We demonstrated that co-administration of C10/C8 was effective at reducing both seizures and mortality, whereas C10 alone only reduced mortality, suggesting that the ratio of C10 to C8 in the MCT is an important factor for efficacy. When C10 and C8 are supplemented at an 80:20 ratio in the diet, C10 accumulates in the brain in high enough concentrations to enhance brain energy metabolism by both stimulating mitochondrial enrichment and increasing its antioxidant status.

Significance: The results from this study indicate that MCT diet therapy may provide therapeutic benefits in DS. Future clinical studies would elucidate whether these positive effects are mirrored in human patients.
Key words
Epilepsy, Dravet syndrome, MCT, octanoic acid, decanoic acid, seizure reduction

Key Points

- MCT dietary intervention prolongs survival and reduces seizure frequency during the critical period of highest seizure occurrence in the $Scn1a^{R1407X/+}$ mice
- MCT dietary intervention has protective effects against hyperthermia-induced seizures in $Scn1a^{R1407X/+}$ mice
- MCT supplementation leads to increased levels of C10 in plasma and brain of $Scn1a^{R1407X/+}$ mice
- MCT supplementation appears to stimulate mitochondrial enrichment and enhanced antioxidant status, providing potential mechanisms of seizure relief
INTRODUCTION

Dravet syndrome (DS) is a drug-resistant epilepsy condition affecting approximately 1:15700 people in America,\(^1\) characterized by generalized tonic-clonic seizures that start within the first year of life, either spontaneously or as part of a febrile event. DS is also associated with developmental delay, speech impairment ataxia and other health problems. The mortality rate in DS is \(~5\)% by adulthood.\(^2\) Variants in SCN1A gene account for \(~80\)% of DS cases.\(^3\) SCN1A encodes the Nav1.1 protein, a voltage-gated sodium channel important for action potential initiation in GABAergic neurons.\(^4\) The Scn1a\(^R1407X/\) mouse model is based on a human DS variant and recapitulates many DS features including spontaneous seizures, increased febrile seizure susceptibility and early death.\(^5\) This makes it a strong preclinical model for assessing potential therapeutic strategies in DS.

Treatment of drug-resistant epilepsy syndromes, including DS, is difficult. First-line treatments (e.g. valproate and clobazam) aim at limiting seizure frequency.\(^6,7\) However, these therapies often fail to control seizures. After a three to four antiepileptic drugs have failed to attenuate seizure activity, second line treatments, such as the ketogenic diet (KD) and its variations, are utilized.\(^8,9\) The medium chain triglyceride (MCT) diet is an alternative therapy to the classic KD. Both the MCT and classic KD have similar efficacy in terms of seizure reduction, but the MCT diet allows for more protein and carbohydrate calories, leading to fewer gastrointestinal side-effects and better compliance.\(^10\) Previous research has assessed the efficacy of the MCT diet on drug-resistant epilepsies, including DS, reporting seizure reduction in 23% of patients after 6 months. An even higher efficacy was observed in patients with
cluster seizures (>5 seizures in a short period of time) where 67% reported seizure reduction.\textsuperscript{11}

Though it is well documented that the KD is an effective management option for drug-resistant epilepsies, its seizure reducing mechanisms remain unclear. It was thought that ketone bodies (KB) play a role in seizure reduction, however plasma KB levels correlate poorly with seizure control.\textsuperscript{12} There is growing evidence suggesting that medium chain fatty acids (MCFA), specifically decanoic acid (C10) and octanoic acid (C8), play an important role in energy metabolism and the consequent reduction of seizures seen in drug-resistant epilepsy patients receiving the MCT diet. Recent \textit{in vitro} studies showed that MCFA, in particular C10, positively influence neuronal energy metabolism, through targeting the nuclear receptor PPAR$\gamma$,\textsuperscript{13} and antioxidant status.\textsuperscript{14} Furthermore, C10 was shown to inhibit the AMPA receptor providing another anti-seizure mechanism.\textsuperscript{15} Although C8 does not have a comparable effect upon energy metabolism or the AMPA receptor,\textsuperscript{13,15} it may exert a “sparing” effect, preventing oxidation of C10 in the brain thereby increasing its availability.\textsuperscript{16}

To date, there is little information on the effects of dietary intervention with C10 on seizure frequency and biochemical changes \textit{in vivo}. We aimed to investigate the effects of diet supplementation with MCTs on convulsive seizure frequency and mortality in the $Scn1a^{R1407X/+}$ mouse model of DS. In addition, due to the reported effects of C10 on mitochondrial content, respiratory chain activity and antioxidant status \textit{in vitro}, these were evaluated along with brain levels of C10, C8 and the ketone body $\beta$-hydroxybutyrate.
2 MATERIALS AND METHODS

2.1 Customized mouse diet containing C10 and C8 fatty acids

The following customized mouse chow containing C10 and C8 MCTs in different ratios were used (Specialty Feeds, Glen Forrest, WA): a) normal mouse diet + C10 (C10), based on conditions utilized in previous *in vitro* studies;\textsuperscript{13,14} b) normal mouse diet + C10 and C8 in an 80:20 ratio (C10/C8-80:20), derived from prior *in vitro* study that suggests a small amount of C8 exerts a protective effect on C10 levels;\textsuperscript{16} c) normal mouse diet + C10 and C8 in a 40:60 ratio (C10/C8-40:60), a condition similar to that in a standard MCT diet.\textsuperscript{10} No group received C8 alone as *in vitro* work has consistently shown no significant alterations in cellular metabolism or seizure models following C8 treatment.\textsuperscript{13,14,15} Normal mouse chow was used as a control (Control). The supplements in diets (a-c) comprised 35% of dietary calories.

2.2 Animals

All animal experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Experiments were performed using both male and female heterozygous $\text{Scn1a}^{R1407X/+}$ DS mice maintained on the 129S1/SvImJ background and were backcrossed onto C57BL/6J (N1) for experimentation. Mice were housed under standard laboratory conditions in a temperature-controlled environment with 12-h light/dark cycle and had access to food and water *ad libitum.*
Mice were randomly assigned to different customized diet groups at post-natal day 18 (P18), and remained on the same diet for 4 weeks, after which they were tested for febrile seizure susceptibility.

Body weights were measured on a weekly basis, and at the end of the study, brains were collected and fresh frozen on liquid nitrogen.

2.3 Spontaneous convulsive seizure frequency monitoring
To examine the effect of MCT diets on spontaneous seizure frequency and behaviour, Scn1a<sup>R1407X+/+</sup> mice (P18) were randomly assigned to mouse chow containing C10 and C8 or a normal mouse diet as a control and placed in transparent plexiglass cages. Video monitoring was done at room temperature for four weeks using a Vivotek video server (VS8102) connected to an infrared day and night digital color camera (EVO2; Pacific Communications). Seizure classification was based on the revised Racine scale and only seizures over Racine score 4 were noted. Score 5 represents generalised tonic-clonic seizure with loss of postural tone, jumping and hind limb extension.<sup>17</sup> The mouse chow was colour coded, therefore the person analysing the videos was not blinded to the treatment. All video recordings were analysed by a trained animal behavioral specialist experienced in the identification and scoring of seizures. In a subset of videos, detection was performed using an automated motion detection custom Matlab script confirming visually identified seizures. The averaged group seizure across cohorts was calculated by dividing the total number of seizures in a treatment group over the number of mice.
2.4 Hyperthermia-induced seizures

After four weeks of MCT-enriched diets administration, the susceptibility to hyperthermia-induced seizures was tested using the thermogenic assay by placing Scn1a^{R1407X/+} mice (P46) in a double walled metal chamber slowly heated from below to constant 42°C. A thick layer of paper was placed on the inner wall of the chamber to prevent the mice from direct contact with the metal. The chamber was covered with a transparent plexiglass lid. The time to first tonic-clonic seizure was recorded and that was considered the experimental end point. WT mice were also used as a control. The effect of C10/C8-80:20 diet on internal body temperature was assessed in a separate cohort of WT mice after 1 week on diet therapy.

2.5 Brain homogenate preparation

Mouse brains were weighed and homogenized 1:10 (w/v) in phosphate buffered saline, 1mM EDTA and 5mM Tris, pH 7.4 using a glass homogenizer.

2.6 Analysis of medium chain fatty acids and β-hydroxybutyrate in brain homogenate

100µL brain homogenate were used to analyse MCFA content, and 50µL used to analyse BOHB via GC-MS, as in 18. Further detail available in supplementary material.

2.7 Citrate synthase, relative mtDNA content and respiratory chain enzymes

Citrate synthase (CS), complexes I, II-III and IV were measured as described previously,19 using a Uvikon 922 spectrophotometer at 30°C. Further detail available in supplementary material. Protein content of mouse brain homogenate was analyzed using a modified Lowry assay.20 Mt-co3 and Gapdh in genomic DNA were analysed
as in Supplementary Material, relative mtDNA content was calculated using the ΔΔCT method. Statistical significance was calculated utilizing ΔCt data.

2.9 Reduced Glutathione and Catalase

Reduced glutathione (GSH) was determined using electrochemical HPLC as detailed in 21. Catalase (CAT) activity was determined fluorometrically as in 22. Further detail available in supplementary material.

2.10 Reagents

TRIzol reagent, Amplex Red and Desalting columns were from Thermo-Fisher Scientific (Loughborough, UK). iQ SYBR Green Supermix was from Bio-Rad Laboratories inc (Hertfordshire, UK). MCT oils were provided by Vitaflo, part of the Nestlé Health Science group. Further details available in supplementary material.

2.11 Statistical analysis

Data are presented either as mean±standard error (SEM) or ± standard deviation (SD). Survival curves were analyzed using the Mantel-Cox method (GraphPad Prism 8). Statistical comparisons between more than two groups were carried out using a One-way ANOVA, followed by Tukey’s Post-hoc test. Analysis for time to hyperaemia induced seizure was completed using one-way ANOVA followed by a Dunnett’s multiple comparison test. Two-way ANOVA followed by Tukey’s post hoc analysis was used for analysing the spontaneous seizure frequency. Results were statistically significant at p<0.05, with a minimum sample size (n) of 6 per test group.
3 RESULTS

Scn1a<sup>R1407X/+</sup> mice fed with C10 and C8 MCTs exhibited normal body weights that progressively increased with time (n=8–12 per group). There were no significant differences in body weight between the mice on control diet and mice on C10 only, C10/C8-80:20 or C10/C8-40:60 during the four weeks of experimentation (Figure 1A).

3.1 Therapy with chronic MCT diets promotes survival and reduces spontaneous convulsive seizure frequency in Scn1a<sup>R1407X/+</sup> mice

Survival of DS mice was significantly reduced when compared to wild type (WT) littermates, with highest mortality between P18 and P26. To test whether chronic treatment with MCT diets would promote survival, Scn1a<sup>R1407X/+</sup> mice were given chow containing C10 (n=26), C10/C8-80:20 (n=28) and C10/C8-40:60 (n=27) from P18, and survival was monitored until P46. Normal chow was used as control (n=34). Early mortality of DS mice on a control diet was highest between P20 and P28, with survival rate of 50% at P46. MCT diets significantly extended survival in Scn1a<sup>R1407X/+</sup> mice, with 82% of C10/C8-80:20, 81% of C10/C8-40:60 and 80% of C10 treated mice surviving to P46 (**P < 0.01) (Figure 1B).

As MCT therapy improved survival, we sought to determine if this correlated with decreased spontaneous convulsive seizure frequency. DS mice develop spontaneous seizures as early as P16 that become much more frequent between P18-P26 (Figure 1C; Figure S1). Video analysis revealed that all deaths are associated with a convulsive seizure. Seizure frequency peaked at P23 for Scn1a<sup>R1407X/+</sup> mice on a control diet. Interestingly, in the C10 group, seizure frequency remained high before falling after P25 (Figure 1C). This is surprising given the improved survival noted on C10 (Figure 1B). In contrast, mice treated with C10/C8-80:20 and C10/C8-40:60 had
significantly reduced seizure frequency during the critical time window of highest seizure incidence, which correlated with decreased mortality (Figure 1C).

3.2 Therapy with chronic MCT diets reduces febrile seizure susceptibility in Scn1a\textsuperscript{R1407X/+} mice

Scn1a\textsuperscript{R1407X/+} mice recapitulate the induction of seizures by elevated body temperature observed in patients with DS. To test susceptibility to hyperthermia-induced seizures in Scn1a\textsuperscript{R1407X/+} mice treated with MCT diets for four weeks, we subjected mice on P46 to a constant 42°C environment and measured the latency to the first tonic-clonic seizure as described previously.\textsuperscript{23} All DS mice developed seizures in this assay. While all MCT diets prolonged the latency to seizure onset when compared to the control (Figure 1D), C10/C8-80:20 and C10/C8-40:60 (***p<0.0001 Log-rank test) were more effective than C10 alone in reducing hyperthermia-induced seizure susceptibility in Scn1a\textsuperscript{R1407X/+} (**p<0.008 Log-rank test). Similarly, average time to clonic-tonic seizure was significantly prolonged in mice on the diets (8.71±0.59; p=0.0474-C10, 9.03±0.74; p=0.0006-C10/C8-80:20 and 9.53±0.54; p<0.0001-C10/C8-40:60) when compared to the control (8.19±0.49). The age matched WT mice did not have febrile seizure phenotype. We used C10/C8-80:20 diet in a separate cohort of mice to show that the MCT administration does not alter the basal body temperature when compared to the control diet in these experiments (Figure S2).

3.3 Accumulation of medium chain fatty acids and β-hydroxybutyrate in mouse brain following MCT diets

There were no significant differences in C8 concentration in control diet, C10/C8-80:20 or C10/C8-40:60 diet mouse brain. However, there was a highly significant decrease
in C8 levels in the brains of the C10 only diet compared to control diet (Figure 2A). C10 was accumulate dramatically in the C10/C8-80:20, C10/C8-40:60 and C10 only diet mice compared to control diet (Figure 2B). Although it has been suggested that ketone body accumulation may bring about a reduction in seizures during the MCT diet therapy, we found there to be no difference in brain BOHB levels between diet groups; BOHB appeared elevated in the C10 only group, but this was not significantly different from control diet mice (p=0.09, Figure 2C).

3.4 Effect of MCT diet on mitochondrial respiratory chain and content in mouse brain

The activity of citrate synthase (CS), a well-established biomarker of mitochondrial content,\textsuperscript{24,25} was significantly increased in brains of mice fed the C10/C8-80:20 diet, however, there were no alterations in C10/C8-40:60 or C10 only diet mice (Figure 3A). The C10/C8-80:20 diet was the only diet associated with a significant increase in mtDNA relative to nuclear DNA (Figure 3B). Similarly, the C10/C8-80:20 diet was associated with significant increases in complex I activity, where the C10/C8-40:60 and C10 only diets lead to increased complex II+III activity (Figure 3C-D). There was no alteration in complex IV activity in any treatment group (Figure 3E). Interestingly, when the activities of the complexes were expressed as a ratio to CS, there was no significant alterations in activity of any of the complexes in the C10/C8-80:20 diet mice (Figure 3F-H). However, the increased activity of complex II+III observed in the C10/C8-40:60 and C10 only diet mice was also associated with an increase in the ratio to CS (Figure 3G).
3.5 Effect of MCT diets on antioxidant markers in mouse brain

Alterations in mitochondrial respiratory chain enzymes have been associated with increased reactive oxygen species (ROS) production and cellular damage. Furthermore, improved antioxidant status has been associated with KDs. We therefore analysed two important antioxidants–GSH and CAT–to assess the antioxidant status of the mouse brains following each MCT diet. We found that only the C10/C8-80:20 MCT diet led to a significant increase in GSH levels (Figure 4A). In contrast, both the C10/C8-40:60 and C10 only diets were associated with highly significant increases in CAT activity, where the C10/C8-80:20 diet was not (Figure 4B).

4 DISCUSSION

KDs have long been utilized as a therapeutic strategy for patients with drug-resistant epilepsy, including DS. However, the mechanisms underlying the therapeutic benefits remain unclear. Previous work suggested that the MCFA components of the diet, in particular C10, play a key role in the beneficial effects associated with the diet. Here we investigated whether diet supplementation with varying ratios of C10 and C8-containing MCTs was associated with prolonged survival, convulsive seizure reduction and biochemical changes in a mouse model of DS.

The MCT diets used in this study were well tolerated by the DS mice and did not induce adverse effects. While it was previously reported that MCT diets can influence body weight and fat mass, we did not observe any differences to the control. This may be related to inclusion of proteins and carbohydrates to create a more balanced diet, along with the ability of the mice to adapt to the new metabolic conditions.
Scn1a<sup>R1407X</sup> mice recapitulate many DS clinical symptoms, including spontaneous seizures and premature mortality.<sup>5</sup> We demonstrated that chronic administration of MCT diet with C10 and C8 fatty acids is associated with prolonged survival and reduced spontaneous convulsive seizure frequency in DS mice. Importantly, we observed that the ratio of C8 to C10 in the diet is a key factor for efficacy, as diets containing C10/C8-80:20 or C10/C8-40:60 were more efficacious than C10 alone in reducing seizures. Interestingly, although C10 only diet did not reduce seizure frequency during the first few days post-administration, survival was still extended, suggesting that C10 can reduce premature mortality by mechanisms not directly associated with anticonvulsant effects. This is consistent with a recent study evaluating the effects of a long-term intervention with KD on mortality and seizure frequency in the same mouse model.<sup>32</sup> Specifically, chronic treatment with KD was able to extend survival in the mice but failed to reduce seizure frequency and severity. Our data indicate that C10/C8 MCT therapy might be an alternative approach for management of DS not only by reducing seizures but also involving mechanisms that go beyond seizure control.

The anticonvulsant effects of C8 and C10 have been previously tested in multiple models of experimental seizures.<sup>33,34</sup> These studies reported that C8 elevates the seizure thresholds for pentylenetetrazole (PTZ)-induced myoclonic and clonic convulsions as well as 6-Hz seizure test. Similarly, C10 exerted anticonvulsant properties by increasing seizure thresholds in the 6-Hz and maximal electroshock seizure tests but was ineffective against PTZ-induced seizures. Here we showed that the susceptibility to hyperthermia-induced seizures in DS mice was reduced by all MCT diets tested, but co-administration of C8 and C10-containing triglycerides
enhanced anticonvulsant properties over C10 alone. These divergent anticonvulsant profiles suggest C8 and C10 may act differently biochemically.

Though \textit{in vitro} experiments have suggested that C10 may elicit a biological response in neuronal cells, including altering mitochondrial content and targeting the AMPA receptor\textsuperscript{13,15}, there are little data available to indicate whether brain accumulation is sufficient to have any effect. In view of this, we aimed to assess accumulation of C8, C10 and BOHB in brains of MCT-supplemented \textit{Scn1a}^R1407X/+ mice.

MCFA accumulation differed greatly between treatment groups. C8 levels in brain remained similar to normal diet mice in the C10/C8-80:20 and C10/C8-40:60-diet mice. There was a highly significant depletion of C8 levels in C10 only diet mice, relative to normal diet mice. C10 levels were increased in all MCT diet groups, with the C10/C8-80:20-diet mice having the highest levels. Previous work\textsuperscript{16} has shown that C8 is preferentially oxidized in the place of C10, which may allow sufficient C10 accumulation to exert biological action(s), such as interaction with AMPA receptors\textsuperscript{15} and activation of PPAR\textsubscript{γ}.\textsuperscript{35,13} In the C10 only diet, β-oxidation of C10 may be increased due to the absence of exogenous C8, limiting accumulation.

Movement of MCFA across the blood brain barrier may involve active transport,\textsuperscript{36,37} which may explain our findings. It has been reported that patients taking a traditional MCT diet as epilepsy therapy have increased plasma KB, but this correlates poorly with seizure reduction.\textsuperscript{38} Although there was a non-significant increase in the C10 only diet group, we found no differences in brain BOHB in any of the treatment group mice,
relative to normal diet mice, suggesting that seizure reduction occurs through a ketone-body independent mechanism.

Alterations to mitochondrial metabolism is commonly associated with the progression of many epilepsy syndromes and has been linked to DS pathophysiology.\(^{39}\) Although in this study we did not compare mitochondrial content of \(\text{Scn1a}^{R1407X/+}\) mice with wild-type mice, a recently published study presented metabolomic data strongly suggestive of impaired mitochondrial function in the \(\text{Scn1a}^{R1407X/+}\) mice.\(^{40}\) Enhanced energy metabolism through mitochondrial biogenesis has been suggested as an anticonvulsant mechanism using the classic KD,\(^{41}\) and an \textit{in vitro} study has suggested C10 may be effective through a similar mechanism.\(^{13}\) We assessed several mitochondrial markers to determine whether MCT supplementation stimulated similar alterations to previous \textit{in vitro} findings. We observed a significant increase in CS activity in the brains of the C10/C8-80:20 diet mice only, compared to normal diet mice, suggesting higher mitochondrial content.\(^{24,25}\) This increase in CS activity, supported by an increase in relative mtDNA content, suggests an increase in mitochondrial content in the brains of C10/C8-80:20 diet mice. We also observe alterations in the mitochondrial respiratory chain complexes, with the C10/C8-80:20 diet associated with an increase in complex I activity, and the C10/C8-40:60 and C10 only diets associated with increases in complex II+III activity. Impairment of the mitochondrial respiratory chain complexes, complex I in particular, has been associated with the propagation of seizure activity.\(^{42}\) This dysfunction of complex I has been suggested to lead to the generation of ROS and epileptogenesis.\(^{43}\) Therefore, higher complex I activity may help prevent propagation of seizure activity. When expressing activity of mitochondrial complexes activity to CS activity, to account for the enrichment of mitochondrial
content, we find no significant difference of complex I activity in C10/C8-80:20 diet mice, indicating that increases of complex I activity are proportional to mitochondrial biogenesis. In contrast, we found that increases in complex II+III activity in C10/C8-40:60 and C10 only diet mice also lead to an increase when expressed as a ratio to CS activity. This suggests that the alteration of complex II+III activity may be independent of mitochondrial enrichment. It is unclear whether this alteration of complex II+III activity may have a beneficial effect in the treatment of epilepsy. However, it has been documented that elevated complex II activity can lead to increased mitochondrial ROS through reverse electron transfer to complex I.\textsuperscript{44}

As alterations to mitochondrial content have been associated with increased ROS production, we measured brain GSH of \textit{Scn1a}\textsuperscript{R1407X/+} mice following MCT supplementation. We found a significant increase in GSH levels in the C10/C8-80:20 diet mice indicating a potential upregulation to combat any increased ROS production resulting from increased mitochondrial content. This would be consistent with previous \textit{in vitro} studies reporting that neural cells can upregulate GSH status in response to oxidant exposure.\textsuperscript{45} In contrast, for mice receiving the C10/C8-40:60 and C10 only diets, no significant difference in GSH status was observed. This finding may suggest that there is no need to up-regulate antioxidant status following administration of these particular diets or other antioxidant systems are being utilised.

To further elucidate the impact of MCT supplementation on antioxidant status, we assessed the activity of CAT, an important antioxidant enzyme that decomposes hydrogen peroxide, which has decreased activity in the plasma of patients with epilepsy.\textsuperscript{46} We found no significant difference in CAT activity in the brains of C10/C8-
80:20 diet mice. Conversely, there was a highly significant increase in both the C10/C8-40:60 and C10 only diet mice. This suggests differential upregulation of GSH and CAT following exposure to the different diets; further studies are now required to ascertain the mechanism(s).

The aim of this study was to test the antiepileptic properties of MCT diets with varying ratios of C8 and C10 in a DS mouse model and further our understanding of the biological changes associated with this. We demonstrated that MCT diet therapy is an effective antiepileptic treatment strategy in the DS mice that improves survival. We found that when supplementing the MCTs C10 and C8 in an 80:20 ratio, C10 may accumulate to sufficient levels in the brain to enhance energy metabolism by stimulating mitochondrial enrichment and enhancing antioxidant status, thereby providing a potential mechanism for the extended survival and seizure reduction in the DS mice. We acknowledge that the absence of a non-seizure control group as a limitation, allowing comparisons only between the Dravet mouse groups; nevertheless we believe that as the biochemical effects are associated with dramatic effects on seizure frequency and mortality, they are likely to be of relevance. The primary aims of this study were to determine the effects of MCTs on seizures and survival in the Dravet mouse, and to determine whether any differences were associated with increases in brain MCFA or BOHB. As secondary findings, mitochondrial content and antioxidant levels were found to be altered in the 80/20 supplemented Dravet mice compared with un-supplemented mice; these findings will now be extended to include comparisons with WT animals and further studies exploring the relevance of these findings to epileptogenesis. The differences in biochemical findings between the three supplemented groups suggest complex effects on brain neurochemistry and ultimately
on seizures and mortality, that are seemingly dependent on the ratio of MCFA supplemented.

The results presented here assessing the effects of MCT supplementation on seizure frequency, mortality and cellular metabolism indicate that supplementing C10 and C8 in an 80/20 ratio may be optimal in patients. In both the current study and our previous in vitro studies,\textsuperscript{13,14,16} 80:20 proved to be effective. We have recently successfully completed an open label feasibility study in adults and children with drug-resistant epilepsy (including 8 patients with DS) of an MCT product based on an 80:20 ratio, which had primary outcomes of acceptability, tolerability, and compliance. There was preliminary evidence for efficacy which correlated with plasma C10 levels, although this was an unpowered secondary outcome.\textsuperscript{18}

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We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

**CONFLICTS OF INTEREST**

N.J. does not have potential conflicts of interest to report.
T.B. has received support from Vitaflo, during the conduct of the study.
M.O. does not have potential conflicts of interest to report.
M.L. does not have potential conflicts of interest to report.
G.D.J. does not have potential conflicts of interest to report.
L.E.B. does not have potential conflicts of interest to report.
T.R. is employed by Vitaflo International, a for-profit company that develops innovative specialized clinical nutrition products.
C.R. does not have potential conflicts of interest to report.
S.H. has received grants from Vitaflo, during the conduct of the study. In addition, S.H. has a patent US2020147025 (A1) - COMPOSITION FOR USE IN THE TREATMENT OF EPILEPSY pending to Vitaflo.
S.E. has received grants from Vitaflo, during the conduct of the study. In addition, S.E. has a patent US2020147025 (A1) - COMPOSITION FOR USE IN THE TREATMENT OF EPILEPSY pending to Vitaflo.
S.P. has received support from Vitaflo, during the conduct of the study. S.P. is founder and equity holder and Chief Scientific Officer of Praxis Precision Medicines and RogCon Bioscience.

AUTHOR CONTRIBUTIONS
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Figure Legends:

FIGURE 1: The effect of MCT diet therapy in Scn1a<sup>R1407X/+</sup> mice. (A) Effect of MCT enriched diets on body weight. Results are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA (ns—denotes no significant difference) (n=8-12 per group). (B) Kaplan-Meier survival curves comparing survival in mice on control diet and mice treated with MCT diets. Treatment commenced at P18 (dashed line). Mantel-Cox log-rank test was used for statistical comparison of survival curves (**P < 0.01) (C10 n=26; C10/C8-80:20 n=28; C10/C8-40:60 n=27; Control n=34). (C) Spontaneous seizure frequency during the period of highest seizure incidence. Statistical significance calculated using two-way ANOVA, followed by Tukey’s post hoc analysis. Data presented as mean ± SEM, (ns—no significant difference; *P < 0.05; ***P<0.001; ****P<0.0001) (C10 n=26; C10/C8-80:20 n=28; C10/C8-40:60 n=27; Control n=34). (D) Hyperthermia-induced tonic-clonic seizure. Kaplan-Meier curves showing time to first tonic-clonic seizure during thermal stress (** P< 0.008 - C10 Mantel-Cox log-rank test compared to control diet) (C10 n=17; C10/C8-80:20 n=20; C10/C8-40:60 n=18; Control n=14; WT n=6).

FIGURE 2: Analysis of MCFA and β-hydroxybutyrate accumulation in mouse brain following MCT diets. (A) There was no significant difference between C8 levels in control diet, C10/C8-80:20 or C10/C8-40:60 diets, however, C8 was significantly lower in C10 diet than control (****P<0.0001). (B) C10 accumulated to significantly higher levels in C10/C8-80:20 (****P<0.0001), C10/C8-40:60 (****P<0.0001) and C10 only (***P<0.01) mice compared with control mice. (C) There were no differences in β-hydroxybutyrate levels between any of the test groups. Statistical significance
calculated using one-way ANOVA, followed by Tukey’s post hoc analysis. Data presented as mean ±SD. (Control diet, n=15); (C10/C8-80:20, n=20); (C10/C8-40:60, n=18); (C10, n=16).

FIGURE 3: The effect of MCT diet on mitochondrial markers in mouse brain. (A) Citrate synthase activity (***P<0.001) and (B) relative mitochondrial DNA (*P<0.05) were increased only in the brains of mice fed the C10/C8-80:20 diet. Changes of activity in the mitochondrial respiratory chain enzymes were assessed and expressed as a ratio to (C-E) protein content and (F-H) citrate synthase activity. (C) We found significant increases in complex I (*P<0.05) activity in the C10/C8-80:20 diet mice, with complex II+III activity significantly increased in the C10/C8-40:60 (**P<0.01) and C10 only diet (****P<0.0001) mice (D). The alterations in complex I activity in the C10/C8-80:20 diet mice were not associated with any significant alterations in the ratio to citrate synthase. However, the increase in complex II+III activity in the C10/C8-40:60 (*P<0.05) and C10 only diet (**P<0.01) mice was associated with significant increases in the ratio to citrate synthase. Statistical significance calculated using one-way ANOVA, followed by Tukey’s post hoc analysis. Data presented as mean ±SD. n=6 for all groups.

FIGURE 4: The effect of MCT diets on markers of antioxidant status in mouse brain. (A) Levels of GSH were found to be significantly increased in the C10/C8-80:20 diet mouse brains (*P<0.05) but not in the other diets. (B) CAT activity was significantly increased in the C10/C8-40:60 (***P<0.001) and C10 only diet (**P<0.01) mouse brains. Statistical significance calculated using one-way ANOVA, followed by Tukey’s post hoc analysis. Data presented as mean ±SD. n=6 for all groups.
Figure 1

(A) Graph showing weight (g) over time (weeks) with different groups indicated.

(B) Graph showing probability of survival over postnatal day.

(C) Graph showing seizures per 24 hours with different groups indicated.

(D) Graph showing percentage without febrile seizures over time to seizure (minutes).

Figure 2

(A) C8 concentration (nmoles/g brain tissue)

(B) C10 concentration (nmoles/g brain tissue)

(C) BOHB concentration (nmoles/g brain tissue)
Figure 3
Figure 4

(A) GST concentration (nmol/g brain tissue)

(B) Catalase activity (μmol/min/mg protein)
Supplementary material

Methods

GC-MS analysis of MCFA

100μL brain homogenate, 30μL internal standard mix (0.33μM each of d\textsubscript{15}-octanoic acid, d\textsubscript{5}-decanoic acid and d\textsubscript{23}-dodecanoic acid) and 400μL water was acidified with 125μL 6N HCL. 3mL ethyl acetate was added, and the upper organic layer was dried under N\textsubscript{2} and derivatized at room temperature for 15 minutes using 50μL 10% (v/v) 2,3,4,5,6-Pentafluorobenzyl bromide in acetonitrile plus 10μL of triethylamine. Samples were dried under N\textsubscript{2}, redissolved in 100μL of ethyl acetate and analysed by GC/MS (Thermo DSQ II with Trace GC, RXI\textsuperscript{®}-5Sil column (30m x 0.25mm I.D, 0.25μM film thickness), inlet temperature 280°C, helium flow rate 0.8mL/min, 2μl injection and 1:100 split ratio. Oven temperature gradient was 110°C to 220°C at 10°C/min, then to 300°C at 30°C/min. Compounds were analysed by negative chemical ionization (methane flow 2mL/min). The following fragment ions were detected in selected ion monitoring mode: m/z 143 (C\textsubscript{8}), 158 (d\textsubscript{15}-C\textsubscript{8}), 171 (C\textsubscript{10}), 176 (d\textsubscript{5}-C\textsubscript{10}), 199 (C\textsubscript{12}), 222 (d\textsubscript{23}-C\textsubscript{12}).

GC-MS based analysis of BOHB

Brain homogenate (50μL), plus 25μL of internal standard (200μM \textsuperscript{13}C\textsubscript{4} β-hydroxybutyrate), were added to 225μL acetonitrile, which was vortexed and centrifuged for 5 minutes at 15000g. The supernatant was dried under N\textsubscript{2}. Samples were resuspended in 200μL 100% ethanol and redried. Samples were derivatised in ethyl acetate (30μL) and N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA)/10% trimethylchlorosilane (TMCS)(30μL) at 37°C for 30 minutes. β-hydroxybutyrate (2μL with a split ratio of 1:8) was analysed as above, oven temperature 60°C for 1 minute,
to 140°C at 10°C/min, then to 240°C at 40°C/min. Samples were analysed by positive chemical ionization with a methane flow rate of 2mL/min, with selected ion monitoring for BOHB at m/z 233, and 237 for $^{13}$C$_4$-BOHB.

**Enzyme assays**

The assay to measure CS follows the reaction between CoASH – formed from CS activity – and the reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), which causes a colorimetric change and can be followed at 412nm. The complex I assay follows the oxidation of NADH at 340nm, where rotenone (a potent complex I inhibitor) is added halfway through the assay to determine the rate of complex I-independent NADH oxidation, which is then subtracted from the initial rate to give the complex I-dependent (i.e. rotenone sensitive) rate. The assay to determine complex II+III activity follows the oxidation of succinate by complex II, leading to the reduction of ubiquinone to ubiquinol. Ubiquinol then reduces cytochrome c, catalysed by complex III, which can be followed at 550nm. Antimycin A, a complex III inhibitor, is used to determine the complex II/III independent reduction of cytochrome c occurring. Complex IV is measured as reoxidation of reduced cytochrome c at 550nm (as a first-order rate constant). In this reaction, catalase decomposes H$_2$O$_2$ to water and oxygen, then Amplex Red reacts with the remaining H$_2$O$_2$ with 1:1 stoichiometry, catalyzed by horseradish peroxidase, producing the highly fluorescent resorufin. This reaction can then be followed with an excitation filter of 560nm and emission of 590nm.

**qPCR analysis**

Specific primers for *Mt-co3* and *Gapdh* were designed for each target gene using Primer3 software (Supplementary Table 1). Melt curve analysis was used to ensure a
single product was formed during amplification. Genomic DNA was extracted from Mouse Brain homogenate using Qiagen DNeasy Blood and Tissue Kit, as per manufacturer’s instructions. Quantification of the target gene (Mt-co3) was carried out using the Sybr-green method for qPCR, as per manufacturer’s instructions (Bio-rad, Hertfordshire, UK), and was normalized to nuclear housekeeping gene Gapdh. Relative mtDNA content was calculated using the ∆∆CT method. Statistical significance was calculated utilizing ∆Ct data.

Reagents
TRIzol reagent was from Thermo-Fisher Scientific (Loughborough, UK). iQ SYBR Green Supermix was from Bio-Rad Laboratories Inc. (Hertfordshire, UK). Desalting columns for reduced cytochrome c purification were from Fisher Scientific (Loughborough, UK). Amplex Red was from Thermo Fisher Scientific. DNeasy blood and tissue kit was from Qiagen (Manchester, UK). Deuterium labelled fatty acids were from CDN Isotopes (Quebec, Canada). MCT oils were provided by Vitaflo, part of the Nestlé Health Science group. All other reagents were from Sigma Aldrich (Poole, UK).
Table 1. Primer design

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**Supplementary Figure S1.** Spontaneous convulsive seizure frequency of MCT treated Scn1a\( ^{R1407X/+} \) mice during the study (P18-P46). The period of highest seizure incidence is also shown in Fig 2B. Statistical significance calculated using two-way ANOVA, followed by Tukey’s post hoc analysis. Data presented as mean ± SEM, (*P < 0.05; ***P<0.001; ****P<0.0001).
**Supplementary Figure S2.** Rectal temperature in WT mice after 1 week on control (n=5) and MCT C10/C8-80:20 diet (n=5). Statistical analysis was performed using unpaired t-test (ns-denotes no significant difference).