Sigrid Pedersen

Dietary treatment in children and adults with drug resistant epilepsy

Epilepsy is a common neurological disease, affecting about 65 million people globally. The mainstay treatment of epilepsy is anti-seizure medications (ASMs). However, 1 in 3 patients do not achieve seizure control with drugs alone. In these patients, high-fat, low-carbohydrate diets, termed ketogenic diets, can be a treatment option. Randomized controlled trials have demonstrated the ketogenic diets' effectiveness in reducing seizures in patients with epilepsy, but the mechanism(s) of action remain elusive.

This thesis has investigated how ketogenic diets influence 1) DNA methylation, and 2) serum concentrations of ASMs in patients with epilepsy.

In adults with epilepsy, we identified widespread changes in DNA methylation following 12 weeks of treatment with a modified ketogenic diet. In children, we found decreased serum concentration of two commonly used ASMs following 12 weeks of treatment with a classical ketogenic diet, which indicate interactions between the dietary treatment and certain ASMs.

Although the clinical implications of our results are yet to be explored, our findings may provide important clues to understand how ketogenic diets reduce seizures in epilepsy.

Dissertation for the Degree of PhD 2023
Institute of Clinical Medicine
Faculty of Medicine

ISBN 978-82-348-0277-5

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UNIVERSITY OF OSLO

Faculty of Medicine

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Thesis for the degree of Philosophiae Doctor (PhD) by

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March, 2023

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo

ISBN 978-82-348-0277-5

Cover: UiO.
Print production: Graphic center, University of Oslo.

"Since Wilder, in 1921, suggested the use of a ketogenic diet in the treatment for epilepsy, this procedure has taken a definite place in the treatment for this type of convulsion. The mechanism of the action of this diet, however, is not yet clear."

Keith MB, American Journal of Diseases of Children, 1931

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Acknowledgements

The work of the present thesis was conducted at the National Centre for Epilepsy, Sandvika, from 2017 to 2022.

First and foremost I wish to express my gratitude to the patients and families that took part in the studies. You have made my work interesting, educational, and meaningful. It has been touching to be with you on a small part of the road.

I would like to thank my main supervisor Kaja Kristine Selmer for all her help and advice with this PhD. Your support has been invaluable. I appreciate all your enthusiasm and dedication. Thank you for all encouragement you have given me throughout this project.

A particular thank you to my co-supervisors Per Ole Iversen and Knut Rudi who has been a part of the project from the very beginning. Thank you Knut, for your friendly and calm nature, and for providing a valuable perspective from outside the field of epilepsy. Per Ole, thank you for welcoming me to your research group, your quick responses, and for standing up when needed. I also like to thank my co-supervisors Benedicte Alexandra Lie for the scientific discussions and valuable inputs in the early stages of the project and Kristina Gervin for performing the bioinformatics analysis.

I would like to thank all my colleagues at the Section for children and youth. A special thanks to my amazing KEF-colleagues Magnhild Kverneland, Kathrine Cammermeyer Haavardsholm, Sara Linn Saunes, and Cecilie Marie Rødbøl for their encouragements, support, academic discussions, and lovely company in the lunch breaks. A big thank you to the wonderful doctors Karin Benan, Anne Våtevik, Carine Valaye, Trine Ingebrigtsen, and Kristine Lossius who were in charge of the dietary treatment. It has been a true pleasure working with you. Also, many thanks to Lina Engeland, Solveig Hauso, Bente Karlsen, Anne-Lise Myrold, Inah Kim, Iris Salicath, Nina Kristiansen, Mari Grindvold, and Irene Bernardo, the outstanding nurses at “Post C”, for their positive attitude, assistance with data collecting, and kind support of the patients and their caregivers. Finally, thank you Melinda Elvheim – you are the very definition of order and structure, without you, everything becomes chaos!

I very much appreciate the funding and support provided by the National Centre for Rare Epilepsy-Related Disorders for this study. I want to thank Ine, Mia, Silje, Kathrine, Åse Katrine, Caroline, Marit, and Edel for all our enjoyable conversations and the moral support throughout the final year of my PhD. A special thank you to Nina Benan. You exhibit the qualities of an excellent leader in that you are committed, empathetic, and knowledgeable.

I would like to thank my co-authors Karl Otto Nakken for all his contributions to the writing and Cecilie Johannesen Landmark for teaching me important pharmacology aspects and rapid responses to my inquiries.

Magne Thoresen and Dag Hofoss deserve special thanks as well for their valuable advices and fruitful discussions regarding statistics. I would also like to thank Manus Vigeland for his help when we wrote the applications for research funding.

Thank you to the Section for Clinical Pharmacology at Oslo University Hospital for the contributions with the laboratory work, especially Gerd Karin Hella, who has had the overall responsibility.

This work was financially supported by the Norwegian Epilepsy Association, Stiftelsen Dam, Novo Nordic foundation, and National Organization for Rare Disorders – thank you! A special thanks to the Norwegian Epilepsy Association for choosing to support the research project. For me, this recognition was an important motivation to work further to achieve full funding. Thank you, Jørn Mandla Sibeko - you have guided us safely through the application processes, reporting and formalities!

I want to thank the director of the National Center for Epilepsy, Grete Almåsbak, and Head of Section for Children and Youth, Marit Bjørnvold, for recognizing the value of research and making it possible for me to finish this doctorate.

Warm thanks to my incredible friends Annalena, Sarah, Kristin, Majuri, Marte, and Racheal for their support and belief in me. I truly appreciate you. I would also like to give a special thanks to “Team Dynamin”. This was where it all started. Thanks for histodisco-Saturdays, endless hours at PK, copious amounts of brownies, cabin trips, and all the lovely dinners throughout the course of my studies. Many thanks to Siril for the support from the very beginning, our great hiking trips, and our good talks. Ingunn, thank you for cheering me up! Thank you Aksel, for your warmth, generosity, and acceptance.

To my parents and my siblings, thank you for all your love, care, and support throughout the years.

Finally, I would like to express my gratitude to my dear Frederik. Thank you for your continuous support, words of encouragement, and patience. You keep impressing me and bright up my life.

I am immensely grateful to all the wonderful people who have each in their own way helped and supported me along the way. I am privileged to be surrounded by such kind people. Thank you.

Oslo, March 2023

Sigrid Pedersen

Sammendrag

Bakgrunn

Epilepsi er en av de vanligste nevrologiske sykdommene i verden og rammer omtrent 65 millioner mennesker globalt. En av grunnpilarene i behandling av epilepsi er anfallsforebyggende legemidler. De fleste pasienter med epilepsi har god effekt av slike legemidler, men omtrent 1 av 3 oppnår ikke tilfredsstillende anfallskontroll med legemidler alene. Hos disse pasientene kan en ernæringsbehandling kalt ketogen diett være et godt behandlingsalternativ. Ketogen diett er en samlebetegnelse på ulike dietter med høyt innhold av fett og lavt innhold av karbohydrat som induserer produksjon av ketonlegemer. Ketogen diett er en internasjonalt etablert epilepsibehandling som har vært brukt i mer enn 100 år og diettens anfallsreduserende effekt er godt dokumentert. Likevel vet man lite om virkningsmekanismene til ketogen diett ved epilepsi.

De siste årene er DNA-metylering foreslått å spille en rolle i ketogen dietts evne til å redusere anfall hos pasienter med epilepsi. DNA-metylering er en kjemisk modifikasjon av DNA som ikke endrer selve DNA-sekvensen, men som kan påvirke genuttrykket. Det er velkjent at ulike miljøfaktorer, som for eksempel kosthold, kan påvirke DNA-metylering. Imidlertid mangler det kunnskap om hvilken innvirkning ketogen diett har på DNA-metylering hos pasienter med epilepsi, samt den potensielle sammenhengen mellom diettutløste endringer i DNA-metylering og diettens anfallsreduserende effekt. Et annet kunnskapshull er hvordan ketogen diett påvirker serumkonsentrasjonen av anfallsforebyggende legemidler. Få studier har undersøkt dette og resultatene fra studiene som finnes er sprikende.

Mål

Det overordnede målet med denne avhandlingen var å bedre vår forståelse av hvordan det ketogen diett reduserer anfallshyppighet hos pasienter med epilepsi ved å undersøke hvordan ketogen diett påvirker kroppen på et molekylært nivå. Spesifikt undersøkte vi effekten av ketogen diett på DNA-metylering og serumkonsentrasjon av anfallsforebyggende legemidler hos pasienter med epilepsi.

Metode

Arbeidet i denne oppgaven er basert på to ulike studiepopulasjoner med litt ulike diettintervensjoner: 1) voksne med legemiddelresistent epilepsi behandlet med modifisert ketogen diett, og 2) barn med legemiddelresistent epilepsi behandlet med klassisk ketogen diett. Pasientene i begge studiepopulasjonene spiste sitt normale kosthold i en baselineperiode før de umiddelbart etter startet behandling med ketogen diett i en 12-ukers intervensjonsperiode. Epileptiske anfall ble registrert systematisk og all annen epilepsibehandling ble holdt uendret gjennom hele studien. Blodprøver for analysering av DNA-metylering og måling av serumkonsentrasjonen av anfallsforebyggende legemidler ble samlet inn før og under diettintervensjonen.

Resultater

I artikkel I viste vi at voksne med legemiddelresistent epilepsi som ble behandlet med en modifisert ketogen diett i 12 uker hadde en signifikant reduksjon i DNA-metylering, både globalt og på spesifikke posisjoner i genomet. En betydelig andel av de differensielt metylerte posisjonene var tilknyttet gener assosiert med epilepsi, regulering av lipid- og glukosemetabolisme, gentranskripsjon,

inositolfosfatmetabolisme eller grunnleggende cellefunksjoner. Vi fant ingen forskjeller i DNA-metylering hos pasienter som opplevde anfallsreduksjon etter diettintervensjonen, sammenlignet med pasienter som ikke hadde anfallsreduksjon. I artikkel II brukte vi data fra samme studiepopulasjon som i artikkel I for å undersøke DNA-metylering på spesifikke posisjoner tilknyttet gener som er kjent å være involvert i metabolismen av anfallsforebyggende legemidler. Vår analyse identifiserte en differensielt metylert posisjon tilknyttet genet *CES1* som er involvert i metabolismen av det anfallsforebyggende legemiddelet rufinamid. I tillegg fant vi en sterk negativ korrelasjon mellom endringer i DNA-metylering på en posisjon annotert til genet *UGT1A4* (som koder for et enzym som metaboliserer legemidler) og endringer i serumkonsentrasjoner av det anfallsforebyggende legemiddelet lamotrigin. I artikkel III avdekket våre analyser av serumkonsentrasjoner av anfallsforebyggende legemidler i den pediatriske studiepopulasjonen en signifikant reduksjon i serumkonsentrasjonen av de to anfallsforebyggende legemidlene klobazam/desmetylklobazam og lamotrigin etter 12-ukers diettintervensjon.

Konklusjon

Vi fant globale endringer i DNA-metylering etter behandling med en modifisert ketogen diett hos voksne med legemiddelresistent epilepsi. Blant våre mest spennende funn var reduksjonen i DNA-metylering av gener assosiert med epilepsi og inositolfosfatmetabolisme. Inositol (*myo*-inositol) har tidligere vært foreslått å ha anfallshemmendeegenskaper og endringer i inositolfosfatmetabolismen kan representere en mulig mekanisme for hvordan ketogen diett virker anfallsreduserende ved epilepsi. Videre fant vi en reduksjon i serumkonsentrasjonen av to hyppig brukte anfallsforebyggende legemidler etter behandling med klassisk ketogen diett hos barn med legemiddelresistent epilepsi, noe som indikerer mulige interaksjoner mellom ketogen diett og visse anfallsreduserende legemidler. Selv om det gjenstår mer forskning for å forstå de kliniske implikasjonene av resultatene våre, kan funnene gi viktige ledetråder for å forstå mekanismene bak ketogen dietts evne til å redusere anfall ved epilepsi.

Summary

Background

Epilepsy is one of the most common neurological diseases, affecting about 65 million people worldwide. Anti-seizure medications (ASMs) are the mainstay of epilepsy treatment and most of the patients respond well to these drugs. However, approximately 1 in 3 patients do not achieve seizure control with drugs alone. In these patients, ketogenic diets can be a treatment option. Ketogenic diets is an umbrella term for various high-fat, low-carbohydrate diets that induce production of ketone bodies. Ketogenic diets is an internationally established treatment in epilepsy that has been used for more than 100 years and its effectiveness in reducing seizures is well documented. Still, little is known about the ketogenic diets' mechanism(s) of action.

In recent years, DNA methylation has been suggested to have a role in ketogenic diets' ability to reduce seizures in patients with epilepsy. DNA methylation is a chemical modification of the DNA that do not change the DNA sequence itself, but may influence the gene expression. It is well known that various environmental factors, such as diet, can affect DNA methylation. However, knowledge about the ketogenic diets impact on DNA methylation in patients with epilepsy, and the relationship between potential diet-induced DNA methylation changes and the diet's seizure-reducing effect, is lacking. Another knowledge gap is the potential influence of ketogenic diets on ASM serum concentrations. Few studies have investigated this topic and the results are inconsistent.

Aims

The overarching aim of this thesis was to improve our understanding of how the ketogenic diet ameliorate seizures in patients with epilepsy by investigating how the ketogenic diet influences the body at a molecular level. Specifically, we investigated the impact of ketogenic diets on DNA methylation and ASM serum concentrations in patients with epilepsy.

Methods

The work in the present thesis is based on two different study populations with slightly different diet interventions: 1) adults with drug resistant epilepsy treated with a modified ketogenic diet, and 2) children with drug resistant epilepsy treated with a classical ketogenic diet. In both studies, the patients ate their normal diet in a baseline period and subsequently a ketogenic diet in a 12-week intervention period. Seizures were recorded systematically and all epilepsy treatments were kept unchanged throughout the study. Blood samples for analysis of DNA methylation and ASM serum concentrations were collected before and during the diet intervention.

Results

In paper I, we showed that adults with drug resistant epilepsy treated with a modified ketogenic diet for 12 weeks had a significant decrease in DNA methylation, both globally and at specific positions in the genome. A considerable proportion of the differentially methylated positions were annotated to genes associated with epilepsy, regulation of lipid and glucose metabolism, gene transcription, inositol phosphate metabolism, or basic cell functions. We found no differences in DNA methylation in patients who experienced seizure reduction following the diet intervention, compared to patients

who did not experience any seizure reduction. In paper II, using data from the same study population as in paper I, we investigated DNA methylation at specific positions annotated to genes known to be involved in the metabolism of ASMs. Our analysis identified one differentially methylated position annotated to the gene *CES1* involved in the metabolism of the ASM rufinamide. In addition, we found a strong negative correlation between changes in DNA methylation of one position annotated to gene *UGT1A4* (encoding a drug-metabolizing enzyme) and changes in serum concentrations of the ASM lamotrigine. Finally, in paper III, our analysis of ASM serum concentrations in the paediatric study population revealed a significant decrease in the serum concentration of the two ASMs clobazam/desmethylclobazam and lamotrigine following 12 weeks of diet intervention.

Conclusion

We found widespread changes in DNA methylation following treatment with a modified ketogenic diet in adults with drug resistant epilepsy. Among the most intriguing findings were the decrease in DNA methylation of genes associated with epilepsy and inositol phosphate metabolism. Inositol (*myo*-inositol) has previously been suggested to have anti-seizure properties, and alterations in inositol phosphate metabolism may represent a possible mechanism for how ketogenic diets attenuate seizures in epilepsy. Further, we found a decrease in the serum concentration of two commonly used ASMs following treatment with a classical ketogenic diet in children with drug resistant epilepsy, indicating potential interactions between ketogenic diets and certain ASMs. Although the clinical implications of our results are yet to be explored, our findings may provide important clues to understand the mechanism(s) behind the ketogenic diets' ability to reduce seizures in epilepsy.

List of publications

Paper I: **Pedersen S**, Kverneland M, Nakken KO, Rudi K, Iversen PO, Gervin K, Selmer KK. Genome-wide decrease in DNA methylation in adults with epilepsy treated with modified ketogenic diet: A prospective study. *Epilepsia* 2022; 63(9): 2413-26.

Paper II: **Pedersen S**, Kverneland M, Nakken KO, Rudi K, Iversen PO, Landmark CJ, Gervin K, Selmer KK. DNA methylation of genes involved in the metabolism of anti-seizure medications in adult epilepsy patients treated with modified ketogenic diet. *Manuscript* (submitted).

Paper III: **Pedersen S**, Kverneland M, Rudi K, Gervin K, Landmark CJ, Iversen PO, Selmer KK. The impact of the ketogenic diet on serum concentrations of anti-seizure medications in children with drug resistant epilepsy. *Manuscript* (submitted).

List of abbreviations

APOB48R	Apolipoprotein B48 receptor
ASM	Anti-seizure medication
ATP	Adenosine triphosphate
CES1	Carboxylesterase 1
CGI	CpG island
CKD	Classical ketogenic diet
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine dinucleotide
CPT1A	Carnitine palmitoyltransferase 1A
CYP	Cytochrome P450
DNMT	DNA methyltransferase
FDR	False discovery rate
HbA_{1c}	Haemoglobin A_{1c}
ILAE	International League Against Epilepsy
IMPA	*Myo*-inositol monophosphatase
IP_3	Inositol 1,4,5-triphosphate
IQR	Interquartile range
KD	Ketogenic diet
LGIT	Low-glycemic index treatment
MAD	Modified ketogenic (Atkins) diet
MCT	Medium-chain triglyceride
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase kinase 4
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SD	Standard deviation
TET	Ten-eleven translocation
THF	Tetrahydrofolate
UGT	UDP-glucuronosyltransferase
WGBS	Whole-genome bisulphite sequencing

1 BACKGROUND

1.1 Epilepsy

1.1.1 What is epilepsy?

Epilepsy is a disease of the central nervous system characterized by unprovoked, recurrent epileptic seizures. An epileptic seizure is a transient event due to electrical disturbances in a dysfunctional cellular network in the brain, causing a variety of signs and/or symptoms[1]. The seizure semiology varies depending on which area of the brain that is affected, and whether the epileptic activity spread to other brain areas. The seizures may vary from a short lasting subjective feeling (e.g. a smell) or a barely noticeable jerk of an arm or leg, to severe convulsive seizures with loss of consciousness.

The International League Against Epilepsy (ILAE) defines a person to have epilepsy if any of the following conditions are fulfilled: 1) At least two unprovoked (or reflex) seizures occurring more than 24 hour apart, 2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years, or 3) diagnosis of an epilepsy syndrome[1].

In addition to the recurrent and unpredictable seizures, it is well recognized that epilepsy may be associated with a high number of somatic and psychosocial comorbidities[2]. In fact, about 50% of people with epilepsy have one or more comorbidities[2]. Moreover, people with epilepsy have decreased life expectancy, and compared to the general population they have 2-3 times increased risk of premature death[3,4]. The World Health Organization estimates that epilepsy accounts for more than 0.5% of the global burden of disease, measured in disability-adjusted life years[5].

1.1.2 Epidemiology

Epilepsy is one of the most common neurological disorders affecting about 65 million people worldwide[6]. The burden of epilepsy is not equally distributed; low-and middle-income countries have an estimated incidence of 139 per 100.000 persons per year, compared to 49 per 100.000 persons per year in high-income countries[7]. In Norway, a high-income country, the prevalence of epilepsy is found to be 0.65%, i.e. about 35.000 Norwegians are affected[8].

Although much progress has been made in identifying the underlying cause of epilepsy, in a large share of the patients the aetiology remains unknown[8,9]. The aetiologies of epilepsy are roughly divided into six categories; structural (e.g. cerebral tumour, stroke), metabolic, infectious, genetic, immune, and unknown[10]. World Health Organization estimates that 25% of the cases of epilepsy are preventable[5].

1.1.3 Classification of seizures, epilepsy types, and epilepsy syndromes

Epilepsy is classified at three levels; seizure type, epilepsy type, and epilepsy syndrome (**Figure 1**) [10].

At the first level, epileptic seizures are classified according to seizure onset: focal, generalized, or unknown. A focal onset seizure originates from a more or less well defined cellular network in one hemisphere in the brain, while a generalized onset seizure involves both hemispheres. If it is not possible to determine the onset of the seizure, it is classified as “unknown”. Further, seizures can be sub-classified based on the degree of awareness, motoric involvement, and other signs or symptoms (e.g. behaviour arrest, emotional, cognitive, or sensory symptoms) [11].

At the second level, the patient’s epilepsy diagnosis is classified as an epilepsy type based on seizure semiology, electroencephalogram (EEG), and magnetic resonance imaging (MRI) findings. The epilepsy types are: generalized, focal, combined generalized and focal, and unknown[10].

The third level of classification are the epileptic syndromes, such as Dravet syndrome, Lennox-Gastaut syndrome, juvenile myoclonic epilepsy[10]. Building from the position paper defining the three

classification levels, in 2022, ILAE launched a series of position papers with the first official classification of epilepsy syndromes[12-15]. An epilepsy syndrome is here defined as "*a characteristic cluster of clinical and EEG features, often supported by specific etiological findings (structural, genetic, metabolic, immune, and infectious)*" [12]. The epilepsy syndromes are divided based on the age of onset; 1) syndromes with onset in neonates and infants (up to two years) [13], 2) syndromes with onset in childhood[14], and 3) syndromes that may begin at a variable age (in both paediatric and adult patients) [15]. Based on the type of seizure, the syndromes are further categorized into generalized, focal, or generalized and focal, as well as a category for syndromes with developmental and epileptic encephalopathy or progressive neurological deterioration[12].

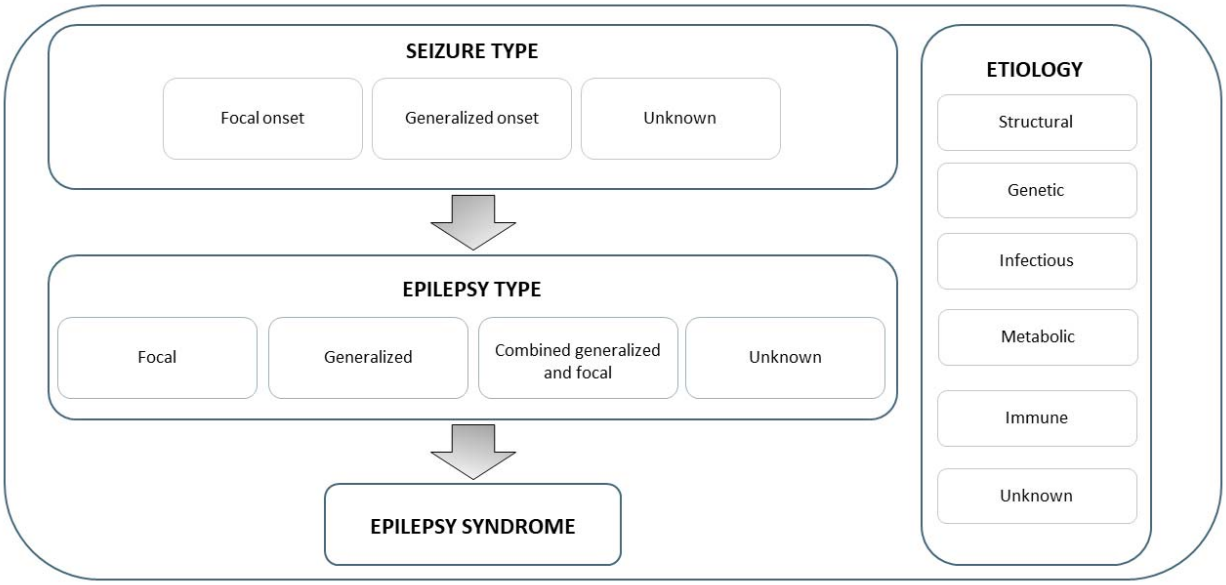


Figure 1: The ILAE classification of epilepsies (2017). Epilepsy is classified at three levels; seizure type, epilepsy type, and epilepsy syndrome. The epilepsy aetiologies are incorporated at all levels. *Figure is modified from[10].*

1.1.4 Diagnosis and treatment

Epilepsy is primarily a clinical diagnosis based on a detailed medical history together with a thorough description of the seizures by either the patient him-/herself or by an eyewitness. Epileptiform activity in EEG provides support for the diagnosis, but a normal EEG does not rule out epilepsy. All

patients with a suspected epileptic seizure must have an MRI investigation of the brain to reveal a potential underlying structural cause. In selected patients, other, specific investigations may be required, e.g. genetic testing[16].

The mainstay of epilepsy treatment is anti-seizure medications (ASMs). ASMs work through various mechanisms to prevent seizures, usually by either decreasing neuronal excitation (anti-glutamatergic drugs) or enhancing inhibition (GABAergic drugs) [17]. Over the past 30 years, multiple new ASMs with different modes of actions, effect- and side effect profile have been developed. Currently, there are about 30 different ASMs approved for treatment of epilepsy[18]. Most of the patients (60-70%) respond well to these drugs and achieve acceptable seizure control. However, about one third of the patients do not achieve seizure control[19]. Patients who continue to experience recurrent seizures after having tried two or more appropriately chosen and tolerated ASMs, are referred to as drug resistant[20]. Although this term may somewhat wrongly give the impression that the patients do not respond to pharmacological treatment at all, most of the patients experience some seizure reduction by the drugs. However, drugs alone are not enough to achieve sustained, adequate seizure control.

If pharmacological treatment does not give a satisfactory result, other treatment options like epilepsy surgery, vagus nerve stimulation, or deep brain stimulation should be considered. However, far from all drug resistant patients are eligible for this kind of treatment and there is a risk of complications[21]. For those patients, treatment with a ketogenic diet can be a favourable alternative.

1.2 Dietary treatment of epilepsy

1.2.1 Ketogenic diet

Ketogenic diets is an umbrella term for various types of high-fat, low-carbohydrate diets that have in common that they induce production of ketone bodies. The ketogenic diet is today an internationally established and documented treatment for epilepsy (see section 1.2.3) [22]. In

accordance with Norwegian guidelines, it is recommended that dietary treatment is considered after 2-3 attempts with appropriately chosen and tolerated drugs without achieving seizure control[23].

The original ketogenic diet, called the classical ketogenic diet, was first introduced to patients with epilepsy in the 1920s by Dr. Russel Wilder[24]. Inspired by Dr. Henry Geyelin's promising results with fasting as a treatment for epilepsy[25], Wilder designed the ketogenic diet to mimic the metabolic state of fasting. Later, more variants of ketogenic diet, including the modified ketogenic diet[26] (also known as the modified Atkins diet), the medium-chain triglyceride (MCT) diet[27], and the low-glycaemic index treatment (LGIT) diet[28] have been introduced attempting to provide diets that are more palatable and easier to adhere to.

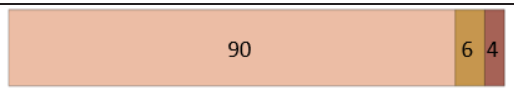
1.2.2 Variants of ketogenic diets

The main differences between variants of ketogenic diets are the macronutrient composition (i.e. proportion of carbohydrate, protein, and fat) and the practical approach to achieve a high-fat, low-carbohydrate diet. An overview of the main features and the macronutrient composition of the various ketogenic diets are given in **Table 1**.

For the classical ketogenic diet, the basic principle is to calculate meal recipes based on pre-specified amounts of fat, protein, and carbohydrate, and during preparation all ingredients are accurately weighed. The ketogenic ratio is defined as the ratio of grams of fat to the sum of grams protein plus carbohydrate. Fat induces production of ketone bodies while carbohydrate and protein inhibit the production of ketone bodies. All meals have the same ketogenic ratio and are eaten at regular time points. No food is allowed between the meals. In contrast, the modified ketogenic diet specify only the daily allowance of carbohydrate (typically 10 grams for children and 20 grams for adults). Foods containing protein and fat are eaten freely and are not weighed. The meals can be eaten whenever desired, although it is recommended to eat regular meals. The classical ketogenic diet is usually more restricted in carbohydrate and protein, and have a higher proportion of fat than the modified ketogenic diet[29].

The MCT and LGIT diets are rarely used as treatment for epilepsy in Norway. Briefly, the MCT diets take advantage of the higher ketone yield per kilocalorie of medium-chain fatty acids, compared to long-chain fatty acids, which means that the patient can eat more carbohydrate and protein, while still preserving the desired ketosis level. LGIT allows a higher amount of carbohydrate (typically 40-60 g per day), but all carbohydrates must have a low glycaemic index[29]. The glycaemic index is a measure of how much the blood glucose increase after consumption of a food item containing carbohydrate[30].

Table 1: The macronutrient composition and the main features of the various types of ketogenic diets.

Diet type	Macronutrient composition (E%)	Ketogenic ratio[a]	Main features
CKD		4:1	A tailor made meal plan is provided by a dietitian. Each meal contains predefined amounts of gram fat, protein, and carbohydrate per meal, based on daily energy requirements. Meals are served at regular hours. Fats are mainly provided as long-chain triglycerides. All food items are weighed.
MAD		2:1	The amount of carbohydrate is usually restricted to 10-20 gram per day. All foods containing carbohydrate are weighed. Foods containing protein and fat are eaten freely, but a high intake of foods rich in fat are recommended.
MCT		1.5:1	Similar to classical ketogenic diet, however 30-60% of total fat is provided as medium-chain triglycerides which have a higher yield of ketones per kilocalorie of energy than long-chain fatty acids. Thus, more protein and carbohydrate are allowed.
LGIT		1:1	The amount of carbohydrate are usually restricted to 40-60 grams per day, including fibres, however, all foods need to have a glycaemic index[b] of <50 compared to glucose. Foods are not weighed, but based on portion sizes. Daily intake of fat, protein, and total energy content are approximately.

The approximate energy percentage from fat, protein, and carbohydrate, as well as the ketogenic ratio of the four main types of ketogenic diets for epilepsy[29].
The energy percentage and ketogenic ratio may vary between patients. *Table inspired by[31,32].*
Abbreviations: CKD, classical ketogenic diet; E%, energy percentage from each macronutrient; MAD, modified ketogenic (Atkins) diet; MCT, medium-chain triglyceride diet; LGIT, low-glycaemic index treatment diet.
[a] The ketogenic ratio is defined as the ratio of grams fat to the sum of grams protein plus carbohydrate.
[b] The glycaemic index is a measure of how much the blood glucose increase after consumption of a food item containing carbohydrate. The glycaemic index value is calculated as the area under the curve for blood glucose during the two first hours after consumption of a food item containing 50 grams of carbohydrate and divided by the area under the curve for blood glucose of the same amount of carbohydrate as glucose[30].

1.2.3 Effectiveness of ketogenic diets in epilepsy

In 2008, the first randomized clinical trial investigating the effectiveness of a ketogenic diet in children with epilepsy was published by Neal et al. [33]. Twenty-eight (38%) of the participants in the diet group achieved ≥50% seizure reduction compared to four (6%) in the control group after 3 months of intervention. Moreover, five (7%) children in the diet group achieved ≥90% seizure reduction, whereas none in the control group. This study was a milestone and confirmed the ketogenic diets' seizure-reducing effectiveness observed in clinical practice and observational studies. Later, these results have been confirmed in several randomized trials comparing the seizure reducing effectiveness of ketogenic diets with no diet intervention[34-37]. In addition, a number of randomized clinical studies have compared the effectiveness of various types of ketogenic diets (e.g. classical versus modified ketogenic diet or a high versus low ketogenic ratio). There is some evidence pointing to a greater effectiveness of the classical ketogenic diet, compared to less restrictive diets such as a modified ketogenic diet or LGIT, as well as diets with a higher ketogenic ratio than to a lower ratio, but the results are inconclusive[36,38-40]. Overall, clinical trials report that the proportion of children achieving at least 50% seizure reduction with a ketogenic diet ranges from 38% to 72%[35,37,38,41,42].

Two randomized trials assessing effectiveness of a ketogenic diet in adults with drug resistant epilepsy have been published. In the first study, published in 2017, Zare et al. [43] reported 50% seizure reduction in 35% (*n* = 12) of participants after 2 months on a modified ketogenic diet. No participants in the control group achieved 50% seizure reduction. None of the patients in either groups were seizure free. In the second study by Kverneland[43], only 11% (*n* = 3) in diet group achieved 50% seizure reduction after 12 weeks on a modified ketogenic diet, compared to 6% (*n* = 2) in the control group. None of the patients in neither the diet group nor the control group became seizure free.

Although limited, based on current evidence, there may be a greater effectiveness of such dietary treatments in drug resistant epilepsy of children than adults.

1.2.4 Biochemical adaptations to ketogenic diets

Originally designed to mimic the metabolic state of fasting, ketogenic diets bring about a wide range of biochemical adaptations. The term "ketogenic diets" comes from the diets ability to induce production of ketone bodies, a biochemical process called "ketogenesis". Ketogenesis generates the endogenous ketone bodies β-hydroxybutyrate, acetoacetate, and acetone. Ketone bodies are predominantly formed in liver cells as a result of breakdown of fatty acids. Since the liver lacks the enzyme β-ketoacyl-CoA transferase necessary to utilize the ketone bodies, the ketone bodies are excreted from the liver and represents an alternative energy source for most other tissues[44]. Although the body is continuously producing small amounts of ketone bodies, the concentration of ketone bodies in the blood is low (typically <0.3 mmol/L) under normal physiological conditions. Elevated blood ketone concentration is a condition referred to as "ketosis". The main physiological causes of elevated blood ketone concentration, a condition referred to as "ketosis", are fasting and treatment with a high-fat, low-carbohydrate diet. This diet-induced ketosis is a physiological adaptation and is not to be confused with diabetic ketoacidosis, which is a potentially life-threatening condition typically seen in type I diabetes mellitus[45].

During the first days of treatment with a ketogenic diet, there is a biochemical shift from the use of glucose (i.e. glycolysis) as the main source of energy to oxidation of fatty acids, and ultimately production of ketone bodies. Ketogenesis mainly occurs in the mitochondria of liver cells. Fatty acids are transported into the mitochondria via carnitine palmitoyltransferase I (CPT1) where they are degraded into acetyl-CoA through β-oxidation. Acetyl-CoA can then be used as a substrate for ketone bodies synthesis. The first step in ketogenesis is the formation of acetoacetyl-CoA from two molecules of

acetyl-CoA by thiolase (also known as acetyl coenzyme A acetyltransferase). Acetoacetyl-CoA is then converted to hydroxyl-β-methylgluaryl-CoA, a reaction catalysed by HMG-CoA synthase. Hydroxyl-β-methylgluaryl-CoA is further metabolized to the ketone body acetoacetate by HMG-CoA lyase. Acetoacetate can then either reversibly be converted to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase or spontaneously degrade to acetone and carbon dioxide[44].

Ketogenesis is predominantly regulated by insulin, although other hormones, including glucagon, cortisol, and catecholamines also play a role. A low insulin level stimulates ketogenesis trough decreased inhibition of hormone-sensitive lipase, which leads to increased levels of free fatty acids, indirect reduced inhibition of CPT1, leading to increased uptake of free fatty acids into the mitochondria, and increased HMG-CoA activity ultimately increasing the production of ketone bodies[44].

During situations where access to glucose is limited, the ketone bodies become an important source of energy, especially for the brain. The breakdown of ketone bodies in extrahepatic tissues to produce energy is referred to as “ketolysis”. Cerebral utilization of ketone bodies is mainly determined by the concentration of ketone bodies in the blood[46]. Ketone bodies are transported over the blood-brain barrier by monocarboxylate transporters and represents a significant energy source when the blood concentration reaches about 4 mmol/L[47].

1.2.5 Mechanism(s) of action of the ketogenic diet

Despite utilizing the ketogenic diet as epilepsy treatment for more than 100 years, it still remains an enigma how the dietary treatment can reduce seizures. Researchers have tried to identify epilepsy aetiologies that respond better to ketogenic dietary treatment than others, but epilepsies of a variety of aetiologies have shown to respond well to the diet[33,48]. In the same way as epilepsy is a complex disease without a single cause, the mechanisms of how the dietary treatment can ameliorate seizures, are likely to be complex and involve overarching mechanisms affecting several aetiologies.

A plethora of hypotheses for the ketogenic diets' mechanism(s) of action have been proposed[49]. Previously, the level of ketosis has received much attention as a possible mediator of the anti-seizure effect. Although the endogenous ketone bodies β-hydroxybutyrate, acetoacetate, and acetone have been shown to have direct anti-seizure effects in animal models of epilepsy[50-52], the results from clinical studies investigating the relationship between ketone body level and seizure control are inconsistent[35,36,42,53,54]. Other suggested mechanisms of action include modulation of neuronal ion channels, enhanced GABAergic neurotransmission, attenuation of reactive oxygen species, increased levels of adenosine which activate inhibitory adenosine A_1 receptors, and enhanced bioenergetics[49]. In recent years, the potential role of gut microbiota and epigenetics in the anti-seizure efficacy of the diets have received much attention[55]. Studies of animal models of epilepsy have demonstrated that administration of ketogenic diet reduces DNA hypermethylation associated with epilepsy and increases seizure threshold[56,57]. The hypothesis of modulation of the gut microbiota as the mediator of the clinical efficacy of ketogenic diets was strengthened by the landmark study of Olson et al. [58]. This study demonstrated that the gut microbiota is required to achieve the beneficial effects of the ketogenic diet in two different mouse models of refractory epilepsy. Moreover, they reported that the anti-seizure effect of the ketogenic diet was mediated by the gut microbiota through modulation of GABA/glutamate ratio of the hippocampus. Although very exciting findings, confirmation in human clinical trials is lacking.

1.2.6 Adverse effects of the ketogenic diet in epilepsy

Potential adverse effects of the ketogenic diet include gastrointestinal symptoms, such as constipation, nausea, vomiting, abdominal pain and diarrhoea, reflux, undesirable weight loss, lack of energy, hypoglycaemia, metabolic acidosis, hyperlipidaemia, impaired growth, hyperuricemia, and kidney stones[59]. Other more rarely reported adverse effects are pancreatitis[60], cardiomyopathy, and prolonged QT intervals[61]. The long-term risk of adverse effects and the impact on overall health of ketogenic diet is currently uncertain[62].

1.3 Epigenetics

1.3.1 What is epigenetics?

Epigenetics literally means “above genetics” and is, in simple words, the study of mechanisms that regulate gene expression. The term was first introduced in the early 1940s by the biologist and geneticist Conrad H. Waddington who defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” [63]. Later, the meaning of the term has evolved and various definitions has been proposed. In general, it is agreed upon that epigenetics are chemical modifications of DNA and histone proteins that affect gene expression. These modifications can be heritable through cell divisions, yet potentially reversible, and do not change the DNA sequence itself (**Figure 2**) [64]. The complete collection of epigenetic changes in a genome is called the epigenome.

There are various epigenetic mechanisms, including DNA methylation, histone modifications, and microRNA expression. Epigenetic modifications are dynamic and potential reversible, thus, enabling the organism to respond to environmental clues[64]. It is well known that various lifestyle factors, such as diet, smoking, alcohol consumption, and physical activity can influence the epigenetic patterns, and thus the expression of genes[65].

Figure 2: The main characteristics of epigenetics. *Figure made with icons from Shutterstock and free images from Servier Medical Art (Creative Commons Attribution License, https://creativecommons.org/licenses/by/4.0/).*

1.3.2 DNA methylation

One of the most studied epigenetic modifications is DNA methylation. DNA methylation is essential for gene regulation, cell differentiation, embryonic development, chromatin structure, X chromosome inactivation, genomic imprinting, and genomic stability[66]. A wide range of diseases, including epilepsy and other neurological disorders, have been shown to be associated with aberrant DNA methylation[67-69].

DNA methylation is the addition of a methyl group (CH_3) to a cytosine nucleotide in the DNA. This reaction is catalysed by a family of enzymes called DNA methyltransferases (DNMTs) that covalently transfer a methyl group from the universal methyl group donor S-adenosyl methionine (SAM) to the fifth carbon of cytosines (**Figure 3**). DNMT1 specifically recognizes hemi-methylated DNA and is responsible for maintaining the DNA methylation, while DNMT3A and DNMT3B catalyse *de novo* methylation of the DNA.

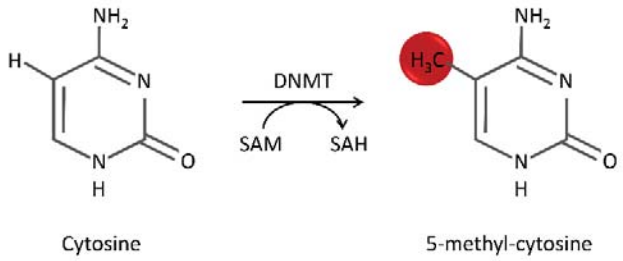


Figure 3: DNA methylation. DNA methylation involves the transfer of a methyl group (CH_3) to the fifth carbon of cytosine, a reaction that is catalysed by DNA methyltransferases (DNMTs).
Abbreviations: DNMT, DNA methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosyl methionine.

Methylation of the DNA primarily occurs at cytosine nucleotides followed by guanine nucleotides, called cytosine-phosphate-guanine dinucleotides (CpGs). In general, DNA methylation is associated with transcriptional silencing, although this depends on genomic location[70]. In total, there are approximately 28 million CpGs in the human genome, of which less than 10% are clustered in short regions with a particular high density of CpGs, called CpG islands (CGIs) [71]. The CGIs are often located within or close to gene promoters and can be directly involved in the regulation of gene expression. The vast majority of CpGs within the mammalian genome are methylated, with the exception of CpGs within CGIs, which usually are unmethylated. However, if methylated within CGI, it tends to silence the gene[70].

Removal of methyl groups from DNA, a process referred to as DNA demethylation, can occur through either passive or active mechanisms. Passive DNA demethylation occurs when the newly synthesized DNA strands are not methylated by DNMT1 after replication. Active DNA demethylation takes place through several sequential enzymatic processes. The first step in active DNA demethylation is the oxidation of 5-methyl cytosine to 5-hydroxymethylcytosine catalysed by the ten-eleven translocation (TET) protein family of enzymes. 5-hydroxymethylcytosine is then further metabolized to 5-formylcytosine and 5-carboxylcytosine. Both 5-formylcytosine and 5-carboxylcytosine can then

eventually be converted back to unmethylated cytosine by either a replication-dependent dilution or by base excision repair mediated by thymine DNA glycosylase[72].

DNA methylation is highly tissue- and cell type specific (i.e. the DNA methylation pattern differs between tissues and cell types) [73]. Differences in the epigenome enable cells with identical genes to maintain a cell-type-specific gene expression pattern[74]. In fact, the intraindividual differences in epigenetic patterns between tissues are greater than the interindividual differences within a given tissue[75]. Hence, this needs to be taken into account when studying the association between DNA methylation and disease phenotype.

1.3.3 Nutrition and DNA methylation

Nutrition is by its continuous exposure throughout life a key environmental factor that influences epigenetic modifications of the genome. Nutriepigenomics, the study of food nutrients and their effects on human health through epigenetic modifications, is a rapidly evolving field of research[76]. Both nutritional status and the dietary intake of micronutrients and bioactive compounds can modify the epigenome and subsequently the gene expression[76,77].

Central to DNA methylation is the so-called “one-carbon metabolism” which encompasses a series of cyclical biochemical reactions that provides one-carbon groups (also referred to as methyl groups) for multiple biological processes, including methylation of DNA. One-carbon metabolism includes both the folate cycle and the methionine cycle, and is dependent upon a number of enzymes and micronutrients. Folate, choline, betaine, and methionine are important dietary derived sources of methyl groups. A number of cofactors, including vitamins B_2, B_6 and B_{12}, are also required in the one-carbon metabolism[77]. The methionine and folate cycles and their relation to dietary micronutrients, are illustrated in **Figure 4**.

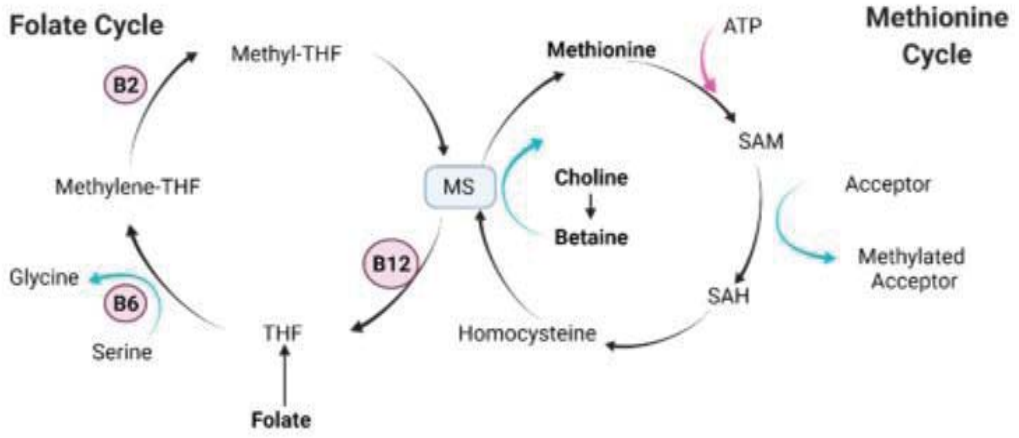


Figure 4: The interlinked folate and methionine cycles and their relation to dietary micronutrients. Dietary derived folate enters the folate cycle after it is first converted into dihydrofolate and then tetrahydrofolate (THF). THF is converted to 5,10-methylene THF. 5,-10-methylene THF is further converted into 5-Methyl-THF by the key enzyme 5,10-methyltetrahydrofolate reductase (MTHFR). In the methionine cycle, methionine is converted into the universal methyl donor S-adenosyl methionine (SAM) which again is converted to S-adenosylhomocysteine (SAH) by SAH hydrolase when a methyl group is transferred to an acceptor (i.e. DNA methylation). This is an ATP-driven reaction. SAH is reversibly metabolized to homocysteine by S-adenosyl-L-homocysteine hydrolase. Homocysteine can be remethylated back to methionine by either the folate cycle or by a betaine-dependent mechanism. Dietary derived choline is oxidized to betaine, which donates a methyl group to homocysteine. This results in the conversion of homocysteine to methionine. B vitamins acts as cofactors for several of the enzymatic processes in the folate and methionine cycle. Figure modified from[78]. (Modifications made: the link between dietary micronutrients and cognitive changes is omitted.) (Creative Commons Attribution License, https://creativecommons.org/licenses/by/4.0/).
Abbreviations: ATP, adenosine triphosphate; B2, vitamin B_2; B6, vitamin B_6; B12, vitamin B_{12}; MS; methionine synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosyl methionine; THF, tetrahydrofolate.

In 2003, a landmark study demonstrating the importance of nutrition for epigenetic regulation of gene expression was published by Waterland and Jirtle[79]. Here, they used the viable yellow agouti (A^{vy}) mouse model to investigate how a diet rich in methyl donors and cofactors of the one-carbon metabolism (i.e. choline, betaine, folic acid, and vitamin B_{12}) during pregnancy affected DNA methylation and phenotype of agouti offspring. When the agouti gene is continuously expressed, this mouse strain has a characteristic phenotype of yellow coat colour, obesity, and a predisposition to diabetes and cancer. However, in offspring of mothers fed a supplemented diet, DNA methylation increased and the agouti gene was silenced, resulting in offspring with brown fur, and a generally healthier phenotype than the offspring of mothers fed an unsupplemented diet.

Besides altering the availability of substrate necessary for DNA methylation through the one-carbon metabolism, certain bioactive food components can have a direct impact on epigenetic enzymes, such as DNMT[80]. For example, *in vitro* studies have shown that the dietary polyphenols genistein (from soybean) and catechin (from tea) inhibit the activity of DNMTs[81]. Other nutrients, such as selenium can also influence DNA methylation. *In vitro* and *in vivo* studies have demonstrated that a reduction in selenium causes a decrease in global DNA methylation and decreased expression of DNMTs[82,83].

Another example underlining the importance of nutritional status for epigenetic regulation of gene expression is the study by Heijmans et al. [84]. Here, they investigated the epigenetic consequences of perinatal exposure to famine in humans six decades later. The authors demonstrated that individuals prenatally exposed to famine during the Dutch Hunger Winter in 1944-45, had reduced DNA methylation of the insulin-like growth factor II gene compared to their unexposed siblings when measured 60 years later. This was the first study to demonstrate that transient exposure to environmental factors during early stages of gestation could lead to persistent and even life-long epigenetic changes. Persistent epigenetic changes induced in early life may have long-term consequences for health and disease[85-87].

1.3.4 DNA methylation and epilepsy

Given the essential role of DNA methylation of normal brain development and plasticity, it is perhaps not surprising that aberrant DNA methylation has been associated with neurological diseases (reviewed in[88]). In recent years, the potential role of DNA methylation in the pathophysiology of epilepsy, as well as a possible therapeutic target, has been increasingly recognized[89].

Abnormal DNA methylation has been found in both experimental animal models and in humans with epilepsy (reviewed in[89]). In general, the majority of studies investigating DNA methylation in epilepsy have reported a preponderance of increased DNA methylation[56,67,69,90-94]. In a genome-wide study of

DNA methylation in blood, Long et al. [67] demonstrated increased DNA methylation at more than 75% of the positions differentially methylated in epilepsy patients compared to healthy controls. Increased DNA methylation in epilepsy have also been reported in studies comparing DNA methylation in brain samples from patients who have undergone resective epilepsy surgery with autopsy material[69,92]. Moreover, the expression of DNMTs in brain tissue from temporal lobe epilepsy patients have been found to be increased compared to controls[93]. However, there might be brain region-specific differences in DNA methylation and DNMT expression[94].

Interestingly, inhibition of DNA methylation in animal models of epilepsy appears to prevent epileptogenesis[95]. Indeed, ketogenic diet has been shown to counteract the hypermethylation in animal models of epilepsy, a finding that was correlated with increased seizure threshold[56,57]. Of note, it can also be mentioned that valproic acid, one of the most commonly used and highly effective ASMs, has been shown to induce a decrease in global DNA methylation[96], supporting the notion that DNA methylation level may be associated with seizure threshold.

1.4 Pharmacokinetics

1.4.1 Pharmacokinetics

Pharmacokinetics can in simple terms be described as “what the body does to the drug” and refers to the various processes from the drug enters the body until it is excreted. The main processes of pharmacokinetics include absorption, tissue distribution, metabolism, and finally excretion of the drug (**Figure 5**).

Various factors such as age, sex, genetics, ethnicity, pregnancy, and pathological states can influence pharmacokinetics and hence the serum concentration of a drug[97]. Moreover, interactions between drugs (drug-drug interactions), i.e. when a drug is taken together with one or more drugs, can also affect

serum concentration. Perhaps less well known is the potential of interactions between food and drugs (food-drug interactions). Intake of specific food components, macronutrient compositions, or nutritional status may alter the pharmacokinetics of the drug and ultimately either increase or decrease the serum concentration of the drug[98]. The recent years, questions about potential pharmacokinetic interactions between the ketogenic diet and ASMs have been raised.

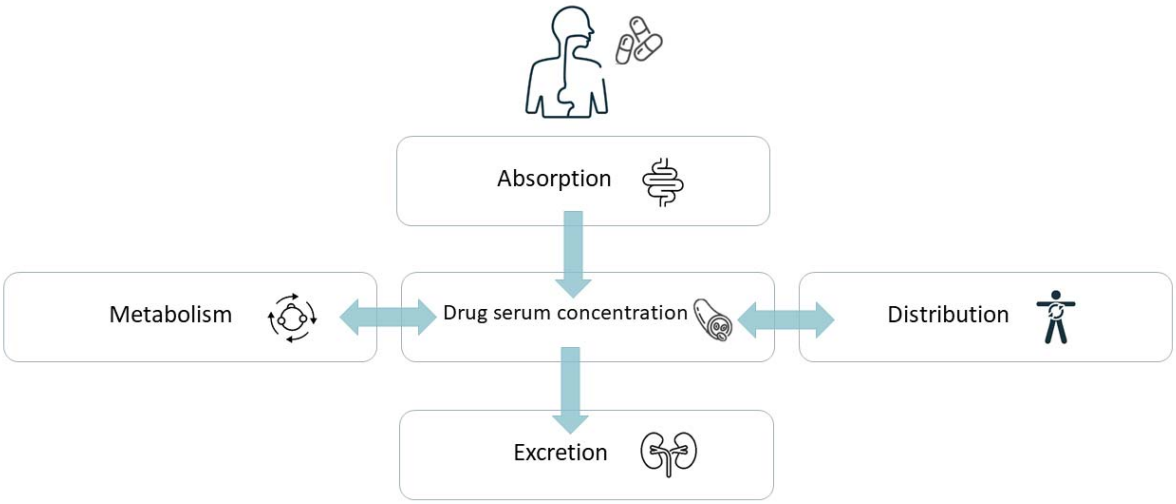


Figure 5: Principles of pharmacokinetics. Pharmacokinetics include the processes of gastrointestinal absorption, tissue distribution, metabolism, and excretion of metabolites. *Figure inspired by[97] and made with icons from Shutterstock.*

1.4.2 Potential pharmacokinetic interactions between ketogenic diet and ASMs

Ketogenic diets are commonly used as an add-on treatment to ASMs. A potential consequence of pharmacokinetic interactions between ketogenic diets and ASMs, are altered serum concentration of the ASMs. Reduced ASM serum concentrations could decrease the efficacy of the ASMs, leaving the patient at risk of being less protected against seizures. An increase in the ASM serum concentrations could increase the risk of toxicity and adverse effects. On the other hand, increased ASM serum concentrations could also theoretically have a positive effect if a patient had a suboptimal serum

concentration in the first place. All of these potential consequences could in turn lead to misinterpretation of the effectiveness and adverse effects of ketogenic diet interventions in clinical trials (**Figure 6**).

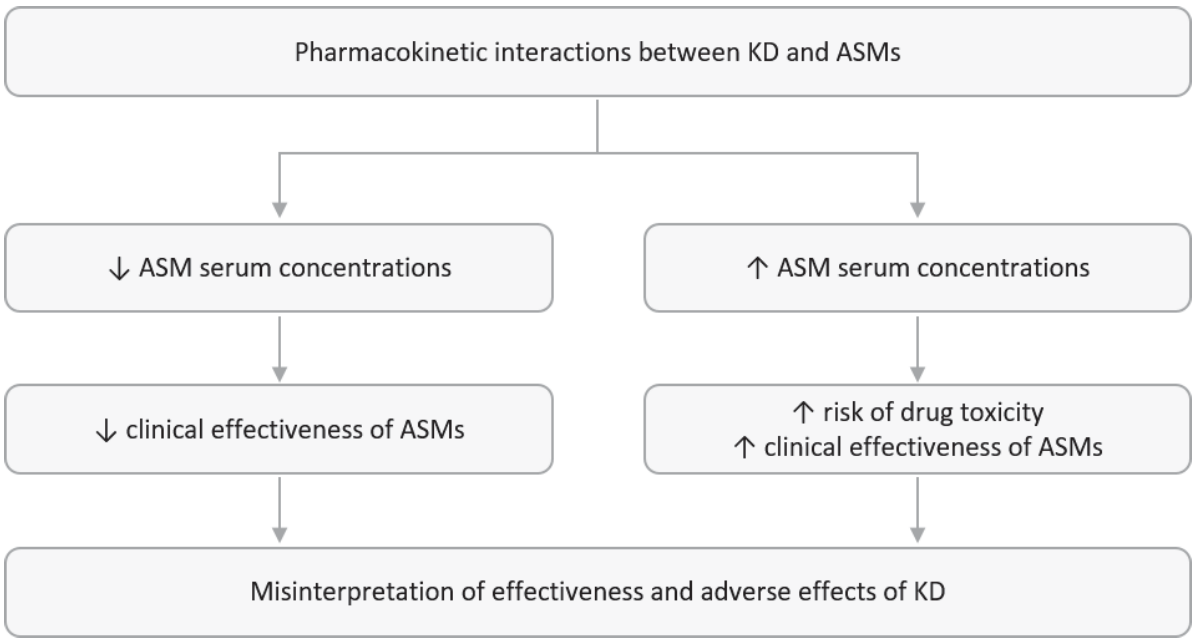


Figure 6: Potential consequences of pharmacokinetic interactions between ketogenic diets and ASMs. Pharmacokinetic interactions between ketogenic diets and ASMs can lead to either a decrease or an increase in ASM serum concentrations. Decreased ASM serum concentration may reduce the clinical effectiveness of the ASMs (i.e. reduced protection against seizures), while increased ASM serum concentration may increase the risk of drug toxicity (i.e. increased adverse effects). On the other hand, increased ASM serum concentration may improve treatment effectiveness if the patient had a suboptimal ASM serum concentration before ketogenic diet initiation. Taken together, unintended alterations in ASM serum concentrations may lead to misinterpretation of effectiveness and adverse effects of the dietary treatment.
Abbreviations: ASM, anti-seizure medication; KD, ketogenic diet.

Our research group has previously reported a substantial decrease in several ASMs in adults treated with a modified ketogenic diet[99]. Other studies investigating this topic in children treated with ketogenic diets have shown conflicting results, but important limitations of these studies include retrospective study design, small sample size, changes in ASMs during the diet intervention, as well as few ASMs examined[100-102]. An overview of the studies investigating the potential pharmacokinetic interactions between ketogenic diet and ASMs and the main results are given in **Table 2**.

Knowledge of potential pharmacokinetic interactions is imperative in order to make recommendations about ASMs that are favourable to combine with a ketogenic diet, as well as recommendations regarding monitoring of ASM serum concentrations during the dietary treatment. Furthermore, since potential interactions could lead to misinterpretation of effectiveness in clinical trials, it is of both great clinical and scientific importance to determine whether there are pharmacokinetic interactions between the ketogenic diet and ASMs.

Table 2: Clinical studies investigating the influence of ketogenic diets on ASM serum concentrations.

Author	Year	Subjects	*n*	Study design	Diet type	Diet duration	Results
Kverneland[99]	2019	Adults	63	Prospective, non-randomized clinical study	MKD	4 and 12 weeks	↓ CBZ, CLB, LCM, LTG, TPM, VPA NS: LEV, ZNS, OXC
Welzel[103]	2020	Infant	1	Case report	CKD	49 days	↓ CBZ
Heo[102]	2017	Children and adolescents	139	Retrospective study	CKD, MKD, and LGIT	≥30 – 120 days[a]	↓ VPA NS: CBZ, LTG, LEV, PB, TPM
Coppola[101]	2010	Children and adolescents	36	Prospective, non-randomized clinical study	CKD	1, 2, 3, and 4 weeks	NS: PB, VPA
Dahlin[100]	2006	Children and adolescents	51	Prospective, non-randomized clinical study	CKD	1 month	NS: CBZ, CLB, LTG, PB, PHT, TPM, VPA

Downward arrow (↓) indicates statistical significant reduction in ASM serum concentration, while "NS" indicates ASMs without statistical significant change in serum concentration.
Abbreviations: ASM, anti-seizure medication; CBZ, carbamazepine; CKD, classical ketogenic diet; CLB, clobazam; LCM, lacosamide; LTG, lamotrigine; LEV, levetiracetam; MKD, modified ketogenic diet; NS, not significant; OXC, oxcarbazepine; PB, phenobarbital; TPM, topiramate; VPA, valproic acid; ZNS, zonisamide.
[a]Variable time on diet, from 30 days to up to 120 days.

1.4.3 Metabolism of ASMs

Most ASMs are mainly metabolized in the liver by cytochrome P450 (CYP) or the UDP-glucuronosyltransferase (UGT) super families of enzymes[18]. The CYP and UGT enzymes convert lipophilic compounds to more hydrophilic compounds, thereby facilitating the elimination of drugs from the body. Together, these enzymes are responsible for the metabolism of more than 90% of drugs that are cleared hepatically[104]. A few ASMs are metabolized extrahepatic or excreted unchanged in the urine[18].

1.4.4 Pharmacoepigenetics

Although genetic factors certainly contribute to pharmacokinetic variability, it has become evident that genetics only determine a minor part of the interpersonal differences in drug response[97,105]. Thus, there must be other factors contributing to the interindividual differences. Pharmacoepigenetics, the study of how epigenetic marks affect individual variations in drug response, may offer an explanation of the pharmacokinetic interpersonal variability which cannot be ascribed to genetic factors[106].

DNA methylation has been found to be involved in the regulation of genes encoding drug-metabolizing enzymes[107]. As mentioned above, diet is an important environmental factor that influence DNA methylation, and preclinical studies have demonstrated that high-fat diets can influence the expression of drug-metabolizing enzymes[108,109].

2 AIMS OF THE THESIS

The overarching aim of this thesis was to improve our understanding of how the ketogenic diet ameliorates seizures in patients with epilepsy by investigating how the ketogenic diet influences the body at a molecular level. The specific objectives of the papers were:

- To investigate the impact of a modified ketogenic diet on DNA methylation in adults with drug resistant epilepsy (Paper I).

- To investigate the impact of a modified ketogenic diet on DNA methylation of candidate genes encoding enzymes involved in the metabolism of ASMs, and to examine the correlation between change in DNA methylation and ASM serum concentrations in adults with drug resistant (Paper II).

- To examine the potential influence of a classical ketogenic diet on serum concentrations of ASMs in children with drug resistant epilepsy (Paper III).

3 MATERIAL AND METHODS

3.1 Study populations and study design

The present work is based on two different study populations:

1) Adults with drug resistant epilepsy treated with a modified ketogenic diet (Paper I and II)

2) Children with drug resistant epilepsy treated with a classical ketogenic diet (Paper III)

Both study populations were recruited at the National Centre for Epilepsy, Oslo University Hospital, Norway.

3.1.1 Adult study population (Paper I and II)

The adult study population consisted of 58 patients (age 16-65 years) merged from one randomized and one non-randomized study by Kverneland et al. [110,111] recruited between March 1, 2011, and February 28, 2017. In the randomized study, all participants were eventually offered the dietary treatment, either immediately after the baseline period (intervention group) or after a 12-week delay (control group). When the study populations were merged, we redefined the control groups' baseline period as the 12 weeks immediately preceding the intervention period. Thus, the study design of the merged cohort can be considered as a prospective, non-randomized clinical trial.

In the baseline period, the participants ate their normal diet, recorded seizures systematically, and all epilepsy treatments were kept unchanged. In the 12-week diet intervention period, the participants ate a modified ketogenic diet (see section 3.2.1), and continued to systematically record seizures. All other epilepsy treatments were unchanged throughout the entire study. Data were collected at baseline (pre-diet), and after 4 and 12 weeks of diet intervention.

The inclusion criteria were generalized or focal epilepsy according to the International ILAE's classification[10], ≥3 countable seizures per month, having tried ≥3 ASMs, age ≥16 years, BMI >18.5 kg/m^2, and the participants had to be motivated and willing to try the dietary treatment for at least 12 weeks.

Exclusion criteria included familial hypercholesterolaemia, cardiovascular disease, kidney disease, psychogenic non-epileptic seizures, diseases which contraindicated the dietary treatment, previous treatment with a ketogenic diet for more than one week during the preceding year, status epilepticus the past six months, epilepsy surgery or vagus nerve stimulator implant the past year, four continuous seizure-free weeks the preceding two months, use of drugs or supplements that may interfere with the diet or ASMs, and pregnancy or planned pregnancy.

3.1.2 Paediatric study (Paper III)

The paediatric study population consisted of 28 children (age 2 – 13 years) recruited in a prospective, non-randomized clinical study between August 15th, 2017, and January 31th, 2022. The participants ate their habitual diet in a 4-week baseline period and subsequently a classical ketogenic diet (see section 3.2.2) in a 12-week intervention period. Seizures were recorded systematically and all other epilepsy treatments were kept unchanged during the 16-week study period.

The inclusion criteria were age between 2 and 18 years, drug resistant epilepsy according to the ILAE's classification[20], two or more countable seizures per week on average, and willingness to try the classical ketogenic diet in at least 12 weeks.

Exclusion criteria were glucose transporter protein 1 deficiency syndrome, pyruvate dehydrogenase deficiency, pyruvate carboxylate deficiency, diseases which contraindicated the dietary treatment, epilepsy surgery, including vagus nerve stimulator implantation the past 6 months before diet initiation, steroid medication the past two months before diet initiation, continuous, prophylactic

antibiotic treatment, breastfeeding, psychogenic non-epileptic seizures, eating disorders, feeding disabilities expected to unable the dietary treatment, inability to follow the study protocol, including lack of motivation by patient or caregivers, previous treatment with a ketogenic diet, medical need to start dietary treatment immediately, and pregnancy or planned pregnancy.

3.2 Dietary interventions

3.2.1 Adult study (Paper I and II)

The modified ketogenic diet was initiated at home after an educational course in the dietary treatment during hospital admission. The diet contained a maximum of 16 g carbohydrate per day (excluding fibres and alcohol). The participants were encouraged to eat food items with a high fat content to replace the carbohydrate in the diet. Protein was eaten *ad libitum*. The total energy content was not restricted. The diet was supplemented with vitamins and minerals. A daily fluid intake of 2-3 L was recommended. The nutritional content of the meals were calculated using the Norwegian Food Composition Database[112]. All food items were weighed on a scale with an accuracy of 1 gram.

3.2.2 Paediatric study (Paper III)

The classical ketogenic diet was initiated during hospital admission using a non-fasting, full-calorie, gradual initiation protocol. The diet was started at a ketogenic ratio (ratio of gram fat: gram carbohydrate plus protein) of 1:1 – 2:1 with a gradual increase up to a maximum of 4:1 depending on the child's tolerability and efficacy. The protein content was minimum 0.9 grams per kg body weight per day. The diet was supplemented with adequate amounts of vitamins, minerals, and trace elements, as well as omega-3 fatty acids. A fluid intake corresponding to the estimated fluid requirement was recommended. The macro nutrient content of the meals was calculated using the electronic meal planner DietistNet (Kost och Näringsdata, Bromma, Sverige) with associated databases, including the

Norwegian Food Composition Database[112]. All food items were weighed on a scale with an accuracy of 0.1 gram.

3.3 Participant adherence (Paper I-III)

Adherence to the study protocols were assessed at each study visit. Details of the assessments are given in each paper, but in short, adherence was assessed by structured questions regarding the diet intervention, prescribed ASMs, and other epilepsy treatments, biochemical measurements (i.e. blood glucose, blood ketones, haemoglobin A_{1c} (HbA_{1c}), and in the adult study also urine ketones), and 3 days' weighed food diaries.

3.4 DNA methylation analysis (Paper I and II)

An overview of the main steps of the DNA methylation analysis from blood sampling to data analysis is presented in **Figure 7**. First, venous blood samples were collected at baseline, and after 4 and 12 weeks of dietary treatment. Next, DNA were extracted from white blood cells, quantified, and analysed for DNA methylation using the Illumina Infinium® MethylationEPIC BeadChip (hereafter EPIC array). The EPIC array offers a comprehensive coverage and measures DNA methylation with a single-nucleotide resolution of approximately 850.000 selected positions across the genome. An essential step in the methylation analysis is bisulfite conversion of DNA. Treatment of DNA with sodium bisulfite converts unmethylated cytosines to uracil, while methylated cytosines remains as cytosines (**Figure 8**). Uracil is later converted to thymine (post amplification) [113]. In this way epigenetic information is transformed into genetic information that enables us to use traditional genotyping methods to assess methylation status by analysing which cytosines that have been converted to thymine following bisulfite treatment[114].

The next steps of the sample preparation are amplification and enzymatic fragmentation of the DNA, to ensure an oligonucleotide-length that is optimal for array hybridization. The fragmented DNA is then applied to the array, which contains millions of tiny silica beads embedded in micro wells. Each bead is

coated with multiple copies of a DNA oligonucleotide probe targeting a specific locus in the genome. Importantly, each bead have a different probe attached to their surface and perform a different[115].

DNA fragments from the sample will attach to their complementary probe oligonucleotide sequence, a process referred to as hybridization. The EPIC array utilizes both Infinium I probes and Infinium II probes. Infinium I probes uses two bead types per position; one methylated bead (M bead) and one unmethylated bead (U bead). The M bead oligonucleotide sequence is designed to match the methylated locus of bisulfite converted DNA, while the U bead oligonucleotide sequence is complementary to the unmethylated locus of the bisulfite converted DNA. The oligonucleotide of the unmethylated probe ends in cytosine-adenine, which is complementary to guanine – thymine. Guanine – thymine is the converted sequence of an unmethylated CpG after bisulfite treatment. A methylated CpG, on the other hand, will not convert to guanine – thymine and thus do not hybridize to the unmethylated probe. Conversely, the oligonucleotide sequence of the methylated probe ends in cytosine-guanine, which is complementary to the methylated locus, thus allowing for hybridization. Hybridization enables extension of the probe by a labelled single base immediately upstream of the CpG of interest. The Infinium II probes uses only one bead (U/M bead) per position. These probes are designed to match both the unmethylated and the methylated locus, and report the methylation status by using two different colours (red and green). The probes ends in cytosine and through a single-base extension reaction, a base that is complementary to either a cytosine that has been converted or not, are incorporated[114].

Adenine and thymine nucleotides are labelled with dinitrophenyl while guanine and cytosine nucleotides are labelled with biotin. After the base extension of the probes, the next step is staining. Green fluorescent streptavidin, which binds to the biotin labelled probes, and red fluorescent anti-dinitrophenyl antibody, which binds to the dinitrophenyl labelled probes, are applied to the array. After

this staining process, the array is imaged in an Illumina scanner to measure the fluorescent intensity signal for each bead. The methylation level at each position can then be calculated by the ratio of the intensity of the fluorescent methylated and the unmethylated signal[115].

There are two ways to express methylation levels at a genomic locus; β-values and M-values. The β-value is defined as the ratio of methylated versus unmethylated allele, using the formula: β = intensity of the methylated signal/ (intensity of the methylated signal + intensity of the unmethylated signal + 100). The β-values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated. The M-value is defined as the $\log_2$ of the β-value. The main advantage of β-values are their intuitive biological interpretation. However, due to heteroscedasticity, the β-values are less applicable for many statistical analysis. Indeed, M-values are recommended to use for differential DNA methylation analysis because they are approximately homoscedastic[116].

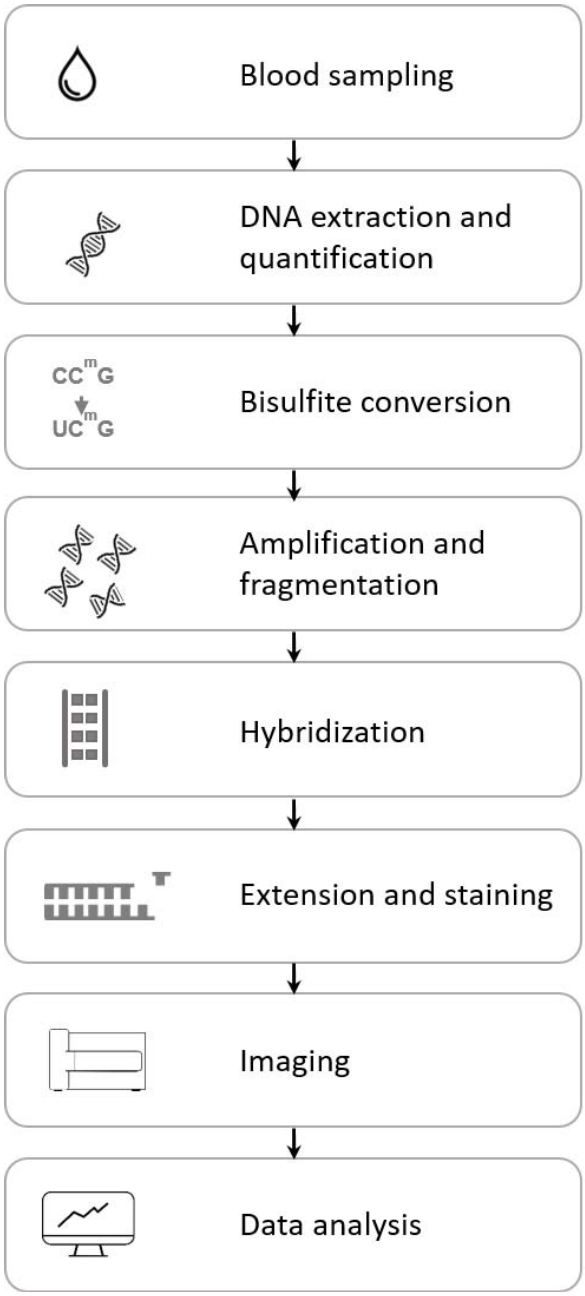


Figure 7: An overview of the DNA methylation analysis workflow from blood sampling to data analysis. First**,** venous blood samples were collected at baseline, and after 4 and 12 weeks of dietary treatment. Next, genomic DNA were extracted from white blood cells and quantified before sodium bisulfite treatment. Bisulfite treatment is a chemical process in which unmethylated cytosines are converted into uracil, while methylated cytosines remain unchanged (**Figure 8**). The bisulfite treated DNA is then used as template in the amplification step, in which the amplicons will have thymine instead if uracil. The next step of the sample preparation is enzymatic fragmentation, before applying the bisulfite treated sample into the microarray chip. The microarray contains millions of tiny silica beads, each coated with hundreds of thousands of copies of a probe specific to a locus of interest. Hybridization of the bisulfite treated sample-DNA and microarray probes allow for extension, meaning that a single, labelled nucleotide is incorporated at the end of the probe sequence. Thereafter, in the staining step, a fluorescent signal specific for the labelled probes are applied. Finally, the array is imaged by the Illumina scanner in which the fluorescent intensity signal for each bead in the array are measured and made available for analysis. *Figure inspired by*[117] *and made with icons from Shutterstock.*

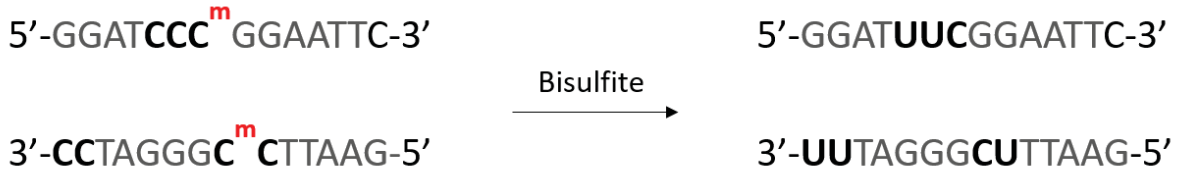


Figure 8: Bisulfite conversion of DNA. Treatment of DNA with sodium bisulfite converts unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. Bisulfite conversion of unmethylated cytosines result in two non-complementary DNA strands. *Bold indicates methylated and unmethylated cytosine positions before and after bisulfite conversion. Figure inspired by*[118].
Abbreviations: A, adenine; C, cytosine; C^m, methylated cytosine, G, guanine, T, thymine; U, uracil.

3.5 ASM serum concentration analysis (Paper II and III)

The ASM serum concentration analyses are described in detail in Paper II and III. A brief overview of the methods used to measure the ASM serum concentrations is given below.

All ASM serum concentration analyses were performed by validated methods at the Section for Clinical Pharmacology, Oslo University Hospital (Oslo, Norway). Since the adult and the paediatric study were conducted in different time periods, the methods for measuring the ASM serum concentrations differ between the two studies.

3.5.1 Adult study

Venous blood samples were drawn food- and drug fasting in the morning at assumed steady-state of the ASMs at baseline (pre-diet), and after 4 and 12 weeks of dietary treatment. ASM serum concentrations were analysed by either an immunoassay (carbamazepine, phenobarbital, phenytoin, topiramate, and valproic acid) or by high-pressure liquid chromatography with ultraviolet detection (HPLC-UV) (clobazam, clonazepam, desmethylclobazam, lacosamide, lamotrigine, levetiracetam, zonisamide, and oxcarbazepine/ eslicarbazepine acetate).

3.5.2 Paediatric study

Venous blood samples were drawn food- and drug fasting in the morning at assumed steady-state of the ASMs at baseline (pre-diet), and after 6 and 12 weeks of dietary treatment. ASM serum concentrations were measured either by high pressure liquid chromatography with ultraviolet detection (HPLC-UV) (clonazepam, clobazam, and desmethylclobazam) or by ultra-high performance liquid chromatography with mass spectrometric detection (UHPLC-MS/MS) (lacosamide, ethosuximide, lamotrigine, levetiracetam, topiramate, oxcarbazepine, rufinamide, valproic acid, zonisamide).

3.6 Statistics

The statistical analyses are described in details in each paper. A brief description of the main analyses is given in the following sections.

3.6.1 Descriptive statistics (Paper I-III)

Normality of continuous data were examined by visual inspection of histograms and quantile-quantile plots (Q-Q plots). Continuous data are presented as mean with standard deviations (SD) (normally distributed variables), or medians and quartiles with interquartile range (IQR) and minimum- maximum (min – max) scores (non-normally distributed variables). Categorical variables are presented as frequencies and percentages (%). Descriptive data were analysed using a one-sample t-test or a paired t test if data were normally distributed. Data with non-normal distribution were analysed using a non-parametric test (Wilcoxon signed-rank test) which does not assume normal distribution. Statistical significance level was set to $P < 0.05$.

3.6.2 Differential DNA methylation analyses (Paper I and II)

To identify intraindividual differentially methylated CpGs between baseline and 4 and 12 weeks of diet intervention, a linear regression model implemented in the R package *limma*[119] was fitted to M-values ($\log_2$ of the β-values) (Paper I and II). Intraindividual differences in global DNA methylation were tested

using paired *t* test (Paper I). To adjust for multiple comparisons, a false discovery rate (FDR) cut-off of less than 5% was applied by using the method of Benjamini and Hochberg[120] implemented in the *limma* package in R[119].

3.6.3 Correlation analyses of DNA methylation and ASM serum concentrations (Paper II)

Spearman's rank correlation coefficient was used to calculated correlations between absolute difference in DNA methylation (β-values) at individual positions (CpGs) and percentage change in serum concentrations of the individual ASMs after 4 weeks of diet intervention. To adjust for multiple comparisons, a FDR with a significance threshold of less than 5% was applied by using the method of Benjamini and Hochberg[120] implemented in the *stats* package in R[121].

3.6.4 Differences in ASM serum concentrations after dietary treatment (Paper III)

Differences in ASM serum concentrations were investigated using a linear mixed effect model analysis of the relationship between serum concentrations of ASMs and time on ketogenic diet. As fixed effect, we entered time on ketogenic diet into the model, and as random effects we used intercepts for subjects.

3.6.5 Statistical software

IBM Statistical package for the social sciences (SPSS, version 26.0 and 28.0, NY, USA) and R (version 4.1.3, Vienna, Austria, www.r-project.org) were used for all statistical analyses.

4 SUMMARY OF RESULTS

4.1 Paper I: Genome-wide decrease in DNA methylation in adults with epilepsy treated with modified ketogenic diet: A prospective study.

Despite the use of ketogenic diets in the treatment of epilepsy for decades, the mechanism(s) underpinning the ketogenic diets ability to ameliorate seizures in epilepsy remain elusive. Recently, DNA methylation has been highlighted as a possible mechanism for how the ketogenic diet can reduce seizures in people with epilepsy. Hence, in Paper I, we aimed to investigate the impact of a modified ketogenic diet on DNA methylation in adults with drug resistant epilepsy.

Our study cohort consisted of 58 adults (34 females) with drug resistant epilepsy aged 16 to 65 years. The patients had a long history of epilepsy, a high number of previously tried ASMs, and multiple current ASMs. We measured methylation of DNA extracted from white blood cells at more than 700.000 positions across the genome before, and after 4 and 12 weeks of dietary treatment. The results demonstrated a genome-wide decrease in DNA methylation following 12 weeks of treatment with modified ketogenic diet. A considerable share of the differentially methylated positions were annotated to genes associated with epilepsy (*n* = 7), lipid metabolism (*n* = 8), and transcriptional regulation (*n* = 10). Also, five of the identified positions were associated with inositol phosphate metabolism, which was an intriguing finding, as *myo*-inositol has been suggested to have anticonvulsant effects. However, we found no significant differences in DNA methylation between patients who experienced seizure reduction and those who did not.

In conclusion, we identified widespread DNA methylation changes following the dietary treatment. However, the implications of these alterations are yet to be explored. Nevertheless, we believe that a better understanding of the diets' influence at a molecular level is central to unravel the mechanism(s) behind the anti-seizure effect of the dietary treatment in epilepsy.

4.2 Paper II: DNA methylation of genes encoding enzymes involved in metabolism of anti-seizure medications in adults with epilepsy treated with modified ketogenic diet.

In Paper I, we demonstrated decreased DNA methylation following treatment with a modified ketogenic diet. In the same study population, our research group has recently identified a significant decrease in the serum concentration of several ASMs following the dietary treatment[99]. Thus, in Paper II, we hypothesized that altered DNA methylation of genes involved in ASM metabolism may be involved in the observed decrease of ASM serum concentrations.

Analysis of in total 131 positions annotated to 13 genes related to ASM metabolism revealed only one differentially methylated position annotated to the gene *carboxylesterase 1* (*CES1*). This position was already identified in Paper I. Although only one positions had a statistically significant change in DNA methylation, even minor alterations may have an impact on gene expression. Hence, we next performed correlation analysis to examine whether changes in DNA methylation correlated with changes in ASM serum concentrations. We found a strong negative correlation between absolute change in DNA methylation of one position annotated to *UGT1A4* and percentage change in serum concentration of lamotrigine (*rho* = -0.85), whereas the remaining positions showed no significant correlation.

In conclusion, our targeted approach in Paper II did not identify additional differentially methylated positions annotated to genes involved in the metabolism of ASMs. Also, at the majority of positions, we did not find any correlation between changes in DNA methylation and changes in ASM serum concentrations.

4.3 Paper III: The impact of ketogenic diet on serum concentrations of anti-seizure medications in children with drug resistant epilepsy.

In a recent work by our research group, a significant decrease in the serum concentration of several ASMs in adults treated with a modified ketogenic diet were demonstrated[99]. Currently, the knowledge about potential pharmacokinetic interactions between ketogenic diets and ASMs are limited and the results are inconsistent. Thus, in Paper III, we aimed to examine the potential influence of classical ketogenic diet on serum concentrations of ASMs in children with drug resistant epilepsy.

The serum concentrations of ASMs were measured before diet initiation, and after 6 and 12 weeks of treatment with classical ketogenic diet. Prospective data from 25 children aged 2 to 13 years (12 females) were included in our analysis. The participants used in total 12 different ASMs and the majority used polypharmacy (92%) during the diet intervention. None of the participants changed the type or dose of the ASMs during the study. We found a significant decrease in the serum concentration of clobazam, desmethylclobazam, and lamotrigine following the diet intervention. Our analysis did not reveal any significant differences in the serum concentrations of levetiracetam, topiramate, and valproic acid following the diet intervention, although there was a non-significant trend towards a decrease in valproic acid.

In conclusion, in Paper III, we demonstrated a significant decrease in the serum concentration of clobazam, desmethylclobazam, and lamotrigine in children with drug resistant epilepsy following treatment with ketogenic diet. Unintended changes in the ASM serum concentrations may render the patient less protected against seizures. In addition, altered ASM serum concentrations might lead to misinterpretation of the effectiveness of the dietary treatment. Hence, we suggest to monitor the ASM serum concentrations in patients starting on a ketogenic diet.

5 DISCUSSION

5.1 Epigenetics as mediating effect of ketogenic diets

5.1.1 DNA methylation following treatment with ketogenic diet

In Paper I, we demonstrated a global decrease in DNA methylation in adults with drug resistant epilepsy treated with a modified ketogenic diet. A number of differentially methylated positions annotated to genes with a wide range of biological functions, including epilepsy, regulation of fatty acid and glucose metabolism, transcription, inositol phosphate metabolism, and various basic cell functions, were identified to be differentially methylated after treatment with a modified ketogenic diet. Strikingly, all of these differentially methylated positions had a decrease in DNA methylation.

It has been proposed that the observed long-lasting seizure-reducing effects of ketogenic diets in epilepsy might be explained by persistent epigenetic changes[55]. The growing evidence suggesting an *increased* DNA methylation in epilepsy made our observations of *decreased* DNA methylation following diet intervention particularly intriguing. Ketogenic diets have been shown to attenuate seizures and counteract the aberrant increase in DNA methylation[56,57], even after diet withdrawal[57]. However, whether the observed decreased DNA methylation following treatment with a ketogenic diet occurs at the same genomic positions as those found to have increased DNA methylation, is yet unknown. Indeed, we were not able to identify any differences in DNA methylation between responders and non-responders in our study (see also section 5.3.6). Thus, it is currently uncertain whether these alterations in DNA methylation are related to the ketogenic diets seizure-reducing effect in epilepsy.

To our knowledge, no previous studies have investigated the impact of ketogenic diet on DNA methylation in humans with epilepsy. Our results corroborate previous studies in animal models of epilepsy demonstrating a decrease in DNA methylation following ketogenic diet treatment[56,57]. Also, in a

study of patients with obesity treated with a very low calorie ketogenic diet found a decrease in DNA methylation following the diet intervention. Similar to our study, they measured the methylation level in DNA extracted from white blood cells using EPIC array[122].

5.1.2 How may ketogenic diet influence DNA methylation?

Generally, DNA methylation is a link between environment and gene expression, by which environmental cues (e.g. changes in dietary intake) change DNA methylation, and in turn, gene expression. Currently, the mechanisms underlying the observed decrease in DNA methylation following the ketogenic diet are unknown. Nutrients, especially those involved in one-carbon metabolism, such as folate, methionine, choline, and betaine, are likely to have an impact on DNA methylation because of their influence on the one-carbon metabolism in which the universal methyl donor SAM is synthesized.

Considering the significant overall reduction in DNA methylation observed in our study, one could speculate whether the ketogenic diet is deficient in methyl-donors and co-factors for the one-carbon metabolism. However, all participants were provided a multivitamin- and mineral supplement containing folic acid and other B vitamins. Thus, we assume that the intake of these nutrients increased, rather than decreased, during the diet intervention. This is supported by blood samples in study participants revealing increased concentrations of both folate and vitamin B_{12}, as well as unchanged homocysteine concentration (plasma level of homocysteine is used as a biomarker of folate and vitamin B_{12} status; homocysteine rises when serum folate and vitamin B_{12} are low[123]) following the diet intervention. Methionine is an amino acid found in protein rich foods like fish, meat, eggs, and dairy products. Based on 3-day weighed food records, the estimated protein intake before and during the diet intervention were similar, indicating that the intake of methionine was not decreased during the dietary treatment. Although we do not have data on the intake of choline and betaine during the study, a study in six patients with Alzheimer's disease on a ketogenic diet with similar protein and carbohydrate

content as in our intervention, demonstrated increased intake of choline[124]. Taken together, there is no evidence indicating that ketogenic diet is a methyl donor deficient diet. Still, this statement is based on the quantitative intake of specific nutrients, and does not take into account the complex interplay in whole-diets.

Another possible mechanism by which the ketogenic dietary treatment can lead to a decrease in DNA methylation is through adenosine. Adenosine is a known endogenous anticonvulsant[125,126], and a proposed mechanism for the seizure reducing effect of ketogenic diets[127]. Moreover, adenosine can indirectly inhibit DNA methylation[95]. It has been suggested that patients with epilepsy have low levels of adenosine in the brain, leading to increased DNMTs activity, and consequently DNA hypermethylation, and potentially enhanced epileptogenesis[95]. In the previously mentioned study on DNA methylation in adults with obesity following a very low calorie ketogenic diet, the decreased DNA methylation was found to be correlated with reduced gene expression of DNMT1, DNMT3a, and DNMT3b[122]. Notably, the ketogenic diet has previously been shown to increase the level of adenosine[95], which might reverse the DNA hypermethylation and halt epileptogenesis[56,57,127]. Even though we did not find any statistically significant changes in genes related to adenosine metabolism, there could be small (but clinically relevant) alterations in adenosine metabolism not captured in our study.

5.1.3 Epilepsy genes with altered DNA methylation

Of particular interest in our findings, was the discovery that a relatively high proportion of the differentially methylated positions were annotated to genes associated - or potentially associated - with epilepsy (*ELMO1*, *FTO*, *GNAO1*, *INPP4A*, *KCNQ1*, *MED13L*, and *ZEB2*) [128]. These genes are abundantly expressed in the CNS and have essential roles in normal brain development and function. However, the genes identified are likely to cause epilepsy through quite different mechanisms, as we could not find any obvious shared pathological mechanism.

The gene *KCNQ1* encodes a voltage-gated potassium channel which plays an essential role in electrical excitability and potassium recycling in many tissues, predominantly in the heart, but also in neuronal networks in the brain[129]. Genetic variants in the *KCNQ1* gene have also been suggested to be linked to sudden unexpected death in epilepsy, although this needs further confirmation[130,131]. *GNAO1* encodes G protein subunit alpha o1, a guanosine nucleotide-binding protein which is involved in regulation of neurotransmission and neuronal excitability. Pathogenic variants of *GNAO1* are associated with early-onset epileptic encephalopathy, movement disorders and severe developmental delay[132].

MED13L and *ZEB2* are both genes that are central in transcriptional regulation. *MED13L* encodes a subunit of the Mediator complex which is involved in transcriptional regulation of almost all genes transcribed by RNA polymerase II[133], while the protein Zinc finger E-box homeobox, encoded by *ZEB2*, is an essential transcriptional repressor[134]. Moreover, *FTO*, which encodes the fat mass and obesity-associated protein was the first mRNA demethylase identified[135]. Notably, FTO has received a lot of attention due to its potential role in regulating energy and food intake and its association with obesity. In line with our results (assuming that decreased DNA methylation leads to increased gene expression), one month of feeding with ketogenic diet led to a transient increased level of *Fto* mRNA and FTO protein in mice[136]. *ELMO1* and *INPP4A* are genes that are putatively associated with epilepsy, however, further verification is required[128].

The knowledge about diet response in epilepsies caused by pathogenic variants in these particular epilepsy associated genes is sparse and it is unknown whether these aetiologies are more likely to be responsive to ketogenic diets than epilepsies of other causes. One study investigating the effectiveness of ketogenic diet for specific genetic mutations reported one individual with a mutation in *ZEB2* to be a responder (≥90% reduction in seizure frequency), whereas one individual with a genetic mutation in

GNAO1 was a non-responder[137]. In another study, one infant with *GNAO1* encephalopathy treated with ketogenic diet achieved seizure freedom for a transient period[132].

5.1.4 Altered inositol phosphate metabolism – a possible anti-seizure mechanism?

IMPA2, *INPP1*, *INPP4A*, *INPP5A*, and *PLCXD2* were another group of genes that attracted our attention. All five genes encode enzymes involved in inositol phosphate metabolism and, to our knowledge, our study is the first to suggest an impact of the ketogenic diet on inositol metabolism. This is an intriguing finding because it has previously been suggested that abnormalities and imbalances in inositol and its derivatives are involved in the pathogenesis of several neurological disorders such as bipolar disorder, Alzheimer disease, and epilepsy[138].

Inositol is a 6-carbon sugar alcohol that exists as nine different stereoisomers, of which *myo*-inositol is the predominant type[139]. *Myo*-inositol can be obtained from the diet (e.g. from fresh fruits, beans, grains, nuts, and many other food items[140]), by *de novo* synthesis (from glucose metabolism), or through recycling in the phosphatidylinositol cycle[138]. Interestingly, *myo*-inositol is abundant in many tissues, but is at especially high concentrations in the brain. Inositol phosphorylated at one or more positions are referred to as inositol phosphates. In the CNS, inositol phosphates such as inositol 1,4,5-triphosphate (IP_3) is important in signal transduction and calcium homeostasis[138]. *INPP1*, *INPP4A*, *INPP5A* and *IMPA2* are key in inositol phosphate metabolism by encoding hydrolases that remove phosphate groups at specific positions of the inositol ring, and together contribute to the conversion of IP_3 into free *myo*-inositol[141].

The *IMPA2* gene encodes a *myo*-inositol monophosphatase (IMPA), which catalyses the conversion of *myo*-inositol monophosphate into *myo*-inositol. IMPA has an important role in regulating the inositol homeostasis trough both *de novo* synthesis from glucose 6-phosphate and recycling of phosphatidylinositol[138]. Interestingly, *IMPA2* has been reported to be a putative febrile seizure

susceptibility gene[142]. Further, the activity of IMPA has been shown to be stimulated by the commonly used ASM carbamazepine[141].

In contrast, lithium, a mood-stabilizing drug used in the treatment of bipolar disorders, inhibits IMPA and have been shown to have a proconvulsive effect in a rat epilepsy model[143]. The proconvulsive effect of lithium was inhibited by administration of *myo*-inositol[143]. Indeed, anticonvulsant effects of *myo*-inositol have been demonstrated in several animal models of epilepsy[144,145]. However, the role of *myo*-inositol in epilepsy and its potential as a therapeutic target is not entirely clear. For example, the *myo*-inositol level in the brain increased following provoked (pilocarpine or kainate) seizures in rats[146,147]. Furthermore, in rats that later develop epilepsy, the elevated level of *myo*-inositol persisted, while the increase was only transient in animals who later did not develop epilepsy[146].

Taken together, diet-induced alterations of the inositol phosphate metabolism may represent a mechanism for how the ketogenic diet attenuates seizures.

5.1.5 Metabolic adaptations in lipid- and glucose metabolism

The transition from a typical Norwegian diet with a high carbohydrate- and a low fat content[148] to a high-fat, low-carbohydrate ketogenic diet necessitates major metabolic adaptations. During treatment with a ketogenic diet, glycolysis decreases, oxidation of fatty acids increases, and ketone bodies are produced. These metabolic changes were evident in the participants in the form of objective measures of decreased blood glucose and concomitantly increased blood ketones. Not so surprisingly, a large share of the differentially methylated positions identified in Paper I were annotated to genes associated with glucose and fatty acid metabolism, including the genes *APOB48R*, *B4GALT5*, *CERS6*, *CES1*, *CPT1A*, *GALNT2*, *PLCXD2*, and *PPAP2B*.

PDK4, encoding the enzyme pyruvate dehydrogenase kinase 4 (PDK4), has an essential role in regulation of glucose and fatty acid metabolism through its ability to phosphorylate the pyruvate dehydrogenase (PDH) complex, the main rate-limiting step in aerobic glucose oxidation. Phosphorylation of two subunits of the PDH complex inhibit the activity of the PDH complex, and hence glycolysis is down-regulated[149]. Along with the down-regulation of glycolysis, fatty acid oxidation increases. *PDK4* gene expression is associated with increased fatty acid oxidation and has been suggested as a surrogate marker for fatty acid oxidation[150].

Another gene known to be involved in fatty acid oxidation that we identified to be differentially methylated at one position was *CPT1A*. *CPT1A* encodes carnitine palmitoyltransferase 1A (CPT1A), which is a key rate-limiting enzyme in fatty acid oxidation through its role in controlling the entry of fatty acids into the mitochondria where β-oxidation takes place. Long-chain fatty acids are unable to cross the inner mitochondrial membrane unless they are attached to the amino acid carnitine. Thus, an essential step in the fatty acid oxidation is the transfer of carnitine to activated long-chain fatty acids catalysed by CPT1A (reviewed in[151]).

Moreover, we identified altered DNA methylation of genes involved in the uptake of lipids, such as *APOB48R*. *APOB48R* encodes apolipoprotein B48 receptor (APOB48R), a macrophage receptor that binds to apolipoprotein B48 of dietary derived triglyceride-rich lipoproteins in blood and has a decisive role in postprandial uptake of lipids in macrophages. Interestingly, a previous study in healthy humans showed that a high-fat meal increased the APOB48R mRNA expression[152]. Unfortunately, the study did not analyse DNA methylation.

Taken together, our findings are in line with what could be expected following treatment with a high-fat, low-carbohydrate diet, and indicate that our method captures important biological adaptations, hence supporting the validity of our results.

5.2 Food-drug interactions in dietary treatment

5.2.1 Ketogenic diets and ASMs - pharmacokinetic interactions

Ketogenic diets, as a part of comprehensive care of patients with severe epilepsy, are commonly used as an add-on treatment to ASMs. In recent years, clinical observations have raised questions about potential pharmacokinetic interactions between ketogenic diets and ASMs. Further, our research group has previously reported a significant decrease in the serum concentration of several ASMs following a ketogenic diet intervention in adults with epilepsy, indicating such pharmacokinetic interactions between diet and ASMs[99].

In Paper III, we investigated the influence of a classical ketogenic diet on the serum concentration of ASMs in children with drug resistant epilepsy. After 12 weeks of diet intervention, there were a statistically significant decrease in the serum concentrations of clobazam, desmethylclobazam, and lamotrigine after 12 weeks of diet intervention. To the best of our knowledge, our study is the first in children with epilepsy to indicate an interaction of the ketogenic diet on the serum concentration of these particular ASMs.

Previous studies that examined the impact of the ketogenic diet on serum concentration of ASMs in patients with epilepsy are summarized in **Table 2** (see section 1.4.2). Contrary to our findings, Dahlin et al. [100] found no evidence of altered ASM serum concentrations in a study of 51 children treated with a classical ketogenic diet for 3 months. Coppola et al. [101] investigated the serum concentrations of valproic acid and phenobarbital in 36 children on a classical ketogenic diet, evaluating the effect after 28 days of diet intervention. Similarly to our results, the authors reported a non-significant trend towards a decline in the serum concentration of valproic acid. No significant change in the serum concentration of phenobarbital was found. In contrast, in a retrospective study of 139 children, Heo and colleagues[102] found a significant decrease in the serum concentration of valproic acid in children treated with

ketogenic diet (classical or modified). In adults, only one study has examined the potential pharmacokinetic interactions between ketogenic diet and ASMs[99]. Consistent with our findings, clobazam and lamotrigine decreased following treatment with a modified ketogenic diet. In addition, they demonstrated a significant decrease in the serum concentration of carbamazepine, lacosamide, topiramate, and valproic acid[99]. Neither Kverneland nor Heo found any evidence of an influence on levetiracetam by the diet intervention, which is in accordance with our results[99,102]. The results from the various studies are thus not entirely consistent and there are perhaps some ASMs that are more prone to food-drug interactions.

This discrepancy between the studies may have several explanations. First, the type of diet and the duration of the diet intervention varied between the studies. Second, it is widely known that there are important differences in pharmacokinetics between children and adults[153]. Third, differences in how the data analyses are performed (e.g. concentration/dose-ratio versus absolute levels of serum concentrations) may influence the results. Fourth, variations in the type of ASMs used, as well as dissimilarities in the combinations of ASMs are likely to have an impact. Finally, in one of the studies, the drug dosages were changed immediately before or during the diet intervention which may have influenced the results[100].

Currently, it remains unknown how ketogenic diet may interact with ASMs. As described in section 1.4.1, pharmacokinetics involves four main processes; absorption from the gastrointestinal tract, distribution to the tissues, metabolism, and eventually elimination from the body. Pharmacokinetic interactions may occur at any of these stages of pharmacokinetics. Since most ASMs are lipophilic, and in general have an extensive absorption and a high bioavailability[97], we would not expect a negative impact on absorption by ketogenic diet. Thus, altered absorption of the ASMs seems less likely to explain the observed decrease in serum concentration.

Changes in ASM protein binding may be another factor of relevance, particularly for drugs with a high degree of protein binding, such as valproic acid and phenytoin. However, we found no evidence of altered concentration of albumin, the most abundant protein in blood, in our study. Changes in body weight during the diet intervention could also potentially influence the serum concentrations of ASMs. However, the caloric content of the diet intervention in our paediatric study (Paper III) was fine-tuned according to each individual's needs during the 12-week diet intervention and the body weights were unchanged. In the previous study by Kverneland[99], quite a few of the participants experienced a weight loss, but this weight change would be expected to cause a higher, rather than a lower, ASM serum concentration.

Another possible mechanism suggested by Kverneland[99], is a transcriptional up-regulation of the hepatic drug-metabolizing enzymes. With a few exceptions, ASMs are predominantly metabolized in the liver by enzymes belonging to either the CYPs or UGT super families[18,104,154]. These enzymes are responsible for the metabolism of the majority of commonly used drugs[104]. In addition to the enzymes' key role in drug metabolism, they are also central in the metabolism of lipids[154,155]. Evidence from animal studies have shown that high-fat diets can influence the expression of genes encoding these drug-metabolizing enzymes, and subsequently their drug-metabolizing activity[108,109]. Indeed, in Paper I, we identified one differentially methylated position annotated to *CES1* encoding the drug-metabolizing enzyme carboxylesterase 1 (CES1), sparking the hypothesis in Paper II, that altered DNA methylation of genes involved in ASM metabolism might be involved in the observed decrease of ASM serum concentrations.

5.2.2 Decreased ASM serum concentration – does it matter?

The decrease in ASM serum concentrations observed in our study (Paper III), especially for clobazam and desmethylclobazam, is likely to be of clinical relevance and can potentially reduce the patients' seizure protection. A minority of patients starting treatment with a ketogenic diet experience a paradoxical

seizure aggravation[110,156]. The reason for this seizure worsening is unknown. We speculate whether this seizure increase may, at least partly, be related to an unintended decrease in the serum concentration of one or more ASMs. In our study, two patients had a substantial seizure increase, of which one of them developed refractory status epilepticus. Both patients discontinued the diet shortly after the seizure exacerbation, thus we do not have any serum concentration measurements while they still were on the diet. However, neither of the two patients used an ASM that was observed to have a significant decrease in serum concentration.

For several reasons it is challenging to investigate the relationship between alterations in ASM serum concentrations and seizure control. For example, if a patient has little or no seizure protection by the drug, a decrease in the serum concentration may not result in a seizure increase. The interpretation of the results are further challenged by the complexity of interactions between several ASMs in individual patients due to polypharmacy. Since ASMs are known for their susceptibility for drug interactions, a diet-induced change of one ASM might, in turn, influence the serum concentration of other ASMs indirectly. Hence, for the above mentioned reasons and a small sample size, we did not perform any statistical analysis to investigate correlations between ASM serum concentration changes and seizure frequency. Indeed, Kverneland et al. [99] found no correlation between change in serum concentrations of ASMs and change in seizure frequency.

5.2.3 DNA methylation of genes involved in ASM metabolism

In contrast to our untargeted approach in Paper I, where we investigated DNA methylation of more than 700.000 positions in the genome, in Paper II, we examined the DNA methylation level of 131 positions annotated to 13 genes with a known central role in ASM metabolism. These genes were: *CES1*, *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2E1*, *CYP3A4*, *CYP3A5*, *UGT1A3*, *UGT1A4*, and *UGT2B6*.

Disappointingly, our targeted approach in Paper II did not discover any additional differentially methylated positions beyond the position annotated to *CES1* already identified. *CES1* is highly expressed in the liver and is involved in the metabolism of numerous drugs, as well as in lipid metabolism. However, the only ASM known to be extensively metabolized by CES1 is rufinamide[157].

Although only one position had a statistically significantly change in methylation status, even minor alterations may impact the gene expression. Thus, we next performed correlation analysis on ASM serum concentrations, to examine whether they were correlated with changes in DNA methylation. Our analysis revealed a strong negative correlation between absolute change in DNA methylation of one position (cg01478198) annotated to *UGT1A4* and (percentage) change in serum concentration of lamotrigine (*rho* = -0.85).

Lamotrigine is a commonly used ASM in the treatment of epilepsy and is predominantly metabolized by the enzyme UGT1A4 (encoded by the *UGT1A4* gene) [97]. UGT1A4 catalyses the covalent binding of glucuronic acid to lamotrigine to convert it to the major inactive metabolite 2-N-glucuronide conjugate, before it is finally eliminated from the body through the kidneys. Previous studies have shown that endogenous compounds like oestrogen, and similarly oestrogen-containing oral contraceptives, are able to induce *UGT1A4*[158,159]. Thus, metabolic alterations in response to the dietary treatment may have an impact on the gene expression of *UGT1A4*. Furthermore, it has been demonstrated that DNA methylation is an important mechanism in the regulation of the hepatic gene expression of *UGT1A1*, a gene encoding an enzyme belonging to the same family[160].

No other significant results were obtained from the correlation analysis after correction for multiple comparisons.

5.3 Methodological considerations and limitations

5.3.1 Study design and sample size

Important limitations of the studies in the present thesis are the non-randomized study design and lack of a control group. Without a control group we cannot be certain that the observed changes in DNA methylation (Paper I and II) or ASM serum concentrations (Paper III) has arisen from the diet intervention. Ideally, we would have included a control group consisting of patients with drug resistant epilepsy continuing with care-as-usual. However, with regard to the DNA methylation studies (Paper I and II), we would argue that the use of intraindividual comparisons before and after treatment intervention also has advantages, as this study design reduces the risk of confounding related to genetic variations or interindividual differences in environmental factors.

As further discussed in section 5.3.8, we chose a non-randomized study design in the paediatric study (Paper III) because we considered it unethical to randomize children to a control group since ketogenic diet had a documented (positive) treatment effect in children with epilepsy and already was an established treatment in clinical use in Norway. Another argument for not choosing a randomized study design included an expected lower willingness to participant and higher drop-out rate, due to risk of being assigned to the control group.

In Paper I and II, data from both a non-randomized and an originally randomized trial was merged (see section 3.1.1) in order to obtain a larger sample size. Still, small sample size is a limitation of the studies in the present thesis. Even though the ketogenic diet has become an established treatment of epilepsy, the dietary treatment is resource demanding and is still a rare treatment. Over the past five years, around 25 children and 20 adults has started on a ketogenic diet per year at the National Centre for Epilepsy, Norway (unpublished data). In Norway, few patients with epilepsy outside the National Centre for Epilepsy start on ketogenic diet, thus, the patient population of interest is limited, and hence it

would be difficult to obtain larger sample size in our studies. Recruitment of patients was further hampered by strict eligibility criteria. As a result, we were not able to reach the wanted number of participants even though the willingness to participate was high.

5.3.2 Common challenges in clinical nutrition research

One of the major challenges when conducting clinical nutrition research is diet adherence[161]. Good adherence is essential for valid results in dietary trials. Given the fact that the ketogenic diets are strict and difficult-to-comply with, diet adherence was recognized as a potential challenge when conducting the studies in the present thesis. To maximize diet adherence, the patients and/or caregivers received comprehensive training, educational booklets, ketogenic food recipes, and close follow-up throughout the study. Indeed, the modified ketogenic diet used in the adult study was originally developed to increase palatability and adherence. For example, not needing to weigh all food simplifies the practical implementation of the diet.

Clinical experience over the years has shown that it is generally easier to implement the dietary treatment in children than in adolescents and adults. Children have caregivers that facilitates the diet implementation, while adults to a larger extent need to take care of the treatment themselves. Also, a high proportion of adults with epilepsy suffer from cognitive impairment[162], making it even more difficult to comply with a comprehensive dietary treatment. In the paediatric study (Paper III), a high proportion of the children had a gastrostomy tube (40%), which made implementation of the dietary treatment easier. We used various methods, including structured interviews, objective biological biomarkers (i.e. blood glucose, HbA_{1c}, blood ketones, urine ketones), and weighed food diaries to capture adherence to the diet intervention. Despite the strict nature of the ketogenic diets, these assessments indicated a good adherence with the diet interventions.

The complex nature of whole diets is also a well-recognized challenge within the field of clinical nutrition research[163]. Modification of the amount of one macronutrient (i.e. protein, fat, or carbohydrate) inevitably either changes the relative proportion of other macronutrients or the total energy intake. For example, the increase in fat in ketogenic diets will necessarily lead to a decrease in the proportion of carbohydrate. Moreover, the intake of micronutrients and various bioactive compounds are difficult to maintain at the same level when changing the macronutrient composition. Currently, the optimal composition of ketogenic diet, and whether it is the high fat content, carbohydrate restriction, specific dietary compounds (e.g. specific fatty acids) or other elements that leads to the diets' positive effects in epilepsy, remains unknown.

Another challenge in clinical nutrition research is participant blinding, especially when studying whole diets (i.e. not an isolated nutrient or supplements). The aim of blinding is to reduce the risk of bias due to awareness of group assignment[164]. However, since ketogenic diets have a very high fat content (typically 80-90% of the energy from fat), we considered it virtually impossible to provide a ketogenic diet disguised as a regular diet. As of today, no studies assessing the seizure-reducing effectiveness have successfully double-blinded the ketogenic diet intervention in a randomized trial. Although Freeman et al. [165] aimed to assess the short-term efficacy of ketogenic diet in a double-blinded, crossover study by giving a ketogenic diet together with either a solution containing glucose (control arm) or an artificial sweetener (saccharin) (intervention arm), the study has been criticized because the amount of glucose given was insufficient to break the diet-induced ketosis. In addition, all patients fasted for 36-hours before randomization, which may have influenced the seizure frequency. Thus, the study failed to adequately differentiate between the intervention and control arm.

5.3.3 Blood as a surrogate tissue in DNA methylation studies

The increased recognition of the potential role of DNA methylation in the development of numerous disease has led to a great increase in studies investigating epigenetic mechanisms in human diseases. Since the tissue of relevance often is unavailable, blood is frequently used as a proxy tissue in human studies. However, DNA methylation patterns are known to be tissue-specific[73], and an important question is how well DNA methylation in blood reflects DNA methylation in the target tissue.

In Paper I, we investigated DNA methylation using blood as a proxy for brain tissue. Results from human studies investigating the correspondence of DNA methylation between blood and brain are inconsistent. In general, it appears like the correlation between DNA methylation in blood and brain samples is higher when using an across-subject, rather than a within-subject design[166,167]. Studies using a within-subject design have focused on the concordance at variable CpGs (i.e. CpGs with a high interindividual variability) as these CpGs are most likely to be of relevance for phenotypic differences. In a study of 27 patients with epilepsy undergoing resective brain surgery, a significant correlation was found at only 2% of the variable CpGs[167]. The lack of significant findings may be because of the study's use of a stringent correction for multiple comparisons (Bonferroni correction). In another study by Walton and colleagues[168], the authors found that approximately 8% of the variable CpGs had a significant correlation between blood and brain tissue. Similarly, Edgar et al. [169] reported that about 10% of the variable CpGs were informative between blood and brain. However, for a subset of loci, DNA methylation in blood may be a strong predictor of DNA methylation in brain[168,169].

Taken together, the most consistent finding of these studies is that – for the majority of loci – the correlation between blood and brain DNA methylation is limited. Thus, the results from our and others' studies using DNA methylation in blood as a surrogate tissue of diseases that manifests in the brain, must be interpreted carefully.

However, an important point is that since it's currently uncertain how the ketogenic diet can ameliorate seizures in people with epilepsy, the mechanisms behind seizure reduction may involve both alterations at a systemic level (e.g. immune or hormonal changes), as well as alterations in the brain.

In Paper II, we investigated DNA methylation of genes involved in ASM metabolism. Metabolism of ASMs primarily occurs in the liver, thus, the tissue presumed to be of primary interest is liver. However, as liver samples, for obvious reasons, were not readily available, blood was used blood as a surrogate tissue. In a study of 27 participants undergoing liver surgery, Olsson and colleagues[170] examined whether DNA methylation in blood could serve as a proxy for DNA methylation in liver by collecting paired liver and blood samples concomitantly. Here, the authors reported that the intraindividual concordance between liver and blood varied greatly across the 10.000 individual CpGs investigated. A moderate to strong correlation were found in approximately 10% of the variable CpGs. Thus, for these positions, DNA methylation in blood could serve as a reliable proxy for the DNA methylation in liver. To our knowledge, no other human studies using a within-subject design have investigated the concordance between DNA methylation in blood and liver.

Another key aspect when studying DNA methylation in blood is to take within tissue cell-type heterogeneity into account. In our study, DNA extracted from white blood cells was used to investigate how a ketogenic diet influences DNA methylation. As white blood cells consist of several different cell types and these have their own cell-type specific DNA methylation pattern, it is important to make sure that any differences in DNA methylation from before to after the intervention are not simply due to alterations in the cell-type composition. To address this issue, we performed white blood cell differential counts which measure the relative proportions of lymphocytes, monocytes, and granulocytes. In addition, we performed a cellular deconvolution (also referred to as cell proportion estimation) and estimated the proportions of subtypes of lymphocytes (B cells, $CD4^+$ and $CD8^+$ T cells, NK cells,

monocytes, and granulocytes) by using the estimateCellCounts2 function in the FlowSorted.Blood.EPIC R package[171]. Apart from a minor increase in NK cells, which we considered too small to be taken into account in the following analyses, there were no significant changes in cell type compositions. Thus, our observed differences in DNA methylation in white blood cells are likely to represent an adaptive change due to the diet intervention, rather than being a result of altered cell-type composition.

5.3.4 Advantages and disadvantages of EPIC array

Both microarray platforms and whole-genome bisulphite sequencing (WGBS) can be used for genome-wide DNA methylation profiling. We measured DNA methylation using a microarray from Illumina; the EPIC array. The EPIC array, like WGBS, is based on bisulphite conversion of the DNA. Although WGBS is considered as the "gold standard" due to its single-nucleotide resolution and whole-genome coverage, the EPIC array has become a popular choice[114]. Advantages of the EPIC array includes single-nucleotide resolution, genome-wide coverage, high reproducibility and reliability, low amount of input material required, and affordable price. In addition, the EPIC array is not dependent on high-throughput sequencing, but are rather based on Illuminas's beadchip technology and requires less bioinformatics handling than WGBS[114].

However, EPIC array also comes with some disadvantages. Compared to WGBS, the EPIC array has limited coverage. While WGBS in principle covers every single CpG in the genome, only approximately 3% (~850 000) of the CpGs are assessed using EPIC array[114]. Another disadvantage of EPIC array is the preselection of CpGs in the array. This represents a selection bias and as noted by Stirzaker[172], "the design is not hypothesis neutral". Furthermore, the EPIC array uses two different types of probe design which must be taken into account during the data analysis[114]. Nevertheless, although, EPIC array are less comprehensive than WGBS, it offers a reliable, user-friendly, and cost-effective tool for epigenetic research.

5.3.5 DNA methylation and gene expression

Another important challenge when studying DNA methylation is how changes in DNA methylation correlate with altered gene expression. Generally, DNA methylation is associated with repressed gene expression, but, as previously mentioned, this depends on the genomic location[70]. We performed differential DNA methylation analysis at individual positions. A significant differentially methylated position may or may not have a functional impact on the expression of the associated gene. The final gene expression may depend on DNA methylation of several positions associated to the gene. It is debated whether one should call a gene differentially methylated if at least one position associated to the gene is differentially methylated[173]. Further, it is important to bear in mind that DNA methylation is only one of several epigenetic mechanisms that regulate gene expression. Since we do not have gene expression data, the functional impact of a single significantly differentially methylated position is not known. It would be very interesting to explore the functional impact of the identified DNA methylation changes on gene expression in future studies.

5.3.6 DNA methylation in responders versus non-responders

We were not able to identify any differences in DNA methylation between responders and non-responders in our study. However, our sample size was small, especially when dividing into subgroups based on change in seizure frequency. Also, in an attempt to limit the risk of bias due to uncertain seizure-response, we did not include patients with a change in seizure frequency between -0.1 and -24.9%. The results may have been different if we had chosen to include the patients with a sparse reduction in seizure frequency in the non-responder category or if we had chosen a different cut-off for defining a patient as a responder (for example at least 50% seizure reduction instead of at least 25% seizure reduction). Another point to emphasize is that the seizure reducing effectiveness in our study population was modest. None of the patients became seizure free and less than 20% of the patients experienced 50% or more seizure reduction. Thus, investigation of the relationship between changes in

DNA methylation and seizure reduction in a study population with a greater seizure reduction may increase the likeliness of identifying potential differences in DNA methylation between responders and non-responders.

5.3.7 Challenges in identifying relationship between ASM serum concentrations and DNA methylation

For unknown reasons, ketogenic diet may lead to decreased ASM serum concentrations. In Paper II, we investigated DNA methylation of 13 candidate genes involved in ASM metabolism before and after a ketogenic diet intervention in order to examine whether altered DNA methylation of these genes may be involved in the decreased ASM serum concentrations. Admittedly, at the majority of positions investigated, we were not able to identify significant changes in DNA methylation or correlations between changes in DNA methylation and ASM serum concentrations. Thus, there might be other mechanisms than DNA methylation modifications of genes encoding hepatic drug-metabolizing genes responsible for the observed reduction in ASM serum concentrations in patients treated with a ketogenic diet. Alternatively, our study hypothesis could be true, yet at most of the positions we were not able to identify associations between changes in DNA methylation and change in ASM serum concentrations for the following reasons.

First, it should be noted that most ASMs are metabolized by several enzymes[97]. Thus, it may be challenging to identify a correlation between change in DNA methylation and altered ASM serum concentration if an enzyme is responsible for only 30-40% of the metabolism of a drug. Second, we investigated the correlation between changes in ASM serum concentrations and DNA methylation level at single positions, while the final gene expression (and eventually the activity of the drug-metabolizing enzyme) may be influenced by the DNA methylation level at several positions annotated to the gene of interest. Third, as discussed in section 5.3.3, blood was used as a surrogate because liver tissue samples were not available. However, blood might not be the best suitable tissue to investigate epigenetic

changes of drug-metabolizing enzymes mainly expressed in the liver, although we did identify altered DNA methylation of *CES1*, a gene which is said to be negligibly expressed in blood[174]. Fourth, the majority of study participants used two or more ASMs. ASMs are known for their frequent interactions with other drugs, which means that drug-drug interactions through other mechanism than DNA methylation may have influenced our results. Lastly, we did not investigate DNA methylation of genes encoding drug transporters and nuclear receptors known to be involved in the regulation of the activity of drug-metabolizing enzymes. Altogether, the combination of small sample size and the number of other factors besides DNA methylation modifications that may also influences the serum concentration level might explain the difficulties with identifying associations between DNA methylation changes and reduction in ASM serum concentration.

5.3.8 Ethical considerations

The studies in the present thesis are all in accordance with the Declaration of Helsinki, and were approved by the Regional Committee for Medical and Health Research in South East of Norway (Paper I and II: 2010/2326, Paper III: 2016/2016). We obtained written informed consent from all participants or parents/legal guardian. The studies were registered with the ClinicalTrials.gov database (Paper I and II: ID: NCT01311440, Paper III: ID: NCT04063007) and HelseNorge.no (https://oslo-universitetssykehus.no/kliniske-studier/diett-hos-barn-ved-epilepsi).

A cornerstone of clinical research ethics is to minimize the risk of participation and to protect particularly vulnerable subjects. Children and patients with intellectual disabilities are vulnerable groups of patients which require special consideration. Patients with disability were included in both the adult study (Paper I and II) and in the paediatric study (Paper III). These particular patients often have a long history of severe epilepsy without responding to the currently available treatment options. Thus, it was considered unethical to not offer this patient group a potentially beneficial treatment. Moreover,

numerous examples throughout medical history have illustrated the risks of extrapolating information from one study population to another, for example from adults to children[175]. As noted by Christensen[175], "The child is not a small adult". Thus, limiting the involvement of vulnerable groups such as children and patients with intellectual disabilities in research may lead to practices that are not evidence-based and potentially harmful[176].

Another key aspect of clinical research ethics is that it is only ethical to conduct clinical trials if there is genuine uncertainty to whether the intervention under investigation is beneficial or not. This uncertainty has been termed clinical equipoise[177]. Because ketogenic diet was an established treatment for epilepsy with proven effect by the time the paediatric study was conducted, we considered it unethical to randomize patients with severe epilepsy to a control group with delayed diet initiation.

Participation in the paediatric study (Paper III) did not entail any risks of adverse effects beyond ordinary treatment. However, we collected some additional blood samples. Since blood sampling may come with some discomfort, all blood samples related to the study were collected at the same time as the ordinary blood samples. In addition, all children were offered a patch with a mild local anaesthetic to prevent pain before drawing blood.

Another ethical concern is the social aspects of being on a strict diet. Especially for older children and adolescents, the strict diet may impair psychosocial well-being and elicit or increase a feeling of being different. The children who participated in the study had very different experience with the diet; the diet transition went smoothly for some of the children, while others expressed that they found it difficult to be on a special diet.

Although we designed the studies to minimize the burden of participation for the patients and their caregivers, participation in the studies inevitably entailed some time consumption. However, in general,

we experienced a very positive attitude towards participating in the studies and that the patients or caregivers found it meaningful to contribute to research. Parents of children who participated also expressed gratitude for us researching their child's diagnosis.

6 CONCLUSION

The overall aim of present thesis was to improve our understanding of how the ketogenic diet ameliorate seizures in patients with epilepsy at a molecular level.

Following the diet intervention, we found widespread DNA methylation changes, globally and at specific positions. Among the most intriguing findings were the decreased DNA methylation at positions annotated to genes associated with epilepsy and inositol phosphate metabolism. The diet's influence on inositol phosphate metabolism was an interesting finding because *myo*-inositol has previously been suggested to have anti-seizure properties, and thus altered inositol phosphate metabolism may represent a possible mechanism for how the ketogenic diet attenuates seizures.

Further, we found a significant decrease in the serum concentration of two commonly used ASMs that are likely to be of clinical importance. Especially clobazam/desmethylclobazam had a pronounced decline in serum concentration following the diet intervention. In addition to the potential consequences for each patients' seizure protection, these unintended ASM serum concentration changes may lead to misinterpretation of the effectiveness of ketogenic diets.

Although the clinical implications of these changes in DNA methylation and ASM serum concentrations are yet to be explored, our findings may provide important clues to understand the mechanism(s) behind the ketogenic diets' ability to reduce seizures in epilepsy.

7 FUTURE PERSPECTIVES

Ketogenic diet is a well-tolerated non-pharmacological treatment option which has shown to be an effective treatment for many patients with drug resistant epilepsy. Still, the ketogenic diets' mechanism(s) of action on epileptic seizures remains elusive. Understanding ketogenic diets influence on the body at a molecular level may be the key to unravel the mechanism(s) by which the diet can ameliorate seizures in epilepsy, which in turn may make it possible to identify patients that are likely to benefit from ketogenic diet, and to optimize the dietary treatment. Indeed, an understanding of the diets' mechanism(s) of action may enable us to identify novel therapeutic targets for epilepsy.

Our findings of widespread alterations in DNA methylation following the diet intervention warrant further investigation. It would be valuable to conduct studies with larger sample size that also include gene expression data. Further, it would be highly relevant to look at epigenetic changes in brain tissue samples. In addition to epigenetic alterations, changes in the gut microbiota is an evolving field of dietary research. Future studies aiming to identify the diets' seizure reducing mechanism(s) should investigate these topics through both experimental and clinical trials.

Other key issues to address in future studies are: 1) whether more liberal variants of the diet may be as effective as the classical ketogenic diet, 2) potential pharmacokinetic interactions between ketogenic diets and ASMs, and 3) identification of predictive biomarkers of treatment response.

Dietary treatment of epilepsy is still a relatively rare treatment. A prerequisite to achieve significant progress in the research of dietary treatment of epilepsy would be to establish international research networks that have the resources to conduct well-designed multicentre studies with larger sample size.

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Papers I-III

I

Received: 28 February 2022 | Revised: 27 June 2022 | Accepted: 27 June 2022

DOI: 10.1111/epi.17351

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RESEARCH ARTICLE

Genome-wide decrease in DNA methylation in adults with epilepsy treated with modified ketogenic diet: A prospective study

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Funding information
EkstraStiftelsen Helse og Rehabilitering (Stiftelsen Dam), Grant/Award Number: FO202918; National Organization for Rare Disorders, Norway; Novo Nordisk Fonden, Grant/Award Number: NNF17OC0027260; The Norwegian Epilepsy Association's Research Fund

Abstract

Objective: The aim of this study was to investigate the impact of the modified ketogenic diet on DNA methylation in adults with epilepsy.

Methods: In this prospective study, we investigated the genome-wide DNA methylation in whole blood in 58 adults with epilepsy treated with the modified ketogenic for 12 weeks. Patients were recruited from the National Center for Epilepsy, Norway, from March 1, 2011 to February 28, 2017. DNA methylation was analyzed using the Illumina Infinium MethylationEPIC BeadChip array. Analysis of variance and paired *t*-test were used to identify differentially methylated loci after 4 and 12 weeks of dietary treatment. A false discovery rate approach with a significance threshold of <5% was used to adjust for multiple comparisons.

Results: We observed a genome-wide decrease in DNA methylation, both globally and at specific sites, after 4 and 12 weeks of dietary treatment. A substantial share of the differentially methylated positions (CpGs) were annotated to genes associated with epilepsy ($n = 7$), lipid metabolism ($n = 8$), and transcriptional regulation ($n = 10$). Furthermore, five of the identified genes were related to inositol phosphate metabolism, which may represent a possible mechanism by which the ketogenic diet attenuates seizures.

Significance: A better understanding of the modified ketogenic diet's influence at the molecular level may be the key to unraveling the mechanisms by which the diet can ameliorate seizures and possibly to identifying novel therapeutic targets for epilepsy.

KEYWORDS

epigenetics, high-fat, Infinium MethylationEPIC BeadChip, low-carbohydrate diet

1 | INTRODUCTION

Epilepsy is a heterogeneous neurological disorder characterized by unprovoked, recurrent seizures. Worldwide, >65 million people are affected and the disease itself, and associated comorbidities represent a huge burden of disease.[1,2] Antiseizure medications (ASMs) are the mainstay of epilepsy treatment. However, about one third of patients do not respond adequately to the currently available ASMs (drug-resistant patients).[3,4] Moreover, treatment with ASMs offers only symptomatic relief by reducing the seizures without affecting the underlying epilepsy mechanisms. Thus, in most cases they are not able to prevent disease progression (epileptogenesis).[5]

The ketogenic diet, a high-fat, low-carbohydrate diet, is an established treatment for patients with drug-resistant epilepsy. The efficacy of the dietary treatment in children with epilepsy is well documented,[6] and recent years' research suggests that adults may also benefit from such diet therapy.[7] However, despite significant efforts to identify the underlying mechanisms behind the diet's seizure-reducing effect, the mechanisms of action still remain elusive. Recent work suggests epigenetic mechanisms as an attractive candidate to explain the reduced neuronal excitability.[8–10]

Epigenetic modification, including DNA methylation, is a dynamic process involved in regulation of gene expression and is essential for normal brain development and plasticity. Abnormal DNA methylation has been reported in a wide range of diseases, including epilepsy and other neurological disorders.[11–13] DNA methylation is the addition of a methyl group (CH_3) at a cytosine base in the DNA. In mammals, this primarily occurs at cytosines followed by guanines, called cytosine–guanine dinucleotides (CpGs). Methylation of DNA is catalyzed by DNA methyltransferases (DNMTs), and methylation of promoters tends to induce gene silencing. Changes in DNA methylation can lead to altered expression of genes involved in neuronal excitability and inhibition, and thereby potentially promote epileptogenesis. "The methylation hypothesis" in epilepsy suggests that seizures themselves can induce DNA methylation changes that sustain, and even exacerbate, the epileptogenic process.[10] Studies both in animal models of epilepsy and in humans with temporal lobe epilepsy have shown a global increase in DNA methylation in epileptic brains compared to healthy controls.[8,9,11,14] Interestingly, inhibition of DNA methylation in animal models of epilepsy appears to prevent epileptogenesis.[8] Furthermore, the increase in DNA methylation has been shown to be counteracted by ketogenic dietary treatment, which also correlated with increased seizure threshold.[8,9]

Key Points

- In this prospective study, we investigated the impact of the modified ketogenic diet on DNA methylation in adults with epilepsy
- Intraindividual comparisons of DNA methylation after the dietary treatment revealed a significant global decrease in DNA methylation
- A substantial share of the differentially methylated CpGs were annotated to genes associated with epilepsy, lipid metabolism, and transcriptional regulation
- Differentially methylated CpGs annotated to genes involved in inositol phosphate metabolism may represent a possible mechanism for the diet's antiseizure efficacy
- Identifying the molecular consequences of the dietary treatment may reveal the diet's mechanisms by which it can ameliorate seizures

Because nutrition is a key environmental factor influencing DNA methylation,[15] we hypothesized that the drastic change in macronutrient composition that the ketogenic diet represents will have an impact on DNA methylation. Thus, we conducted a longitudinal genome-wide DNA methylation study and investigated whether treatment with a modified ketogenic diet is associated with changes in DNA methylation in patients with drug-resistant epilepsy.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

Patients were recruited from the National Center for Epilepsy, Norway, between March 1, 2011, and February 28, 2017. The study cohort consisted of patients with focal epilepsy included in the randomized clinical trial by Kverneland et al.[16] and patients with generalized epilepsy included in an associated prospective, non-randomized study by the same research group.[17] All participants followed the same diet intervention protocol. The baseline period was defined as the 12 weeks immediately preceding the 12-week diet intervention period. In the baseline period, the participants ate their normal diet and recorded seizures systematically, and no changes in epilepsy treatment were allowed. In the intervention period, the participants ate a modified ketogenic diet and continued to keep a systematic record of seizures. All other epilepsy treatments were kept unchanged.

Inclusion criteria were generalized or focal epilepsy according to the International League Against Epilepsy classification,[18] ≥3 countable seizures per month, having tried ≥3 ASMs, age ≥ 16 years, body mass index > 18.5 kg/m^2 (no upper limit), and the participants had to be motivated and capable of adhering to the diet for at least 12 weeks. Exclusion criteria were familial hypercholesterolemia, cardiovascular disease, kidney disease, treatment with a ketogenic diet for >1 week during the preceding year, status epilepticus in the past 6 months, epilepsy surgery (including vagus nerve stimulator implant in the past year), 4 continuous weeks free of seizures in the preceding 2 months, psychogenic nonepileptic seizures, known disease in which the dietary treatment is contraindicated, use of drugs or supplements that may interfere with the diet or ASMs, change of ASMs in the past 3 months before baseline, and pregnancy or planned pregnancy.

2.2 | Procedures

2.2.1 | Diet

The dietary intervention was previously described in detail.[16] Briefly, the diet contained a maximum of 16 g carbohydrate per day (excluding fibers), and the participants were encouraged to eat high-fat foods to replace the carbohydrates in the diet. Proteins were eaten ad libitum, and the total energy content was not restricted. The diet was supplemented with one multivitamin and mineral tablet (Nycoplus Multi, Takeda) and 800 mg calcium (calcium carbonate, Takeda). A daily fluid intake of 2–3 L was recommended. To calculate the nutritional content of the meals, the participants used the Norwegian Food Composition Database.[19]

2.2.2 | Diet adherence

To assess adherence to the diet, the participants performed a 3-day weighed food record prior to starting on the diet and before the hospital admissions at the 4- and 12-week time points. In addition, the participants recorded urine ketones (acetoacetate) twice daily (morning and evening) at home during the diet intervention using urine dipsticks (Ketostix, Bayer Healthcare). Blood glucose and blood ketones (β-hydroxybutyrate) were measured morning and evening during the hospital admissions (FreeStyle Precision Neo, Freestyle Precision Blood Glucose Test Strips, and FreeStyle Precision Xtra Blood β-Ketone Test Strips, Abbott). The data have previously been reported by Kverneland et al. and indicate good compliance with the dietary treatment.[16,17,20]

2.2.3 | Biochemical analyses

Venous blood samples were collected after an overnight food and drug fast at baseline, and after 4 and 12 weeks of dietary treatment. All biochemical routine analyses were performed at Oslo University Hospital (Oslo, Norway). Folate, vitamin B_{12}, and homocysteine serum (before June 6, 2012) or plasma concentrations were measured on a Roche Diagnostics platform using the Elecsys Folate III assay (Roche Diagnostics), the Elecsys Vitamin B_{12} II assay (Roche Diagnostics), and the Axis Homocysteine Enzyme Immunoassay (Axis-Shield Diagnostics), respectively, according to the manufacturer's instructions.

DNA methylation analysis: Microarray preprocessing and quality control

Whole blood for DNA extraction was collected in VACUETTE K_2EDTA blood collection tubes (Greiner Bio-One International). DNA methylation was analyzed using the Infinium MethylationEPIC BeadChip, which quantitatively interrogates DNA methylation at >850 000 positions (CpGs) genome-wide with single nucleotide resolution. The EPIC BeadChips were processed at the LIFE & BRAIN laboratory according to the manufacturer's instructions. All DNA methylation analyses were carried out using the R programming language (http://www.r-project.org/). Preprocessing and quality assessment were performed using functions implemented in the minfi package.[21] First, normalization was performed with *precrocessQuantile*. Then, the data were preprocessed and filtered to remove probes with unreliable measurements (detection p-values > .01, n = 20 283), probes located on the sex chromosomes (n = 18 654), probes with overlapping single nucleotide polymorphisms (n = 27 348), cross-reactive probes (n = 39 112), and non-CpG probes (n = 2458),[22,23] resulting in a final dataset consisting of 760 462 probes and 172 samples.

2.3 | Statistical analysis

2.3.1 | Statistical data analysis tools and presentation of data

DNA methylation analyses were carried out in R using packages specifically developed to analyze Illumina EPIC DNA methylation array data. Statistical analyses of other background variables were carried out in SPSS Statistics version 26 (IBM). Data are presented as mean (±SD) or median (quartiles), and minimum–maximum, or frequency (%), as appropriate. We tested for differences in blood biochemistry from baseline to 4 and 12 weeks of dietary treatment using paired t-test.

2.3.2 | Cell type composition

White blood cell differential counts consisting of relative proportions of lymphocytes, monocytes, and granulocytes were measured by standard methods at Oslo University Hospital. To explore potential differences in the lymphocytes, we also performed whole-blood deconvolution and estimated proportions of $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, B cells, monocytes, and granulocytes using the estimateCellCounts2 function in the FlowSorted.Blood.EPIC R package.[24] Deconvolution estimates were evaluated by calculating R2 and root mean square error comparing estimates to matched cell counts.

2.3.3 | Differential DNA methylation analyses

A linear regression model implemented in limma[25] was fitted to M-values (log2 of the β-values) to identify intraindividual differentially methylated positions before and after treatment with the modified ketogenic diet. Intraindividual differences in global DNA methylation were tested using a paired *t*-test between time points on mean DNA methylation across all CpGs in patients containing complete data from all time points. To adjust for multiple testing, a false discovery rate cutoff of <5% was used for genome-wide significance by using the method of Benjamini and Hochberg.[26]

2.4 | Study outcomes

The primary outcome was changes in DNA methylation associated with 4 and 12 weeks treatment with the modified ketogenic diet. The secondary outcome was changes in DNA methylation associated with seizure response comparing responders with nonresponders after 12 weeks of dietary treatment. Because a threshold of 25% seizure reduction has been proposed as the lowest clinically relevant outcome of dietary treatment[27] and the seizure reduction in our study population was modest (Table S1), we chose to define participants who achieved ≥25% seizure reduction after 12 weeks of dietary treatment as responders. A nonresponder was defined as a participant with no seizure reduction (including participants with an increase in seizure frequency). Participants with .1%–24.9% seizure reduction at 12 weeks were included in neither the responder nor the nonresponder category, in an attempt to limit bias from participants with an uncertain seizure response to the dietary treatment.

3 | RESULTS

3.1 | Baseline demographics and clinical characteristics

Samples from 58 participants (age = 16–65 years) were included in the study (Figure 1). In general, the participants had a long history of epilepsy (mean = 25.0 ± SD 11.9 years), a high number of previously tried ASMs (mean = 8.7 ± SD 4.1), and multiple current ASMs (mean = 2.1 ± SD .9). Also, two thirds of the participants were occupationally disabled, indicating a high burden of disease. An overview of the main baseline demographics and clinical characteristics of the participants is presented in Table 1.

3.2 | Energy and macronutrient intakes

Table 2 shows the estimated dietary intake of energy and macronutrients at baseline and 4 and 12 weeks after diet initiation based on the 3-day weighed food records. On the ketogenic diet, the intake of fat was increased to about twice that of their baseline diet, whereas the intake of carbohydrate was greatly reduced to an average of only 13 g per day. The ketogenic ratio (grams fat to the sum of grams protein plus carbohydrate) was 1.7:1 at both 4 and 12 weeks after diet initiation as opposed to .3:1 at baseline. Thus, the macronutrient intake during the intervention period was in line with the study protocol.

3.3 | Folate, vitamin B12, and homocysteine status

Folate and vitamin B_{12} are essential micronutrients that together with homocysteine play an important role in the metabolism of methyl groups. The blood values for vitamin B_{12}, folate, and homocysteine are given in Table 3. There was a significant increase in vitamin B_{12} and folate at both 4 and 12 weeks of dietary treatment compared to baseline ($p < .001$), whereas homocysteine was unchanged ($p = .07$ and $p = .23$ between baseline and 4 weeks and 12 weeks of dietary treatment, respectively). Hence, the folate and vitamin B_{12} status was improved after the diet intervention.

3.4 | Cell type composition

As DNA methylation is highly tissue and cell type specific, changes in cell type composition can be a confounding factor. Therefore, we investigated potential alterations in cell type composition. There were no significant changes in the relative proportions of lymphocytes, monocytes,

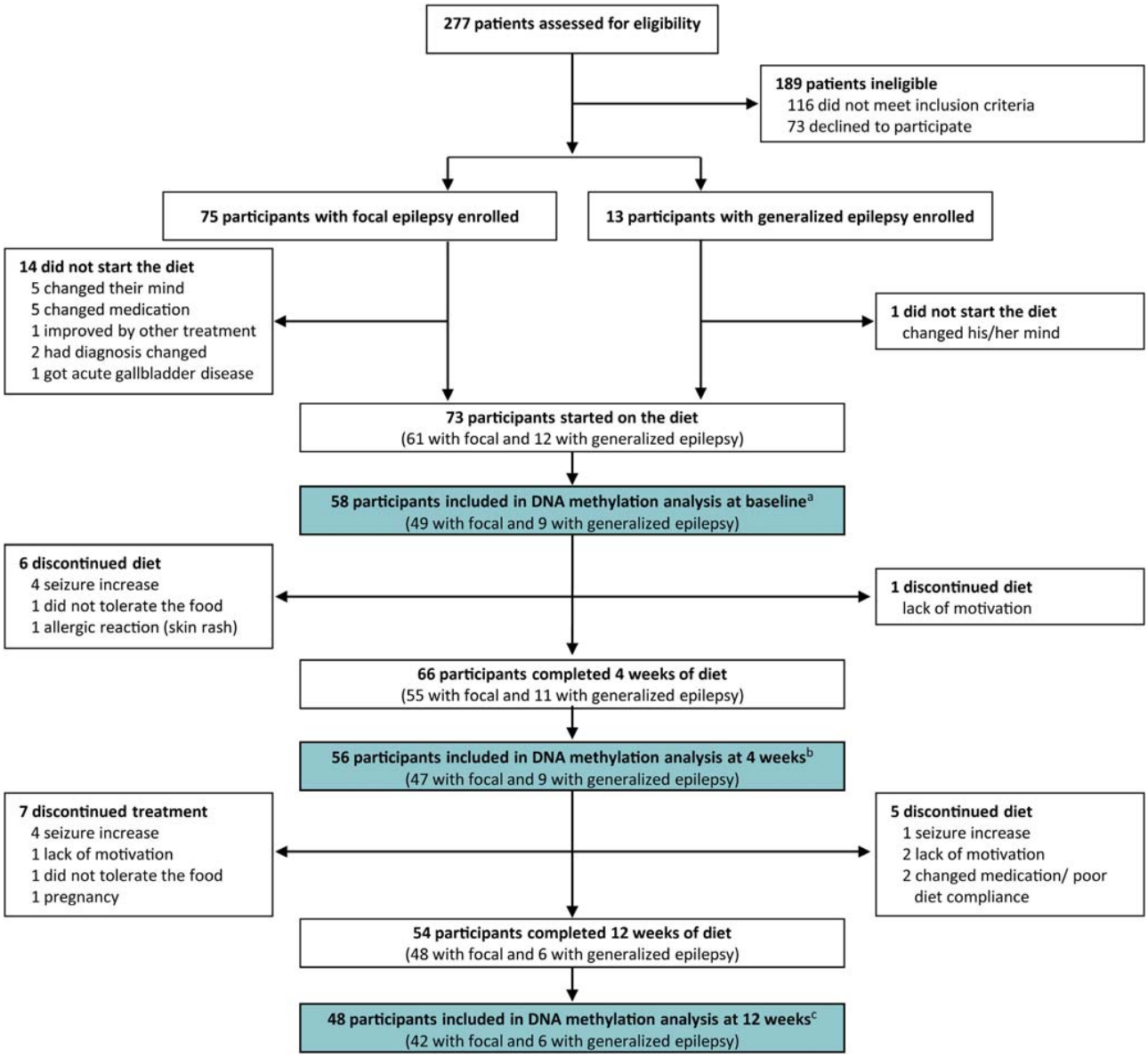

FIGURE 1 Study profile. [a]Two participants did not give permission for analysis of the samples abroad, two participants were excluded from analysis due to poor diet compliance and change in medication, and 11 were missing blood samples or their blood samples were not analyzed because of lack of sample for DNA methylation analysis at 4 and 12 weeks of dietary treatment. [b]Two participants did not give permission for analysis of the samples abroad, two participants were excluded from analysis due to poor diet compliance and change in medication, and six were missing blood samples. [c]Two participants did not give permission for analysis of the samples abroad and four were missing blood samples.

and granulocytes from baseline to 4 and 12 weeks of dietary treatment measured by routine cell counts (data not shown). To increase the precision, we also estimated the relative proportions of subpopulations of white blood cells. There were no significant changes in the estimated relative proportions of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, monocytes, and granulocytes, except a small increase in NK cells after 4 weeks of dietary treatment ($.01 \pm SD$ .02, $p = .001$). NK cells constitute only a small proportion of white blood cells, and we considered this minor change insufficient to be taken into account in the downstream analyses.

3.5 | Epilepsy treatments

In accordance with the study protocol, none of the participants had any changes in epilepsy treatments, including type or doses of ASMs, neither during the baseline nor during 12 weeks of dietary intervention.

TABLE 1 Demographic and clinical characteristics of the participants at baseline, $n = 58$

Characteristic	Mean (±SD) or median (quartiles)	Frequency (%)	Min–Max
Gender			
Male		24 (41%)	
Female		34 (59%)	
Age, years	36.5 (11.8)		16–65
Epilepsy classification			
Focal		49 (85%)	
Generalized		9 (16%)	
Age at first seizure, years	7 (2–16)		0–55
Epilepsy etiology			
Structural		16 (27.6%)	
Genetic		7 (12.1%)	
Infectious		4 (6.9%)	
Unknown		31 (53.4%)	
Years with epilepsy	25.0 (11.9)		7–58
Seizure frequency per week	3.5 (1.5–14.1)		.1–351.8
Intellectual disability		21 (36%)	
VNS, previous or current		24 (41%)	
Employment			
Paid employment		13 (22%)	
Occupationally disabled		37 (64%)	
Other		8 (14%)	
Total number of ASMs tried	8.7 (4.1)		3–23
ASMs at diet initiation	2.1 (.9)		0–4

Note: Data are presented as mean (±SD) or median (quartiles) and Min–Max for continuous variables, and frequency (percentage) for discrete variables.

Abbreviations: ASM, antiseizure medication; Min–Max, minimum–maximum; VNS, vagus nerve stimulator.

TABLE 2 Estimated intake of energy and macronutrients based on 3-day weighed diet records at baseline, and after 4 and 12 weeks of treatment with modified ketogenic diet

	Baseline		4 weeks on diet		12 weeks on diet	
	Mean (±SD)	n[a]	Mean (±SD)	n[a]	Mean (±SD)	n[a]
Energy, kcal	1856 (380)	15	1987 (670)	51	2007 (657)	42
Fat, g	79 (19)	15	170 (64)	51	174 (61)	42
Fat, E%	39 (7)	15	76 (7)	51	77 (6)	42
Protein, g	81 (17)	15	89 (31)	51	92 (38)	42
Protein, E%	18 (3)	15	19 (5)	51	18 (4)	42
Carbohydrates, g	194 (58)	15	13 (4)	51	13 (3)	42
Carbohydrates, E%	41 (8)	15	3 (1)	51	3 (1)	42
Ketogenic ratio[b]	.3:1 (.1)	15	1.7:1 (.5)	51	1.7:1 (.5)	42

Abbreviation: E%, energy percentage.

[a]Variation in n is due to missing values.

[b]The ketogenic ratio defined as the ratio of grams fat to the sum of grams protein plus carbohydrate.

TABLE 3 Blood biochemistry at baseline, and after 4 and 12 weeks of treatment with modified ketogenic diet

	Baseline		4 weeks on diet		12 weeks on diet	
	Mean (±SD)	n[a]	Mean (±SD)		Mean (±SD)	n[a]
Folate, nmol·L^{-1}[b]	19.3 (9.6)	57	26.9 (8.0)[c]	55	28.2 (9.4)[c]	49
Vitamin B_{12}, pmol·L^{-1}[b]	409.0 (172.6)	58	527.0 (275.9)[c]	55	473.0 (197.8)[c]	49
Homocysteine, μmol·L^{-1}[b]	11.8 (6.7)	58	10.6 (5.2)	54	11.0 (5.8)	49

[a]Variation in *n* is due to missing values.

[b]Paired t-test was used as the statistical test.

[c]Significantly different from baseline values ($p < .001$).

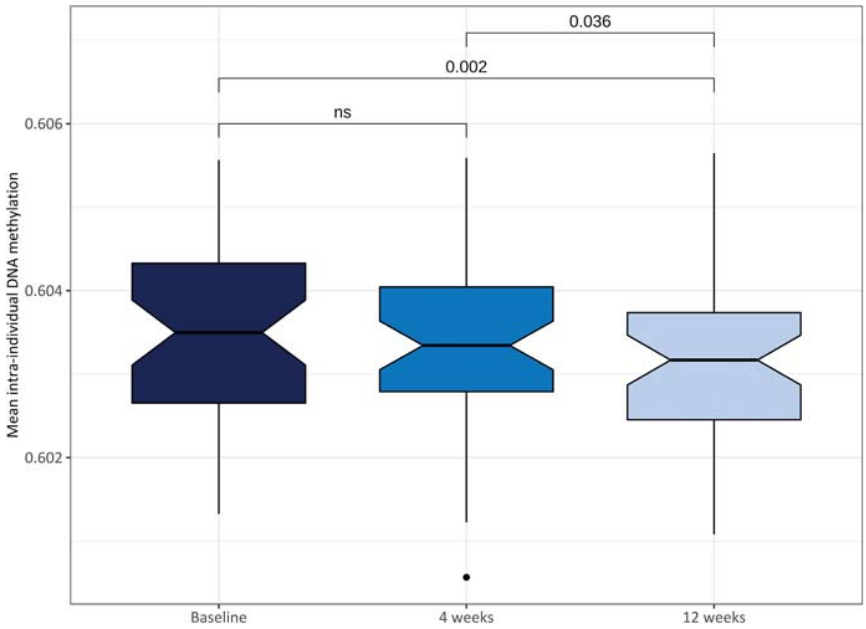

FIGURE 2 Intraindividual differences in mean global DNA methylation level across all time points ($n = 47$). Global DNA methylation levels were reduced after 4 and 12 weeks of dietary treatment; this was statistically significant between baseline and 12 weeks ($p = .002$), and between 4 and 12 weeks of dietary treatment ($p = .036$). Values are shown as boxplots (center lines, medians; notches, 95% confidence interval of medians; box limits, upper and lower quartiles; whiskers, 1.5 × interquartile range; points, outliers). ns, not significant.

3.6 | Global decrease in DNA methylation following treatment with the modified ketogenic diet

To investigate the influence of a modified ketogenic diet on global DNA methylation, we performed an intraindividual comparison of mean DNA methylation across all CpGs between baseline, and 4 and 12 weeks of dietary treatment, measuring the total content of DNA methylation at all CpGs included in this study ($n = 760\,462$). This analysis revealed a significant decrease in global DNA methylation between baseline and 12 weeks, and between 4 and 12 weeks (paired *t*-test $p = .002$ and .036, respectively; Figure 2).

3.7 | Treatment with the modified ketogenic diet is associated with DNA methylation changes in genes related to epilepsy, metabolism, transcription, and various basic cell functions

Next, we performed analyses at a single nucleotide resolution to investigate whether the modified ketogenic diet was associated with changes in DNA methylation at specific CpGs. First, we performed an analysis of variance (ANOVA) in DNA methylation associated with the modified ketogenic diet across all time points. We identified 100 differentially methylated CpGs annotated to 75 genes (Table S1). Consistent with the observed reduction in global DNA methylation following dietary treatment, all CpGs displayed a decrease in DNA methylation compared to baseline. The genomic distribution of the differentially methylated CpGs in relation to genes shows that a large proportion are located in gene bodies ($n = 54$ sites, 54%) and gene promoters ($n = 27$ sites, 27%). With respect to CpG island context, the majority of differentially methylated CpGs were located outside CpG islands (i.e., open sea regions, $n = 76$ sites, 76%), whereas a smaller proportion were annotated to CpG islands ($n = 22$, 22%, in shores and shelfs, and $n = 2$, 2%, in the core islands).

To examine whether the changes in DNA methylation occur at specific times during the diet intervention, we performed pairwise comparisons between the different time points. These analyses identified 33 CpGs annotated to 21 genes from baseline to 4 weeks of dietary

treatment (Figure 3A, Table S2), and 31 CpGs annotated to 24 genes from baseline to 12 weeks of dietary treatment (Figure 3B, Table S3). All differentially methylated CpGs showed a decrease in DNA methylation compared to baseline. There were no significant changes in DNA methylation between 4 and 12 weeks on diet (data not shown). The distribution of genomic locations of the differentially methylated CpGs at 4 and 12 weeks of dietary treatment was similar to the CpGs identified with the ANOVA analysis; the majority of the CpGs were located within or in close proximity to genes (52% in gene bodies, 21% in the promoters, and 2% in 3′ untranslated region). In the relation to CpG islands, most CpGs were located outside CpG islands (80% in open sea regions) and a smaller proportion within CpG islands (18% in shores and shelfs and 2% in core islands).

An overview of all genes to which the differentially methylated CpGs are annotated is given in Table 4. Overall, the identified genes encode proteins involved in a broad range of biological functions, including

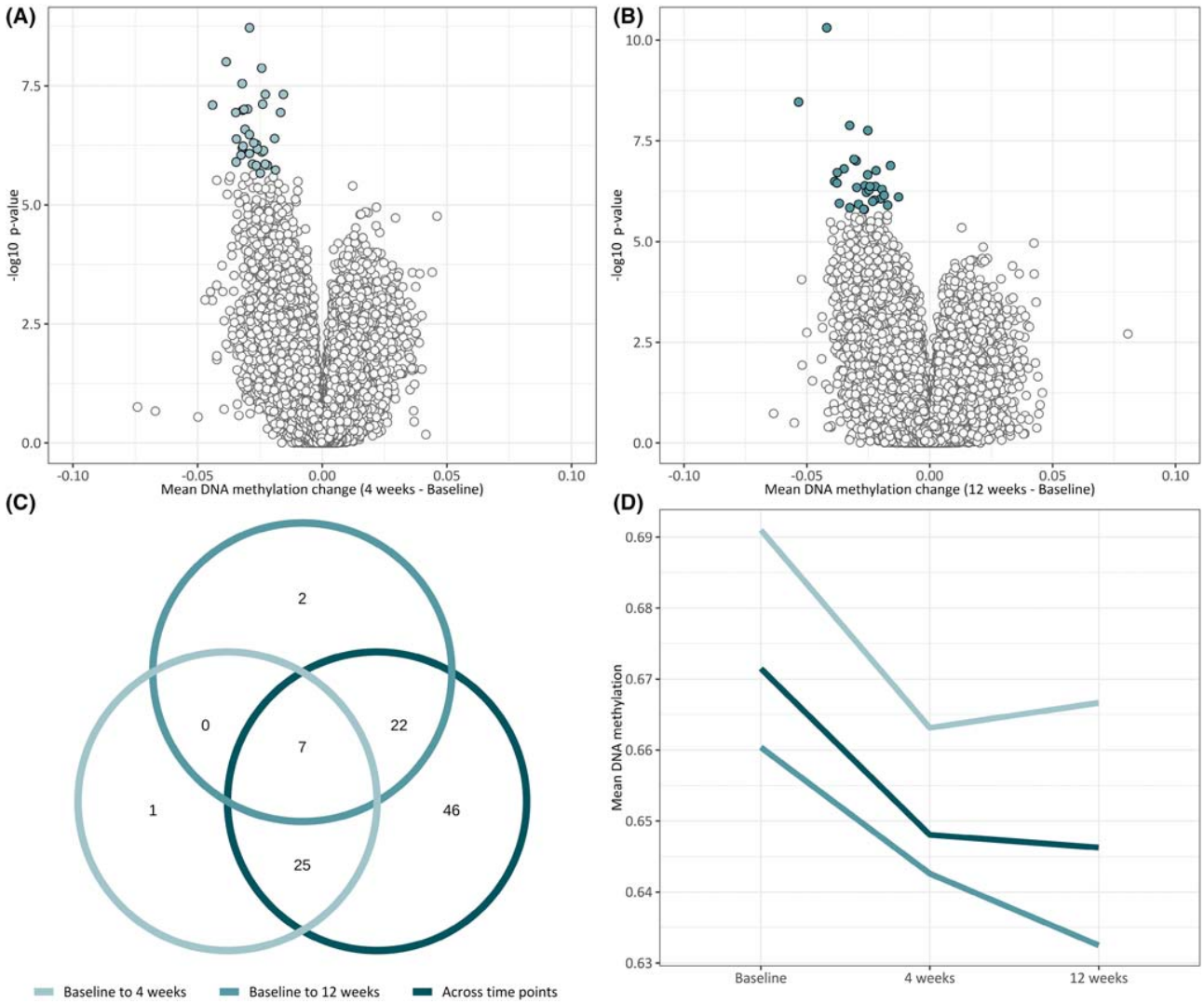

FIGURE 3 Differential DNA methylation from before to after treatment with a modified ketogenic diet. (A, B) volcano plot of log10 (*p*-value) against mean delta–beta change, representing difference in DNA methylation from before to after treatment with the modified ketogenic diet. White or green circles indicate CpGs with significant differential DNA methylation. Thirty-three CpGs were differentially methylated after 4 weeks of dietary treatment, and 31 CpGs were differentially methylated after 12 weeks of dietary treatment. (C) Venn diagram showing the overlap of CpGs differentially methylated in the three statistical analyses: analysis of variance, paired *t*-test between baseline and after 4 weeks of dietary treatment, and paired *t*-test between baseline and after 12 weeks of dietary treatment. (D) Mean linear trend of DNA methylation at differentially methylated CpGs during the intervention period. The decrease in DNA methylation at 4 weeks was slightly reversed after 12 weeks, whereas there was a linear decrease in DNA methylation of CpGs identified at 12 weeks of dietary treatment and across all time points.

epilepsy, lipid metabolism, transcriptional regulation, inositol phosphate metabolism, and regulation of cell growth and apoptosis.

The majority of the differentially methylated CpGs identified between time points did not overlap (79% and 77% from baseline to 4 weeks and 12 weeks, respectively) and showed a time-dependent change in DNA methylation during the diet intervention (Figure 3C). Only a small number of CpGs ($n = 7$), annotated to five genes (*C5orf27*, *CD93*, *HEPN1*, *KCNQ1*, and *NAT8*, two CpGs without gene annotation), were significant at both 4 and 12 weeks. Interestingly, there was a distinct difference in the mean linear trend of DNA methylation changes along the time course of the diet (Figure 3D). Whereas the decrease in DNA methylation identified at 4 weeks was slightly reversed after 12 weeks (light green line), the linear trend in decreased DNA methylation was consistent across the whole intervention period for the CpGs identified between baseline and 12 weeks or across time in general (medium green and dark green lines, respectively). These results may reflect rapid short-term metabolic adaptations that are partly reversed after 4 weeks, whereas other metabolic adaptations occur more gradually and take more time.

3.8 | No differences in DNA methylation between responders and nonresponders

As we suggest that DNA methylation may play a key role in exerting the seizure-reducing effect in patients with epilepsy, we wanted to examine possible differences in DNA methylation between responders and nonresponders. However, intraindividual comparison of DNA methylation changes from baseline to 12 weeks of dietary treatment between responders ($n = 20$) and nonresponders ($n = 21$) did not reveal any significant differentially methylated CpGs between the two groups (data not shown).

4 | DISCUSSION

This is the first report of the impact of the modified ketogenic diet on DNA methylation in humans with epilepsy. Adult epilepsy patients treated with the modified ketogenic diet had a decrease in DNA methylation, both globally and at specific genes associated with epilepsy, metabolism, transcriptional regulation, and various basic cell functions.

Despite the use of the ketogenic diet in epilepsy treatment for approximately 100 years, the mechanisms behind the diet's seizure-reducing effect remain elusive. Evidence from preclinical studies suggests that epigenetic mechanisms, including DNA methylation, play a central role in disease development, as well as in successful dietary treatment of epilepsy.[8,9,28,29] Interestingly, a global increase in DNA methylation has been demonstrated in both animal models and in humans with epilepsy.[8,9,28,30] Moreover, studies in animal models of epilepsy have shown that treatment with the ketogenic diet counteracts the increased DNA methylation and attenuates seizures.[8,9] However, it is still unknown whether the observed reduction in DNA methylation after dietary treatment occurs at the same positions as those found to have increased DNA methylation, and whether these DNA methylation alterations are linked to the seizure-reducing effect of the dietary treatment. Also, DNA methylation has been shown in experimental studies to be etiology-dependent[31]; thus, the baseline DNA methylation pattern of the participants may be influenced by etiology. However, even if baseline patterns differ between the participants, the diet-induced DNA methylation alterations may be independent of epilepsy etiology, and the within-subject design in our study may limit this potential bias.

Interestingly, we found that about 10% ($n = 7$) of the genes containing differentially methylated CpGs were associated or potentially associated with epilepsy (*ELMO1*, *FTO*, *GNAO1*, *INPP4A*, *KCNQ1*, *MED13L*, and *ZEB2*).[32] The identified genes are highly expressed in the central nervous system (CNS) and have essential roles in normal brain development and function. Of note, three of these genes play key roles in transcriptional regulation (*MED13L*, *ZEB2*) or posttranscriptional modifications (*FTO*). *MED13L* encodes a subunit of the Mediator complex, which is involved in transcriptional regulation of almost all genes transcribed by RNA polymerase II[33]; the protein zinc finger E-box homeobox, encoded by *ZEB2*, is an essential transcriptional repressor[34]; and *FTO* was the first mRNA demethylase identified.[35] A large share of the identified genes encode proteins with transcriptional regulation as their main biological function.

Ten (12%) of the identified genes encode proteins involved in transcriptional regulation (*CIITA*, *FOXN3*, *KIAA1267*, *LDB2*, *MED13L*, *PLAGL1*, *RERE*, *TCF25*, *TCFL5*, and *ZEB2*). *FOXN3*, *RERE*, *TCF25*, and *ZEB2* encode proteins that act as transcriptional repressors or corepressors,[34,36–38] whereas *PLAGL1* and *CIITA* encode a transcriptional activator and coactivator, respectively.[39,40] Moreover, LIM domain binding 2, encoded by *LDB2*, is an adapter molecule that allows assembly of transcriptional regulatory complexes.[41] In addition, *NUKCS1* is involved in chromatin remodeling and thereby may influence transcription.[42] Taken together, alterations in DNA methylation by these genes are likely to have a far-reaching

TABLE 4 Overview of the genes (sorted alphabetically by gene name) to which the differentially methylated CpGs are annotated

Gene	Full gene name	Freq	Gene	Full gene name	Freq
ANXA11	Annexin A11		*LEPREL1*	Prolyl 3-hydroxylase 2	
APOB48R	Apolipoprotein B48 receptor		*LTBP1*	Latent transforming growth factor beta binding protein 1	
ARHGEF28	Rho guanine nucleotide exchange factor 28		*LY86*	Lymphocyte antigen 86	
B4GALT5	Beta-1.4-galactosyltransferase 5		*MED13L*	Mediator complex subunit 13 like	
BCKDHB	Branched chain keto acid dehydrogenase E1 subunit beta		*NAT8*	N-acetyltransferase 8	
BLNK	B cell linker		*NET1*	Neuroepithelial cell transforming 1	
CD93	CD93 molecule		*NMUR1*	Neuromedin U receptor 1	
CERS6	Ceramide synthase 6		*NUCKS1*	Nuclear casein kinase and cyclin dependent kinase substrate 1	
CES1	Carboxylesterase 1		*PDE4D*	Phosphodiesterase 4D	2
CIITA	Class II major histocompatibility complex transactivator		*PDK4*	Pyruvate dehydrogenase kinase 4	
CLASP1	Cytoplasmic linker associated protein 1		*PDZD8*	PDZ domain containing 8	
CPSF4L	Cleavage and polyadenylation specific factor 4 like	2	*PLAGL1*	PLAG1 like zinc finger 1	
CPT1A	Carnitine palmitoyltransferase 1A		*PLCXD2*	Phosphatidylinositol specific phospholipase C X domain containing 2	
CSGALNACT1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1		*PPAP2B*	Phospholipid phosphatase 3	
DLGAP1	DLG associated protein 1		*PRKCA*	Protein kinase C alpha	
DTD1	D-tyrosyl-tRNA deacylase 1		*PSTPIP2*	Proline-serine-threonine phosphatase interacting protein 2	
DZIP1L	DAZ interacting zinc finger protein 1 like		*PTH2R*	Parathyroid hormone 2 receptor	
EFNA5	Ephrin A5		*RERE*	Arginine-glutamic acid dipeptide repeats	
EHD1	EH domain containing 1		*RNF166*	Ring finger protein 166	
EIF4E3	Eukaryotic translation initiation factor 4E family member 3		*RNF19A*	Ring finger protein 19A	
ELMO1	Engulfment and cell motility 1		*SLC22A23*	Solute carrier family 22 member 23	
FAM198B	Family with sequence similarity 198 member B		*SNTB1*	Syntrophin beta 1	
FOXN3	Forkhead box N3		*STARD9*	StAR related lipid transfer domain containing 9	
FTO	Fat mass and obesity-associated protein		*SUSD1*	Sushi domain containing 1	
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	2	*SWT1*	SWT1, RNA endoribonuclease homolog	
GNAO1	G protein subunit alpha o1		*TCF25*	Transcription factor 25	
HAL	Histidine ammonia-lyase		*TCFL5*	Transcription factor like 5	
HEPN1	Hepatocellular carcinoma down-regulated 1		*TEC*	Tec protein tyrosine kinase	

TABLE 4 (Continued)

Gene	Full gene name	Freq	Gene	Full gene name	Freq
IMPA2	Inositol monophosphatase 2		*TM4SF20*	Transmembrane 4 L six family member 20	
INPP1	Inositol polyphosphate-1-phosphatase		*TMEM45A*	Transmembrane protein 45A	
INPP4A	Inositol polyphosphate-4-phosphatase type I A	2	*TPD52L1*	Tumor protein D52 like 1	
INPP5A	Inositol polyphosphate-5-phosphatase A	2	*TSPAN2*	Tetraspanin 2	
KCNQ1	Potassium voltage-gated channel subfamily Q member 1	2	*TSSC1*	EARP complex and GARP complex interacting protein 1	
KIAA1267	KAT8 regulatory NSL complex subunit 1		*TULP4*	Tubby like protein 4	
LDB2	LIM domain binding 2		*ZEB2*	Zinc finger E-box binding homeobox 2	

Note: Genes of uncertain function are not listed; these can be found in Tables S2–S4.

Abbreviations: Freq, frequency (the number of unique differentially methylated CpGs annotated to the gene concerned).

downstream effect on gene expression of a wide range of other genes.

Another group of genes with potential important effects in the CNS consists of five genes encoding for enzymes involved in inositol phosphate metabolism (*IMPA2*, *INPP1*, *INPP4A*, *INPP5A*, and *PLCXD2*). Inositol phosphate has important roles in signal transduction and Ca^{2+} homeostasis in the CNS, and imbalances in the inositol phosphate metabolism have been suggested to have a role in several neurological disorders, including epilepsy.[43] *IMPA2* encodes inositol monophosphatase 2, an enzyme that catalyzes the conversion of myo-inositol monophosphate to myo-inositol.[44] Interestingly, Nakayama et al. reported *IMPA2* to be a putative susceptibility gene for febrile seizures.[45] Furthermore, carbamazepine, a common ASM, has previous been shown to stimulate IMPA2 enzyme activity.[46] On the other hand, lithium, which is used in the treatment of bipolar disorders, inhibits IMPA2[46] and has a proconvulsive effect in rat lithium–pilocarpine-induced seizures.[47] Interestingly, this effect can be reversed by administration of myo-inositol.[47] Anticonvulsant effects of myo-inositol have also been demonstrated in rats with pentylenetetrazol- or kainic acid-induced seizures.[48,49] To our knowledge, an impact of the ketogenic diet on the inositol phosphate metabolism has not been described before, and may represent a plausible mechanism by which the ketogenic diet attenuates seizures.

As expected from the major shift in the whole-body metabolism induced by the dietary treatment, a large proportion of the genes identified in our study are involved in lipid metabolism (*APOB48R*, *B4GALT5*, *CERS6*, *CES1*, *CPT1A*, *GALNT2*, *PLCXD2*, and *PPAP2B*) and regulation of carbohydrate metabolism (*PDK4*). Particularly interesting are *CPT1A* and *PDK4*, which play key roles in the regulation of fatty acid beta oxidation and glycolysis, respectively, as well as *APOB48R*, encoding the macrophage receptor apolipoprotein B48, which is decisive in postprandial uptake of lipids in macrophages.[50] Collectively, these findings demonstrate that our method captures important biological adaptations, thus underlining the validity of our results.

This study has some limitations. The dietary treatment was given as an adjunctive treatment, and all participants, except one, used ASMs. Although none of the participants changed type or dose of the ASMs during the 24-week study period, the serum concentration of several ASMs was reduced during the diet intervention.[20] ASMs could potentially influence the DNA methylation profile either directly or indirectly through their influence on one-carbon metabolism nutrients.[51] For instance, valproic acid, one of the most commonly used ASMs,[52] has been shown to inhibit DNMTs and induce a decrease in global DNA methylation. Thus, we cannot exclude that the unintentional drop in serum concentration could have influenced the DNA methylation.

Another limitation of the study is the lack of a control group, which means that we do not know whether continued epileptogenesis might have affected our results. However, our study population consists of patients with a very long history of epilepsy (on average 25 years). From this perspective, 12 weeks of continued epileptogenesis is a very short period of time, which we do not expect to constitute a relevant difference with regard to DNA methylation changes. In addition, we argue that the longitudinal study design with intraindividual comparisons of DNA methylation before and after the diet intervention

also has the advantage of reducing the likelihood of potential confounding effects of genetic variations and interindividual differences in lifestyle and environmental exposures. Furthermore, the large proportion of differentially methylated sites annotated to genes associated with lipid metabolism supports that our study identifies meaningful biological changes that are genuine effects of the diet intervention.

We applied a pragmatic study design to investigate the impact of the dietary treatment on DNA methylation in a real-life setting. Although the amount of carbohydrate was restricted to a maximum of 16 g per day, the ratio of fat to protein and the calorie intake were not specified. Hence, variations in the macronutrient compositions or the weight reduction[16,17] experienced by several of the participants may have influenced the results.

Our study used whole blood as a surrogate tissue for a disease that manifests in the brain. Currently, it is still unknown whether seizure-associated DNA methylation changes occur in blood and how well diet-induced DNA methylation alterations in blood correspond to DNA methylation changes in the brain. However, the ketogenic diet's antiseizure effect may be ascribed to a combination of mechanisms, involving both alterations at a systemic level and changes directly in the brain.[53] Finally, we were not able to detect any differences in DNA methylation between responders and nonresponders. However, our sample size was small, and the antiseizure effect of the dietary treatment in our study population was modest.[16]

Importantly, we also find that our study has significant strengths. Compliance is a well-known challenge in nutrition research, and in this study the intervention represents a significant change in the patients' diet and everyday life. However, we have robust objective measures of ketosis, regular follow-up, and dietary assessments based on 3-day weighed food records documenting compliance in our study.

In conclusion, we have identified a genome-wide decrease in DNA methylation both globally and at specific loci in adult epilepsy patients treated with the modified ketogenic diet. Interestingly, a substantial share of the identified genes were associated with epilepsy and inositol phosphate metabolism. However, we were not able to identify any differences between responders and nonresponders; thus, the clinical implications of these findings remain to be elucidated. We believe that understanding the ketogenic diet's influence at the molecular level may be the key to unraveling the mechanisms by which the diet can ameliorate seizures and possibly to identifying novel therapeutic targets for epilepsy. Further studies, with larger sample size and with a control group of epilepsy patients eating their habitual diet while all epilepsy treatments are kept unchanged, are needed to elucidate the role of DNA methylation in successful dietary treatment of epilepsy.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Data collection was performed by Magnhild Kverneland and Karl Otto Nakken. Data analysis of DNA methylation was performed by Kristina Gervin; all other data analyses were performed by Sigrid Pedersen. The first draft of the manuscript was written by Sigrid Pedersen, and all authors commented on the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the Dam Foundation, the Norwegian Epilepsy Association's Research Fund, the Novo Nordisk Foundation, and the National Advisory Unit on Rare Disorders, Norway. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit the manuscript for publication. We kindly thank the patients who, despite their load of disease, took part in the study. We thank Erik Taubøll for his continued support in planning and conducting the clinical trial. We would also like to thank the leadership and staff at the National Center for Epilepsy for all contributions.

CONFLICT OF INTEREST

M.K. has received two honoraria from Nutricia, which had no influence on data collection, analysis, or writing of the manuscript. The remaining authors have nothing to disclose.

ETHICAL APPROVAL

The study was approved by the Regional Committee for Medical and Health Research (2010/2326). All participants or parents/caregivers provided written informed consent before enrollment. All procedures in this study were in accordance with the Helsinki Declaration. The randomized clinical trial was registered at ClinicalTrials.gov (ID: NCT01311440). 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.

DATA AVAILABILITY STATEMENT

Raw data from this project are not available due to privacy and ethical restrictions of the project approval and consent forms. Metadata generated in the study and code used in the analysis are available from the corresponding author upon reasonable request in accordance

with the privacy policy of the informed consent by the participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Pedersen S, Kverneland M, Nakken KO, Rudi K, Iversen PO, Gervin K, Genome-wide decrease in DNA methylation in adults with epilepsy treated with modified ketogenic diet: A prospective study. Epilepsia. 2022;63:2413–2426. https://doi.org/10.1111/epi.17351

Epilepsia

Supporting Information

Genome-wide decrease in DNA methylation in adults with epilepsy treated with modified ketogenic diet: A prospective study.

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Table S1 Mean percentage change in seizure frequency after 12 weeks of dietary treatment (n = 49)

Mean percentage change in seizure frequency*	Number of patients (%)
Seizure freedom	0 (0%)
90 – 99.9% seizure reduction	1 (2.0%)
50 – 89.9% seizure reduction	8 (16.3%)
25 – 49.9% seizure reduction	11 (22.4%)
<25% seizure reduction	29 (59.2%)

*Mean percentage change in seizure frequency is calculated comparing the seizure frequency in week 5-12 of the intervention period to the seizure frequency in week 1-12 of the baseline period.

Table S2 Differentially methylated CpGs (sorted alphabetically by gene name and ascending by cgID for CpGs with no gene annotation) across all time points (n = 47)

							Mean beta			Δ beta				
cgID	**Gene**	**Full gene name**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-value**	**FDR**
cg17828988	ANXA11	Annexin A11	10	81951504	5'UTR	OpenSea	0.725	0.699	0.696	-0.026	-0.029	-0.003	4.0E-06	0.038
cg14237301	APOB48R	Apolipoprotein B48 receptor	16	28506477	Body	N_Shelf	0.675	0.647	0.652	-0.028	-0.023	0.005	5.6E-06	0.043
cg18019618	ARHGEF28	Rho guanine nucleotide exchange factor 28	5	72964090	5'UTR	OpenSea	0.600	0.573	0.570	-0.027	-0.030	-0.003	2.7E-06	0.032
cg07380641	B4GALT5	Beta-1.4-galactosyltransferase 5	20	48295033	Body	OpenSea	0.509	0.477	0.478	-0.032	-0.031	0.001	1.2E-06	0.023
cg10077346	BCKDHB	Branched chain keto acid dehydrogenase E1 subunit beta	6	80920458	Body	OpenSea	0.748	0.716	0.728	-0.032	-0.020	0.012	2.1E-06	0.029
cg07397033	BLNK	B cell linker	10	98031201	5'UTR; 1stExon	OpenSea	0.573	0.555	0.547	-0.019	-0.027	-0.008	3.5E-06	0.035
cg04871807	C5orf27	NA	5	95186505	TSS1500	OpenSea	0.536	0.508	0.506	-0.028	-0.030	-0.002	3.3E-08	0.003
cg14928764	CD93	CD93 molecule	20	23064608	Body	OpenSea	0.821	0.799	0.796	-0.023	-0.026	-0.003	1.1E-07	0.004
cg20601482	CERS6	Ceramide synthase 6	2	169337576	Body	OpenSea	0.737	0.711	0.714	-0.026	-0.023	0.003	8.7E-07	0.019
cg08077617	CES1	Carboxylesterase 1	16	55868196	TSS1500	S_Shore	0.639	0.602	0.605	-0.037	-0.034	0.003	1.3E-06	0.024
cg02554361	CIITA	Class II major histocompatibility complex transactivator	16	11001757	Body	Island	0.912	0.902	0.902	-0.010	-0.009	0.001	4.5E-06	0.040
cg04075184	CLASP1	Cytoplasmic linker associated protein 1	2	122290763	Body	S_Shelf	0.744	0.732	0.718	-0.012	-0.025	-0.014	1.4E-06	0.025
cg08813944	CPSF4L	Cleavage and polyadenylation specific factor 4 like	17	71258589	TSS1500	OpenSea	0.746	0.721	0.715	-0.025	-0.031	-0.006	2.8E-06	0.033
cg19393755	CPSF4L	Cleavage and polyadenylation specific factor 4 like	17	71258101	TSS200	OpenSea	0.707	0.689	0.680	-0.018	-0.027	-0.009	4.4E-06	0.040
cg22911054	CPT1A	Carnitine palmitoyltransferase 1A	11	68603379	5'UTR	N_Shelf	0.770	0.745	0.728	-0.025	-0.042	-0.017	2.0E-10	0.000
cg11095122	CSGALNACT1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	8	19540734	TSS1500	OpenSea	0.673	0.642	0.653	-0.031	-0.019	0.012	3.8E-06	0.037
cg06070496	DLGAP1	DLG associated protein 1	18	3706638	Body	OpenSea	0.634	0.610	0.601	-0.024	-0.033	-0.009	1.9E-06	0.028
cg12359298	DTD1	D-tyrosyl-tRNA deacylase 1	20	18735103	3'UTR	OpenSea	0.683	0.656	0.653	-0.026	-0.029	-0.003	4.0E-07	0.012
cg15546194	DZIP1L	DAZ interacting zinc finger protein 1 like	3	137800724	Body	OpenSea	0.685	0.664	0.666	-0.021	-0.018	0.002	3.0E-06	0.034
cg07019857	EFNA5	Ephrin A5	5	106822849	Body	OpenSea	0.707	0.715	0.679	0.008	-0.028	-0.037	1.7E-06	0.028
cg18518074	EHD1	EH domain containing 1	11	64642316	Body	N_Shelf	0.495	0.464	0.467	-0.031	-0.027	0.003	1.0E-06	0.021
cg03457142	EIF4E3	Eukaryotic translation initiation factor 4E family member 3	3	71804859	TSS1500	S_Shore	0.498	0.484	0.478	-0.014	-0.019	-0.006	2.6E-06	0.032
cg23752007	ELMO1	Engulfment and cell motility 1	7	37429500	5'UTR	OpenSea	0.742	0.717	0.720	-0.025	-0.022	0.003	1.9E-06	0.028
cg02314596	FOXN3	Forkhead box N3	14	89852725	Body	OpenSea	0.744	0.720	0.727	-0.024	-0.017	0.007	1.8E-07	0.006
cg02479755	FTO	Fat mass and obesity-associated protein	16	54097517	Body	OpenSea	0.830	0.808	0.804	-0.022	-0.026	-0.004	3.7E-07	0.011
cg03702011	GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	1	230193513	TSS200	OpenSea	0.732	0.701	0.708	-0.031	-0.024	0.007	5.2E-07	0.015
cg20703242	GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	1	230279135	Body	OpenSea	0.510	0.490	0.487	-0.020	-0.023	-0.003	4.3E-06	0.040
cg06783533	GNAO1	G protein subunit alpha o1	16	56388908	Body	OpenSea	0.843	0.822	0.820	-0.021	-0.023	-0.002	4.9E-06	0.041
cg27246571	HAL	Histidine ammonia-lyase	12	96389588	Body	Island	0.720	0.693	0.694	-0.028	-0.026	0.001	1.0E-06	0.021
cg17439422	HEPN1	Hepatocellular carcinoma down-regulated 1	11	124788656	TSS1500	N_Shelf	0.836	0.820	0.818	-0.017	-0.019	-0.002	2.5E-08	0.002
cg06865026	IMPA2	Inositol monophosphatase 2	18	12015910	Body	OpenSea	0.850	0.820	0.831	-0.029	-0.019	0.010	1.1E-08	0.002

Table S2 (continued)

							Mean beta			Δ beta				
cgID	**Gene**	**Full gene name**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-value**	**FDR**
cg00416922	INPP1	Inositol polyphosphate-1-phosphatase	2	191207560	TSS1500	N_Shore	0.712	0.691	0.691	-0.021	-0.021	0.000	1.8E-06	0.028
cg11555067	INPP4A	Inositol polyphosphate-4-phosphatase type I A	2	99081350	5'UTR	OpenSea	0.291	0.262	0.275	-0.029	-0.017	0.013	2.1E-06	0.029
cg10967866	INPP5A	Inositol polyphosphate-5-phosphatase A	10	134362164	Body	S_Shore	0.744	0.712	0.716	-0.032	-0.028	0.004	8.3E-08	0.004
cg19137806	INPP5A	Inositol polyphosphate-5-phosphatase A	10	134362170	Body	S_Shore	0.709	0.677	0.678	-0.031	-0.030	0.001	4.5E-08	0.003
cg05993525	KCNQ1	Potassium voltage-gated channel subfamily Q member 1	11	2769184	Body	OpenSea	0.567	0.534	0.539	-0.033	-0.027	0.005	1.0E-06	0.021
cg08376310	KCNQ1	Potassium voltage-gated channel subfamily Q member 1	11	2858621	Body	OpenSea	0.902	0.887	0.886	-0.016	-0.016	0.000	5.4E-09	0.001
cg21214508	KIAA1267	KAT8 regulatory NSL complex subunit 1	17	44248233	1stExon	OpenSea	0.865	0.849	0.851	-0.017	-0.015	0.002	2.1E-06	0.029
cg04460609	LDB2	LIM domain binding 2	4	16532808	Body	OpenSea	0.514	0.490	0.481	-0.024	-0.033	-0.009	4.1E-08	0.003
cg24133663	LEPREL1	Prolyl 3-hydroxylase 2	3	189679585	Body	OpenSea	0.088	0.088	0.075	-0.001	-0.013	-0.012	6.3E-07	0.016
cg09954473	LOC101927156	NA	2	182245509	Body	OpenSea	0.826	0.805	0.810	-0.021	-0.017	0.005	4.7E-06	0.040
cg01164919	LOC101927588	NA	8	125214930	Body	OpenSea	0.855	0.847	0.833	-0.008	-0.022	-0.014	2.6E-06	0.032
cg04232816	LOC285033	NA	2	96905390	TSS1500	OpenSea	0.703	0.682	0.674	-0.022	-0.029	-0.008	6.4E-06	0.049
cg18394235	LTBP1	Latent transforming growth factor beta binding protein 1	2	33359059	TSS1500; Body	OpenSea	0.686	0.663	0.659	-0.023	-0.027	-0.004	5.3E-06	0.042
cg06022811	LY86;LY86-AS1	Lymphocyte antigen 86;NA	6	6599919	Body	OpenSea	0.725	0.698	0.697	-0.027	-0.028	-0.001	4.7E-06	0.040
cg09427959	MED13L	Mediator complex subunit 13 like	12	116520303	Body	OpenSea	0.813	0.800	0.791	-0.013	-0.023	-0.010	3.6E-06	0.036
cg14728609	MIR3134;SUSD1	NA; Sushi domain containing 1	9	114933506	Body	N_Shelf	0.679	0.659	0.656	-0.020	-0.023	-0.003	1.3E-06	0.024
cg13617154	NAT8	N-acetyltransferase 8	2	73868507	Body	OpenSea	0.792	0.768	0.770	-0.024	-0.022	0.002	2.7E-09	0.001
cg26879421	NET1	Neuroepithelial cell transforming 1	10	5455710	Body	S_Shore	0.720	0.699	0.698	-0.021	-0.022	0.000	4.5E-06	0.040
cg22069247	NMUR1	Neuromedin U receptor 1	2	232393256	Body	N_Shore	0.665	0.627	0.638	-0.039	-0.027	0.011	2.1E-08	0.002
cg05262369	NUCKS1	Nuclear casein kinase and cyclin dependent kinase substrate 1	1	205716365	Body	N_Shore	0.738	0.709	0.713	-0.030	-0.025	0.005	7.0E-08	0.003
cg14499189	PDE4D	Phosphodiesterase 4D	5	59492401	5'UTR	OpenSea	0.806	0.787	0.783	-0.019	-0.023	-0.003	2.4E-06	0.031
cg23987137	PDE4D	Phosphodiesterase 4D	5	58653624	Body	OpenSea	0.800	0.777	0.778	-0.023	-0.022	0.001	1.1E-06	0.021
cg17075888	PDK4	Pyruvat dehydrogenase kinase 4	7	95225339	Body	N_Shore	0.595	0.594	0.568	-0.001	-0.027	-0.026	1.8E-06	0.028
cg07599432	PDZD8	PDZ domain containing 8	10	119126318	Body	OpenSea	0.770	0.740	0.740	-0.029	-0.029	0.000	8.2E-07	0.019
cg12368246	PLAGL1	PLAG1 like zinc finger 1	6	144286757	5'UTR	OpenSea	0.824	0.802	0.805	-0.022	-0.019	0.003	2.1E-06	0.029
cg16615151	PLCXD2	Phosphatidylinositol specific phospholipase C X domain containing 2	3	111409324	Body	OpenSea	0.545	0.510	0.514	-0.035	-0.030	0.004	1.0E-07	0.004
cg26683198	PPAP2B	Phospholipid phosphatase 3	1	57039392	Body	OpenSea	0.519	0.493	0.498	-0.026	-0.021	0.005	3.5E-06	0.035
cg15438481	PRKCA	Protein kinase C alpha	17	64499496	Body	OpenSea	0.877	0.867	0.859	-0.009	-0.017	-0.008	3.2E-06	0.034
cg02641339	PSTPIP2	Proline-serine-threonine phosphatase interacting protein 2	18	43635048	Body	OpenSea	0.609	0.589	0.570	-0.020	-0.039	-0.019	1.7E-06	0.028
cg03189652	PTH2R	Parathyroid hormone 2 receptor	2	209224422	TSS200	N_Shore	0.763	0.740	0.735	-0.022	-0.028	-0.006	5.3E-06	0.042
cg08047233	RERE	Arginine-glutamic acid dipeptide repeats	1	8578167	Body	OpenSea	0.696	0.671	0.666	-0.026	-0.031	-0.005	4.9E-06	0.041
cg07268595	RNF166	Ring finger protein 166	16	88766501	Body; 5'UTR	S_Shore	0.584	0.561	0.555	-0.023	-0.029	-0.006	5.8E-06	0.044
cg04161193	RNF19A	Ring finger protein 19A	8	101338327	5'UTR	OpenSea	0.551	0.525	0.526	-0.026	-0.025	0.001	3.4E-06	0.035
cg21882620	SLC22A23	Solute carrier family 22 member 23	6	3379971	Body	OpenSea	0.752	0.736	0.718	-0.017	-0.035	-0.018	8.5E-07	0.019

Table S2 (continued)

cgID	Gene	Full gene name	Chr	Position	Gene context	CpG context	Mean beta			Δ beta			P-value	FDR
							B	4W	12W	4W-B	12W-B	12W-4W		
cg03492107	SNTB1	Syntrophin beta 1	8	121715913	Body	OpenSea	0.644	0.612	0.613	-0.032	-0.031	0.001	2.5E-07	0.009
cg15835363	STARD9	StAR related lipid transfer domain containing 9	15	42894942	Body	OpenSea	0.759	0.727	0.731	-0.032	-0.028	0.004	1.9E-08	0.002
cg14957855	SWT1	SWT1. RNA endoribonuclease homolog	1	185234541	Body	OpenSea	0.857	0.833	0.865	-0.025	0.007	0.032	2.6E-06	0.032
cg02949436	TCF25	Transcription factor 25	16	89940853	Body	S_Shore	0.068	0.058	0.059	-0.010	-0.009	0.000	2.9E-06	0.033
cg19933954	TCFL5	Transcription factor like 5	20	61494242	TSS1500	S_Shore	0.630	0.588	0.591	-0.042	-0.039	0.003	3.4E-06	0.035
cg22744398	TEC	Tec protein tyrosine kinase	4	48226070	Body	OpenSea	0.650	0.622	0.612	-0.027	-0.038	-0.010	5.9E-07	0.016
cg27549551	TM4SF20	Transmembrane 4 L six family member 20	2	228245419	TSS1500	OpenSea	0.783	0.765	0.752	-0.018	-0.031	-0.012	3.2E-07	0.010
cg10288625	TMEM45A	Transmembrane protein 45A	3	100289748	Body	OpenSea	0.824	0.805	0.823	-0.019	-0.001	0.018	1.9E-06	0.028
cg25124205	TPD52L1	Tumor protein D52 like 1	6	125519976	5'UTR; Body	OpenSea	0.730	0.698	0.676	-0.032	-0.053	-0.022	9.2E-09	0.002
cg23999170	TSPAN2	Tetraspanin 2	1	115628111	Body	N_Shelf	0.715	0.688	0.687	-0.027	-0.028	-0.002	1.5E-06	0.026
cg11166303	TSSC1	EARP complex and GARP complex interacting protein 1	2	3320529	Body	N_Shore	0.650	0.624	0.620	-0.026	-0.030	-0.004	2.3E-06	0.031
cg06096446	TULP4	Tubby like protein 4	6	158744008	Body	OpenSea	0.700	0.686	0.680	-0.014	-0.020	-0.005	5.2E-06	0.042
cg20347155	ZEB2	Zinc finger E-box binding homeobox 2	2	145218951	Body	OpenSea	0.634	0.597	0.604	-0.037	-0.030	0.007	3.4E-06	0.035
cg00095930	NA	NA	12	109569116	NA	OpenSea	0.455	0.431	0.425	-0.024	-0.030	-0.006	6.5E-08	0.003
cg01119319	NA	NA	7	38356808	NA	OpenSea	0.596	0.552	0.558	-0.044	-0.038	0.006	1.2E-07	0.004
cg02146228	NA	NA	14	75401904	NA	OpenSea	0.528	0.511	0.503	-0.017	-0.025	-0.009	5.8E-08	0.003
cg05303899	NA	NA	2	9919494	NA	OpenSea	0.692	0.674	0.673	-0.018	-0.019	-0.001	5.0E-06	0.041
cg05329280	NA	NA	20	5192635	NA	OpenSea	0.726	0.699	0.697	-0.028	-0.030	-0.002	6.9E-07	0.017
cg05999592	NA	NA	11	72925008	NA	N_Shelf	0.630	0.608	0.608	-0.022	-0.022	0.000	4.5E-06	0.040
cg07418291	NA	NA	15	58553346	NA	OpenSea	0.757	0.733	0.738	-0.024	-0.019	0.005	2.5E-06	0.032
cg08698943	NA	NA	10	3509758	NA	OpenSea	0.412	0.390	0.391	-0.023	-0.021	0.001	1.6E-06	0.027
cg10042645	NA	NA	11	2308589	NA	OpenSea	0.651	0.622	0.625	-0.029	-0.027	0.003	2.8E-06	0.032
cg10629173	NA	NA	10	5576434	NA	OpenSea	0.772	0.745	0.745	-0.027	-0.026	0.000	3.4E-06	0.035
cg12582317	NA	NA	17	55822272	NA	OpenSea	0.770	0.748	0.748	-0.022	-0.022	0.000	4.3E-06	0.040
cg14165660	NA	NA	10	112889349	NA	OpenSea	0.811	0.789	0.792	-0.023	-0.019	0.004	7.3E-08	0.003
cg15256315	NA	NA	8	135791732	NA	OpenSea	0.317	0.283	0.294	-0.035	-0.024	0.011	5.4E-06	0.042
cg15295322	NA	NA	3	178689645	NA	OpenSea	0.769	0.752	0.748	-0.016	-0.021	-0.004	4.6E-06	0.040
cg16797699	NA	NA	12	109569084	NA	OpenSea	0.708	0.682	0.682	-0.026	-0.026	0.000	3.2E-07	0.010
cg19815228	NA	NA	10	134232489	NA	OpenSea	0.615	0.581	0.575	-0.034	-0.040	-0.006	4.1E-06	0.038
cg20025086	NA	NA	12	109569130	NA	OpenSea	0.502	0.481	0.476	-0.021	-0.025	-0.005	7.5E-07	0.018
cg21156054	NA	NA	21	40225493	NA	OpenSea	0.464	0.453	0.439	-0.011	-0.025	-0.014	3.1E-06	0.034
cg22768761	NA	NA	15	58551412	NA	OpenSea	0.877	0.858	0.863	-0.019	-0.014	0.005	1.7E-06	0.028
cg26129664	NA	NA	10	134232399	NA	OpenSea	0.464	0.429	0.426	-0.034	-0.038	-0.003	2.6E-08	0.002
cg26645302	NA	NA	14	22891244	NA	OpenSea	0.254	0.231	0.217	-0.023	-0.037	-0.014	3.8E-06	0.037
cg26835531	NA	NA	18	47980832	NA	OpenSea	0.822	0.811	0.798	-0.011	-0.024	-0.013	2.6E-06	0.032

Abbreviations: 4W, 4 weeks of dietary treatment; 12W, 12 weeks of dietary treatment; B, Baseline; Chr, Chromosome; FDR, False discovery rate; NA, No information available. FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele ($\beta = M/(M + U + 100)$). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

Table S3 Differentially methylated CpGs (sorted alphabetically by gene name and ascending by cgID for CpGs with no gene annotation) from baseline to 4 weeks of dietary treatment (n=56)

							Mean beta			Δ beta		
cgID	**Gene**	**Full gene name**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**P-value**	**FDR**
cg17951488	AGAP7	NA	10	230193513	TSS1500	N_Shore	0.891	0.872	0.879	-0.019	1.8E-06	0.044
cg10077346	BCKDHB	Branched chain keto acid dehydrogenase E1 subunit beta	6	2308589	Body	OpenSea	0.748	0.716	0.728	-0.032	5.8E-07	0.022
cg04871807	C5orf27	NA	5	23064608	TSS1500	OpenSea	0.536	0.508	0.506	-0.028	1.4E-06	0.036
cg14928764	CD93	CD93 molecule	20	112889349	Body	OpenSea	0.821	0.799	0.796	-0.023	1.4E-06	0.036
cg20601482	CERS6	Ceramide synthase 6	2	58553346	Body	OpenSea	0.737	0.711	0.714	-0.026	6.6E-07	0.023
cg12359298	DTD1	D-tyrosyl-tRNA deacylase 1	20	95186505	3'UTR	OpenSea	0.683	0.656	0.653	-0.026	1.5E-06	0.036
cg23752007	ELMO1	Engulfment and cell motility 1	7	2769184	5'UTR	OpenSea	0.742	0.717	0.720	-0.025	2.2E-06	0.050
cg02314596	FOXN3	Forkhead box N3	14	18735103	Body	OpenSea	0.744	0.720	0.727	-0.024	7.6E-08	0.007
cg03702011	GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	1	135791732	TSS200	OpenSea	0.732	0.701	0.708	-0.031	2.6E-07	0.014
cg27246571	HAL	Histidine ammonia-lyase	12	109569116	Body	Island	0.720	0.693	0.694	-0.028	5.0E-07	0.021
cg17439422	HEPN1	Hepatocellular carcinoma down-regulated 1	11	38356808	TSS1500	N_Shelf	0.836	0.820	0.818	-0.017	1.1E-07	0.007
cg06865026	IMPA2	Inositol monophosphatase 2	18	73868507	Body	OpenSea	0.850	0.820	0.831	-0.029	1.9E-09	0.001
cg11555067	INPP4A	Inositol polyphosphate-4-phosphatase type I A	2	205716365	5'UTR	OpenSea	0.291	0.262	0.275	-0.029	3.3E-07	0.017
cg10967866	INPP5A	Inositol polyphosphate-5-phosphatase A	10	134232399	Body	S_Shore	0.744	0.712	0.716	-0.032	1.0E-07	0.007
cg19137806	INPP5A	Inositol polyphosphate-5-phosphatase A	10	96389588	Body	S_Shore	0.709	0.677	0.678	-0.031	9.8E-08	0.007
cg05993525	KCNQ1	Potassium voltage-gated channel subfamily Q member 1	11	134362164	Body	OpenSea	0.567	0.534	0.539	-0.033	9.0E-07	0.026
cg08376310	KCNQ1	Potassium voltage-gated channel subfamily Q member 1	11	99081350	Body	OpenSea	0.902	0.887	0.886	-0.016	4.8E-08	0.006
cg13617154	NAT8	N-acetyltransferase 8	2	12015910	Body	OpenSea	0.792	0.768	0.770	-0.024	1.3E-08	0.003
cg22069247	NMUR1	Neuromedin U receptor 1	2	121715913	Body	N_Shore	0.665	0.627	0.638	-0.039	9.9E-09	0.003
cg05262369	NUCKS1	Nuclear casein kinase and cyclin dependent kinase substrate 1	1	89852725	Body	N_Shore	0.738	0.709	0.713	-0.030	9.7E-08	0.007
cg16615151	PLCXD2	Phosphatidylinositol specific phospholipase C X domain containing 2	3	2858621	Body	OpenSea	0.545	0.510	0.514	-0.035	1.1E-07	0.007
cg03492107	SNTB1	Syntrophin beta 1	8	80920458	Body	OpenSea	0.644	0.612	0.613	-0.032	6.4E-07	0.023
cg15835363	STARD9	StAR related lipid transfer domain containing 9	15	55822272	Body	OpenSea	0.759	0.727	0.731	-0.032	2.8E-08	0.005
cg00095930	NA	NA	12	134362170	NA	OpenSea	0.455	0.431	0.425	-0.024	7.8E-07	0.025
cg01119319	NA	NA	7	42894942	NA	OpenSea	0.596	0.552	0.558	-0.044	7.9E-08	0.007
cg07418291	NA	NA	15	232393256	NA	OpenSea	0.757	0.733	0.738	-0.024	7.2E-07	0.024
cg10042645	NA	NA	11	51487759	NA	OpenSea	0.651	0.622	0.625	-0.029	8.3E-07	0.025
cg12582317	NA	NA	17	37429500	NA	OpenSea	0.770	0.748	0.748	-0.022	1.5E-06	0.036
cg14165660	NA	NA	10	109569084	NA	OpenSea	0.811	0.789	0.792	-0.023	4.8E-08	0.006
cg15256315	NA	NA	8	111409324	NA	OpenSea	0.317	0.283	0.294	-0.035	1.3E-06	0.035
cg16797699	NA	NA	12	169337576	NA	OpenSea	0.708	0.682	0.682	-0.026	5.4E-07	0.022
cg22768761	NA	NA	15	58551412	NA	OpenSea	0.877	0.858	0.863	-0.019	4.0E-07	0.018
cg26129664	NA	NA	10	124788656	NA	OpenSea	0.464	0.429	0.426	-0.034	4.1E-07	0.018

Abbreviations: 4W, 4 weeks of dietary treatment; 12W, 12 weeks of dietary treatment; B, Baseline; Chr, Chromosome; FDR, False discovery rate; NA, No information available. FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele ($\beta = M/(M + U + 100)$). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

Table S4 Differentially methylated CpGs (sorted alphabetically by gene name and ascending by cgID for CpGs with no gene annotation) from baseline to 12 weeks of dietary treatment (n= 49)

							Mean beta			Δ beta		
cgID	**Gene**	**Full gene name**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**12W-B**	**P-value**	**FDR**
cg04871807	C5orf27	NA	5	95186505	TSS1500	OpenSea	0.536	0.508	0.506	-0.030	9.9E-08	0.013
cg14928764	CD93	CD93 molecule	20	23064608	Body	OpenSea	0.821	0.799	0.796	-0.026	5.9E-07	0.023
cg04075184	CLASP1	Cytoplasmic linker associated protein 1	2	122290763	Body	S_Shelf	0.744	0.732	0.718	-0.025	2.2E-07	0.015
cg19393755	CPSF4L	Cleavage and polyadenylation specific factor 4 like	17	71258101	TSS200	OpenSea	0.707	0.689	0.680	-0.027	1.6E-06	0.039
cg22911054	CPT1A	Carnitine palmitoyltransferase 1A	11	68603379	5'UTR	N_Shelf	0.770	0.745	0.728	-0.042	4.9E-11	0.000
cg03457142	EIF4E3	Eukaryotic translation initiation factor 4E family member 3	3	71804859	TSS1500	S_Shore	0.498	0.484	0.478	-0.019	5.1E-07	0.021
cg24170465	FAM198B	Family with sequence similarity 198 member B	4	159094277	TSS200	OpenSea	0.682	0.670	0.649	-0.033	1.4E-06	0.038
cg02479755	FTO	Fat mass and obesity-associated protein	16	54097517	Body	OpenSea	0.830	0.808	0.804	-0.026	4.1E-07	0.020
cg17439422	HEPN1	Hepatocellular carcinoma down-regulated 1	11	124788656	TSS1500	N_Shelf	0.836	0.820	0.818	-0.019	7.1E-07	0.026
cg08376310	KCNQ1	Potassium voltage-gated channel subfamily Q member 1	11	2858621	Body	OpenSea	0.902	0.887	0.886	-0.016	1.3E-07	0.014
cg04460609	LDB2	LIM domain binding 2	4	16532808	Body	OpenSea	0.514	0.490	0.481	-0.033	1.3E-08	0.003
cg24133663	LEPREL1	Prolyl 3-hydroxylase 2	3	189679585	Body	OpenSea	0.088	0.088	0.075	-0.013	7.8E-07	0.027
cg01164919	LOC101927588	NA	8	125214930	Body	OpenSea	0.855	0.847	0.833	-0.022	4.2E-07	0.020
cg09427959	MED13L	Mediator complex subunit 13 like	12	116520303	Body	OpenSea	0.813	0.800	0.791	-0.023	9.3E-07	0.029
cg14728609	MIR3134; SUSD1	NA; Sushi domain containing 1	9	114933506	Body	N_Shelf	0.679	0.659	0.656	-0.023	1.0E-06	0.031
cg13617154	NAT8	N-acetyltransferase 8	2	73868507	Body	OpenSea	0.792	0.768	0.770	-0.022	1.7E-07	0.015
cg17075888	PDK4	Pyruvat dehydrogenase kinase 4	7	95225339	Body	N_Shore	0.595	0.594	0.568	-0.027	1.6E-06	0.039
cg15438481	PRKCA	Protein kinase C alpha	17	64499496	Body	OpenSea	0.877	0.867	0.859	-0.017	1.3E-06	0.034
cg02641339	PSTPIP2	Proline-serine-threonine phosphatase interacting protein 2	18	43635048	Body	OpenSea	0.609	0.589	0.570	-0.039	3.2E-07	0.020
cg21882620	SLC22A23	Solute carrier family 22 member 23	6	3379971	Body	OpenSea	0.752	0.736	0.718	-0.035	1.6E-07	0.015
cg22744398	TEC	Tec protein tyrosine kinase	4	48226070	Body	OpenSea	0.650	0.622	0.612	-0.038	3.5E-07	0.020
cg27549551	TM4SF20	Transmembrane 4 L six family member 20	2	228245419	TSS1500	OpenSea	0.783	0.765	0.752	-0.031	9.1E-08	0.013
cg25124205	TPD52L1	Tumor protein D52 like 1	6	125519976	5'UTR. Body	OpenSea	0.730	0.698	0.676	-0.053	3.4E-09	0.001
cg06096446	TULP4	Tubby like protein 4	6	158744008	Body	OpenSea	0.700	0.686	0.680	-0.020	8.7E-07	0.029
cg00095930	NA	NA	12	109569116	NA	OpenSea	0.455	0.431	0.425	-0.030	1.8E-08	0.020
cg02146228	NA	NA	14	75401904	NA	OpenSea	0.528	0.511	0.503	-0.025	1.2E-06	0.003
cg14783355	NA	NA	5	10530621	NA	OpenSea	0.721	0.706	0.692	-0.029	5.2E-07	0.033
cg21156054	NA	NA	21	40225493	NA	OpenSea	0.464	0.453	0.439	-0.025	1.9E-07	0.021
cg26129664	NA	NA	10	134232399	NA	OpenSea	0.464	0.429	0.426	-0.038	1.1E-06	0.015
cg26645302	NA	NA	14	22891244	NA	OpenSea	0.254	0.231	0.217	-0.037	4.3E-07	0.033
cg26835531	NA	NA	18	47980832	NA	OpenSea	0.822	0.811	0.798	-0.024	9.9E-08	0.020

Abbreviations: 4W, 4 weeks of dietary treatment; 12W, 12 weeks of dietary treatment; B, Baseline; Chr, Chromosome; FDR, False discovery rate; NA, No information available. FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele (β = M/(M + U + 100)). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

II

DNA methylation of genes involved in the metabolism of anti-seizure medications in adult epilepsy patients treated with modified ketogenic diet.

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Acknowledgments
The authors want to thank the patients who took their time to participate in the study. We also want to thank Erik Taubøll for his support in planning and conducting the clinical trial and Ellen Molteberg for her contribution in the data collection. The Section for Clinical Pharmacology, Oslo University Hospital (Oslo, Norway) is acknowledged for the analyses of all ASMs. Finally, we thank our colleagues at the National Centre for Epilepsy for all contributions. This work was supported by the Dam Foundation, the Norwegian Epilepsy Association's Research Fund, the Novo Nordisk Foundation, and the National Advisory Unit on Rare Disorders, Norway. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit the manuscript for publication.

Ethical disclosures
The study was approved by the Regional Committee for Medical and Health Research (2010/2326). All participants or parents/caregivers provided written informed consent before enrollment. All procedures in this study were in accordance with the Helsinki Declaration. The randomized clinical study was registered at ClinicalTrials.gov (ID:NCT01311440). MK has received two honoraria from Nutricia. CJL has received speakers' or expert group honoraria from Angelini, Eisai, Jazz and UCB Pharma. KKS has been reimbursed for travel and accommodation as a speaker at a sponsored workshop by Kolfarma. KON has received lecture honoraria from Desitin and Eisai. The remaining authors have nothing to disclose. 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

Data and code availability
The raw data from this project is not available due to privacy and ethical restrictions of the project approval and consent forms. Metadata generated in the study and code used in the analysis are available from the corresponding author upon reasonable request within the privacy policy of the informed consent by the participants.

Abstract

Objective: In this candidate gene study, we examined the impact of a modified ketogenic diet on DNA methylation of genes involved in the metabolism of ASMs in adults with epilepsy. In addition, we investigated whether changes in DNA methylation were correlated with changes in ASM serum concentrations.

Methods: Fifty-eight patients with focal or generalized drug resistant epilepsy (age 16 – 65 years) were included in the study. Relevant enzymes of ASM metabolism were identified. DNA methylation was analysed using the Illumina Infinium MethylationEPIC array. Differentially methylated positions after 4 and 12 weeks of dietary treatment were identified using a linear regression model and ANOVA. Correlation between changes in DNA methylation and changes in serum concentrations of ASMs were investigated using Spearman's correlation coefficient. We used the Benjamini-Hochberg procedure with a false discovery rate (FDR) of <5% to correct for multiple comparisons.

Results: We examined DNA methylation at 131 CpGs annotated to 13 genes involved in the metabolism of ASMs. After the diet intervention, we identified a significant change in DNA methylation of one position annotated to *CES1*. In addition, we found a strong correlation (rho= -0.85) between absolute difference in DNA methylation of *UGT1A4* and percentage change in lamotrigine serum concentration.

Significance: These results suggest that altered DNA methylation associated with treatment with a modified ketogenic diet may contribute to observed decreases in ASM serum concentrations in epilepsy patients treated with the modified ketogenic diet.

Key words: Food-drug interactions, high-fat, low-carbohydrate diet, modified Atkins diet

Key points

- We hypothesized that changes in DNA methylation of genes encoding drug-metabolizing enzymes may contribute to the observed reduction in ASM serum concentrations in patients on the diet.
- In this candidate gene study, we investigated the impact of the modified ketogenic diet on DNA methylation of 131 CpGs annotated to 13 genes involved in the metabolism of ASMs in adult epilepsy patients.
- DNA methylation of the *CES1* gene encoding the drug-metabolizing enzyme carboxylesterase 1 was significantly reduced after the diet intervention.
- We identified a strong, negative correlation between change in DNA methylation of *UGT1A4* and change in lamotrigine serum concentration following the diet intervention.

1. Introduction

Epilepsy is one of the most common neurological disorders, affecting more than 65 million people worldwide (1). The disease is characterized by unprovoked, recurrent seizures, frequently associated with a high burden of neurological, psychiatric, cognitive, and biological comorbidities (2). The mainstay of epilepsy treatment is ASMs. However, about one-third of the patients do not achieve adequate seizure control with the currently available ASMs (i.e. they have drug resistant epilepsy) (3, 4). The ketogenic diet, an umbrella term for various types of high-fat, low-carbohydrate diets that induces ketosis, has for decades been used successfully in the treatment of patients with drug resistant epilepsy (5-7). The modified ketogenic diet (also known as the modified Atkins diet) is a less strict and more flexible variant of the ketogenic diet (8), often used by adult epilepsy patients. Commonly, the diet is used as an add-on treatment to ASMs.

Recently, there have been concerns about potential pharmacokinetic interactions between the ketogenic diets and ASMs (9-12). Heo et al. reported a significant decrease in the serum concentration of valproic acid in children treated with a ketogenic diet (9), while Coppola et al. found a non-significant trend towards reduced serum concentration of valproic acid during the first month of dietary treatment (10). In accordance, we previously demonstrated a substantial decrease in the serum concentration of several ASMs in adults treated with a modified ketogenic diet despite no change in the drug doses, suggesting a pharmacokinetic food-drug interaction (13).

Evidence from preclinical studies have revealed that high-fat diets can alter the gene expression of drug-metabolizing enzymes, including the cytochrome P450s (CYPs) and UDP-glucuronosyltransferases (UGTs) superfamilies of enzymes (14, 15). These enzymes are mainly expressed in the liver and have a key role in the metabolism of a variety of commonly used drugs, including several ASMs (16-18). Together, the CYP and UGT enzymes are responsible for the clearance of the majority of drugs with hepatic clearance (18). Importantly, in addition to their role in

drug metabolism, the CYPs are also involved in the synthesis of steroids, cholesterol, and other lipids (16, 19, 20). Therefore, a high-fat diet may interact with enzymes involved in ASM metabolism.

DNA methylation, an epigenetic modification in which a methyl group (CH_3) is added to a cytosine nucleotide in the DNA, is one of the most studied epigenetic modifications. In humans, methylation of the DNA mainly occurs on cytosine nucleotides followed by guanine nucleotides, called cytosine-phosphate-guanines dinucleotides (CpGs). DNA methylation is involved in regulation of gene expression, and the effect is dependent on genomic location of the DNA methylation (21). Several environmental factors, including diet can influence DNA methylation (22). We recently reported a genome-wide decrease in DNA methylation in patients with epilepsy treated with the modified ketogenic diet (23). Interestingly, DNA methylation has been linked to the regulation of genes encoding drug-metabolizing enzymes (24). We hypothesize that diet-induced alterations in DNA methylation may explain the observed reduction in ASM serum concentrations in patients with epilepsy treated with a ketogenic diet.

The aim of the present study was to investigate the impact of the modified ketogenic diet on DNA methylation of candidate genes encoding enzymes involved in the metabolism of ASMs in adults with drug resistant epilepsy. In addition, we investigated whether changes in DNA methylation were correlated with changes in ASM serum concentrations.

2. Methods

2.1. Study design and participants

We studied 58 patients recruited from the National Centre for Epilepsy, Norway, between March 1, 2011, and February 28, 2017. The study cohort consisted of patients with focal epilepsy included in a randomized clinical study (25) and patients with generalized epilepsy included in an associated prospective, non-randomized study (26). Our previous studies on genome-wide DNA methylation (23) and diet–drug interactions (13) were based on the same population.

Inclusion criteria were generalized or focal epilepsy according to the classification by the International League Against Epilepsy's (ILAE) (27), ≥3 countable seizures per month, having tried ≥3 ASMs, age ≥16 years, BMI >18.5 kg/m^2, and the participants had to be motivated and willing to try the dietary treatment for at least 12 weeks. Exclusion criteria included familial hypercholesterolemia, cardiovascular disease, kidney disease, psychogenic non-epileptic seizures, other diseases which contraindicated the dietary treatment, previous treatment with a ketogenic diet for more than one week during the preceding year, status epilepticus the past six months, epilepsy surgery (including vagus nerve stimulator implant the past year), four continuous seizure-free weeks the preceding two months, use of drugs or supplements that may interfere with the diet or ASMs, and pregnancy or planned pregnancy.

The study consisted of a 12-week baseline and a 12-week intervention period. In the baseline period, the participants ate their habitual diet and recorded seizures systematically in a seizure diary. In the intervention period, the participants ate the modified ketogenic diet (see description of the diet below) and continued to recorded seizures systematically. All other epilepsy treatments were kept unchanged during the 24-week study period.

2.2. Diet intervention

The dietary intervention is previously described in detail (25). Briefly, the diet contained a maximum of 16 g carbohydrate per day (excluding fibre). The participants were encouraged to eat high-fat foods to replace the carbohydrate in the diet, while protein was eaten *ad libitum*. The total energy content was not restricted. The diet was supplemented with one multivitamin- and mineral tablet (Nycoplus multi, Takeda, Asker, Norway) and 800 mg calcium (Calcium carbonate, Takeda, Asker, Norway). A daily fluid intake of 2-3 L was recommended. The nutritional content of the meals were calculated using the Norwegian Food Composition Database (28). The macro nutrient intake during the study has previously been presented and shows that the participants, in accordance with the study protocol, switched from carbohydrate to fat as the main energy source (23).

2.3. Assessment of compliance with the diet intervention

The dietary intake was assessed using 3 days' weighed food records before the participants started on the diet, and after 4 and 12 weeks of dietary treatment. Urine ketones (acetoacetate) were measured twice daily at home (Ketostix, Bayer Healthcare, Leverkusen). Blood ketones (β-hydroxybutyrate) and blood glucose were measured morning and evening at hospital admissions prior to diet initiation, and after 4 and 12 weeks of dietary treatment (FreeStyle Precision Neo, FreeStyle Precision Xtra Blood β-Ketone Test Strips, and Freestyle Precision Blood Glucose Test Strips, Abbott, UK). The data has previously been reported and supports a high compliance with the diet intervention (13, 25, 26).

2.4. *ASM serum concentration analysis*

Venous blood samples were drawn food- and drug fasting in the morning at assumed steady-state of serum concentrations of ASMs at baseline, and after 4 and 12 weeks of dietary treatment. The analyses of all ASMs were based on routine measurements by validated methods at the Section for Clinical Pharmacology, Oslo University Hospital (Oslo, Norway).

Carbamazepine, phenobarbital, phenytoin, topiramate, and valproic acid were measured by a validated immunoassay on a COBAS C111 instrument (Roche Diagnostics, Oslo, Norway). The remaining ASMs were analysed by high-pressure liquid chromatography with ultraviolet detection (HPLC-UV). Lamotrigine was analysed by an AgilentA 1200 instrument (Oslo, Norway), with a 250 x 3.0 mm 4 µm Synergy 4u Hydro-RP 80A C18 column. Clonazepam, clobazam, desmethylclobazam, levetiracetam, lacosamide, zonisamide, pregabaline, and oxcarbazepine/eslicarbazepine acetate were measured on a Dionex Ulitimate 3000 instrument with the following columns: Clonazepam, clobazam, and desmethylclobazam was analysed with a 4.6 x 30 mm 3.5 µm ZORBAX Eclipse Plus C18 column. Levetiracetam analyses were performed with a Varian Omnisphere 250 x 3 mm 5 µm C18 column. For lacosamide, a 125 x 3 mm 3 µm Hypersil BDS C-18 column was used. Zonisamide, pregabalin and oxcarbazepine/eslicarbazepine acetate was analysed with a 250 x 3 mm 5µm Intersil

C-18 column. For the determination of oxcarbazepine and eslicarbazepine, we applied a non-stereospecific method for the racemic monohydroxy-derivative licarbazepine.

Analyses of acetozolamide, nitrazepam, retigabine, rufinamide, tiagabine, and vigabatrin were either not available or were not routinely analysed at the time the data collection of the study was performed.

2.5. Selection of candidate genes

We performed a non-systematic literature search and reviewed relevant literature to identify genes encoding drug-metabolizing enzymes involved in the metabolism of ASMs. We identified 14 genes (*CES1*, *CYP1A2, CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2E1*, *CYP3A4*, *CYP3A5*, *UGT1A3*, *UGT1A4*, *UGT2B6*, and *UGT2B7*) with a central role in the metabolism of ASMs (16, 17, 29-31). All, except one of the genes (*UGT2B6*), were available in the EPIC array used for DNA methylation analyses, resulting in a total of 13 genes in our analyses.

2.6. Selection of genes and ASMs for correlation analyses

An overview of the procedure of selecting genes and ASMs used in the correlation analyses is given in **Figure 1**. Included genes had to be an intermediate (>30-40%) or major (>50%) route of elimination of the ASM of interest (16, 17, 29-31) and available for DNA methylation analysis in EPIC array. Next, we included ASMs with reported change in serum concentration following treatment with the modified ketogenic diet were included (13). For pairs of DNA methylation and serum concentrations with less than six measurements, the sample size was considered too small to allow for meaningful analysis. Analysis was only performed on the 4-week time point due to the observation that most changes in DNA methylation occurred during these first weeks of dietary treatment (23), and because we expected the pharmacokinetic alterations to occur within a short time.

2.7. DNA methylation analysis

The procedures for blood sampling and DNA methylation analysis have been reported previously (23). Briefly, DNA methylation in white blood cells was analysed using the Illumina Infinium MethylationEPIC array, which investigates DNA methylation at >850 000 positions (CpGs). The arrays were analysed by the LIFE & BRAIN laboratory according to the manufacturer's instructions, and subsequent data analyses performed in R (http://www.r- project.org/). The data included in this candidate gene study is based on analysis of DNA methylation at 131 CpGs annotated to 13 genes involved in metabolism of ASMs based on UCSC composite gene track using the R package IlluminaHumanMethylationEPICanno.ilm10b3.hg19 (32).

2.8. Statistical analysis

The differential DNA methylation analyses were performed on the M values (log2 of the β-values). Identification of differentially methylated positions associated with ketogenic diet after 4 and 12 weeks within patients was done by fitting a linear model regression implemented in limma (33), and across the diet intervention using an ANOVA like method.

Spearman's rank correlation coefficient, rho, was used to calculated correlations between absolute difference in DNA methylation (β-values) per CpG and percentage change in serum concentrations of the individual ASMs after 4 weeks of dietary treatment. To adjust for multiple comparisons, a false discovery rate (FDR) with a significance threshold of less than 5% was applied by using the method of Benjamini and Hochberg (34). Statistical package for the social sciences (SPSS, v.28, IBM) and R (v. 4.1.3) was used for the statistical analysis.

3. Results

3.1. Baseline characteristics

Baseline demographics and clinical characteristics of the participants are presented in **Table 1**. The data comprise 58 adults (34 women) aged 16 – 65 years, of which 49 had focal and nine had

generalized epilepsy (**Figure 2**). The participants had a long history of epilepsy with a median age at first seizure of 7 (interquartile range 2 – 6) years, and a high number of previously tried ASMs (mean 8.7 ±SD 4.1). One-third of the participants had intellectual disability (*n* = 21). In addition, the majority were occupationally disabled (*n* = 37), underscoring the severity of the disease and associated comorbidities.

3.2. *ASMs used during the study*

All participants, except one, used ASMs. Sixteen participants used one ASM, 20 participants used two ASMs, 19 participants used three ASMs, and two participants used four ASMs. Thus, 71% (*n* = 41) used polytherapy. Eighteen different types of ASMs were in use during the study. The most frequently used ASMs were levetiracetam (*n* = 15), oxcarbazepine (*n* = 15), and valproic acid (*n* = 15), followed by lamotrigine (*n* = 14), clobazam (*n* = 10), and topiramate (*n* = 10). None of the participants changed the type or dose of the ASMs during the 24-weeks study period. An overview of the ASMs used during the study, their main route of elimination, and the percentage change in serum concentration after 4 weeks of dietary treatment is given in **Table 2**.

3.3. Differential DNA methylation

In this candidate gene study, we investigated DNA methylation of 131 CpGs annotated to 13 genes (*CES1*, *CYP1A2, CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2E1*, *CYP3A4*, *CYP3A5*, *UGT1A3*, *UGT1A4*, *UGT2B6*, and *UGT2B7*) encoding drug-metabolizing enzymes involved in the metabolism of ASMs (16, 17, 29-31).

First, an analysis of variance (ANOVA) of DNA methylation per CpG was performed across all time points (**Table S1**). An ANOVA identified differential DNA methylation at one CpG (cg08077617) annotated to *CES1* (*p* value < 0.001) after the dietary treatment. This is the same position as previously identified in our genome-wide analysis published recently (23). Next, we examined changes in DNA methylation after 4 (**Table S2**) and 12 weeks (**Table S3**) of dietary treatment compared to baseline. As expected, these analyses identified differential DNA methylation at one

CpG (cg08077617) annotated to *CES1* after both 4 and 12 weeks of dietary treatment (*p* value < 0.001 and *p* value = 0.002, respectively). The number of CpGs with an increase or decrease in DNA methylation after 4 weeks of dietary treatment in relation to gene- and CpG island context is given in **Figure 3**. Overall, the proportion of CpGs with an increase or decrease in DNA methylation following the dietary treatment was approximately equal.

3.4. Correlation between change in DNA methylation and change in serum concentration of ASMs

Next, to investigate the potential interplay and regulatory role of DNA methylation on drug-metabolizing enzymes, we estimated the correlation (Spearman's) between changes in DNA methylation and change in serum concentrations of ASMs after 4 weeks of dietary treatment. Six genes (*CYP2C19*, *CYP3A4*/*3A5*, *UGT1A3*, *UGT1A4*, and *UGT2B7*) and six ASMs (carbamazepine, clobazam/desmethylclobazam, lacosamide, lamotrigine, topiramate, and valproic acid) were included in the analyses, resulting in a total of 112 comparisons. These analyses revealed a moderate to strong correlation between absolute difference in DNA methylation (β-values) and percentage change in the serum concentrations of four ASMs (carbamazepine, lacosamide, lamotrigine, topiramate) at ten CpGs annotated to four genes (*CYP3A4*, *CYP2C19*, *UGT1A3*, and *UGT1A4*). However, after correction for multiple comparisons (FDR <5%) only one CpG (cg01478198) annotated to *UGT1A4* remained statistically significant (*p* value = 0.049) (**Figure 4**). At this position we found a strong, negative correlation between absolute differences in DNA methylation and percentage changes in lamotrigine serum concentration, rho = -0.85. Patients with the highest increase in DNA methylation had a greater reduction in the serum concentration of lamotrigine compared to patients with a decrease in DNA methylation after 4 weeks of dietary treatment. In general, for the CpGs displaying a moderate to high correlation of changes in DNA methylation and change in ASM serum concentrations there was a consistent linear relationship for the most of patients. However, for the majority of positions, we were not able to identify any relationship between change in DNA methylation and change in serum concentrations of ASMs. The correlation coefficient, rho, and corresponding unadjusted and adjusted *p* values of all correlation analyses can be found in the supporting information (**Table S4**).

4. Discussion

To our knowledge, this is the first candidate gene study investigating the impact of the modified ketogenic diet on DNA methylation of genes involved in ASM metabolism in humans. After the diet intervention, we identified a significant change in DNA methylation of *CES1*. In addition, our correlation analyses revealed a strong negative correlation between change in DNA methylation of *UGT1A4* and percentage change in lamotrigine serum concentration.

DNA methylation is a central mechanism for regulating gene expression. In an earlier publication, we reported a genome-wide decrease in DNA methylation in epilepsy patients treated with a modified ketogenic diet (23). Moreover, in the same study population, we also observed a marked decrease in the serum concentration of several ASMs (13). Interestingly, DNA methylation has previously been linked to the regulation of genes encoding drug-metabolizing enzymes (24) and preclinical studies have demonstrated the ability of high-fat diets to alter the expression of a variety of hepatic drug-metabolizing enzymes (14, 15, 35).

In the present study, *CES1*, encoding the drug-metabolizing enzyme carboxylesterase 1 (CES1), was the only differentially methylated gene following diet intervention. The gene is expressed in most tissues and is particularly abundant in the liver. The enzyme plays an important role in the metabolism of a wide range of drugs, pesticides, and endogenous compounds, including lipids (36). Although important in the metabolism of numerous drugs, the only ASM extensively metabolized by CES1 is rufinamide (37). Unfortunately, the possible relationship between change in DNA methylation of *CES1* and change in rufinamide serum concentration could not be investigated in the present study due to the low number of patients treated with rufinamide (n = 2) and lack of serum concentration measurements.

Our second main finding was a strong correlation between change in DNA methylation and change in the serum concentration of lamotrigine at one position annotated to *UGT1A4*. Lamotrigine is a commonly used ASM in epilepsy treatment and is mainly metabolized by the enzyme UGT1A4 (29).

UGT1A4 catalyses the covalent linkage of glucuronic acid to lamotrigine to convert it to the major inactive metabolite 2-N-glucuronide conjugate, before final renal elimination of lamotrigine. *UGT1A4* has previously been shown to be induced by endogenous substances such as estrogen and similarly estrogen-containing oral contraceptives (38-40), thus metabolic changes in response to the dietary treatment may influence the gene expression of *UGT1A4*. Moreover, DNA methylation has been shown to be an important mechanism for regulating the gene expression of UGT1A1, an enzyme of the same family, in the liver (41).

Admittedly, at the majority of positions investigated, we were not able to identify significant changes in DNA methylation or correlation between changes in DNA methylation and changes in serum concentrations of ASMs. There may be several reasons for this lack of significant findings. First, DNA methylation patterns are organ-, tissue- and even cell specific. We investigated DNA methylation in blood, although the major organ of drug metabolism is the liver. Currently, there is limited knowledge about how well DNA methylation patterns in blood reflect hepatic DNA methylation. Yet, we were able to identify DNA methylation changes of *CES1*, a gene considered to be negligibly expressed in blood (36), suggesting that DNA methylation changes in other relevant tissues may be reflected and captured in blood. Second, the sample size of the study was small, especially upon division into subgroups of ASMs. Third, most ASMs are metabolized by several enzymes. Hence, if one enzyme is responsible for only 30-40% of the metabolism of a drug (intermediate route of elimination), the impact of a significant change in DNA methylation on the gene's expression could be difficult to detect. Lamotrigine is one of few ASMs mainly metabolised by only one enzyme, the UGT1A4, which may explain why we were able to identify the correlation between changes in lamotrigine serum concentration and DNA methylation of *UGT1A4*. Fourth, the majority of the study participants used polypharmacy. ASMs are a heterogeneous group of drugs known for their extensive propensity for drug interactions. The high number of different combinations of ASMs, as well as variability in co-medications in addition to ASMs, may have influenced our results. Finally, although DNA methylation is an important gene regulation mechanism, we have not assessed other

mechanisms involved in regulating gene expression, such as histone modifications and non-coding RNAs. Moreover, we did not include genes encoding drug transporters and nuclear receptors known to regulate the activity of drug-metabolizing enzymes.

This study also had several strengths, including the prospective study design with intra-individual comparisons of DNA methylation before and after the diet intervention. This study design reduces the likelihood of potential confounding effects of inter-individual differences in genetics and environmental exposures. We have reliable measures of diet adherence including measurements of ketosis and dietary intake based on weighed food records, indicating a good compliance with the diet intervention. Furthermore, all epilepsy treatments besides the diet intervention were kept unchanged by all participants during the entire 24-week study period.

As a final note, although we have focused on the impact of a high-fat diet on DNA methylation of genes encoding enzymes involved in the metabolism of ASMs in epilepsy patients, high-fat diets are also used for treatment of other conditions (42-44). Since these enzymes are involved in the metabolism of numerous commonly prescribed drugs, the results might have implications beyond the epilepsy population.

5. Conclusions

In the present candidate gene study, we identified a significant change in DNA methylation of *CES1*, as well as a strong correlation between change in DNA methylation of *UGT1A4* and lamotrigine serum concentration. Altered DNA methylation of genes involved in the metabolism of ASMs may thus contribute to the observed decrease in serum concentrations of ASMs following treatment with ketogenic diets. These findings warrant larger studies, including a control group, to elucidate the role of DNA methylation as a mediating mechanism in the complex interaction of diet and drug metabolism.

Author contributions

All authors contributed to the study conception and design. Data collection were performed by MK and KON. Analyses of DNA methylation were performed by KG, all other data analyses were performed by SP. CJL contributed with pharmacological evaluations of drug-metabolizing enzymes and analytical procedures. The first draft of the manuscript was written by SP and all authors provided critical feedback and commented on previous versions of the manuscript. All authors read and contributed to the final manuscript.

Supporting information

Table S1 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) across all time points *(n* = 46)

Table S2 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) comparing values at baseline and 4 weeks of dietary treatment (*n* = 56)

Table S3 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) comparing values at baseline and 12 weeks of dietary treatment (*n* = 49)

Table S4 Spearman's correlation between change in DNA methylation and change in ASM serum concentration after 4 weeks of dietary treatment

Table 1 Baseline demographics and clinical characteristics (n = 58).

		Min – max
Gender, n (%)		
Male	24 (41.4%)	
Female	34 (58.6%)	
Age, years, mean (±SD)	36.5 (±11.8)	16 – 65
Epilepsy classification, n (%)		
Focal	49 (84.5%)	
Generalized	9 (15.5%)	
Age at first seizure, median (quartiles)	7 (2 – 16)	0 – 55
Years with epilepsy, mean (±SD)	25.0 (±11.9)	7.0 – 58·0
Intellectual disability, n (%)	21 (36.2%)	
Occupational disability, n (%)	37 (64%)	
Total number of ASMs tried, mean (±SD)	8.7 (±4.1)	3 – 23
ASMs at diet initiation, mean (±SD)	2.1 (±0.9)	
ASMs at diet initiation, n (%)		0 – 4
0	1 (1.7%)	
1	16 (27.6%)	
2	20 (34.5%)	
3	19 (32.8%)	
4	2 (3.4%)	

ASM = anti-seizure medication; SD = standard deviation; VNS = vagus nerve stimulator
Data are presented as mean (± SD) or median (quartiles) and min – max for continues variables, and frequency (%) for discrete variables.

Table 2 An overview of ASMs used during the study, their intermediate to major route of elimination, and percentage change in serum concentration after 4 weeks of dietary treatment

			4 weeks of dietary treatment		
ASM	***n*[a]**	**Intermediate or major route of elimination[b]**	**Percentage change in serum concentration Median (IQR)**	**Min – max**	***n*[c]**
Carbamazepine	8	CYP3A4	-11.0 (18.6)	-31.4 – 5.7	8
Clobazam[d]	10	CYP3A4	-38.5 (21.3)	-64.7 – -11.5	10
Desmethylclobazam[d]	10	CYP2C19	-28.3 (24.7)	-45.3 – -4.6	10
Clonazepam	2	CYP3A4	-0.2 (-)	-4.4 – 4.0	2
Lacosamide	8	CYP2C19	-7.7 (25.0)	-30.8 – 0.0	6
Lamotrigine	14	UGT1A4	-9.1 (19.6)	-38.7 – 1.8	13
Levetiracetame	15	Type B esterase, non-CYP dependent	0.0 (31.5)	-45.2 – 40.2	13
Licarbazepine[e]	17	Arylketone reductase, UGTs	0.0 (17.9)	-33.7 – 34.1	17
Phenobarbital	2	CYP2C9	-15.1 (-)	-23.6 – -6.7	2
Phenytoin	4	CYP2C9	-29.8 (-)	-35.0 – -6.1	3
Topiramate	10	CYP3A4	-21.2 (33.5)	-57.0 – 21.0	10
Valproic acid	15	UGT1A3, UGT2B7	-20.7 (14.3)	-35.2 – 11.6	15
Zonisamide	8	CYP3A4	-24.0 (25.7)	-33.9 – 0.0	5

ASM = anti-seizure medication; IQR = interquartile range. Data are presented as median with interquartile range, and minimum and maximum values. Serum concentration data of pregabalin (*n* = 2), retigabine (*n* =1), rufinamide (*n* = 2), tiagabine (*n* = 1), and vigabatrin (*n* = 1) were not available and thus not included.

[a]Number of participants who used the ASM.

[b]Based on references (16, 17, 29-31).

[c]Number of serum concentration measurements available. The discrepancies between the number of ASMs used and the number of serum concentrations measurements are due to missing blood samples.

[d]Clobazam and desmethylclobazam are both pharmacologically active metabolites of the same drug.

[e]Oxcarbazepine and eslikarbazepine analysed as the pharmacologically active metabolite licarbazepine

Figure 1 Overview of procedure of selecting genes and ASMs used in the Spearman's correlation analyses

Fourteen genes were identified as central in the metabolism of ASMs (16, 17, 29-31). In order to be included in the analyses, the genes had to be available in Illumina EPIC array used to analyse DNA methylation and defined as an intermediate or major route of elimination of the ASM of interest (16, 17, 29-31). Eighteen different ASMs were in use during the present study. Only ASMs with reported change in serum concentration following dietary treatment were included (13). For pairs of DNA methylation and serum concentrations with less than six measurements, the sample size was considered too small to allow for meaningful analysis. Number of CpGs annotated to each of the genes included: *CYP3A4* (n = 8), *CYP3A5* (n = 3), *CYP2C19* (n = 7), *UGT1A3* (n = 29), *UGT1A4* (n = 32), and *UGT2B7* (n = 4).

Abbreviation: ASM, anti-seizure medication

[a]*CES1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP3A4, CYP3A5, UGT1A3, UGT1A4, UGT2B6, and UGT2B7*

[b]Carbamazepine, clobazam, clonazepam, eslicarbazepine, lacosamide, lamotrigine, levetiracetame, oxcarbazepine, phenobarbital, phenytoin, pregabalin, retigabine, rufinamide, tiagabine, topiramate, valproic acid, vigabatrine, and zonisamide

[c]Clobazam and desmethylclobazam are both pharmacologically active metabolites of the same drug.

[d]Correlation analysis of DNA methylation at individual positions (CpGs) and individual ASMs, in total 112 analyses.

Figure 2 Study profile

[a]Two participants did not give permission for analysis of the samples abroad, 2 participants excluded from analysis due to poor diet compliance and change in medication, and 11 had missing blood samples or blood samples not analysed because of lack of sample for DNA methylation analysis at 4 and 12 weeks of dietary treatment.

[b]Two participants did not give permission for analysis of the samples abroad, 2 participants excluded from analysis due to poor diet compliance and change in medication, and 6 had missing blood samples.

[c]Two participants did not give permission for analysis of the samples abroad, 4 had missing blood samples.

Figure 3 Direction of DNA methylation change of all CpGs analysed (*n* = 131). A) Number of CpGs with loss or gain of DNA methylation after 4 weeks of dietary treatment and their respective locations with regard to gene context. B) Number of CpGs with loss or gain of DNA methylation after 4 weeks of dietary treatment and their locations with regard to CpG context.

Figure 4 A strong correlation between changes in DNA methylation of CpG annotated to *UGT1A4* and changes in lamotrigine serum concentrations (*n* = 13) The scatterplot shows a strong, negative, linear relationship between absolute differences in DNA methylation (β-values) at the CpG cg01478198 annotated to *UGT1A4* and percentage change in serum concentration of lamotrigine after 4 weeks of dietary treatment (ρ = -0.85). Each dot represents the pairwise data of one study participant. Data are Spearman's correlation coefficient, rho (ρ), and corresponding unadjusted and adjusted *p* value (FDR <5%).

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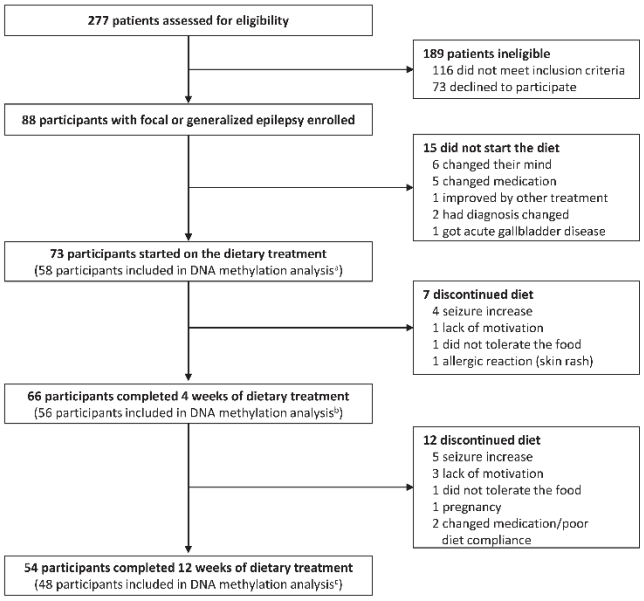
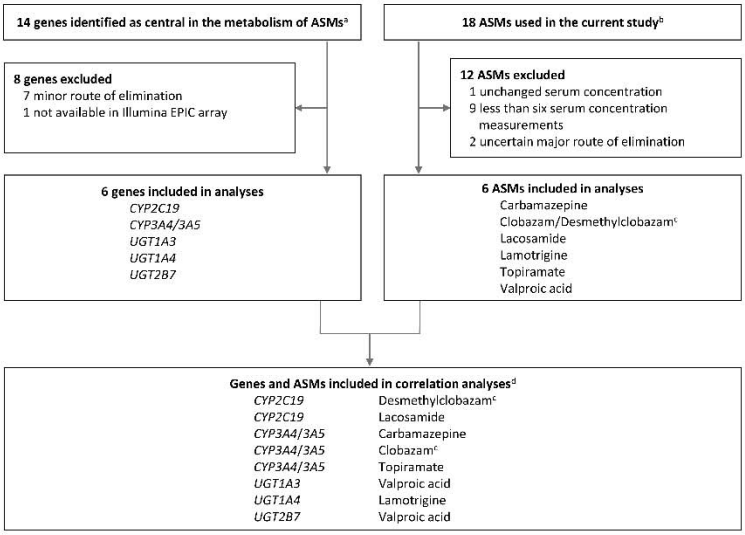
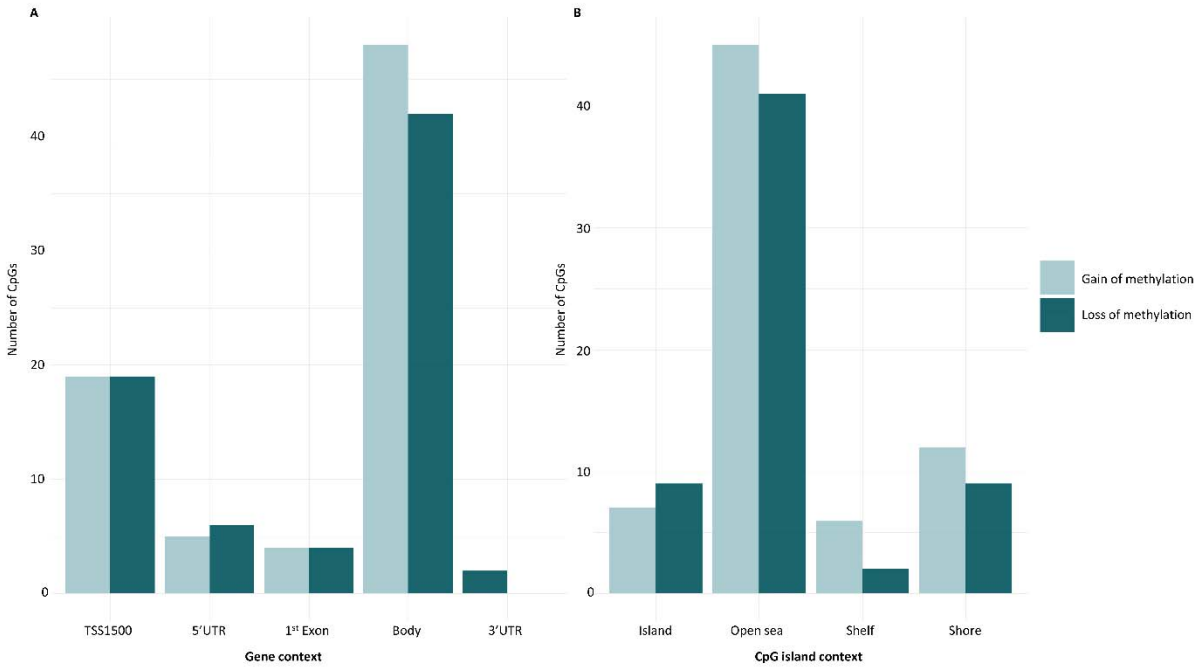

277 patients assessed for eligibility
189 patients ineligible
116 did not meet inclusion criteria
73 declined to participate
88 participants with focal or generalized epilepsy enrolled
15 did not start the diet
6 changed their mind
5 changed medication
1 improved by other treatment
2 had diagnosis changed
1 got acute gallbladder disease
73 participants started on the dietary treatment
(58 participants included in DNA methylation analysis[a])
7 discontinued diet
4 seizure increase
1 lack of motivation
1 did not tolerate the food
1 allergic reaction (skin rash)
66 participants completed 4 weeks of dietary treatment
(56 participants included in DNA methylation analysis[b])
12 discontinued diet
5 seizure increase
3 lack of motivation
1 did not tolerate the food
1 pregnancy
2 changed medication/poor diet compliance
54 participants completed 12 weeks of dietary treatment
(48 participants included in DNA methylation analysis[c])


14 genes identified as central in the metabolism of ASMs[a]
18 ASMs used in the current study[b]
8 genes excluded
7 minor route of elimination
1 not available in Illumina EPIC array
12 ASMs excluded
1 unchanged serum concentration
9 less than six serum concentration measurements
2 uncertain major route of elimination
6 genes included in analyses
CYP2C19
CYP3A4/3A5
UGT1A3
UGT1A4
UGT2B7
6 ASMs included in analyses
Carbamazepine
Clobazam/Desmethylclobazam[c]
Lacosamide
Lamotrigine
Topiramate
Valproic acid
Genes and ASMs included in correlation analyses[d]
CYP2C19 Desmethylclobazam[c]
CYP2C19 Lacosamide
CYP3A4/3A5 Carbamazepine
CYP3A4/3A5 Clobazam[c]
CYP3A4/3A5 Topiramate
UGT1A3 Valproic acid
UGT1A4 Lamotrigine
UGT2B7 Valproic acid


A
B
Number of CpGs
0
10
20
30
40
TSS1500
5'UTR
1st Exon
Body
3'UTR
Gene context
Island
Open sea
Shelf
Shore
CpG island context
Gain of methylation
Loss of methylation

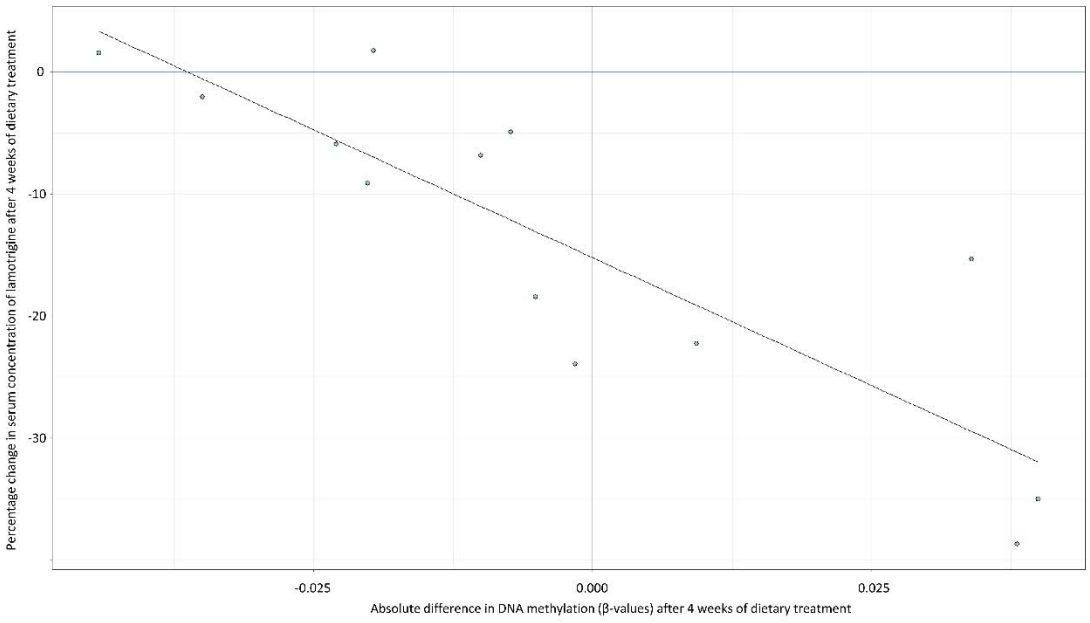

Percentage change in serum concentration of lamotrigine after 4 weeks of dietary treatment
0
-10
-20
-30
-0.025
0.000
0.025
Absolute difference in DNA methylation (β-values) after 4 weeks of dietary treatment

Table S1 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) across all time points (n = 46)

						Mean beta			Δ beta				
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-Value[a]**	**FDR**
cg02301920	CES1	16	55866890	Body	Island	0,649	0,644	0,652	-0,005	0,003	0,009	0,745	0,937
cg03744383	CES1	16	55867072	1stExon;5'UTR	S_Shore	0,380	0,376	0,386	-0,004	0,006	0,010	0,549	0,937
cg03880033	CES1	16	55866757	Body	Island	0,667	0,653	0,659	-0,013	-0,008	0,005	0,298	0,937
cg03993839	CES1	16	55851398	Body	OpenSea	0,788	0,787	0,778	0,000	-0,010	-0,009	0,153	0,937
cg04521981	CES1	16	55850985	Body	OpenSea	0,864	0,854	0,860	-0,010	-0,004	0,006	0,039	0,822
cg06549901	CES1	16	55851316	Body	OpenSea	0,655	0,641	0,631	-0,014	-0,024	-0,010	0,027	0,719
cg08077617	CES1	16	55868196	TSS1500	S_Shore	0,634	0,602	0,603	-0,031	-0,031	0,000	<0,001	**<0,001**
cg09863930	CES1	16	55847064	Body	OpenSea	0,626	0,637	0,638	0,011	0,012	0,001	0,394	0,937
cg09955858	CES1	16	55862851	Body	N_Shelf	0,834	0,831	0,833	-0,003	-0,001	0,002	0,612	0,937
cg13627659	CES1	16	55845971	Body	OpenSea	0,599	0,612	0,610	0,013	0,011	-0,001	0,498	0,937
cg16744664	CES1	16	55863633	Body	N_Shelf	0,872	0,876	0,874	0,004	0,002	-0,002	0,226	0,937
cg18247128	CES1	16	55847762	Body	OpenSea	0,851	0,848	0,853	-0,003	0,001	0,004	0,438	0,937
cg23196985	CES1	16	55866997	1stExon;5'UTR	Island	0,491	0,482	0,483	-0,008	-0,008	0,000	0,757	0,937
cg24155826	CES1	16	55859188	Body	OpenSea	0,619	0,618	0,614	-0,001	-0,005	-0,004	0,519	0,937
cg26880539	CES1	16	55862478	Body	OpenSea	0,665	0,668	0,670	0,003	0,005	0,002	0,464	0,937
cg01359532	CYP1A2	15	75045126	Body	OpenSea	0,687	0,688	0,683	0,001	-0,003	-0,004	0,871	0,951
cg03353233	CYP1A2	15	75042673	Body	OpenSea	0,764	0,765	0,763	0,001	-0,001	-0,002	0,985	0,993
cg04968473	CYP1A2	15	75040734	TSS1500	OpenSea	0,781	0,782	0,774	0,001	-0,007	-0,008	0,293	0,937
cg05849935	CYP1A2	15	75039788	TSS1500	OpenSea	0,889	0,893	0,892	0,004	0,003	-0,001	0,656	0,937
cg07330330	CYP1A2	15	75043256	Body	OpenSea	0,783	0,789	0,789	0,005	0,005	0,000	0,401	0,937
cg09207718	CYP1A2	15	75041386	5'UTR	OpenSea	0,851	0,855	0,852	0,004	0,001	-0,002	0,152	0,937
cg11473616	CYP1A2	15	75041150	TSS200	OpenSea	0,891	0,899	0,893	0,008	0,002	-0,006	0,321	0,937
cg14503537	CYP1A2	15	75044351	Body	OpenSea	0,799	0,800	0,801	0,001	0,002	0,001	0,798	0,937
cg18256630	CYP1A2	15	75040318	TSS1500	OpenSea	0,795	0,794	0,797	0,000	0,002	0,002	0,972	0,987
cg23022457	CYP1A2	15	75040503	TSS1500	OpenSea	0,796	0,789	0,797	-0,006	0,001	0,008	0,242	0,937
cg24182584	CYP1A2	15	75047385	Body	OpenSea	0,952	0,953	0,953	0,002	0,001	0,000	0,484	0,937
cg25235648	CYP1A2	15	75040320	TSS1500	OpenSea	0,837	0,838	0,842	0,002	0,006	0,004	0,907	0,951
cg04653029	CYP2A6	19	41356919	TSS1500	S_Shelf	0,819	0,820	0,821	0,001	0,002	0,001	0,194	0,937
cg01124941	CYP2B6	19	41496076	TSS1500	OpenSea	0,847	0,853	0,850	0,006	0,004	-0,002	0,787	0,937
cg02073501	CYP2B6	19	41508852	Body	OpenSea	0,871	0,868	0,870	-0,003	-0,001	0,002	0,604	0,937
cg04039479	CYP2B6	19	41514693	Body	OpenSea	0,705	0,704	0,706	-0,001	0,001	0,002	0,893	0,951
cg07151194	CYP2B6	19	41507455	Body	OpenSea	0,925	0,926	0,928	0,001	0,003	0,002	0,311	0,937
cg07291917	CYP2B6	19	41495868	TSS1500	OpenSea	0,832	0,836	0,836	0,004	0,004	0,000	0,628	0,937
cg08852641	CYP2B6	19	41510098	Body	OpenSea	0,631	0,636	0,629	0,006	-0,001	-0,007	0,390	0,937
cg09286079	CYP2B6	19	41510059	Body	OpenSea	0,911	0,908	0,911	-0,002	0,001	0,003	0,352	0,937
cg10322876	CYP2B6	19	41496749	TSS1500	OpenSea	0,865	0,865	0,863	-0,001	-0,003	-0,002	0,456	0,937
cg11539883	CYP2B6	19	41496043	TSS1500	OpenSea	0,826	0,830	0,830	0,004	0,004	0,000	0,904	0,951
cg18115248	CYP2B6	19	41495942	TSS1500	OpenSea	0,880	0,880	0,882	0,000	0,002	0,002	0,651	0,937

Table S1 (continued)

						Mean beta			Δ beta				
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-Value[a]**	**FDR**
cg24426371	CYP2B6	19	41510243	Body	OpenSea	0,879	0,880	0,882	0,001	0,003	0,002	0,759	0,937
cg25028129	CYP2B6	19	41509736	Body	OpenSea	0,457	0,460	0,455	0,002	-0,003	-0,005	0,795	0,937
cg00051662	CYP2C19	10	96521086	TSS1500	OpenSea	0,849	0,840	0,847	-0,009	-0,002	0,007	0,547	0,937
cg02808805	CYP2C19	10	96521820	TSS1500	OpenSea	0,824	0,823	0,833	-0,002	0,009	0,010	0,063	0,822
cg04189838	CYP2C19	10	96523347	Body	OpenSea	0,863	0,862	0,864	-0,001	0,000	0,002	0,283	0,937
cg05991489	CYP2C19	10	96521680	TSS1500	OpenSea	0,881	0,885	0,883	0,005	0,002	-0,002	0,427	0,937
cg20031717	CYP2C19	10	96523248	Body	OpenSea	0,691	0,696	0,698	0,005	0,007	0,002	0,784	0,937
cg24857560	CYP2C19	10	96521152	TSS1500	OpenSea	0,725	0,718	0,727	-0,007	0,002	0,009	0,813	0,937
cg27505447	CYP2C19	10	96522264	TSS200	OpenSea	0,757	0,765	0,757	0,007	0,000	-0,007	0,283	0,937
cg00782158	CYP2C8	10	96830284	TSS1500	OpenSea	0,924	0,922	0,926	-0,001	0,002	0,004	0,494	0,937
cg04164578	CYP2C8	10	96798765	Body	OpenSea	0,852	0,850	0,850	-0,001	-0,001	0,000	0,780	0,937
cg12759420	CYP2C8	10	96798865	Body	OpenSea	0,901	0,900	0,904	-0,001	0,002	0,004	0,764	0,937
cg24828322	CYP2C8	10	96830111	TSS1500	OpenSea	0,868	0,878	0,876	0,010	0,008	-0,002	0,288	0,937
cg09550024	CYP2C9	10	96698366	TSS200	OpenSea	0,843	0,843	0,843	0,000	0,000	0,000	0,820	0,937
cg12253859	CYP2C9	10	96748928	3'UTR	OpenSea	0,791	0,789	0,794	-0,003	0,003	0,005	0,569	0,937
cg14191040	CYP2C9	10	96745681	Body	OpenSea	0,778	0,778	0,785	0,000	0,007	0,007	0,432	0,937
cg23202385	CYP2C9	10	96723929	Body	OpenSea	0,105	0,107	0,106	0,002	0,002	0,000	0,746	0,937
cg24140586	CYP2C9	10	96697436	TSS1500	OpenSea	0,868	0,871	0,873	0,003	0,005	0,002	0,602	0,937
cg00321709	CYP2E1	10	135341933	Body	Island	0,218	0,205	0,211	-0,013	-0,007	0,006	0,321	0,937
cg00436603	CYP2E1	10	135340740	TSS200	N_Shore	0,769	0,771	0,772	0,002	0,003	0,001	0,557	0,937
cg00720244	CYP2E1	10	135347434	Body	OpenSea	0,874	0,867	0,872	-0,006	-0,002	0,004	0,661	0,937
cg01355198	CYP2E1	10	135347330	Body	OpenSea	0,895	0,896	0,898	0,002	0,003	0,001	0,328	0,937
cg01465364	CYP2E1	10	135340721	TSS200	N_Shore	0,795	0,795	0,799	0,000	0,004	0,004	0,252	0,937
cg03134882	CYP2E1	10	135341463	Body	Island	0,235	0,231	0,240	-0,003	0,006	0,009	0,156	0,937
cg05194426	CYP2E1	10	135343193	Body	S_Shore	0,308	0,315	0,326	0,007	0,018	0,012	0,115	0,937
cg05417377	CYP2E1	10	135350807	Body	OpenSea	0,706	0,704	0,710	-0,002	0,005	0,007	0,312	0,937
cg05473257	CYP2E1	10	135341443	Body	Island	0,195	0,192	0,190	-0,003	-0,004	-0,001	0,894	0,951
cg07381788	CYP2E1	10	135340445	TSS1500	N_Shore	0,874	0,880	0,870	0,006	-0,004	-0,010	0,071	0,827
cg08472147	CYP2E1	10	135340583	TSS1500	N_Shore	0,616	0,615	0,611	-0,001	-0,005	-0,004	0,591	0,937
cg09208540	CYP2E1	10	135340467	TSS1500	N_Shore	0,870	0,867	0,870	-0,003	0,000	0,003	0,971	0,987
cg10862468	CYP2E1	10	135342218	Body	Island	0,232	0,231	0,233	-0,001	0,001	0,001	0,501	0,937
cg10986462	CYP2E1	10	135340539	TSS1500	N_Shore	0,868	0,871	0,857	0,003	-0,011	-0,014	0,136	0,937
cg11445109	CYP2E1	10	135343248	Body	S_Shore	0,157	0,157	0,158	0,000	0,000	0,001	0,823	0,937
cg13315147	CYP2E1	10	135341528	Body	Island	0,258	0,257	0,267	-0,001	0,009	0,009	0,106	0,937
cg14250048	CYP2E1	10	135340785	TSS200	N_Shore	0,843	0,841	0,837	-0,002	-0,006	-0,004	0,705	0,937
cg16538390	CYP2E1	10	135344917	Body	S_Shelf	0,802	0,806	0,801	0,004	-0,001	-0,005	0,715	0,937
cg18984983	CYP2E1	10	135342936	Body	S_Shore	0,137	0,137	0,140	0,001	0,003	0,003	0,804	0,937
cg19469447	CYP2E1	10	135341870	Body	Island	0,147	0,151	0,150	0,004	0,003	-0,001	0,633	0,937
cg19571004	CYP2E1	10	135340850	TSS200	N_Shore	0,829	0,828	0,828	-0,001	-0,001	0,000	0,886	0,951

Table S1 (continued)

						Mean beta			Δ beta				
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-Value[a]**	**FDR**
cg19721068	CYP2E1	10	135346592	Body	OpenSea	0,832	0,838	0,841	0,005	0,009	0,003	0,060	0,822
cg19837601	CYP2E1	10	135340871	5'UTR;1stExon	N_Shore	0,716	0,716	0,716	0,000	-0,001	0,000	0,927	0,963
cg21024264	CYP2E1	10	135341025	1stExon	N_Shore	0,821	0,821	0,818	0,000	-0,003	-0,003	0,695	0,937
cg23400446	CYP2E1	10	135342560	Body	Island	0,164	0,157	0,159	-0,007	-0,004	0,002	0,573	0,937
cg24530264	CYP2E1	10	135342620	Body	S_Shore	0,129	0,130	0,129	0,001	0,000	0,000	0,799	0,937
cg25330361	CYP2E1	10	135342413	Body	Island	0,137	0,130	0,139	-0,008	0,002	0,009	0,299	0,937
cg26065573	CYP2E1	10	135339469	TSS1500	N_Shore	0,858	0,859	0,861	0,001	0,004	0,002	0,772	0,937
cg01526453	CYP3A4	7	99381523	Body	OpenSea	0,878	0,885	0,878	0,008	0,001	-0,007	0,062	0,822
cg04358264	CYP3A4	7	99355204	3'UTR	OpenSea	0,826	0,819	0,828	-0,008	0,002	0,010	0,482	0,937
cg09914773	CYP3A4	7	99383224	TSS1500	OpenSea	0,925	0,921	0,926	-0,003	0,002	0,005	0,378	0,937
cg14770351	CYP3A4	7	99376495	Body	OpenSea	0,839	0,843	0,843	0,004	0,004	0,001	0,642	0,937
cg20572918	CYP3A4	7	99383250	TSS1500	OpenSea	0,939	0,937	0,938	-0,002	-0,001	0,002	0,783	0,937
cg22821554	CYP3A4	7	99383155	TSS1500	OpenSea	0,852	0,849	0,848	-0,003	-0,004	-0,001	0,626	0,937
cg23326197	CYP3A4	7	99382370	TSS1500	OpenSea	0,731	0,735	0,736	0,004	0,004	0,000	0,633	0,937
cg24014584	CYP3A4	7	99360886	Body;Body	OpenSea	0,868	0,878	0,874	0,010	0,006	-0,004	0,013	0,580
cg01243068	CYP3A5	7	99274390	5'UTR;Body	OpenSea	0,814	0,812	0,815	-0,002	0,001	0,003	0,589	0,937
cg05867406	CYP3A5	7	99275340	5'UTR;Body	OpenSea	0,920	0,920	0,919	0,000	-0,001	-0,001	0,854	0,951
cg14260643	CYP3A5	7	99277394	Body	OpenSea	0,564	0,559	0,559	-0,006	-0,005	0,000	0,329	0,937
cg17872990	UGT1A3;UGT1A4	2	234637301	TSS1500;Body	OpenSea	0,818	0,818	0,821	0,000	0,003	0,003	0,758	0,937
cg00764099	UGT1A3;UGT1A4	2	234650765	Body;Body	N_Shore	0,748	0,747	0,751	-0,001	0,003	0,005	0,713	0,937
cg00963071	UGT1A3;UGT1A4	2	234662401	Body;Body	N_Shore	0,910	0,913	0,917	0,002	0,006	0,004	0,116	0,937
cg01478198	UGT1A3;UGT1A4	2	234670284	Body;Body	OpenSea	0,883	0,884	0,882	0,001	-0,002	-0,003	0,755	0,937
cg01764553	UGT1A3;UGT1A4	2	234673101	Body;Body	OpenSea	0,744	0,744	0,766	0,000	0,022	0,022	0,021	0,690
cg02227331	UGT1A3;UGT1A4	2	234663937	Body;Body	Island	0,787	0,786	0,784	-0,001	-0,003	-0,002	0,870	0,951
cg02789126	UGT1A3;UGT1A4	2	234667777	Body;Body	S_Shelf	0,903	0,902	0,904	-0,001	0,000	0,002	0,952	0,982
cg03607648	UGT1A3;UGT1A4	2	234668930	Body;Body	OpenSea	0,908	0,910	0,908	0,002	-0,001	-0,002	0,568	0,937
cg04314609	UGT1A3;UGT1A4	2	234652466	Body;Body	Island	0,934	0,931	0,931	-0,003	-0,003	0,000	0,609	0,937
cg05542967	UGT1A3;UGT1A4	2	234667988	Body;Body	S_Shelf	0,938	0,939	0,936	0,001	-0,002	-0,002	0,518	0,937
cg06566087	UGT1A3;UGT1A4	2	234655709	Body;Body	S_Shelf	0,833	0,825	0,830	-0,008	-0,004	0,005	0,765	0,937
cg06772514	UGT1A3;UGT1A4	2	234668349	Body;Body	OpenSea	0,903	0,899	0,904	-0,004	0,001	0,005	0,412	0,937
cg07823755	UGT1A3;UGT1A4	2	234668355	Body;Body	OpenSea	0,897	0,896	0,896	0,000	-0,001	0,000	0,904	0,951
cg08697797	UGT1A3;UGT1A4	2	234668834	Body;Body	OpenSea	0,851	0,852	0,849	0,001	-0,003	-0,003	0,710	0,937
cg09339841	UGT1A3;UGT1A4	2	234649204	Body;Body	N_Shelf	0,895	0,899	0,897	0,004	0,002	-0,002	0,367	0,937
cg09505117	UGT1A3;UGT1A4	2	234637306	TSS1500;Body	OpenSea	0,903	0,903	0,905	0,000	0,002	0,002	0,514	0,937
cg11272349	UGT1A3;UGT1A4	2	234637253	TSS1500;Body	OpenSea	0,829	0,830	0,829	0,001	0,001	0,000	0,997	0,997
cg11811840	UGT1A3;UGT1A4	2	234669166	Body;Body	OpenSea	0,867	0,867	0,865	0,000	-0,002	-0,002	0,725	0,937
cg12177266	UGT1A3;UGT1A4	2	234653082	Body;Body	S_Shore	0,890	0,885	0,900	-0,005	0,010	0,015	0,001	0,074
cg14323797	UGT1A3;UGT1A4	2	234662894	Body;Body	N_Shore	0,829	0,813	0,834	-0,016	0,004	0,021	0,121	0,937
cg18109081	UGT1A3;UGT1A4	2	234665615	Body;Body	S_Shore	0,795	0,793	0,797	-0,002	0,002	0,004	0,268	0,937

Table S1 (continued)

						Mean beta			Δ beta				
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**12W**	**4W-B**	**12W-B**	**12W-4W**	**P-Value[a]**	**FDR**
cg19435015	UGT1A3;UGT1A4	2	234672508	Body;Body	OpenSea	0,896	0,896	0,893	-0,001	-0,003	-0,002	0,669	0,937
cg21620495	UGT1A3;UGT1A4	2	234670519	Body;Body	OpenSea	0,921	0,919	0,923	-0,002	0,002	0,004	0,271	0,937
cg22145666	UGT1A3;UGT1A4	2	234663900	Body;Body	Island	0,801	0,795	0,804	-0,006	0,003	0,009	0,312	0,937
cg23229568	UGT1A3;UGT1A4	2	234663729	Body;Body	Island	0,928	0,929	0,927	0,001	-0,001	-0,002	0,459	0,937
cg23714547	UGT1A3;UGT1A4	2	234668865	Body;Body	OpenSea	0,911	0,913	0,925	0,002	0,014	0,012	0,049	0,822
cg24335340	UGT1A3;UGT1A4	2	234636389	TSS1500;Body	OpenSea	0,845	0,843	0,846	-0,002	0,001	0,003	0,584	0,937
cg25726425	UGT1A3;UGT1A4	2	234637574	TSS200;Body	OpenSea	0,864	0,866	0,860	0,002	-0,004	-0,006	0,683	0,937
cg27361577	UGT1A3;UGT1A4	2	234662759	Body;Body	N_Shore	0,720	0,720	0,718	0,000	-0,003	-0,003	0,819	0,937
cg02234120	UGT1A4	2	234626351	TSS1500	OpenSea	0,805	0,805	0,810	0,000	0,005	0,004	0,470	0,937
cg08964597	UGT1A4	2	234627295	TSS200	OpenSea	0,733	0,737	0,743	0,004	0,010	0,007	0,199	0,937
cg22147092	UGT1A4	2	234627744	1stExon	OpenSea	0,921	0,923	0,918	0,002	-0,002	-0,004	0,195	0,937
cg02363364	UGT2B7	4	69968620	Body	OpenSea	0,778	0,783	0,782	0,005	0,004	-0,001	0,890	0,951
cg04558553	UGT2B7	4	69960869	TSS1500	OpenSea	0,862	0,861	0,855	-0,002	-0,008	-0,006	0,076	0,827
cg14688178	UGT2B7	4	69976406	Body	OpenSea	0,732	0,733	0,740	0,001	0,008	0,007	0,535	0,937
cg15935333	UGT2B7	4	69964573	Body	OpenSea	0,868	0,876	0,873	0,008	0,006	-0,002	0,521	0,937

Abbreviations: 4W, 4 weeks of dietary treatment; 12W, 12 weeks of dietary treatment, B, Baseline; Chr, Chromosome; FDR, False discovery rate; FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele (β = M/(M + U + 100)). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

[a]Comparing DNA methylation values across all time points using an analysis of variance (ANOVA)

Table S2 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) comparing values at baseline and 4 weeks of dietary treatment (n = 56)

						Mean beta		Δ beta		
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**4W**	**4W-B**	**P-Value[a]**	**FDR**
cg02301920	CES1	16	55866890	Body	Island	0.658	0.660	0.002	0.529	0.998
cg03744383	CES1	16	55867072	1stExon;5'UTR	S_Shore	0.387	0.382	-0.004	0.666	0.999
cg03880033	CES1	16	55866757	Body	Island	0.668	0.658	-0.010	0.130	0.998
cg03993839	CES1	16	55851398	Body	OpenSea	0.788	0.787	0.000	0.861	0.999
cg04521981	CES1	16	55850985	Body	OpenSea	0.866	0.855	-0.011	0.012	0.505
cg06549901	CES1	16	55851316	Body	OpenSea	0.655	0.643	-0.012	0.099	0.998
cg08077617	CES1	16	55868196	TSS1500	S_Shore	0.639	0.602	-0.037	<0.001	**<0.001**
cg09863930	CES1	16	55847064	Body	OpenSea	0.651	0.656	0.005	0.435	0.998
cg09955858	CES1	16	55862851	Body	N_Shelf	0.834	0.831	-0.003	0.335	0.998
cg13627659	CES1	16	55845971	Body	OpenSea	0.601	0.609	0.008	0.295	0.998
cg16744664	CES1	16	55863633	Body	N_Shelf	0.868	0.874	0.006	0.091	0.998
cg18247128	CES1	16	55847762	Body	OpenSea	0.851	0.849	-0.002	0.387	0.998
cg23196985	CES1	16	55866997	1stExon;5'UTR	Island	0.489	0.486	-0.003	0.756	0.999
cg24155826	CES1	16	55859188	Body	OpenSea	0.620	0.618	-0.002	0.853	0.999
cg26880539	CES1	16	55862478	Body	OpenSea	0.663	0.668	0.005	0.372	0.998
cg01359532	CYP1A2	15	75045126	Body	OpenSea	0.686	0.690	0.004	0.881	0.999
cg03353233	CYP1A2	15	75042673	Body	OpenSea	0.763	0.762	-0.002	0.973	0.999
cg04968473	CYP1A2	15	75040734	TSS1500	OpenSea	0.781	0.781	0.000	0.963	0.999
cg05849935	CYP1A2	15	75039788	TSS1500	OpenSea	0.891	0.893	0.003	0.361	0.998
cg07330330	CYP1A2	15	75043256	Body	OpenSea	0.785	0.788	0.003	0.255	0.998
cg09207718	CYP1A2	15	75041386	5'UTR	OpenSea	0.850	0.856	0.005	0.055	0.998
cg11473616	CYP1A2	15	75041150	TSS200	OpenSea	0.892	0.897	0.004	0.166	0.998
cg14503537	CYP1A2	15	75044351	Body	OpenSea	0.798	0.798	0.000	0.999	0.999
cg18256630	CYP1A2	15	75040318	TSS1500	OpenSea	0.796	0.798	0.001	0.812	0.999
cg23022457	CYP1A2	15	75040503	TSS1500	OpenSea	0.798	0.791	-0.007	0.164	0.998
cg24182584	CYP1A2	15	75047385	Body	OpenSea	0.951	0.953	0.002	0.268	0.998
cg25235648	CYP1A2	15	75040320	TSS1500	OpenSea	0.837	0.838	0.000	0.858	0.999
cg04653029	CYP2A6	19	41356919	TSS1500	S_Shelf	0.818	0.823	0.005	0.088	0.998
cg01124941	CYP2B6	19	41496076	TSS1500	OpenSea	0.849	0.851	0.002	0.491	0.998
cg02073501	CYP2B6	19	41508852	Body	OpenSea	0.872	0.869	-0.002	0.324	0.998
cg04039479	CYP2B6	19	41514693	Body	OpenSea	0.705	0.702	-0.003	0.636	0.999
cg07151194	CYP2B6	19	41507455	Body	OpenSea	0.926	0.926	0.000	0.952	0.999
cg07291917	CYP2B6	19	41495868	TSS1500	OpenSea	0.831	0.834	0.003	0.422	0.998
cg08852641	CYP2B6	19	41510098	Body	OpenSea	0.631	0.636	0.005	0.267	0.998
cg09286079	CYP2B6	19	41510059	Body	OpenSea	0.911	0.909	-0.002	0.324	0.998

Table S2 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	4W	4W-B		
cg10322876	CYP2B6	19	41496749	TSS1500	OpenSea	0.866	0.864	-0.002	0.511	0.998
cg11539883	CYP2B6	19	41496043	TSS1500	OpenSea	0.827	0.828	0.001	0.792	0.999
cg18115248	CYP2B6	19	41495942	TSS1500	OpenSea	0.878	0.880	0.002	0.559	0.999
cg24426371	CYP2B6	19	41510243	Body	OpenSea	0.880	0.881	0.000	0.867	0.999
cg25028129	CYP2B6	19	41509736	Body	OpenSea	0.464	0.464	0.000	0.995	0.999
cg00051662	CYP2C19	10	96521086	TSS1500	OpenSea	0.848	0.842	-0.006	0.319	0.998
cg02808805	CYP2C19	10	96521820	TSS1500	OpenSea	0.827	0.823	-0.004	0.459	0.998
cg04189838	CYP2C19	10	96523347	Body	OpenSea	0.866	0.860	-0.006	0.116	0.998
cg05991489	CYP2C19	10	96521680	TSS1500	OpenSea	0.882	0.886	0.004	0.199	0.998
cg20031717	CYP2C19	10	96523248	Body	OpenSea	0.693	0.693	0.000	0.987	0.999
cg24857560	CYP2C19	10	96521152	TSS1500	OpenSea	0.723	0.722	0.000	0.955	0.999
cg27505447	CYP2C19	10	96522264	TSS200	OpenSea	0.752	0.767	0.014	0.116	0.998
cg00782158	CYP2C8	10	96830284	TSS1500	OpenSea	0.923	0.922	-0.002	0.444	0.998
cg04164578	CYP2C8	10	96798765	Body;	OpenSea	0.852	0.849	-0.003	0.486	0.998
cg12759420	CYP2C8	10	96798865	Body	OpenSea	0.900	0.900	0.000	0.929	0.999
cg24828322	CYP2C8	10	96830111	TSS1500	OpenSea	0.869	0.878	0.009	0.207	0.998
cg09550024	CYP2C9	10	96698366	TSS200	OpenSea	0.841	0.841	0.000	0.849	0.999
cg12253859	CYP2C9	10	96748928	3'UTR	OpenSea	0.791	0.788	-0.003	0.549	0.998
cg14191040	CYP2C9	10	96745681	Body	OpenSea	0.778	0.779	0.001	0.901	0.999
cg23202385	CYP2C9	10	96723929	Body	OpenSea	0.103	0.106	0.003	0.484	0.998
cg24140586	CYP2C9	10	96697436	TSS1500	OpenSea	0.869	0.870	0.001	0.737	0.999
cg00321709	CYP2E1	10	135341933	Body	Island	0.207	0.200	-0.007	0.146	0.998
cg00436603	CYP2E1	10	135340740	TSS200	N_Shore	0.769	0.772	0.003	0.388	0.998
cg00720244	CYP2E1	10	135347434	Body	OpenSea	0.872	0.868	-0.004	0.374	0.998
cg01355198	CYP2E1	10	135347330	Body	OpenSea	0.895	0.898	0.003	0.223	0.998
cg01465364	CYP2E1	10	135340721	TSS200	N_Shore	0.791	0.795	0.004	0.317	0.998
cg03134882	CYP2E1	10	135341463	Body	Island	0.225	0.227	0.002	0.860	0.999
cg05194426	CYP2E1	10	135343193	Body	S_Shore	0.301	0.306	0.005	0.319	0.998
cg05417377	CYP2E1	10	135350807	Body	OpenSea	0.707	0.705	-0.002	0.953	0.999
cg05473257	CYP2E1	10	135341443	Body	Island	0.185	0.186	0.001	0.835	0.999
cg07381788	CYP2E1	10	135340445	TSS1500	N_Shore	0.876	0.880	0.004	0.120	0.998
cg08472147	CYP2E1	10	135340583	TSS1500	N_Shore	0.612	0.613	0.001	0.884	0.999
cg09208540	CYP2E1	10	135340467	TSS1500	N_Shore	0.870	0.869	-0.002	0.811	0.999
cg10862468	CYP2E1	10	135342218	Body	Island	0.218	0.222	0.004	0.280	0.998
cg10986462	CYP2E1	10	135340539	TSS1500	N_Shore	0.868	0.870	0.001	0.759	0.999
cg11445109	CYP2E1	10	135343248	Body	S_Shore	0.146	0.149	0.003	0.539	0.998
cg13315147	CYP2E1	10	135341528	Body	Island	0.251	0.255	0.004	0.541	0.998
cg14250048	CYP2E1	10	135340785	TSS200	N_Shore	0.835	0.838	0.003	0.837	0.999
cg16538390	CYP2E1	10	135344917	Body	S_Shelf	0.801	0.803	0.003	0.864	0.999

Table S2 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	4W	4W-B		
cg18984983	CYP2E1	10	135342936	Body	S_Shore	0.129	0.129	0.000	0.781	0.999
cg19469447	CYP2E1	10	135341870	Body	Island	0.139	0.142	0.003	0.629	0.999
cg19571004	CYP2E1	10	135340850	TSS200	N_Shore	0.826	0.825	-0.001	0.624	0.999
cg19721068	CYP2E1	10	135346592	Body	OpenSea	0.833	0.836	0.003	0.220	0.998
cg19837601	CYP2E1	10	135340871	5'UTR;1stExon	N_Shore	0.711	0.713	0.002	0.725	0.999
cg21024264	CYP2E1	10	135341025	1stExon	N_Shore	0.816	0.822	0.006	0.596	0.999
cg23400446	CYP2E1	10	135342560	Body	Island	0.155	0.148	-0.007	0.293	0.998
cg24530264	CYP2E1	10	135342620	Body	S_Shore	0.122	0.123	0.001	0.523	0.998
cg25330361	CYP2E1	10	135342413	Body	Island	0.126	0.124	-0.003	0.961	0.999
cg26065573	CYP2E1	10	135339469	TSS1500	N_Shore	0.859	0.857	-0.002	0.830	0.999
cg01526453	CYP3A4	7	99381523	Body	OpenSea	0.878	0.884	0.006	0.036	0.998
cg04358264	CYP3A4	7	99355204	3'UTR	OpenSea	0.824	0.818	-0.006	0.425	0.998
cg09914773	CYP3A4	7	99383224	TSS1500	OpenSea	0.924	0.922	-0.002	0.355	0.998
cg14770351	CYP3A4	7	99376495	Body	OpenSea	0.839	0.844	0.005	0.384	0.998
cg20572918	CYP3A4	7	99383250	TSS1500	OpenSea	0.938	0.937	-0.002	0.490	0.998
cg22821554	CYP3A4	7	99383155	TSS1500	OpenSea	0.851	0.850	-0.001	0.665	0.999
cg23326197	CYP3A4	7	99382370	TSS1500	OpenSea	0.735	0.737	0.003	0.469	0.998
cg24014584	CYP3A4	7	99360886	Body	OpenSea	0.869	0.878	0.009	0.003	0.223
cg01243068	CYP3A5	7	99274390	5'UTR;Body	OpenSea	0.815	0.812	-0.003	0.465	0.998
cg05867406	CYP3A5	7	99275340	5'UTR;Body	OpenSea	0.919	0.920	0.001	0.666	0.999
cg14260643	CYP3A5	7	99277394	Body	OpenSea	0.568	0.559	-0.009	0.155	0.998
cg00764099	UGT1A3;UGT1A4	2	234669166	Body;Body	N_Shore	0.746	0.746	0.000	0.958	0.999
cg00963071	UGT1A3;UGT1A4	2	234652466	Body;Body	N_Shore	0.911	0.912	0.002	0.692	0.999
cg01478198	UGT1A3;UGT1A4	2	234626351	Body;Body	OpenSea	0.885	0.884	-0.001	0.915	0.999
cg01764553	UGT1A3;UGT1A4	2	234627295	Body;Body	OpenSea	0.741	0.747	0.006	0.470	0.998
cg02227331	UGT1A3;UGT1A4	2	234670519	Body;Body	Island	0.791	0.789	-0.002	0.653	0.999
cg02789126	UGT1A3;UGT1A4	2	234673101	Body;Body	S_Shelf	0.903	0.903	0.000	0.967	0.999
cg03607648	UGT1A3;UGT1A4	2	234670284	Body;Body	OpenSea	0.908	0.910	0.002	0.377	0.998
cg04314609	UGT1A3;UGT1A4	2	234667777	Body;Body	Island	0.935	0.932	-0.003	0.382	0.998
cg05542967	UGT1A3;UGT1A4	2	234672508	Body;Body	S_Shelf	0.938	0.938	0.000	0.981	0.999
cg06566087	UGT1A3;UGT1A4	2	234668834	Body;Body	S_Shelf	0.830	0.827	-0.003	0.530	0.998
cg06772514	UGT1A3;UGT1A4	2	234668349	Body;Body	OpenSea	0.903	0.901	-0.003	0.246	0.998
cg07823755	UGT1A3;UGT1A4	2	234668865	Body;Body	OpenSea	0.897	0.896	-0.001	0.680	0.999
cg08697797	UGT1A3;UGT1A4	2	234668355	Body;Body	OpenSea	0.849	0.850	0.001	0.687	0.999
cg09339841	UGT1A3;UGT1A4	2	234650765	Body;Body	N_Shelf	0.896	0.900	0.004	0.159	0.998
cg09505117	UGT1A3;UGT1A4	2	234663900	TSS1500;Body	OpenSea	0.905	0.903	-0.002	0.376	0.998
cg11272349	UGT1A3;UGT1A4	2	234663729	TSS1500;Body	OpenSea	0.829	0.828	-0.001	0.964	0.999
cg11811840	UGT1A3;UGT1A4	2	234668930	Body;Body	OpenSea	0.865	0.866	0.000	0.691	0.999
cg12177266	UGT1A3;UGT1A4	2	234637301	Body;Body	S_Shore	0.889	0.887	-0.002	0.598	0.999

Table S2 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	4W	4W-B		
cg14323797	UGT1A3;UGT1A4	2	234662894	Body;Body	N_Shore	0.825	0.813	-0.012	0.149	0.998
cg17872990	UGT1A3;UGT1A4	2	234637306	Body;TSS1500	OpenSea	0.820	0.821	0.000	0.975	0.999
cg18109081	UGT1A3;UGT1A4	2	234665615	Body;Body	S_Shore	0.798	0.793	-0.005	0.174	0.998
cg19435015	UGT1A3;UGT1A4	2	234627744	Body;Body	OpenSea	0.894	0.897	0.002	0.467	0.998
cg21620495	UGT1A3;UGT1A4	2	234667988	Body;Body	OpenSea	0.921	0.919	-0.003	0.353	0.998
cg22145666	UGT1A3;UGT1A4	2	234662401	Body;Body	Island	0.801	0.796	-0.005	0.356	0.998
cg23229568	UGT1A3;UGT1A4	2	234663937	Body;Body	Island	0.929	0.930	0.000	0.574	0.999
cg23714547	UGT1A3;UGT1A4	2	234653082	Body;Body	OpenSea	0.913	0.915	0.001	0.491	0.998
cg24335340	UGT1A3;UGT1A4	2	234662759	TSS1500;Body	OpenSea	0.846	0.843	-0.003	0.499	0.998
cg25726425	UGT1A3;UGT1A4	2	234649204	TSS200;Body	OpenSea	0.860	0.864	0.003	0.591	0.999
cg27361577	UGT1A3;UGT1A4	2	234655709	Body;Body	N_Shore	0.720	0.721	0.001	0.935	0.999
cg02234120	UGT1A4	2	234637574	TSS1500	OpenSea	0.806	0.803	-0.002	0.720	0.999
cg08964597	UGT1A4	2	234637253	TSS200	OpenSea	0.735	0.735	0.000	0.662	0.999
cg22147092	UGT1A4	2	234636389	1stExon	OpenSea	0.920	0.922	0.003	0.210	0.998
cg02363364	UGT2B7	4	69968620	Body	OpenSea	0.780	0.781	0.002	0.781	0.999
cg04558553	UGT2B7	4	69960869	TSS1500	OpenSea	0.858	0.859	0.001	0.878	0.999
cg14688178	UGT2B7	4	69976406	Body	OpenSea	0.735	0.736	0.001	0.994	0.999
cg15935333	UGT2B7	4	69964573	Body	OpenSea	0.867	0.872	0.005	0.400	0.998

Abbreviations: 4W, 4 weeks of dietary treatment; B, Baseline; Chr, Chromosome; FDR, False discovery rate; FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele (β = M/(M + U + 100)). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

[a] Comparing values at baseline and 4 weeks of dietary treatment using paired *t* test

Table S3 DNA methylation of CpGs annotated to genes involved in the metabolism of anti-seizure medications (sorted by gene and cgID) comparing values at baseline and 12 weeks of dietary treatment (n = 49)

						Mean beta		Δ beta		
cgID	**Gene**	**Chr**	**Position**	**Gene context**	**CpG context**	**B**	**12W**	**12W-B**	**P-Value[a]**	**FDR**
cg02301920	CES1	16	55866890	Body	Island	0.658	0.659	0.001	0.500	0.902
cg03744383	CES1	16	55867072	1stExon;5'UTR	S_Shore	0.387	0.387	0.001	0.485	0.902
cg03880033	CES1	16	55866757	Body	Island	0.668	0.660	-0.008	0.319	0.902
cg03993839	CES1	16	55851398	Body	OpenSea	0.788	0.778	-0.010	0.073	0.864
cg04521981	CES1	16	55850985	Body	OpenSea	0.866	0.861	-0.005	0.349	0.902
cg06549901	CES1	16	55851316	Body	OpenSea	0.655	0.631	-0.023	0.008	0.278
cg08077617	CES1	16	55868196	TSS1500	S_Shore	0.639	0.605	-0.034	<0.001	**0.002**
cg09863930	CES1	16	55847064	Body	OpenSea	0.651	0.640	-0.012	0.177	0.902
cg09955858	CES1	16	55862851	Body	N_Shelf	0.834	0.834	0.000	0.806	0.941
cg13627659	CES1	16	55845971	Body	OpenSea	0.601	0.609	0.008	0.337	0.902
cg16744664	CES1	16	55863633	Body	N_Shelf	0.868	0.873	0.005	0.622	0.902
cg18247128	CES1	16	55847762	Body	OpenSea	0.851	0.852	0.000	0.652	0.902
cg23196985	CES1	16	55866997	1stExon;5'UTR	Island	0.489	0.483	-0.005	0.457	0.902
cg24155826	CES1	16	55859188	Body	OpenSea	0.620	0.615	-0.005	0.276	0.902
cg26880539	CES1	16	55862478	Body	OpenSea	0.663	0.669	0.006	0.242	0.902
cg01359532	CYP1A2	15	75045126	Body	OpenSea	0.686	0.681	-0.006	0.705	0.907
cg03353233	CYP1A2	15	75042673	Body	OpenSea	0.763	0.764	0.001	0.893	0.955
cg04968473	CYP1A2	15	75040734	TSS1500	OpenSea	0.781	0.774	-0.007	0.158	0.902
cg05849935	CYP1A2	15	75039788	TSS1500	OpenSea	0.891	0.892	0.001	0.630	0.902
cg07330330	CYP1A2	15	75043256	Body	OpenSea	0.785	0.789	0.005	0.243	0.902
cg09207718	CYP1A2	15	75041386	5'UTR	OpenSea	0.850	0.853	0.003	0.276	0.902
cg11473616	CYP1A2	15	75041150	TSS200	OpenSea	0.892	0.893	0.000	0.915	0.959
cg14503537	CYP1A2	15	75044351	Body	OpenSea	0.798	0.801	0.003	0.552	0.902
cg18256630	CYP1A2	15	75040318	TSS1500	OpenSea	0.796	0.796	-0.001	0.932	0.969
cg23022457	CYP1A2	15	75040503	TSS1500	OpenSea	0.798	0.796	-0.001	0.837	0.941
cg24182584	CYP1A2	15	75047385	Body	OpenSea	0.951	0.953	0.002	0.357	0.902
cg25235648	CYP1A2	15	75040320	TSS1500	OpenSea	0.837	0.843	0.005	0.661	0.902
cg04653029	CYP2A6	19	41356919	TSS1500	S_Shelf	0.818	0.822	0.004	0.188	0.902
cg01124941	CYP2B6	19	41496076	TSS1500	OpenSea	0.849	0.850	0.002	0.734	0.907
cg02073501	CYP2B6	19	41508852	Body	OpenSea	0.872	0.869	-0.002	0.770	0.925
cg04039479	CYP2B6	19	41514693	Body	OpenSea	0.705	0.706	0.001	0.852	0.946
cg07151194	CYP2B6	19	41507455	Body	OpenSea	0.926	0.929	0.003	0.166	0.902
cg07291917	CYP2B6	19	41495868	TSS1500	OpenSea	0.831	0.836	0.005	0.402	0.902
cg08852641	CYP2B6	19	41510098	Body	OpenSea	0.631	0.629	-0.001	0.835	0.941
cg09286079	CYP2B6	19	41510059	Body	OpenSea	0.911	0.911	0.000	0.625	0.902

Table S3 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	12W	12W-B		
cg10322876	CYP2B6	19	41496749	TSS1500	OpenSea	0.866	0.862	-0.003	0.212	0.902
cg11539883	CYP2B6	19	41496043	TSS1500	OpenSea	0.827	0.831	0.004	0.839	0.941
cg18115248	CYP2B6	19	41495942	TSS1500	OpenSea	0.878	0.882	0.004	0.366	0.902
cg24426371	CYP2B6	19	41510243	Body	OpenSea	0.880	0.882	0.002	0.473	0.902
cg25028129	CYP2B6	19	41509736	Body	OpenSea	0.464	0.455	-0.009	0.550	0.902
cg00051662	CYP2C19	10	96521086	TSS1500	OpenSea	0.848	0.847	-0.001	0.964	0.987
cg02808805	CYP2C19	10	96521820	TSS1500	OpenSea	0.827	0.834	0.007	0.097	0.865
cg04189838	CYP2C19	10	96523347	Body	OpenSea	0.866	0.864	-0.002	0.588	0.902
cg05991489	CYP2C19	10	96521680	TSS1500	OpenSea	0.882	0.883	0.001	0.694	0.907
cg20031717	CYP2C19	10	96523248	Body	OpenSea	0.693	0.697	0.004	0.541	0.902
cg24857560	CYP2C19	10	96521152	TSS1500	OpenSea	0.723	0.729	0.006	0.552	0.902
cg27505447	CYP2C19	10	96522264	TSS200	OpenSea	0.752	0.756	0.003	0.588	0.902
cg00782158	CYP2C8	10	96830284	TSS1500	OpenSea	0.923	0.925	0.002	0.647	0.902
cg04164578	CYP2C8	10	96798765	Body	OpenSea	0.852	0.851	-0.001	0.689	0.907
cg12759420	CYP2C8	10	96798865	Body	OpenSea	0.900	0.904	0.004	0.491	0.902
cg24828322	CYP2C8	10	96830111	TSS1500	OpenSea	0.869	0.878	0.009	0.157	0.902
cg09550024	CYP2C9	10	96698366	TSS200	OpenSea	0.841	0.845	0.004	0.536	0.902
cg12253859	CYP2C9	10	96748928	3'UTR	OpenSea	0.791	0.793	0.003	0.613	0.902
cg14191040	CYP2C9	10	96745681	Body	OpenSea	0.778	0.783	0.005	0.230	0.902
cg23202385	CYP2C9	10	96723929	Body	OpenSea	0.103	0.106	0.003	0.554	0.902
cg24140586	CYP2C9	10	96697436	TSS1500	OpenSea	0.869	0.872	0.004	0.319	0.902
cg00321709	CYP2E1	10	135341933	Body	Island	0.207	0.208	0.001	0.314	0.902
cg00436603	CYP2E1	10	135340740	TSS200	N_Shore	0.769	0.773	0.003	0.331	0.902
cg00720244	CYP2E1	10	135347434	Body	OpenSea	0.872	0.872	0.000	0.810	0.941
cg01355198	CYP2E1	10	135347330	Body	OpenSea	0.895	0.898	0.003	0.186	0.902
cg01465364	CYP2E1	10	135340721	TSS200	N_Shore	0.791	0.799	0.008	0.102	0.865
cg03134882	CYP2E1	10	135341463	Body	Island	0.225	0.237	0.012	0.103	0.865
cg05194426	CYP2E1	10	135343193	Body	S_Shore	0.301	0.326	0.025	0.038	0.553
cg05417377	CYP2E1	10	135350807	Body	OpenSea	0.707	0.712	0.005	0.184	0.902
cg05473257	CYP2E1	10	135341443	Body	Island	0.185	0.188	0.002	0.637	0.902
cg07381788	CYP2E1	10	135340445	TSS1500	N_Shore	0.876	0.871	-0.005	0.416	0.902
cg08472147	CYP2E1	10	135340583	TSS1500	N_Shore	0.612	0.609	-0.003	0.333	0.902
cg09208540	CYP2E1	10	135340467	TSS1500	N_Shore	0.870	0.871	0.000	0.894	0.955
cg10862468	CYP2E1	10	135342218	Body	Island	0.218	0.230	0.012	0.368	0.902
cg10986462	CYP2E1	10	135340539	TSS1500	N_Shore	0.868	0.858	-0.011	0.106	0.865
cg11445109	CYP2E1	10	135343248	Body	S_Shore	0.146	0.154	0.008	0.708	0.907
cg13315147	CYP2E1	10	135341528	Body	Island	0.251	0.263	0.013	0.038	0.553
cg14250048	CYP2E1	10	135340785	TSS200	N_Shore	0.835	0.836	0.000	0.531	0.902
cg16538390	CYP2E1	10	135344917	Body	S_Shelf	0.801	0.799	-0.001	0.527	0.902

Table S3 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	12W	12W-B		
cg18984983	CYP2E1	10	135342936	Body	S_Shore	0.129	0.138	0.009	0.685	0.907
cg19469447	CYP2E1	10	135341870	Body	Island	0.139	0.147	0.009	0.341	0.902
cg19571004	CYP2E1	10	135340850	TSS200	N_Shore	0.826	0.827	0.002	0.841	0.941
cg19721068	CYP2E1	10	135346592	Body	OpenSea	0.833	0.842	0.009	0.018	0.399
cg19837601	CYP2E1	10	135340871	5'UTR;1stExon	N_Shore	0.711	0.717	0.006	0.990	0.992
cg21024264	CYP2E1	10	135341025	1stExon	N_Shore	0.816	0.816	0.000	0.726	0.907
cg23400446	CYP2E1	10	135342560	Body	Island	0.155	0.160	0.005	0.648	0.902
cg24530264	CYP2E1	10	135342620	Body	S_Shore	0.122	0.127	0.005	0.639	0.902
cg25330361	CYP2E1	10	135342413	Body	Island	0.126	0.137	0.011	0.160	0.902
cg26065573	CYP2E1	10	135339469	TSS1500	N_Shore	0.859	0.862	0.003	0.608	0.902
cg01526453	CYP3A4	7	99381523	Body	OpenSea	0.878	0.879	0.001	0.992	0.992
cg04358264	CYP3A4	7	99355204	3'UTR	OpenSea	0.824	0.829	0.005	0.657	0.902
cg09914773	CYP3A4	7	99383224	TSS1500	OpenSea	0.924	0.926	0.002	0.612	0.902
cg14770351	CYP3A4	7	99376495	Body	OpenSea	0.839	0.842	0.003	0.476	0.902
cg20572918	CYP3A4	7	99383250	TSS1500	OpenSea	0.938	0.939	0.000	0.816	0.941
cg22821554	CYP3A4	7	99383155	TSS1500	OpenSea	0.851	0.848	-0.003	0.334	0.902
cg23326197	CYP3A4	7	99382370	TSS1500	OpenSea	0.735	0.735	0.001	0.377	0.902
cg24014584	CYP3A4	7	99360886	Body	OpenSea	0.869	0.874	0.005	0.132	0.902
cg01243068	CYP3A5	7	99274390	5'UTR;Body	OpenSea	0.815	0.816	0.001	0.755	0.925
cg05867406	CYP3A5	7	99275340	5'UTR;Body	OpenSea	0.919	0.919	0.000	0.904	0.955
cg14260643	CYP3A5	7	99277394	Body	OpenSea	0.568	0.559	-0.009	0.298	0.902
cg00764099	UGT1A3;UGT1A4	2	234650765	Body;Body	N_Shore	0.746	0.751	0.005	0.480	0.902
cg00963071	UGT1A3;UGT1A4	2	234662401	Body;Body	N_Shore	0.911	0.916	0.005	0.046	0.608
cg01478198	UGT1A3;UGT1A4	2	234670284	Body;Body	OpenSea	0.885	0.881	-0.004	0.479	0.902
cg01764553	UGT1A3;UGT1A4	2	234673101	Body;Body	OpenSea	0.741	0.764	0.023	0.007	0.278
cg02227331	UGT1A3;UGT1A4	2	234663937	Body;Body	Island	0.791	0.786	-0.005	0.653	0.902
cg02789126	UGT1A3;UGT1A4	2	234667777	Body;Body	S_Shelf	0.903	0.904	0.001	0.768	0.925
cg03607648	UGT1A3;UGT1A4	2	234668930	Body;Body	OpenSea	0.908	0.907	-0.001	0.904	0.955
cg04314609	UGT1A3;UGT1A4	2	234652466	Body;Body	Island	0.935	0.931	-0.004	0.412	0.902
cg05542967	UGT1A3;UGT1A4	2	234667988	Body;Body	S_Shelf	0.938	0.936	-0.002	0.303	0.902
cg06566087	UGT1A3;UGT1A4	2	234655709	Body;Body	S_Shelf	0.830	0.828	-0.002	0.536	0.902
cg06772514	UGT1A3;UGT1A4	2	234668349	Body;Body	OpenSea	0.903	0.903	0.000	0.958	0.987
cg07823755	UGT1A3;UGT1A4	2	234668355	Body;Body	OpenSea	0.897	0.896	-0.001	0.730	0.907
cg08697797	UGT1A3;UGT1A4	2	234668834	Body;Body	OpenSea	0.849	0.849	0.000	0.648	0.902
cg09339841	UGT1A3;UGT1A4	2	234649204	Body;Body	N_Shelf	0.896	0.897	0.000	0.585	0.902
cg09505117	UGT1A3;UGT1A4	2	234637306	TSS1500;Body	OpenSea	0.905	0.905	0.000	0.801	0.941
cg11272349	UGT1A3;UGT1A4	2	234637253	TSS1500;Body	OpenSea	0.829	0.830	0.001	0.975	0.990
cg11811840	UGT1A3;UGT1A4	2	234669166	Body;Body	OpenSea	0.865	0.865	0.000	0.661	0.902
cg12177266	UGT1A3;UGT1A4	2	234653082	Body;Body	S_Shore	0.889	0.900	0.011	0.002	0.157

Table S3 (continued)

cgID	Gene	Chr	Position	Gene context	CpG context	Mean beta		Δ beta	P-Value[a]	FDR
						B	12W	12W-B		
cg14323797	UGT1A3;UGT1A4	2	234662894	Body;Body	N_Shore	0.825	0.834	0.009	0.511	0.902
cg17872990	UGT1A3;UGT1A4	2	234637301	Body;TSS1500	OpenSea	0.820	0.821	0.000	0.518	0.902
cg18109081	UGT1A3;UGT1A4	2	234665615	Body;Body	S_Shore	0.798	0.798	0.000	0.865	0.952
cg19435015	UGT1A3;UGT1A4	2	234672508	Body;Body	OpenSea	0.894	0.895	0.000	0.894	0.955
cg21620495	UGT1A3;UGT1A4	2	234670519	Body;Body	OpenSea	0.921	0.923	0.002	0.449	0.902
cg22145666	UGT1A3;UGT1A4	2	234663900	Body;Body	Island	0.801	0.805	0.004	0.507	0.902
cg23229568	UGT1A3;UGT1A4	2	234663729	Body;Body	Island	0.929	0.928	-0.002	0.462	0.902
cg23714547	UGT1A3;UGT1A4	2	234668865	Body;Body	OpenSea	0.913	0.925	0.012	0.016	0.399
cg24335340	UGT1A3;UGT1A4	2	234636389	TSS1500;Body	OpenSea	0.846	0.847	0.001	0.696	0.907
cg25726425	UGT1A3;UGT1A4	2	234637574	TSS200;Body	OpenSea	0.860	0.861	0.001	0.715	0.907
cg27361577	UGT1A3;UGT1A4	2	234662759	Body;Body	N_Shore	0.720	0.716	-0.004	0.553	0.902
cg02234120	UGT1A4	2	234626351	TSS1500	OpenSea	0.806	0.809	0.004	0.378	0.902
cg08964597	UGT1A4	2	234627295	TSS200	OpenSea	0.735	0.744	0.009	0.081	0.865
cg22147092	UGT1A4	2	234627744	1stExon	OpenSea	0.920	0.919	-0.001	0.552	0.902
cg02363364	UGT2B7	4	69968620	Body	OpenSea	0.780	0.782	0.002	0.634	0.902
cg04558553	UGT2B7	4	69960869	TSS1500	OpenSea	0.858	0.853	-0.005	0.037	0.553
cg14688178	UGT2B7	4	69976406	Body	OpenSea	0.735	0.739	0.004	0.319	0.902
cg15935333	UGT2B7	4	69964573	Body	OpenSea	0.867	0.874	0.007	0.286	0.902

Abbreviations: 12W, 12 weeks of dietary treatment; B, Baseline; Chr, Chromosome; FDR, False discovery rate; FDR <0.05. The beta value is defined as the ratio of methylated versus unmethylated allele (β = M/(M + U + 100)). The beta values are between 0 and 1 and can be interpreted as 0 being unmethylated and 1 being methylated.

[a] Comparing values at baseline and 12 weeks of dietary treatment using paired *t* test

Table S4 Spearman's correlation between change in DNA methylation and change in ASM serum concentration after 4 weeks of dietary treatment. Spearman's correlation coefficient, rho, were calculated to estimate the correlation between absolute difference in DNA methylation (beta values) at individual positions (CpGs) and percentage change in serum concentration of the individual ASMs. To correct for multiple comparisons, a false discovery rate of less than 5% was applied by using the method of Benjamini and Hochberg (1).

cgID	Gene	ASM	n	Rho	P-value	FDR
cg01526453	CYP3A4	Carbamazepine	8	0.36	0.389	0.842
cg04358264	CYP3A4	Carbamazepine	8	0.43	0.299	0.842
cg09914773	CYP3A4	Carbamazepine	8	-0.36	0.389	0.842
cg14770351	CYP3A4	Carbamazepine	8	0.05	0.935	0.988
cg20572918	CYP3A4	Carbamazepine	8	0.76	0.037	0.531
cg22821554	CYP3A4	Carbamazepine	8	0.26	0.536	0.976
cg23326197	CYP3A4	Carbamazepine	8	0.33	0.428	0.858
cg24014584	CYP3A4	Carbamazepine	8	0.74	0.046	0.531
cg01243068	CYP3A5	Carbamazepine	8	0.57	0.151	0.841
cg05867406	CYP3A5	Carbamazepine	8	0.02	0.977	1.000
cg14260643	CYP3A5	Carbamazepine	8	-0.40	0.327	0.842
cg01526453	CYP3A4	Clobazam	9	-0.25	0.521	0.939
cg04358264	CYP3A4	Clobazam	9	0.38	0.313	0.842
cg09914773	CYP3A4	Clobazam	9	0.05	0.912	0.988
cg14770351	CYP3A4	Clobazam	9	0.25	0.521	0.975
cg20572918	CYP3A4	Clobazam	9	0.38	0.313	0.842
cg22821554	CYP3A4	Clobazam	9	-0.55	0.133	0.783
cg23326197	CYP3A4	Clobazam	9	-0.12	0.776	0.986
cg24014584	CYP3A4	Clobazam	9	0.13	0.744	0.986
cg01243068	CYP3A5	Clobazam	9	-0.30	0.437	0.865
cg05867406	CYP3A5	Clobazam	9	0.00	1.000	1.000
cg14260643	CYP3A5	Clobazam	9	-0.38	0.313	0.842
cg00051662	CYP2C19	Desmethylclobazam	9	-0.37	0.336	0.842
cg02808805	CYP2C19	Desmethylclobazam	9	0.13	0.744	0.986
cg04189838	CYP2C19	Desmethylclobazam	9	-0.07	0.880	0.986
cg05991489	CYP2C19	Desmethylclobazam	9	-0.37	0.336	0.842
cg20031717	CYP2C19	Desmethylclobazam	9	0.13	0.744	0.986
cg24857560	CYP2C19	Desmethylclobazam	9	-0.45	0.230	0.842
cg27505447	CYP2C19	Desmethylclobazam	9	-0.08	0.843	0.986
cg00051662	CYP2C19	Lacosamide	6	-0.12	0.827	0.986
cg02808805	CYP2C19	Lacosamide	6	0.81	0.050	0.531
cg04189838	CYP2C19	Lacosamide	6	0.81	0.050	0.531
cg05991489	CYP2C19	Lacosamide	6	-0.14	0.784	0.986
cg20031717	CYP2C19	Lacosamide	6	0.00	1.000	1.000
cg24857560	CYP2C19	Lacosamide	6	0.58	0.228	0.842
cg27505447	CYP2C19	Lacosamide	6	-0.23	0.658	0.986
cg00764099	UGT1A4	Lamotrigine	13	0.10	0.751	0.986
cg00963071	UGT1A4	Lamotrigine	13	0.03	0.921	0.988
cg01478198	UGT1A4	Lamotrigine	13	-0.85	0.000	0.049
cg01764553	UGT1A4	Lamotrigine	13	0.12	0.696	0.986
cg02227331	UGT1A4	Lamotrigine	13	-0.53	0.068	0.580
cg02234120	UGT1A4	Lamotrigine	13	-0.84	0.255	0.842
cg02789126	UGT1A4	Lamotrigine	13	0.63	0.024	0.443
cg03607648	UGT1A4	Lamotrigine	13	-0.07	0.835	0.986
cg04314609	UGT1A4	Lamotrigine	13	0.64	0.022	0.443
cg05542967	UGT1A4	Lamotrigine	13	0.07	0.821	0.986
cg06566087	UGT1A4	Lamotrigine	13	0.05	0.878	0.986
cg06772514	UGT1A4	Lamotrigine	13	0.26	0.383	0.842
cg07823755	UGT1A4	Lamotrigine	13	-0.02	0.949	0.994
cg08697797	UGT1A4	Lamotrigine	13	0.18	0.506	0.926
cg08964597	UGT1A4	Lamotrigine	13	0.37	0.217	0.842
cg09339841	UGT1A4	Lamotrigine	13	-0.10	0.737	0.986
cg09505117	UGT1A4	Lamotrigine	13	0.23	0.448	0.906
cg11272349	UGT1A4	Lamotrigine	13	-0.28	0.353	0.842
cg11811840	UGT1A4	Lamotrigine	13	0.40	0.176	0.842
cg12177266	UGT1A4	Lamotrigine	13	0.35	0.247	0.842

Table S4 (continued)

cg14323797	UGT1A4	Lamotrigine	13	0.04	0.906	0.988
cg17872990	UGT1A4	Lamotrigine	13	-0.05	0.020	0.443
cg18109081	UGT1A4	Lamotrigine	13	-0.45	0.125	0.783
cg19435015	UGT1A4	Lamotrigine	13	0.21	0.493	0.926
cg21620495	UGT1A4	Lamotrigine	13	0.05	0.878	0.986
cg22145666	UGT1A4	Lamotrigine	13	-0.21	0.493	0.906
cg22147092	UGT1A4	Lamotrigine	13	0.49	0.093	0.743
cg23229568	UGT1A4	Lamotrigine	13	-0.13	0.669	0.986
cg23714547	UGT1A4	Lamotrigine	13	0.13	0.683	0.986
cg24335340	UGT1A4	Lamotrigine	13	-0.26	0.383	0.842
cg25726425	UGT1A4	Lamotrigine	13	0.17	0.579	0.976
cg27361577	UGT1A4	Lamotrigine	13	-0.41	0.163	0.841
cg01526453	CYP3A4	Topiramate	10	-0.12	0.759	0.986
cg04358264	CYP3A4	Topiramate	10	0.78	0.012	0.443
cg09914773	CYP3A4	Topiramate	10	-0.10	0.785	0.986
cg14770351	CYP3A4	Topiramate	10	0.07	0.865	0.986
cg20572918	CYP3A4	Topiramate	10	0.04	0.919	0.988
cg22821554	CYP3A4	Topiramate	10	-0.32	0.368	0.842
cg23326197	CYP3A4	Topiramate	10	-0.30	0.407	0.844
cg24014584	CYP3A4	Topiramate	10	-0.38	0.279	0.842
cg01243068	CYP3A5	Topiramate	10	-0.52	0.133	0.783
cg05867406	CYP3A5	Topiramate	10	0.38	0.279	0.842
cg14260643	CYP3A5	Topiramate	10	-0.75	0.018	0.443
cg00764099	UGT1A3	ValproicAcid	14	0.16	0.584	0.986
cg00963071	UGT1A3	ValproicAcid	14	0.44	0.116	0.783
cg01478198	UGT1A3	ValproicAcid	14	-0.39	0.165	0.841
cg01764553	UGT1A3	ValproicAcid	14	0.06	0.856	0.986
cg02227331	UGT1A3	ValproicAcid	14	-0.44	0.120	0.783
cg02789126	UGT1A3	ValproicAcid	14	0.30	0.295	0.842
cg03607648	UGT1A3	ValproicAcid	14	0.06	0.844	0.986
cg04314609	UGT1A3	ValproicAcid	14	-0.35	0.227	0.842
cg05542967	UGT1A3	ValproicAcid	14	0.37	0.197	0.842
cg06566087	UGT1A3	ValproicAcid	14	0.01	0.964	0.999
cg06772514	UGT1A3	ValproicAcid	14	0.01	0.988	1.000
cg07823755	UGT1A3	ValproicAcid	14	-0.52	0.062	0.574
cg08697797	UGT1A3	ValproicAcid	14	0.25	0.391	0.842
cg09339841	UGT1A3	ValproicAcid	14	0.07	0.820	0.986
cg09505117	UGT1A3	ValproicAcid	14	0.06	0.844	0.986
cg11272349	UGT1A3	ValproicAcid	14	-0.10	0.739	0.986
cg11811840	UGT1A3	ValproicAcid	14	0.15	0.616	0.986
cg12177266	UGT1A3	ValproicAcid	14	-0.34	0.233	0.842
cg14323797	UGT1A3	ValproicAcid	14	0.20	0.492	0.906
cg17872990	UGT1A3	ValproicAcid	14	-0.03	0.928	0.988
cg18109081	UGT1A3	ValproicAcid	14	-0.35	0.221	0.842
cg19435015	UGT1A3	ValproicAcid	14	-0.26	0.366	0.842
cg21620495	UGT1A3	ValproicAcid	14	-0.06	0.856	0.986
cg22145666	UGT1A3	ValproicAcid	14	-0.10	0.739	0.986
cg23229568	UGT1A3	ValproicAcid	14	0.13	0.660	0.986
cg23714547	UGT1A3	ValproicAcid	14	0.24	0.417	0.856
cg24335340	UGT1A3	ValproicAcid	14	-0.13	0.648	0.986
cg25726425	UGT1A3	ValproicAcid	14	0.24	0.400	0.844
cg27361577	UGT1A3	ValproicAcid	14	-0.53	0.052	0.531
cg02363364	UGT2B7	ValproicAcid	14	0.25	0.415	0.850
cg04558553	UGT2B7	ValproicAcid	14	-0.28	0.353	0.842
cg14688178	UGT2B7	ValproicAcid	14	0.36	0.232	0.842
cg15935333	UGT2B7	ValproicAcid	14	-0.16	0.604	0.986

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III

Decreased serum concentrations of anti-seizure medications in children with drug resistant epilepsy following treatment with ketogenic diet

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Acknowledgments
This work was supported by the Dam Foundation, the Norwegian Epilepsy Association's Research Fund, the Novo Nordisk Foundation, and the National Advisory Unit on Rare Disorders, Norway. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the paper. All authors had full access to all the data in the study and had final responsibility for the decision to submit the manuscript for publication. We are grateful to the patients and their caregivers who participated in the study. We would like to thank Magne Thoresen and Dag Hofoss for valuable advice regarding the statistical analysis. The Section for Clinical Pharmacology, Oslo University Hospital, Norway, is acknowledged for the analyses of all ASMs. We also acknowledge the National Centre for Rare Epilepsy-Related Disorders for their support throughout the study. Finally, we would like to thank our colleagues at the National Centre for Epilepsy, Norway, for their contributions. MK has received two honoraria from Nutricia. CJL has received speakers' or expert group honoraria from Angelini, Eisai, Jazz and UCB Pharma. KKS has been reimbursed for travel and accommodation as a speaker at a sponsored workshop by Kolfarma and has received speaker's honoraria from Roche and Eisai. The remaining authors have nothing to disclose. 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

Ethical disclosures
The study was approved by the Regional Committee for Medical and Health Research in South East of Norway (2016/2016). All participants or parents/caregivers provided written informed consent before enrollment. All procedures in this study were in accordance with the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (ID: NCT04063007) and HelseNorge.no (https://oslo-universitetssykehus.no/kliniske-studier/diett-hos-barn-ved-epilepsi).

Data and code availability

The raw data from this project is not available due to privacy and ethical restrictions of the project approval. Metadata generated in the study and code used in the analysis are available from the corresponding author upon reasonable request within the privacy policy of the informed consent by the participants.

Abstract

Objective: To examine the potential influence of a ketogenic diet on serum concentrations of anti-seizure medications (ASMs) in children with drug resistant epilepsy.

Methods: We investigated the serum concentrations of ASMs in 25 children with drug resistant epilepsy, 2 to 13 years of age, treated with a classical ketogenic diet for 12 weeks. The patients were recruited from the National Centre for Epilepsy from August 15th, 2017, to January 24th, 2022. Changes in ASM serum concentrations were analyzed using a mixed effect model analysis. Significance level was set at P <0.05 for all comparisons.

Results: The participants used 12 different ASMs during the study. The mean number of ASMs was 2.4 (±SD 0.7). None of the participants changed the type or dose of the ASMs during the intervention period. The serum concentrations of clobazam (n = 9, P = 0.002), desmethylclobazam (n = 9, P = 0.010), and lamotrigine (n = 6, P = 0.016) decreased significantly during the dietary treatment. The analytes with the largest reduction in serum concentration after 12 weeks of dietary treatment were clobazam (mean change -38%) and desmethylclobazam (mean change -37%). We found no significant change in the serum concentrations of levetiracetam, topiramate, and valproic acid.

Significance: We identified a significant decrease in the serum concentrations of clobazam, desmethylclobazam, and lamotrigine following a 12-week ketogenic diet intervention in children with drug resistant epilepsy. An unintended decrease in the serum concentrations of ASMs may render the patient prone to seizures. Hence, we suggest to closely monitor the ASM serum concentrations in patients starting on a ketogenic diet.

Key words: High-fat, low-carbohydrate diet, dietary treatment, food-drug interactions, therapeutic drug monitoring, antiseizure medications

Key points

- In this prospective study we examined the influence of a ketogenic diet on serum concentrations of ASMs in children with drug resistant epilepsy.
- We identified a significant decrease in the serum concentrations of clobazam, desmethylclobazam, and lamotrigine following 12 weeks of dietary treatment.
- Unintended alterations in ASM serum concentrations may reduce the seizure protection and increase the risk of adverse effects.
- We suggest to closely monitor the ASM serum concentrations in patients starting on a ketogenic diet.

1. Introduction

Epilepsy is a common brain disorder that causes recurrent, unprovoked seizures. Worldwide, more than 65 million people are affected (1). The mainstay of epilepsy treatment is anti-seizure medications (ASMs). However, despite an increasing number of available ASMs, about 30% of the epilepsy patients do not achieve seizure control (2, 3). Other treatment options include brain surgery and vagus nerve stimulator (VNS) therapy. Unfortunately, a large share of the patients are not eligible for epilepsy surgery or VNS therapy. Another treatment option is a dietary therapy known as the ketogenic diet.

The ketogenic diet is a collective term for various diets high in fat and low in carbohydrate, originally designed to mimic the metabolic state of fasting (4). The term “ketogenic” comes from the diets’ ability to induce “ketosis” (elevated level of ketone bodies in the blood). However, as the ketogenic diet is a comprehensive treatment mainly used by patients with severe epilepsy, the majority use the dietary treatment in combination with ASMs.

In recent years, clinical observations have raised questions about potential pharmacokinetic interactions between the ketogenic diet and ASMs. We have previously reported a substantial decrease in the serum concentration of several commonly used ASMs in adults treated with a modified ketogenic diet (5). Other studies show mixed result, but are hampered by a retrospective design, few ASMs investigated, or changes in ASM dose during the study duration (6-8).

Potential consequences of interactions between the ketogenic diet and ASMs includes reduced efficacy of the ASMs, risk of adverse effects, as well as misinterpretation of the efficacy of the ketogenic diet in clinical trials. In order to optimize epilepsy treatment, it is therefore important to know if the dietary treatment interact with the ASMs. At the National Centre for Epilepsy, Norway, we measure the serum concentrations of ASM in all patients regularly (therapeutic drug monitoring) (9). In the present study, we aimed to examine the potential influence of a ketogenic diet on serum concentrations of ASMs in children with drug resistant epilepsy.

2. Methods

2.1. Participants

The study participants were recruited from the National Center for Epilepsy, Norway, between August 15th, 2017, and January 24th, 2022. Patients between 2 and 18 years of age, with a diagnosis of drug resistant epilepsy according to the classification by the International League Against Epilepsy's (ILAE) (10), with two or more seizures per week on average, and willingness to try the classical ketogenic diet for at least 12 weeks were eligible to participate in this study. If one or more of the following exclusion criteria were present, the patient was excluded from the study: glucose transporter protein 1 deficiency syndrome, pyruvate dehydrogenase deficiency, pyruvate carboxylate deficiency, diseases which contraindicated the dietary treatment, epilepsy surgery, including VNS implantation the past 6 months before diet initiation, steroid medication the past 2 months before diet initiation, prophylactic antibiotic treatment, breastfeeding, psychogenic non-epileptic seizures, eating disorders, feeding disabilities expected to unable the dietary treatment, inability to follow the study protocol, lack of motivation by patient or caregivers, previous treatment with a ketogenic diet, medical need to start dietary treatment immediately, and pregnancy or planned pregnancy.

2.2. Study design and diet intervention

The participants ate their habitual diet during a 4-week baseline period and subsequently a classical ketogenic diet in a 12-week diet intervention period. No changes in epilepsy treatments were allowed during the 16-week study.

The classical ketogenic diet was initiated during a 16-day hospital admission without prior fasting. A registered dietitian tailored all diets individually. The diet was started at a ketogenic ratio (ratio of gram fat: gram carbohydrate plus gram protein) of 1:1 – 2:1 with a gradual increase up to a maximum of 4:1 according to efficacy and tolerability. All diets were supplemented with vitamins and minerals according to the child's need. A daily fluid intake based on standard pediatric guidelines was recommended. The meals consisted of regular food items and/or special medical foods for epilepsy.

The macro nutrient composition of the meals was estimated using the electronic meal planner DietistNet (Kost och Näringsdata, Bromma, Sverige) with associated databases, including the Norwegian Food Composition Database (11). All food items were weighed on a scale with an accuracy of 0.1 gram.

2.3. Seizure recording

All parents/caregivers received training in how to record seizures systematically in a seizure diary. The seizure frequency during the 4-week baseline period was compared with the seizure frequency during the last 4 weeks before the 12-week study visit. We defined patients as a responder when the seizure frequency was reduced with ≥50% compared to the seizure frequency at baseline.

2.4. Participant adherence

Adherence to the study protocol was assessed at each study visit by structured questions regarding compliance with the diet intervention and the prescribed ASMs. The importance of not doing any changes in the pharmacological treatment during the study was underlined to all participants and caregivers. In addition, fasting blood glucose, hemoglobin A_{1c} (HbA_{1c}), and blood ketones (β-hydroxybutyrate) was measured at baseline (pre-diet), and after 6 and 12 weeks of dietary treatment.

2.5. ASM serum concentration measurements

Analyses of serum concentrations of all ASMs were performed as part of the follow-up to all patients. The standard blood-sampling time for serum concentration measurements of ASMs was food- and drug fasting in the morning at assumed steady-state of the ASMs. Blood samples were collected at baseline (pre-diet), and after 6 and 12 weeks of dietary treatment. The analyses of all ASMs were based on routine measurements by validated methods at the Section for Clinical Pharmacology, Oslo University Hospital (Oslo, Norway).

Clonazepam, clobazam, and its active metabolite desmethylclobazam, were measured by high pressure liquid chromatography with ultraviolet detection (HPLC-UV) on a Dionex Ulitimate 3000 instrument with a 4.6 x 30 mm 3.5 µm ZORBAX Eclipse Plus C18 column.

The other ASMs were analysed by ultra-high performance liquid chromatography with mass spectrometric detection (UHPLC-MS/MS) on a Prelude MD HPLC/Endura MD mass spectrometer, using the Antiepileptic Drugs ClinMass TDM Platform Kit System (MS9000, MS9200) from Recipe (Munich, Germany) (https://www.recipe.de/en/products_ms_tdm_ms09000-ms09200_ord.html#MS9200).

2.6. Statistics

Data are presented as means with standard deviations (SD) or medians with interquartile ranges (IQR) and minimum – maximum (min – max) scores for continuous variables. Categorical variables are presented as frequencies and percentages (%). We ran a linear mixed effect model analysis of the relationship between serum concentrations of ASMs and time on ketogenic diet. As fixed effect, we entered time on ketogenic diet into the model and as random effects intercepts for patients. Visual inspection of residual plots did not reveal obvious deviations from normality. Three cases with large residuals were identified, however, re-runs of the model without these cases did not change the results. Of the 12 ASMs, seven were used by fewer than six patients, which was considered too few to allow for meaningful comparisons over time. One-sample *t*-tests were used to compare percentage changes in the serum concentration of all ASMs combined after 6 and 12 weeks of dietary treatment. Levels of blood glucose, HbA_{1c}, β-hydroxybutyrate, albumin, and body weight after 6 and 12 weeks of dietary treatment were compared with baseline values by either paired *t*-test (normally distributed data) or Wilcoxon signed rank test (non-normally distributed data). All tests were two-sided. For all comparisons, significance was set at p value <0.05. Statistical analyses were performed with SPSS (v.28, IBM).

3. Results

3.1. Patient characteristics

The study material consisted of 25 children (age 2 – 13 years) with drug resistant epilepsy (**Figure 1**). An overview of the baseline characteristics and demographics is given in **Table 1**. Seventeen children had generalized epilepsy, three children had focal epilepsy, and five children had combined generalized and focal epilepsy. Fifteen children had a known epilepsy etiology, while the etiology was unknown in ten children. Median years with epilepsy before starting the ketogenic diet was 4.0 (IQR 3.3). Ten children had a gastrostomy tube used either for total enteral feeding or for supplemental feeding, fluids, and medications.

3.2. Diet intervention

The ketogenic ratio after 6 and 12 weeks of dietary treatment is presented in **Table 2**. Twenty-two children completed the 12-week diet intervention. Median ketogenic ratio was 3:1 at both 6 and 12 weeks of diet intervention. The ketogenic ratios ranged from 2:1 to 3.5:1.

3.3. Blood biochemistry

Blood biochemistry results are presented in **Table 3**. Fasting β-hydroxybutyrate was, as expected, almost negligible (median 0.1 mmol/L (IQR 0.1)) at baseline, but increased during the dietary treatment (median 3.5 mmol/L (IQR 1.7) at 6 weeks (P <0.001), and 3.0 mmol/L (IQR 2.3) at 12 weeks (P <0.001). Fasting blood glucose decreased significantly from mean score of 4.7 mmol/L (±SD 0.5) at baseline to a mean of 4.1 mmol/L (±SD 0.5) at 12 weeks (P <0.001). In accordance, HbA_{1c}, which reflects the blood glucose concentration over the past six to eight weeks, declined significantly during the dietary treatment (mean 29.7 mmol/mol (±SD 4.0) at baseline vs. mean 23.8 mmol/mol (±SD 3.3) at 12 weeks; P <0.001). Together, these results indicate a good adherence with the diet intervention.

3.4. Body weight

No significant difference in body weight was observed during the study period (median 23.2 kg (IQR 21.3) at baseline vs. median 23.2 kg (IQR 20.9) at 6 weeks (P = 0.77), and median 24.0 kg (IQR 21.5) at 12 weeks (P = 0.17)).

3.5. Seizure outcomes

Nine (36%) patients had 50% or more seizure reduction, including one (4%) that became seizure free. Sixteen patients (64%) had less than 50% seizure reduction, including three patients that did not complete the 12-week diet intervention due to seizure exacerbation (*n* = 2) or lack of effectiveness and adverse effects (*n* = 1).

3.6. ASM serum concentrations

An overview of the ASMs used in the study, the number of samples, and the mean serum concentrations are given in **Table 4**. The participants used in total 12 different ASMs during the study, and none changed the type or dose of ASMs during the study period. All, except two participants, used two or more ASMs (*n* = 23, 92%), accounting for 22 different ASM combinations. The mean number of ASMs was 2.4 (±SD 0.7). The most frequently used ASMs were valproic acid (*n* = 14), clobazam (which also includes the active metabolite desmethylclobazam) (*n* = 9), topiramate (*n* = 8), and levetiracetam (*n* = 8).

Serum concentrations of clobazam and desmethylclobazam decreased significantly during the diet intervention (clobazam: *P* = 0.002, desmethylclobazam: *P* = 0.010). The mean serum concentration of clobazam declined from 0.54 µmol/L (±SD 0.12) at baseline to 0.34 µmol/L (±SD 0.09) at 6 weeks, and 0.31 µmol/L (±SD 0.16) at 12 weeks. The mean change of clobazam from baseline to 6 and 12 weeks of dietary treatment was -44% and -38%, respectively. All participants experienced a reduction in clobazam serum concentration during the diet intervention. The changes ranged from –81% to –18% at 6 weeks and –54% to –18% at 12 weeks compared to baseline. Correspondingly, the mean serum concentration of desmethylclobazam decreased from 6.60 µmol/L (±SD 8.66) at baseline to 5.55 µmol/L (±SD 7.10) at 6 weeks, and 2.63 µmol/L (±SD 3.04) at 12 weeks. The mean change of desmethylclobazam from baseline was -33.4% at 6 weeks and -37.9% at 12 weeks of dietary treatment. Similar to clobazam, all participants had a decrease in the serum concentration of desmethylclobazam during the diet intervention. However, the degree of decline varied greatly

across individuals, ranging from -56% to -10% at 6 weeks, and from -59% to -13% at 12 weeks of dietary treatment. One participant using clobazam/desmethylclobazam was identified as a cytochrome P450 2C19 (CYP2C19) poor metabolizer (i.e. the CYP2C19 enzyme has limited activity). Desmethylclobazam is mainly metabolized by CYP2C19, and consequently, the serum concentration was high (28 µmol/L, reference range 1- 10 µmol/L) in this patient. However, the patient also experienced a reduction in the serum concentration and exclusion of this participants' data gave similar results (data not shown). Clobazam and its metabolite desmethylclobazam had the largest reduction after 12 weeks of dietary treatment.

Also, the serum concentration of lamotrigine decreased significantly after diet initiation ($P = 0.016$). Mean serum concentration of lamotrigine was reduced from 23.3 µmol/L (±SD 9.9) at baseline to 20.7 µmol/L (±SD 7.8) at 6 weeks of dietary treatment. After 12 weeks on the diet, the mean serum concentration decreased further to 19.5 µmol/L (±SD 8.4). Compared to baseline, the mean change of lamotrigine serum concentration was -8% after 6 weeks and -15% after 12 weeks. The changes ranged from -27% to 13% at 6 weeks, and from -27% to 0% at 12 weeks of diet intervention.

We found no significant change in the serum concentrations of levetiracetam ($P = 0.36$), topiramate ($P = 0.09$), or valproic acid ($P = 0.13$) during the diet intervention. However, there was a trend towards a decreased serum concentration of valproic acid after 12 weeks of dietary treatment. Mean serum concentration of valproic acid was reduced from 470 µmol/L (±SD 121) at baseline to 397 µmol/L (±SD 140) at 12 weeks. In addition to the total valproic acid concentration, the free valproic acid concentration was measured and the free fraction was calculated. Free concentration of valproic acid did not change significantly during the diet intervention ($P = 0.22$). However, there was a trend towards an increase in the free fraction ($P = 0.06$), from mean 12% (±SD 5.1) at baseline to 17% (±SD 6.6) at 6 weeks, and 15% (±SD 3.4) at 12 weeks of dietary treatment.

Due to the low sample size, we did not perform statistical analyses of changes in serum concentrations of clonazepam (*n* = 4), lacosamide (*n* = 3), ethosuximide, (*n* = 1), oxcarbazepine (*n* = 1), perampanel (*n* = 1), rufinamide (*n* = 1), and zonisamide (*n* = 1).

Overall, the majority of ASM serum concentrations were reduced during the diet intervention. Compared to baseline, the mean change in serum concentration of all ASMs combined was -19% (±SD 23%) at 6 weeks (*P* <0.001), and -18% (±SD 23%) at 12 weeks (*P* <0.001) of dietary treatment. In nearly a third of the measurements (*n* = 16, 29%), the serum concentrations declined more than 30% after 12 weeks.

4. Discussion

In the present study, we examined the influence of a ketogenic diet on serum concentrations of ASMs in children with epilepsy. The main findings were statistically significant reductions in the serum concentrations of clobazam, desmethylclobazam, and lamotrigine during the diet intervention.

Our study is the first to demonstrate a decrease in the serum concentrations of clobazam and its active metabolite desmethylclobazam in children following treatment with a ketogenic diet. The results are in line with our previous prospective trial on adults treated with a modified ketogenic diet (5). Importantly, approximately half of the patients had around 50% decrease in serum concentration – a clinically significant decline that may increase the risk of seizure aggravation. In all participants using clobazam, the serum concentration of both clobazam and desmethylclobazam decreased. Another finding consistent with our previous study in adults (5) was the significant decrease in the serum concentration of lamotrigine. Although the decrease in lamotrigine serum concentration was smaller than the decline observed for clobazam and desmethylclobazam, this may still be clinically important for some patients, especially for those who initially had a low serum concentration.

Currently, the mechanisms that underpin the observed decrease in the serum concentration of ASMs are poorly understood. Studies investigating food-drug interactions have traditionally focused on changes in drug absorption and bioavailability. In general, the ASMs are lipophilic and has a high bioavailability (12). Thus, we would not expect any pronounced alterations in the ASM serum concentrations when starting on a ketogenic diet.

Clobazam, desmethylclobazam, and lamotrigine are all metabolized by hepatic enzymes belonging to either the cytochrome P450s (CYPs) or the UDP-glucuronosyltransferase (UGT) superfamilies, which are major pathways for the metabolism of numerous commonly used drugs (9, 13, 14). Importantly, in addition to their pivotal role in drug metabolism, they also have a central role in lipid metabolism (15). High-fat diets have been shown to influence the gene expression and activity of hepatic drug-metabolizing enzymes in experimental animal studies (16). Thus, up-regulation of the drug-metabolizing enzymes may represent a mechanism for the observed decline in ASM serum concentrations.

Unlike most ASMs, levetiracetam is not metabolized in the liver, but is either metabolized in the blood by esterases (24%) or excreted unchanged in the urine (66%) (17). Therefore, perhaps levetiracetam is less prone to interactions than drugs dependent on hepatic metabolism. Indeed, our results and previous studies did not find any change in the serum concentration of levetiracetam during treatment with the ketogenic diet (5, 6).

Contrary to our previous findings in adults (5), we were not able to demonstrate a significant change in the serum concentration of valproic acid. However, consistent with the results of Coppola (8), we found a trend towards a decrease in total valproic acid. Moreover, there was a trend towards an increase in free fraction of valproic acid. Free valproic acid is responsible for the pharmacological effect. Since valproic acid is highly bound to protein (typically 90 – 95%) (18), changes in the albumin level may increase the free concentration of valproic acid. However, albumin levels did not change during the dietary treatment in our study. Moreover, with regard to the trend towards a decrease in

total valproic acid we would expect a decrease in the free fraction, as higher total concentrations (<415 µmol/L) lead to a higher unbound fraction (19). Other factors that may influence the free fraction of valproic acid are the levels of free fatty acids (18), which have been shown to increase in individuals on the ketogenic diet (20). Thus, increased levels of free fatty acids may represent a possible mechanism for increased free fraction of valproic acid. However, more studies are needed to clarify the impact of the ketogenic diet on the serum concentration levels of both total and free valproic acid.

There are several possible explanations for the disparity of results between the studies investigating the influence of the ketogenic diet on ASM serum concentrations (5-8), including differences in the type and duration of the diet intervention, pediatric versus adult study populations, variations in the type of ASMs used, as well as dissimilarities in the combinations of ASMs. In addition, changes in drug dosage during the diet intervention and trial follow-up may influence the results (7).

For unknown reasons, a minority of epilepsy patients experience a paradoxical seizure aggravation when starting on a ketogenic diet (21, 22). Thus, one may speculate whether this, at least partly, might be related to a decrease in ASM serum concentration levels. In the present study, two patients experienced a substantial seizure aggravation. One of them developed refractory status epilepticus. The other patient had a less severe seizure exacerbation, but the diet was tapered off after two weeks of treatment. None of them used the ASMs that decreased significantly. Indeed, in our adult study, we did not find any correlation between change in seizure frequency and change in serum concentrations of ASMs (5).

Strengths of our study include the prospective study design, complete patient follow-up, and that all ASMs were kept unchanged throughout the entire study period. Control group data from our previous adult trial showed negligible changes in ASM serum concentration during a 12-week period on their habitual diet when epilepsy treatments were kept unchanged (5). Thus, it seems unlikely

that the changes observed in our study are a result of random variation. Our systematic and objective measures of diet adherence confirmed good compliance with the diet intervention.

Limitations of the study include lack of a control group and few patients with several different ASMs, many of them in combinations. ASMs are known for their susceptibility for drug interactions, thus a diet-induced change of one ASM may influence the serum concentration of other ASMs. However, none of the participants used carbamazepine, phenytoin, or phenobarbital known to be strong inducers of several hepatic enzymes (23). Also, we cannot exclude that the decrease in ASM serum concentrations is not due to participants not taking the medication as prescribed. However, all parents/caregivers confirmed adherence with the prescribed ASMs at each study visit. Also, since the children in this study had a long history of severe epilepsy, we believe the parents and caregivers were highly motivated to follow the ASM treatment recommendations.

5. Significance

We identified a significant decrease in the ASM serum concentrations following a 12-week ketogenic diet intervention. An unintended decrease in the serum concentrations of ASMs may render the patient prone to seizures. Hence, we suggest to closely monitor the ASM serum concentrations in patients starting on a ketogenic diet.

Author contributions

POI, KR, KKS, and SP contributed to the study conception and design. Data collection were performed by SP and CJL. Statistical analyses were performed by SP. The first draft of the manuscript was written by SP and all authors provided critical feedback and commented on the manuscript.

Table 1: Clinical and demographic characteristics of the study participants at baseline.

		Min – max
Number of patients	25	
Gender, male/female n (%)	13/12 (48/52%)	
Age at diet start, years, median (IQR)	6.0 (7.0)	2.4 – 13.3
Epilepsy classification, n (%)		
Focal	3 (12%)	
Generalized	17 (68%)	
Combined focal and generalized	5 (20%)	
Epilepsy etiology, n (%)		
Structural	5 (21%)	
Genetic	6 (25%)	
Genetic/structural	2 (8%)	
Structural/infectious	1 (4%)	
Unknown	10 (40%)	
Age at first seizure, years, median (IQR)	2.8 (4.2)	0.25 – 8.0
Years with epilepsy, median (IQR)	4.0 (3.3)	0.35 – 11.4
Gastrostomy tube, yes/no (%)	10/15 (40%/60%)	
Number of ASMs, mean (±SD)	2.4 (0.7)	1 – 4
Number of ASMs, n (%)		
1	2 (8%)	
2	12 (48%)	
3	10 (40%)	
4	1 (4%)	

Abbreviations: ASM, anti-seizure medications; IQR, interquartile range; SD, standard deviation.

Table 2: Ketogenic ratio on the diet.

	6 weeks (*n* = 23)	**12 weeks (*n* = 22)**
Ketogenic ratio[a], median (min – max)	3:1 (2:1 – 3.5:1)	3:1 (2:1 – 3.5:1)
Ketogenic ratio[a], n (%)		
2:1 – 2.25:1	6 (26%)	4 (18%)
2.5:1 – 2.75:1	5 (22%)	5 (23%)
3:1 – 3.25:1	11 (48%)	10 (45%)
3.5:1	1 (4%)	3 (14%)

[a]The ketogenic ratio is the ratio of grams fat to the sum of grams of protein and carbohydrate.

Table 3: Blood biochemistry. Blood biochemistry at baseline and after 6 and 12 weeks of dietary treatment.

Analysis	**Baseline**	***n***	**6 weeks**	***n***	***P* value**	**12 weeks**	***n***	***P* value**
Glucose, mmol/L, mean (±SD)	4.7 (0.5)	25	NA		NA	4.1 (0.5)	21	**<0.001**[a]
HbA_{1c}, mmol/mol, mean (±SD)	29.7 (4.0)	25	NA		NA	23.8 (3.3)	21	**<0.001**[a]
β-hydroxybutyrate, mmol/L, median (IQR)	0.1 (0.1)	24	3.5 (1.7)	14	**0.001**[b]	3.0 (2.3)	22	**<0.001**[b]
Albumin, g/L, mean (±SD)	42.7 (3.5)	25	NA		NA	42.9 (3.6)	21	0.910[a]

Abbreviations: HbA_{1c}, Hemoglobin A_{1c}; NA, not applicable; SD, standard deviation.

[a]Paired *t* test.

[b]Wilcoxon signed rank test.

Paired *t* test was used to compare values at baseline with values after 6 and 12 weeks of dietary treatment of all variables except β-hydroxybutyrate in which we used the non-parametric Wilcoxon signed rank test. Bold indicates $p < 0.05$.

Table 4: Serum concentration of ASMs at baseline and after 6 and 12 weeks of dietary treatment.

ASM (μmol/L)	***n***	**Baseline** **Mean (±SD)**	**6 weeks** **Mean (±SD)**	**12 weeks** **Mean (±SD)**	***P* value**
Clobazam	9	0.54 (0.12)	0.34 (0.09)	0.31 (0.16)	**0.002**
Desmethylclobazam[a]	9	6.60 (8.66)	5.55 (7.10)	2.63 (3.04)	**0.010**
Lamotrigine	6	23.3 (9.9)	20.7 (7.8)	19.5 (8.4)	**0.016**
Levetiracetam	8	77.6 (32.5)	65.0 (33.6)	81.0 (25.4)	0.342
Topiramate	8	18.8 (9.5)	19.3 (3.1)	16.5 (7.6)	0.091
Valproic acid, total	14	470 (121)	415 (135)	397 (140)	0.104
Valproic acid, free	12	59.9 (36.0)	64.0 (32.2)	58.5 (24.1)	0.215
Valproic acid, free (%)	12	12.3 (5.1)	16.8 (6.6)	14.6 (3.4)	0.061

Abbreviations: ASM, anti-seizure medication; SD, standard deviation.

[a]Desmethylclobazam is the pharmacologically active metabolite of clobazam.

[b]nmol/L

Data are analyzed using a mixed effect model analysis. Bold indicates p <0.05. Due to the low sample size, we did not perform statistical analyses of the serum concentrations of clonazepam (n = 4), lacosamide (n = 3), ethosuximide (n = 1), oxcarbazepine (n = 1), perampanel (n = 1), rufinamide (n = 1), and zonisamide (n =1).

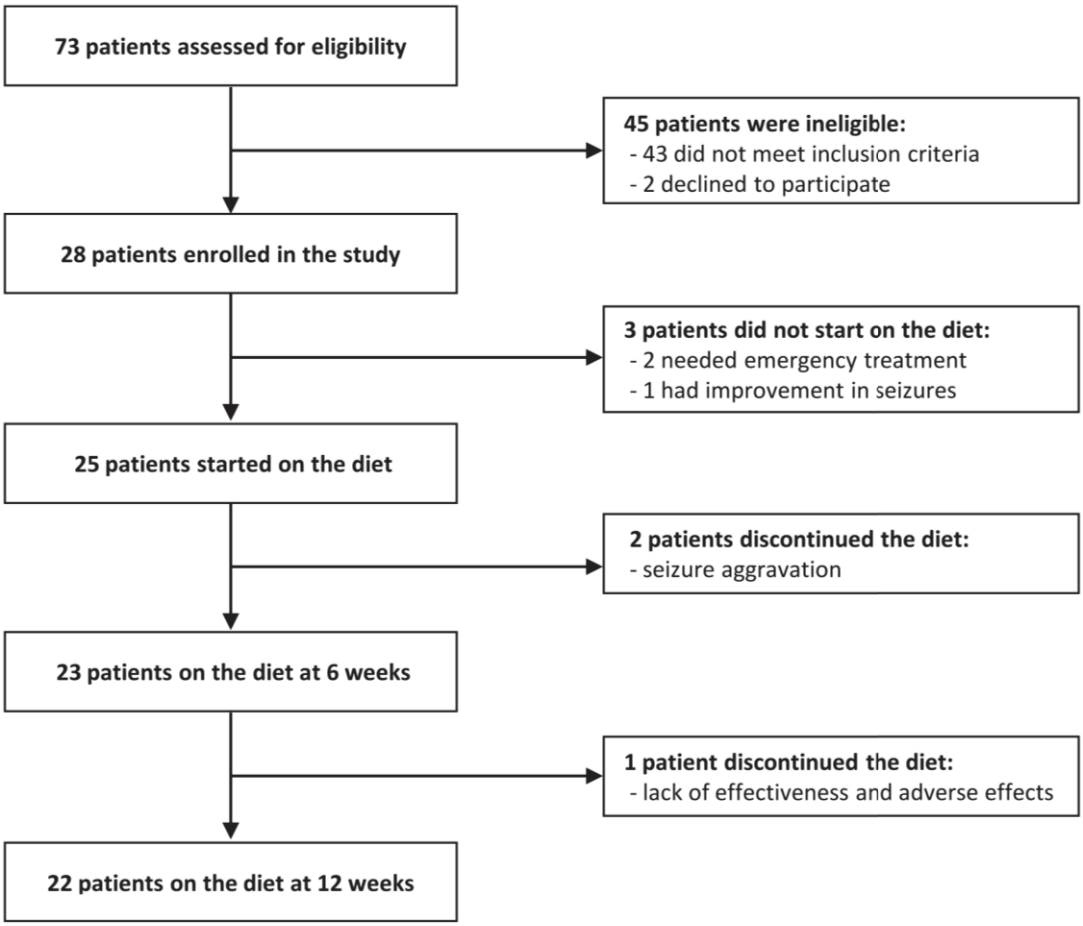

Figure 1: Flow chart illustrating the recruitment process of participants into the study.

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